Phthalate Exposures and Hormonal Disruption in Relation to Birth Outcomes

by

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Abstract

Preterm birth affects 1 out of every 10 pregnancies in the United States and is one of the leading causes of infant death. Other negative birth outcomes, including preeclampsia and gestational diabetes, are associated with comorbidities for the mother and fetus later in life. Widespread exposures to environmental contaminants, such as phthalates, have been hypothesized as playing a casual role in the risk for adverse birth outcomes. Phthalates are endocrine disrupting chemicals, which interfere with hormone levels and regulation inside the body. Regulation of numerous endocrine pathways is essential for maintaining a healthy pregnancy. Exposures to phthalate chemicals may elicit an endocrine response deleterious to the pregnancy, resulting in elevated risk for adverse birth outcomes. This dissertation sought to investigate whether phthalate exposures were associated with disruption of various classes of hormone concentrations including thyroid and reproductive hormones, and whether hormone disruption mediated the association between exposure to mixtures of phthalate metabolites and adverse birth outcomes.

Aim 1 of this dissertation assessed associations between repeated measures of urinary phthalate metabolites and serum hormones in the PROTECT pregnancy cohort. In aim 1, we observed numerous significant associations between phthalate metabolites and hormones that were consistent based on molecular weight of the phthalate. Of note, low molecular weight metabolites were positively associated with testosterone while high molecular weight metabolites were inversely associated with testosterone, pointing to possible mechanistic differences. Aim 1 also revealed effect modification by timing of study visit and fetal sex across many observed associations, which showed phthalate exposure resulting in decreased hormone concentrations among pregnancies with a female fetus and increased hormone concentrations among pregnancies with a male fetus. Aim 2 investigated associations between repeated

measures of hormone concentrations and adverse birth outcomes. Various associations were observed which highlighted the importance of progesterone, estriol, and thyroxine (T4). Progesterone was inversely associated with gestational age at birth, and thyroid hormones were positively associated with risk of spontaneous preterm birth. Few differences were observed by timing of study visit, but many differences were present between fetal sexes which suggested elevated risk of birth outcomes among male pregnancies with increases in most hormone concentrations. Finally, aim 3 explored the mediating effects of hormone concentrations on the associations between mixtures of phthalate metabolites and adverse birth outcomes. Among pregnancies with a male fetus, an interquartile range increase in the mixture of low molecular weight (LMW) metabolites was associated with increased odds of preterm birth at visit 2 (OR: 1.82, 95% CI: 1.01, 3.31) and with spontaneous preterm birth at visit 3 (OR: 2.74, 95% CI: 1.23, 6.13). We observed 17.3% of the association between LMW phthalate exposure at visit 3 and preterm birth was mediated by TSH. CRH, progesterone, and testosterone also mediated 28%, 18%, and 29% of the association between LMW phthalate exposure at visit 1 and spontaneous preterm birth.

Overall, this dissertation advances our understanding of the relationship between environmental phthalate exposure and risk of adverse birth outcomes. We have explored the possible mechanisms by which phthalates may elicit deleterious effects on pregnancy in an endocrine framework. Our findings may be useful in early detection of pregnancies at elevated risk for delivering preterm. Future work should seek to utilize higher case numbers of adverse pregnancy outcomes to substantiate these findings and to broaden our understanding of environmental endocrine disruption during pregnancy.

Chapter I. Introduction

Adverse birth outcomes pose a significant public health threat

Preterm birth is the leading cause of infant mortality in most high- and middle-income countries, including the United States. Of 7.6 million children that died before the age of 5 in 2010 globally, 14% died from complications of preterm birth. Neonatal mortality accounted for 48% of all childhood deaths in the Americas at that time, and 17% of those were attributable to preterm birth[1]. The United States experiences a preterm birth rate higher than most other developed countries[2]. Following a steady decline from 2007 to 2014, the preterm birth rate increased over two consecutive years to 9.85% from 2014 to 2016. This increase was primarily driven by late preterm births, which are those occurring between 34 and 36 weeks gestation[3]. Being born preterm increases the risk of future morbidities including developmental disability, neurological impairments, vision and hearing loss, cerebral palsy, asthma, and attention deficit disorder[4–7].

The causal mechanisms surrounding preterm birth are largely unknown. Infection, inflammation, placental hemorrhage, and stress are all thought to play critical roles, and so environmental contaminants associated with these risk factors may also play a causal role[8]. Preterm birth rates are typically around 10% higher among Black women compared to white women[9,10]. Groups of low socioeconomic status, low educational status and young/old maternal ages are also at an elevated risk for delivering preterm[11–13]. A previous study indicated that an interval of less than 6 months between pregnancies was associated with more than a two-fold increased risk of preterm birth[14]. Obesity is associated with preeclampsia, gestational diabetes and development of congenital abnormalities, all of which are positively associated with preterm birth[15]. Women experiencing extreme external stressors such as housing instability and economic hardship are also at a higher risk for delivering preterm[16]. Understanding the roles that different risk factors

may have in the causal pathway of preterm birth will help in the development of targeted intervention and prevention strategies.

Other rare birth outcomes, about which much less is known, are also important public health concerns. The etiology of preterm birth is complex and some have suggested that subcategorizing preterm deliveries based on obstetric presentation is more informative than assessing all preterm births together. Spontaneous preterm births are those occurring from spontaneous premature initiation of labor or rupture of membranes, in contrast to medically indicated preterm deliveries. McElrath and colleagues have shown that the spontaneous subtype of preterm delivery is generally marked by a state of intrauterine inflammation that is not present in the non-spontaneous type[17]. Thus, the upstream causative factors and biological pathways implicated may be distinct between these two types. Preeclampsia, another rare birth outcome, is implicated in the non-spontaneous subtype of preterm birth. Characterized by new-onset hypertension and proteinuria during pregnancy[18], preeclampsia affects about 6% of pregnancies worldwide[19] and is the leading cause of maternal mortality, cesarean sections, and preterm delivery in the United States[20,21]. The mechanisms of abnormal placentation observed in early stages of preeclampsia are poorly understood, but environmental factors could play a role. Gestational diabetes mellitus (GDM) is diabetes associated specifically with pregnancy that was not present prior to pregnancy. High maternal glucose levels easily cross the placenta and elicit a response from the fetal pancreas. Infants born to mothers with GDM are at elevated risk for macrosomia and metabolic dysfunctions, and mothers become more likely to develop diabetes later in life[22]. Established risk factors for GDM include family history, obesity, advanced maternal age, and cigarette smoking, but epidemiology studies assessing interventions of diet and lifestyle factors report inconsistent results[23]. Understanding the implications of environmental toxicant exposures for risk of developing these negative pregnancy outcomes is important for future environmental policy and protection of this uniquely susceptible population.

Widespread exposure to phthalate compounds

Phthalates are a class of synthetic plasticizers commonly used in the manufacturing of consumer products[24,25] and have been implicated in numerous adverse health effects in animal and human studies, including reproductive and pregnancy outcomes. High-molecular weight (HMW) phthalates, including DEHP, are most commonly used in flexible plastic contained in flooring, medical equipment and food storage containers. Alternatively, low-molecular weight (LMW) phthalates, including DBP and DiBP, are used in personal care products such as shampoos, lotions and fragrances, and lacquers and varnishes. Phthalates are not covalently bound to the products they are used in and can easily leach into the environment; thus their widespread use results in ubiquitous human exposure[26]. Exposure to HMW phthalates usually occurs via ingestion because of their uses in food packaging, while LMW phthalate exposure occurs mostly via dermal absorption and inhalation from personal care product use[27].

Once inside the body, phthalates are rapidly metabolized into their bioactive forms. LMW phthalates typically undergo hydrolysis via phase I biotransformation into their respective monoesters, which are then excreted in urine. HMW phthalates additionally undergo several conjugation steps via phase II biotransformation. The conjugated products are much more hydrophilic than the original diester and are easily excreted in urine[28,29]. While most LWM phthalates are metabolized into only one major hydrolytic monoester, HMW phthalates additionally possess multiple secondary oxidized metabolites and thus can be more difficult to measure.

A study utilizing NHANES to analyze temporal trends in phthalate exposures in the general United States population suggested that since 2001, exposures to DiBP and DiNP have profoundly increased, while exposures to DEP, DnBP, BBzP and DEHP have decreased[30]. The ban on use of DnBP, BBzP and DEHP in the production of children's toys and medical devices may help to explain the decreasing exposure to these chemicals, however significant gaps in available data make it difficult to fully explain trends for other phthalates. Rises in exposures to high molecular weight phthalates like DiBP, in addition to emergent phthalate replacement chemicals, could be a result of their use in place of DEHP.

Di-2-ethyl hexyl terephthalate (DEHTP) and diisononyl 1,2-cyclohexanedicarboxylic acid (DINCH) are commonly considered "safe" alternatives to DEHP and have replaced it in the production of many consumer products, including flexible PVC and children's toys[31,32]. Phthalate replacement chemical metabolites can be widely detected in urine and may be increasing[33–35]. However, limited animal studies have been conducted to rigorously test the potential health effects of terephthalate exposure, and human studies are almost non-existent. Animal studies have indicated general toxicity[36] and changes in liver weight[37] with exposure to DEHTP. Another study exposed male and female rats to DEHTP over 4 weeks and found no effect on any outcomes assessed, including reproductive measures[38]. These studies exposed adult animals to dietary DEHTP, while most animal research on DEHP has indicated that gestational exposure is particularly important in determining reproductive toxicity. Developmental animal studies have found no significant effects of exposures to DEHTP[39], but altered reproductive organ function and decreased circulating testosterone levels were found with developmental DINCH exposure[40].

Human studies assessing adverse health effects associated with exposure to phthalate replacements have increased in number over the past several years, but most studies are plagued by low sample sizes and/or detection rates of metabolites. Metabolites of DINCH have been shown to be associated with an increase in oxidative stress metabolites[41] and differential sperm DNA methylation[42]. Other studies have shown increases in blood pressure among adolescents[43] and increased risk of croup among infants[44] with greater DEHTP exposure. As the use of phthalate replacement chemicals becomes more common, it will be increasingly important to understand the new health threats they pose.

Challenges in phthalate exposure assessment

Phthalates have been studied extensively in relation to many human health endpoints, but comparisons between studies can be difficult due to differences in exposure assessment methods. These differences have contributed to inconsistent findings between studies, and can

also prevent researchers from combining results to draw aggregate conclusions about the true health risks that phthalates pose.

The biological media utilized to measure phthalate concentrations can have a significant impact on the reliability and utility of measurements. The most common types of media are urine and serum, but other types include hair, saliva, umbilical cord blood, sweat, semen, amniotic fluid, and breast milk. Urine is used for most epidemiology studies and confers advantages over other types of media because it is easy to collect in large volumes and usually contains higher concentrations of phthalate metabolites than other media. Serum is used in a small number of epidemiology studies, but the half-life of phthalate metabolites is very short in blood and thus provides a small window of opportunity to obtain accurate sample measurements[45]. Discrepancies between phthalate measurements in different media make it challenging to compare exposure distributions or associations observed across cohorts.

Studies utilizing repeated measures of phthalate metabolites have shown high intra-individual variability between measurements taken at different times[46], suggesting that individual phthalate measurements are better indicators of recent exposure rather than long-term exposure. Most metabolites are excreted from the body within 24 hours of initial exposure[29], likely contributing to this variability. However, phthalate exposures typically result from habitual product use, that is, product use that may vary day by day but not substantially over time. Thus, assuming stable microenvironmental phthalate concentrations, studies that utilize more than one phthalate measurement over time gain a much more meaningful understanding of an individual's phthalate exposures when compared to studies utilizing only one phthalate measurement[46].

Particularly for birth outcome studies, measurement of phthalates during developmental windows of susceptibility could be important for uncovering true associations. Previous studies assessing phthalate exposures and risk of preterm birth have shown strong associations late in the second trimester[47] and early in the third trimester[48], relative to other points during gestation.

It is possible that phthalate exposures occurring during specific windows of fetal growth, placental remodeling, or endocrine changes result in a cascade of events which increase the risk for adverse birth outcomes, and that measuring phthalates outside of these windows returns misleading null findings. This is of particular concern because of significant heterogeneity in the timing of exposure measurements between phthalate epidemiology studies.

Socioeconomic status (SES) and lifestyle factors play a significant role in determining one's exposure level to phthalates, limiting the generalizability of results from one study to another. One study found that higher concentrations of DBP and DEP metabolites were associated with higher SES in a Mexico City birth cohort[35]. In contrast, higher SES and education level was associated with higher concentrations of MCOP, MCNP, and DEHTP metabolites in the PROTECT pregnancy cohort in Puerto Rico[49], and lower SES was associated with elevated phthalate metabolites in pregnant women living in Charleston, South Carolina[50]. Because phthalate exposures predominantly result from consumer product use, inconsistencies in product usage patterns across cohorts could drive significant differences in exposure distributions between populations. Further, if complex (i.e. nonlinear) associations are present, studying populations with exposure levels at different points along the distribution may return inconsistent results. Education and financial instability are also likely to influence product usage. Consumers who are educated on the potential adverse health risks of phthalate exposures, and who have the financial means to make healthier, and often times more expensive, choices, are more likely to avoid products with high levels of phthalates, while other consumers may not have that option.

Phthalate effects on pregnancy outcomes

Historically, the majority of animal studies assessing the health effects of phthalate exposures have focused on DEHP. According to systematic reviews published within the last 2 years, there have been a total of 19 animal toxicology studies assessing impacts of DiBP exposures on various broad health outcome categories[51], while that same number of studies have been published assessing DEHP effects on anogenital distance alone[52]. As human exposure levels to metabolites of DEHP continue to fall, it becomes increasingly important to broaden our understanding of the

health threats posed by the phthalate compounds that have replaced it. Further, animal and human studies assessing the health effects of any HMW phthalate, including DEHP, are challenged by the fact that oxidized secondary metabolites make up the majority of total urinary metabolites being excreted, and so studying only the hydrolytic monoester (MEHP, for example) does not provide adequate data on the true health impacts of the parent compound[27,53].

Human studies aimed at determining the reproductive health threats posed by phthalate exposures have returned inconsistent results and thus have not contributed to a solid understanding of true relationships or biological mechanisms. Findings from a recent systematic review of phthalate effects on male reproductive outcomes highlight the potential for true biological associations, but also an incongruency between classes of phthalates and outcomes[54]. Even when robust associations were observed in studies with which the review authors placed high confidence, results were not consistent across studies and so general conclusions about phthalate toxicity could not be drawn. A similar obstacle was encountered by Yaghjyan and colleagues while reviewing the literature on effects of DEHP on adverse pregnancy outcomes[55]. Even when the scope of study is narrowed to one parent phthalate and one class of health outcomes such as this, significant differences between study protocols persist which impeded the ability to draw solid conclusions.

The challenges present when assessing adverse effects of phthalates on pregnancy outcomes is well illustrated when comparing studies that reported significant associations between phthalate exposure and timing of delivery. One study conducted among women in the PROTECT birth cohort observed positive associations between concentrations of DBP and DiBP metabolites and odds of preterm and spontaneous preterm birth. Results of that study also suggested that phthalate exposures late in the second trimester were most important for determining risk of preterm birth[47], supporting the idea that timing of exposure assessment is important for uncovering biological relationships. A study by Watkins et al similarly found a significant reduction in gestational age at birth with increased concentrations of the sum of DBP metabolites, but this relationship was only significant among female pregnancies when phthalate

concentrations were averaged between measurements at the first trimester and at delivery[56]. While the previous studies did not show significant findings for metabolites of DEHP, a study in Mexico City showed a positive association between risk of preterm delivery and MECPP (a secondary oxidized metabolite of DEHP), as well as MBP and MCPP, in the third trimester[57]. Weinberger and colleagues also found an association between increased concentrations of another secondary DEHP metabolite, MEHHP, and reduced gestational age at birth. However, in that study, MEHHP was measured late in pregnancy and stratification by fetal sex revealed that the association was only significant among male pregnancies[58]. Adibi and colleagues also assessed metabolites of DEHP for associations with timing of delivery in a multicenter pregnancy cohort, but conversely showed that increasing DEHP metabolites were associated with reduced odds of preterm delivery and increased risk of delivering after 41 weeks gestation[59], contradicting findings from the previously mentioned studies. To add even more discrepancies, a pregnancy outcome study in China found null relationships between odds of preterm delivery and all aforementioned phthalate metabolites measured throughout pregnancy, and instead found a significant positive association between MMP and preterm birth[60]. Clearly, the current state of the literature is inconsistent and suggests that study heterogeneity may be driving some differences in results, but also that inherent differences between phthalate metabolites may result in differential associations with adverse birth outcomes. Further, the current literature suggests that different phthalate metabolites may exert their effects on birth outcomes uniquely between fetal sexes and at varying time points through gestation.

Phthalate endocrine disruption during pregnancy

Phthalates may elicit their biological activity by interfering with the body's endocrine system. Previous animal studies have indicated numerous endocrine-related health effects from phthalate exposures. Among male rodents, gestational and/or lactational exposure to phthalates has been shown to result in reproductive malformations, reduced anogenital distance, reduction of testosterone production, reduced testis weight and lower sperm counts[61–66]. Animal studies have also demonstrated potential endocrine disrupting effects of phthalate exposures including altered concentrations of serum reproductive[62,67–69] and thyroid hormones[70,71] and reduced

fertility[72–74]. Given the importance of numerous hormones during pregnancy, understanding the endocrine disrupting potential of phthalates during this sensitive time frame is paramount. Various classes of hormones are potential targets for phthalate disruption and could subsequently have negative effects on pregnancy, including:

Thyroid hormones: Thyroid hormones are critical early in pregnancy for proper brain and skeletal development of the fetus[75]. The maternal supply of thyroxine (T4) is particularly important in the first half of pregnancy, before the fetal thyroid gland has matured enough to produce adequate hormones[76]. At that time, the fetus relies solely on maternal T4, which crosses the placenta via thyroid hormone transporters[77]. Sufficient maternal iodine intake is especially important during the first half of pregnancy to facilitate the increased demand for thyroid hormones by the fetus, and to maintain proper thyroid hormone concentrations within maternal circulation. Throughout gestation, thyroid hormones are important for fetal growth and have been shown to be correlated with infant weight and length at birth. Low thyroid hormones have also been observed in cases of intrauterine growth restriction and small for gestational age (SGA) infants[77].

A number of previous studies have demonstrated altered thyroid hormone concentrations with increases in phthalate exposures during gestation. Results from a pregnancy cohort in Boston suggested that concentrations of free T4 were positively associated with MCPP, a metabolite originating from multiple HMW parent compounds. They also found total T4 to be positively associated with MEHP[78]. Romano and colleagues observed an inverse association between MEP and T4 at 16 weeks gestation[79], in contrast to the Boston study which observed a positive association between total triiodothyronine (T3) and MEP. Various studies in Taiwan have observed inverse associations between MBP and fT4, but the significance was dependent on gestational age at the timing of biomarker measurements[80,81]. Differences in timing of exposure and outcome assessment, low sample sizes, and varying geographical locations all contribute to the lack of consistency between studies.

Testosterone: Roles of androgens during pregnancy are not well understood. Elevated testosterone concentrations have been observed in women with polycystic ovary syndrome (PCOS). Women with recurrent miscarriages have also demonstrated higher circulating testosterone concentrations, regardless of PCOS status. Androgens may elicit their effects via a decrease in the production of various proteins which result in detrimental effects on the pregnancy. Androgens could also affect the endometrium via antagonistic action against estrogens[82].

Assessments of the effects of phthalate exposures on testosterone concentrations have been heavily studied among males and occupationally exposed groups, but this relationship has not been well established among pregnant women. A multicenter pregnancy study found increased testosterone concentrations with higher MEP among male pregnancies, but lower testosterone concentrations with higher MBP among female pregnancies. They also observed an inverse association between testosterone and DEHP metabolites regardless of fetal sex[83]. The same research group later found similar inverse associations with testosterone and MCNP and DEHP metabolites in the TIDES cohort[84]. Further research is clearly warranted to substantiate these findings.

Progesterone and estriol: Concentrations of both progesterone and estriol rise steadily throughout pregnancy, and the coordination between them is critical for the timing of labor. Through gestation, progesterone functions to attenuate the maternal immune system and promote quiescence of the uterine wall[85,86]. Conversely, estriol acts to ready the uterus for labor by increasing expression of prostaglandin and oxytocin receptors, gap junctions, and enzymes responsible for muscle contractions[87]. Over time, estriol concentrations act as a kind of “gas pedal” for the progression of pregnancy and eventual onset of labor. Progesterone concentrations simultaneously act as the “brake pedal” to keep the pro-labor functions of estriol in check. As labor approaches, the maternal response to progesterone is dampened and estriol actions begin to dominate[88], allowing the onset of labor. Because of the coordination of these two hormones, some have hypothesized that studying the ratio of

progesterone to estriol is more meaningful for pregnancy outcomes. However, because the weakened maternal response to progesterone at labor is not due to a change in progesterone concentrations, but rather likely a change in receptor expression levels[89], studying the ratio still may not uncover true biological mechanisms.

Despite the obvious importance of progesterone and estriol during gestation, very little epidemiologic work has been done to understand the effects of environmental toxicants on their concentrations during pregnancy. One pilot study in the PROTECT cohort found that progesterone was inversely associated with MEP consistently across three study visits[90], but this study was very limited with a sample size of only 106 women. Another study among Czech women found a positive association between MBP and estriol during the 37th week of pregnancy[91]. However, this study was also limited by very small sample size (N=18) and the fact that they measured phthalates in maternal plasma rather than urine.

Corticotropin releasing hormone: CRH is normally involved in stress responses, but during pregnancy it's production from the maternal hypothalamus and placenta combine in circulation[92] to perform a unique role. CRH concentrations remain low during early pregnancy and then begin to exponentially rise starting around 20 weeks, peaking at birth[93]. Women who deliver preterm experience a more rapid increase of CRH that can be detected early in the second trimester[94], leading researchers to believe that CRH is involved in a sort of placental clock to determine the timing of labor from a relatively early point during gestation. CRH receptors are present in the myometrium to promote contractile and relaxatory responses of myometrial cells[95]. The fetal zone of the fetal adrenal gland possesses CRH receptors and also produces DHEA-S, which is a precursor for placental estrogen production. CRH entering fetal circulation from the placenta could target these receptors and stimulate placental steroidogenesis[96].

Despite the clear importance of CRH during pregnancy, no epidemiologic work has been done to investigate the potential of phthalates to disrupt CRH concentrations in humans. One

previous *in vitro* study did find that treatment of term human placental cells with MEHP increased levels of CRH protein and mRNA[97]. An animal study in zebra fish also observed increased CRH mRNA expression with DEHP treatment in a dose-dependent manner[98]. Disruption of CRH by environmental toxicants presents a critical gap in the pregnancy outcomes literature and needs to be further assessed.

Studying phthalate mixtures instead of individual metabolites

The vast majority of epidemiology studies on phthalate exposures tend to focus on single metabolites or parent compounds. Humans are rarely exposed to individual phthalates, but rather complex mixtures that vary depending on the sources of exposure. A study conducted in NHANES assessed the percent contribution of six different parent phthalate compounds among a sample of individuals with a median level of total phthalate exposure. They observed that most individuals were exposed to modest concentrations of metabolites from all six parent compounds[99]. This finding suggests that studying associations between individual phthalate metabolites and health effects may not provide an accurate understanding of true biological relationships. Additionally, studying mixtures allows one to investigate the possibility of additive or antagonistic interactions between metabolites and the effects they may have on health outcomes. Despite the fact that human phthalate exposure always occurs in complex mixtures, very few epidemiology studies have investigated the effects of phthalate mixtures on adverse health outcomes, particularly pregnancy outcomes.

Studying environmental toxicants as individual biomarkers can present exposure assessment issues when mixtures methods would be better suited for observed human exposure profiles. Phthalate metabolites originating from the same parent compound or the same exposure sources pose problems of multicollinearity which can contribute to biased effect estimates. Previous research has shown that multiple metabolites from a single parent compound can be highly correlated with one another, particularly those of DEHP, which have shown a correlation coefficient upward of 0.9. Metabolites which likely arise from similar exposure sources, such as those from DBP and DiBP (both LMW phthalates likely originating from personal care products)

also show moderate correlations with one another (R up to 0.7). Further, modest correlations can also be present between metabolites which neither come from the same parent phthalate nor originate from the same exposure sources, such as MEP and DEHP metabolites (R up to 0.3)[46].

Multicollinearity of phthalate metabolites also poses a problem for regression analyses. If one sets out to explore an association between MBP and a particular health outcome, but MiBP actually has a causal relationship with the outcome that MBP does not, MiBP will confound the results because of its high degree of correlation with MBP. Issues of multicollinearity do not exist exclusively within phthalate metabolites, but possibly between other classes of environmental contaminants as well. Other chemicals that are used in the same consumer products as phthalates including bisphenol A, heavy metals, parabens, and polychlorinated biphenyls can also confound associations if they are not accounted for.

One possible way to account for confounding is to adjust statistical models for phthalate metabolites aside from the target metabolite of interest. For example, one could include a whole panel of phthalate metabolites as covariates in a regression model, and then assess each metabolite's association with the outcome while controlling for the rest of the panel. However, this method would mask any additive or multiplicative interactions present between metabolites and would not allow the investigator to determine how the overall mixture of phthalates effects their outcome of interest. Preferred mixtures methods allow the investigator to control for correlation between exposure measures while assessing effects of each metabolite, interactions between metabolites, and effects of the mixture as a whole. A small number of studies utilizing such methods to assess phthalate mixture associations with birth outcomes have been conducted[100,101], but a more in-depth exploration into this emerging area of environmental epidemiology is necessary to understand true biological relationships.

In conclusion, exposures to complex mixtures of phthalates pose significant public health risks, particularly during pregnancy. Many epidemiology studies have found significant associations between phthalate exposures and adverse pregnancy outcomes, but findings are mixed and

warrant further exploration. Further, there is no clear understanding of the mechanism(s) by which phthalates may exert their effects on pregnancy. The maternal, placental, and fetal endocrine milieu are critically important throughout gestation and may be targets for disruption by phthalate metabolites. We currently have significant evidence that phthalates possess endocrine disrupting capabilities, but further research is needed within the context of human pregnancy. Investigating how phthalates may interfere with hormone concentrations through gestation, and the subsequent effects that endocrine dysregulation may have on the pregnancy, is paramount for understanding the biological mechanisms associated with environmental exposures to phthalates.

Specific Aims

This dissertation deepens our understanding of the etiology of adverse pregnancy outcomes by investigating how mixtures of phthalate metabolites are related to changes in hormone concentrations and downstream pregnancy outcomes. Data from the Puerto Rico Testsite for Exploring Contamination Threats (PROTECT) cohort will be utilized. PROTECT is a longitudinal prospective birth cohort which was initiated to explore the effects of environmental contamination on the high rates of preterm birth observed on the island. The study site is situated amongst many Superfund waste sites atop a karst aquifer system, which allows contaminated drinking water to move freely over long distances, exacerbating an already-present environmental pollution problem. The following specific aims address critical gaps in the epidemiology literature regarding phthalate effects on hormones important during pregnancy, effects of phthalate mixtures, and mediation by hormones on the relationships between phthalate mixture exposures and adverse birth outcomes.

Specific Aim 1: To evaluate the associations between concentrations of 16 urinary phthalate/phthalate replacement metabolites and 9 serum hormones measured at two time points through pregnancy (16-20 weeks and 24-28 weeks). I will utilize linear mixed models to estimate associations between repeated measures of biomarkers. I will also conduct sensitivity analyses to investigate differences in associations between time points and between fetal sexes.

Hypothesis 1: Exposures to phthalate metabolites will result in varying and significant changes in maternal hormone concentrations. Varying androgen- and estrogen-like activity will contribute to observed associations. Differential actions during windows of susceptibility, as well as influence by fetal physiology, will result in unique associations at each study visit and between fetal sexes.

Specific Aim 2: To investigate the associations between serum hormone concentrations and measures of preterm birth and adverse birth outcomes including gestational age at birth, birth weight z-score, preterm and spontaneous preterm birth, preeclampsia, gestational diabetes, small for gestational age, and large for gestational age over two time points during pregnancy (16-20 weeks and 24-28 weeks). I will utilize multivariate linear and logistic regression analyses with visit-specific measures of hormone concentrations among all mothers and between male and female pregnancies.

Hypothesis 2: Established functions of various classes of hormones in maintaining pregnancy suggest that many significant associations will be observed. Differential risk of adverse birth outcomes between male and female pregnancies will contribute to differences between fetal sexes, and gestational age-specific changes in hormone concentrations will contribute to differences in associations between study visits.

Specific Aim 3: To investigate the mediating effect of hormone concentrations on the relationships between exposure to phthalate mixtures and adverse birth outcomes. I will utilize ridge regression to determine the relative importance of phthalate metabolites for prediction of adverse birth outcomes, and then create environmental risk scores (ERS) as weighted sums of each individual's total phthalate exposure. I will then use ERS as exposure variables in causal mediation analyses.

Hypothesis 3: Varying properties of phthalate metabolites, including molecular weight and sources of exposure, will result in differential importance between birth outcomes. These differences will also manifest in varying endocrine pathways being implicated as mediators on the causal pathway from phthalate mixtures exposure to adverse birth

outcomes. Given the established importance of CRH, progesterone, and estriol on the timing of labor, we expect these hormones to significantly mediate the association between phthalate mixtures and preterm and spontaneous preterm birth.

Chapter II. Associations of Phthalates and Phthalate Replacements with CRH and Other Hormones Among Pregnant Women in Puerto Rico

Abstract

Background: Phthalates are endocrine disrupting chemicals that may be associated with adverse birth outcomes. Dysregulation of maternal endocrine homeostasis could be a possible biological pathway between phthalates and birth outcomes.

Objective: Examine associations between 19 maternal urinary phthalate or phthalate replacement metabolites and 9 serum hormones measured over two time points during pregnancy.

Methods: In the PROTECT longitudinal pregnancy cohort, we conducted linear mixed effects models among 879 women to determine associations between urinary phthalates and serum hormones measured at 16-20 weeks and 24-28 weeks gestation. We also conducted analyses specific to study visit (16-20 week N=734; 24-28 week N=509) and fetal sex (male N=454; female N=414).

Results: CRH was positively associated with MHiBP (%Δ: 15.4, 95% CI: 2.12, 30.4), MCNP (%Δ: 6.82, 95% CI: -0.02, 14.1), MCOP (%Δ: 14.7, 95% CI: 7.28, 22.7), and MEP (%Δ: 10.5, 95% CI: 1.96, 19.8). Positive associations were found between fT4 and MCNP (%Δ: 1.37, 95% CI: 0.21, 2.52), MCOP (%Δ: 1.51, 95% CI: 0.34, 2.67), and MCPP (%Δ: 2.02, 95% CI: 0.74, 3.31). Testosterone was positively associated with MHBP (%Δ: 17.0, 95% CI: 3.68, 32.1) and inversely associated with MCNP (%Δ: -7.72, 95% CI: -13.5, -1.57), MCOP (%Δ: -9.52, 95% CI: -15.4, -3.21), and MCPP (%Δ: -10.6, 95% CI: -17.0, -3.66). Notably, directions of associations tended to follow trends based on molecular weight of the phthalate metabolite. Various positive associations were observed with thyroid hormones at 16-20 weeks only. Finally, increases in phthalate concentrations tended to result in decreases in hormone concentrations among female pregnancies and increases in hormone concentrations among male pregnancies.

Introduction

Maternal hormonal homeostasis during gestation is critical to maintaining a healthy pregnancy and ensuring proper development of the fetus[102–104]. Human studies have shown that abnormal thyroid hormone levels, including hyper-and hypothyroidism, are associated with preterm birth[105–111] and low birth weight[112–114]. Corticotropin releasing hormone (CRH) is thought to play a major role in the timing of labor and has been shown to be associated with preterm birth in human studies[94,115–120]. Women who have hyperandrogenic conditions such as polycystic ovarian syndrome have higher circulating levels of testosterone, and these types of conditions have been shown to be associated with preterm birth[121]. Additionally, elevated testosterone levels are associated with *in utero* growth restriction, development of gestational diabetes, and preeclampsia[122–125].

Phthalates are a class of synthetic plasticizers commonly found in consumer products that have been shown to be associated with numerous human health effects[24,25]. Because phthalates are not chemically bound to the products in which they are used, they commonly leach into foods and beverages, dust, and air, creating multiple routes of potential human exposure[26]. Consequently, phthalates are ubiquitous in the environment and can be widely detected in humans, specifically pregnant women[57,126–129]. Because pregnant women represent a uniquely susceptible population, it is important to understand the potential effects of phthalate exposures on maternal and fetal physiology during pregnancy.

Animal studies have shown phthalate exposure to be associated with altered concentrations of serum reproductive[62,67–69] and thyroid hormones[70,71] and reduced fertility[72–74]. Numerous human pregnancy studies have suggested that phthalates may play integral roles in determining birth weight, birth length, head circumference, gestational age, and risk of spontaneous abortion and preterm birth[56,57,130–138]. Because of the growing body of evidence suggesting adverse effects of phthalate exposure on hormonal homeostasis and birth outcomes, we aimed to assess the relationships of maternal urinary phthalate and phthalate replacement metabolites with serum hormone concentrations over two time points during pregnancy in PROTECT (Puerto Rico Testsite

for Exploring Contamination Threats), our ongoing pregnancy cohort in Puerto Rico. Phthalate replacement chemical metabolites can be widely detected in urine among the United States population and may be increasing[33], yet few previous epidemiology studies have considered them. Additionally, to our knowledge no epidemiology studies have assessed the relationship between phthalate exposure and serum CRH concentrations, broadening the novelty and importance of the present study.

Methods

Study Participants

The present analysis builds upon a previous pilot study[90] and includes more participants and broader coverage of phthalate metabolites and hormone biomarkers, notably terephthalate metabolites and CRH. Participants were part of the PROTECT ongoing prospective birth cohort. Details on the study recruitment protocol are described elsewhere[129,139]. Briefly, pregnant women living in the Northern karst region of Puerto Rico were recruited from 2012 to 2017 from seven hospitals and prenatal clinics at 14±2 weeks gestation. Eligible participants were 18-40 years old, had their first clinic visit before 20 weeks gestation, did not use oral contraceptives within 3 months of getting pregnant, did not use *in vitro* fertilization to get pregnant, and did not have any known medical or obstetric conditions. Participating women provided blood and spot urine samples for analysis at two time points during pregnancy coinciding with periods of rapid fetal growth: 16-20 weeks and 24-28 weeks gestation. Demographics information was collected from all participants at the first study visit. The present analysis included 879 women who had complete data on at least 1 phthalate-hormone concentration pair for at least one of the two study visits. This study was approved by the research and ethics committees of the University of Michigan School of Public Health, University of Puerto Rico, Northeastern University, and participating hospitals and clinics. All study participants provided full informed consent prior to participation.

Urinary Phthalate Measurement

All spot urine samples were frozen at -80^{o}C and shipped over night on dry ice to the CDC for analysis. All samples were initially analyzed for 15 phthalate metabolites: mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), monobenzyl phthalate (MBzP), mono-isobutyl phthalate (MiBP), mono-hydroxyisobutyl phthalate (MHiBP), mono-3-carboxypropyl phthalate (MCPP), mono carboxyisononyl phthalate (MCNP), mono carboxyisooctyl phthalate (MCOP), mono-hydroxybutyl phthalate (MHBP), mono isononyl phthalate (MNP), and mono oxononyl phthalate (MONP). Four additional phthalate replacement metabolites were later added to the analytical panel: cyclohexane-1,2-dicarboxylic acid monohydroxy isononyl ester (MHiNCH), cyclohexane-1,2-dicarboxylic acid monocarboxy isooctyl ester (MCOCH), mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP), and mono-2-ethyl-5-hydrohexyl terephthalate (MEHHTP). Urine samples were analyzed using solid phase extraction high-performance liquid chromatography-isotope dilution tandem mass spectrometry, the details of which are described elsewhere[140]. Values detected below the limit of detection (LOD) were assigned a value of the LOD divided by the square root of two[141]. Differences in urinary dilution between samples was accounted for using specific gravity, which was measured using a digital handheld refractometer (AtagoCo., Ltd., Tokyo, Japan). Specific gravity correction for all urinary biomarkers was carried out using the formula $P_C = P\ [(SG_m - 1) / (SG_i - 1)]$, where P_c is the specific gravity-corrected biomarker concentration (ng/mL), P is the measured biomarker concentration, SG_m is the median specific gravity value of the study population (1.019), and SG_i is the specific gravity value for each individual[57].

Serum Hormone Measurement

All serum samples collected were analyzed at the Central Ligand Assay Satellite Services (CLASS) laboratory in the department of Epidemiology at the University of Michigan School of Public Health. Progesterone, sex hormone-binding globulin (SHBG), testosterone, total triiodothyronine (T3), total thyroxine (T4), free thyroxine (fT4) and thyroid-stimulating hormone (TSH) were measured using a chemiluminescence immunoassay. Estriol and corticotropin-releasing

hormone (CRH) were measured using an enzyme immunoassay. Some hormone concentrations were not available for all participants due to volume limitations. The ratios of progesterone to estriol (Prog/E3) and T3 to T4 (T3/T4) were assessed in addition to measured hormones. Previous research has indicated that these ratios may be a better indication of adverse pregnancy outcomes than single hormone measurements[142–144]. Two samples had TSH values of zero and were thus dropped from the analysis due to biological implausibility. Five samples had testosterone levels below the LOD and were thus replaced by the LOD divided by the square root of two.

Statistical Analyses

Summary demographic characteristics of the population over the entire study period and at each visit were assessed including maternal age, maternal education, current job status, marital status, number of children, smoking status, environmental tobacco smoke exposure, alcohol use, number of previous pregnancies, and maternal pre-pregnancy BMI.

Distributions of all phthalate metabolites were heavily right-skewed and thus were natural log transformed for all analyses. Distributions of CRH, estriol, progesterone, TSH, testosterone, and SHBG were also right skewed and natural log transformed for all analyses. Distributions of fT4, T3 and T4 were approximately normal and thus were not transformed. Descriptive statistics for all phthalate metabolite and hormone distributions were calculated using specific gravity-adjusted values for all urinary biomarkers among the total study sample and for each study visit. Significant differences in concentrations of biomarkers between study visits were assessed using paired t-tests with natural log transformation to achieve normality where appropriate.

Relationships between exposure and outcome variables and potential confounders were assessed using ANOVA to test for differences between categories of covariates, and then using linear regression to test for linear trends across categories of covariates. Final repeated measures analysis utilized linear mixed models (LMMs) to regress hormones/hormone ratios on phthalate metabolites and included random intercepts for each study participant to account for intra-

individual correlation of exposure and outcome measures. Significance level of the univariate relationship between exposures and outcomes, *a priori* knowledge, and changes in the main effect estimate by at least 10% were criteria used when determining which potential covariates to include in final models. In addition to specific gravity, maternal age and maternal education were selected as covariates to include in final models. Beta estimates for categories of maternal age did not change linearly in final models and thus maternal age was treated as a categorical variable for all analyses. Conversely, beta estimates for categories of maternal education did change linearly and thus maternal education was treated as an ordinal variable for subsequent analyses. To investigate potential windows of susceptibility, additional analyses were run which added an interaction term between study visit number and urinary phthalate biomarkers to the previously described LMM in order to obtain effects estimates specific to each study visit. The same method was utilized to assess effects specific to fetal sexes.

For ease of interpretability, all results were transformed to indicate percent changes and 95% confidence intervals in hormone concentrations associated with an interquartile range (IQR) increase in urinary phthalate metabolite concentration. We calculated q-values using the Benjamini and Hochberg method[145] to address the issue of potential false-positive results from running many statistical tests. Each hormone biomarker was treated as a family of tests (total of 16 tests with phthalate metabolite biomarkers per hormone). High q-values were seen as having a greater risk of being false-positives, while q-values below 0.1 were interpreted with higher confidence. An alpha level of 0.05 was used to indicate statistical significance. All statistical analyses were run using R version 3.4.4.

Results

Demographics and Confounders

A total of 879 pregnant women were included in the present analysis. Of those, 734 and 509 women at visits 1 and 3, respectively, contributed blood and urine samples. Most women were younger than 30 years (67%), either married or cohabitating (79.8%), lived in a home earning less than $30,000 per year (54.8%), were non-smokers (85.3%), did not consume alcohol during their

pregnancy (92.7%), had a BMI less than 30 (78.3%), had less than 2 previous children (73%) and reported no exposure to environmental tobacco smoke (83.3%). About 47% of pregnancies were female and 52% were male. Distributions of education level and employment status were relatively even between categories. Distributions of all demographic characteristics stratified by study visit were similar.

Distributions, geometric means (GM) and geometric standard deviations (GSD) of all urinary phthalate metabolite and serum hormone biomarkers are shown in Table II.1. Concentrations of E3, progesterone, testosterone, and SHBG were all generally higher at visit 3 than at visit 1 ($p<0.001$). Most phthalate metabolite biomarkers were detected in at least 80% of samples. MCOCH, MNP and MHiNCH were detected in less than 35% of samples and were dropped from further analyses. Biomarker concentrations of all phthalate metabolites did not differ significantly between study visits.

Over the duration of the study, number of children, smoking status and alcohol use did not show significant associations with most phthalate metabolites and hormones assessed. Categorical maternal age and ordinal maternal education were significantly associated with the largest number of phthalate metabolites and hormones and thus were retained in final models. Employment status and annual household income were both significantly associated with most hormones but were highly correlated with maternal education ($R=0.560$, $p<0.001$ and $R=0.571$, $p<0.001$, respectively; data not shown) and thus were not considered in further analyses. Self-reported environmental tobacco smoke exposure was also associated with many phthalate metabolites but was not associated with the majority of hormones and was not considered in further analyses.

CRH and Reproductive Hormones

Results from linear mixed models indicating associations between phthalate metabolite biomarkers and serum hormones over the study period are shown in Table II.2. Results from sensitivity analyses showing significant differences by study visit and by fetal sex are shown in

Figures II.1 and II.2, respectively. An increase in CRH concentration was associated with IQR increases in MHiBP (%Δ: 15.4, 95%CI: 2.12, 30.4), MCNP (%Δ: 6.82, 95%CI: -0.02, 14.1), MCOP (%Δ: 14.7, 95%CI: 7.28, 22.7), and MEP (%Δ: 10.5, 95%CI: 1.96, 19.8) over the study period. While the association between MiBP and CRH was not significant in repeated measures analysis, the association became significant when assessing only male pregnancies (%Δ: 12.4, 95%CI: 0.87, 25.3).

An increase in serum testosterone was observed with an IQR increase in MHBP (%Δ: 17.0, 95% CI: 3.68, 32.1), but decreases in testosterone were observed with IQR increases in MCNP (%Δ: -7.72, 95% CI: -13.5, -1.57), MCOP (%Δ: -9.52, 95% CI: -15.4, -3.21), and MCPP (%Δ: -10.6, 95% CI: -17.0, -3.66) over the study period. There were no significant differences observed for testosterone between study visits or fetal sexes. Reductions in SHBG concentrations were observed with IQR increases in MEHHP (%Δ: -4.50, 95% CI: -6.85, -2.10), MEOHP (%Δ: -3.83, 95% CI: -6.26, -1.33), MECPP (%Δ: -3.71, 95% CI: -6.26, -1.09), MBP (%Δ: -3.31, 95% CI: -5.83, -0.72), and MEHHTP (%Δ: -3.49, 95% CI: -6.34, -0.56). Most of these associations were significant at both study visits and only among male fetuses, but differences between visits and fetal sexes were not statistically significant. The association between SHBG and MEHP was significantly different between male and female pregnancies and became significant when assessing only male pregnancies (%Δ: -5.99, 95% CI: -9.26, -2.61).

There were no significant associations between estriol and any phthalate metabolites across the study period, but some associations became significant at specific study visits (MBzP V3 %Δ: -6.32, 95% CI: -11.7, -0.63; MHBP V3 %Δ: -7.51, 95% CI: -14.1, -0.48; MCNP V1 %Δ: 7.40, 95% CI: 2.76, 12.3; MCOP V1 %Δ: 4.83, 95% CI: 0.33, 9.53). An IQR increase in MEHHTP was associated with a 7.25% (95% CI: -13.2, -0.91) decrease in progesterone across the study, which was significant only among male pregnancies (%Δ: -8.75, 95%CI: -16.7, -0.11). The association between progesterone and MHBP also became significant when assessing only male pregnancies (%Δ: -11.9, 95% CI: -19.5, -3.53). No phthalates were associated with the ratio of progesterone to estriol across the study, but inverse associations were observed with MECPP (%Δ: -5.69, 95%CI:

-10.4, -0.70) and MCPP (%Δ: -5.62, 95%CI: -10.2, -0.81) at study visit 1 only. The effects of MECPP on the ratio of progesterone to estriol were significantly different between study visits, but neither association was significant on its own.

Thyroid Hormones

TSH was positively associated with MEHHP (%Δ: 5.21, 95%CI: 0.12, 10.6), MEOHP (%Δ: 5.45, 95%CI: 0.22, 11.0), MECPP (%Δ: 6.69, 95%CI: 1.19, 12.5), MHiBP (%Δ: 10.5, 95%CI: 2.29, 19.4), and MCPP (%Δ: 5.05, 95%CI: 0.03, 10.3). Though no differences between study visits were statistically significant, positive associations with DEHP metabolites were significant only at visit 3, while the positive association with MHiBP was significant only at visit 1. A significant positive association was also present with MCOP at visit 1 only (%Δ: 6.29, 95%CI: 0.81, 12.1).

IQR increases in MCNP, MCOP and MCPP were significantly associated with 1.37% (95% CI: 0.21, 2.52), 1.51% (95% CI: 0.34, 2.67) and 2.02% (95% CI: 0.74, 3.31) increases in fT4 concentrations over the study period, respectively. Conversely, MEOHP was associated with a 1.43% (95% CI: 2.77, 0.08) decrease in fT4. Associations with MCOP, MCPP, and MEP were significantly different between study visits, with positive effects estimates observed at the first study visit only.

A reduction in total T4 was observed with an IQR increase in MEHHP (%Δ: -1.24, 95%CI: -2.42, -0.06), while an increase in total T4 was observed with an IQR increase in MCPP (%Δ: 1.57, 95%CI: 0.41, 2.74). The resulting decrease in T4 with exposure to MEHHP was significant only among female pregnancies. Associations with MCOP and MONP at each study visit were not significant, but they were significantly different from one another, with positive associations at visit 1 and inverse associations at visit 3.

Changes in T3 were significantly associated with IQR increases in MHBP (%Δ: -5.85, 95%CI: -10.3, -1.41), MCNP (%Δ: 3.33, 95%CI: 0.77, 5.88), MCOP (%Δ: 4.41, 95%CI: 1.80, 7.03), and MCPP (%Δ: 4.25, 95%CI: 1.34, 7.15). Assessments by study visit revealed that the inverse association with MHBP persisted only at visit 3 (%Δ: -7.21, 95%CI: -12.7, -1.75), while positive associations with

MCNP (%Δ: 4.62, 95%CI: 1.53, 7.71), MCOP (%Δ: 6.34, 95%CI: 3.21, 9.48), and MCPP (%Δ: 4.92, 95%CI: 1.39, 8.45) persisted only at visit 1. The inverse association with MHBP was also significant among female pregnancies only (%Δ: -11.1, 95%CI: -17.1, -5.00), and a marginal inverse association was observed with MBP (%Δ: -4.02, 95%CI: -8.09, 0.04) among female pregnancies only. Male pregnancies showed unique positive associations with MHiBP (%Δ: 7.57, 95%CI: 1.96, 13.2), MCNP (%Δ: 5.08, 95%CI: 1.54, 8.61), and MCPP (%Δ: 6.34, 95%CI: 2.34, 10.4).

The ratio of T3/T4 increased by 3.67% (95% CI: 0.99, 6.36) and 4.22% (95% CI: 1.48, 6.96) with IQR increases in MCNP and MCOP over the study period, respectively. Conversely, the ratio decreased by 6.33% (95% CI: -10.9, -1.73) with an IQR increase in MHBP. No significant difference between study visits were observed, however positive associations with MCNP (%Δ: 4.57, 95%CI: 1.33, 7.81) and MCOP (%Δ: 5.48, 95%CI: 2.21, 8.76) were present only at visit 1, and an inverse association with MHBP (%Δ: -8.63, 95%CI: -14.3, -2.94) was present only at visit 3. Significant differences were, however, observed by fetal sex which showed an inverse association with MHBP among female pregnancies (%Δ: -10.8, 95%CI: -17.1, -4.49) and a positive association with MHiBP among male pregnancies (%Δ: 7.87, 95%CI: 2.06, 13.7). Other significant associations were observed by fetal sex, but differences between sexes did not reach statistical significance: positive associations with MCNP (%Δ: 5.71, 95%CI: 2.01, 9.41), MCOP (%Δ: 5.31, 95%CI: 1.62, 9.00), and MCPP (%Δ: 4.97, 95%CI: 0.79, 9.14) among male pregnancies, and an inverse association with MONP among female pregnancies (%Δ: -6.28, 95%CI: -11.8, -0.80).

Discussion

Here we investigated the longitudinal associations between gestational phthalate biomarker concentrations and maternal serum hormones measured at two time points during pregnancy. Four phthalate metabolites were significantly associated with increased concentrations of CRH across pregnancy, with most effects being stronger at visit 1 and among male pregnancies. Findings for thyroid hormones were mostly positive, but significance levels between study visits and fetal sexes were highly variable. Generally, increased phthalate exposure resulted in decreased thyroid hormones among female pregnancies, but increased thyroid hormones among

male pregnancies. Associations with progesterone and estriol were largely null, but sensitivity analyses by study visit and fetal sex did reveal some significant associations. Interestingly, findings for most reproductive and thyroid hormones displayed patterns by molecular weight of phthalate metabolites, pointing to potential differences in target biological pathways by phthalate side chain length.

Thyroid Hormone Discussion

We previously conducted a case-control study at Brigham and Women's Hospital in Boston among 439 women recruited between 2006 and 2008 to assess longitudinal associations between urinary phthalate concentrations through pregnancy and maternal serum thyroid hormones[146]. That study is consistent with our finding that fT4 concentrations were higher when measured at earlier points in gestation, as well as finding a positive association between MCPP and fT4. While the present study suggested mostly positive associations between phthalates and T3, the former study found T3 to be positively associated with only mEP, a relationship that was not significant in the present study. In contrast to our current results, the earlier study indicated inverse associations between TSH and several phthalate metabolites, as well as a significant positive relationship between MEHP and T4. While some aspects of the two studies were similar, they were conducted on distinct populations and at differing recruitment times (2006-2008 vs. 2012-2017) and thus may reflect distinct phthalate usage and exposure patterns.

Romano et al. conducted a prospective birth cohort analysis looking at maternal phthalate metabolites and their relationships with thyroid hormones among 202 women in Cincinnati, Ohio[79]. They utilized urinary phthalate metabolite and maternal serum thyroid hormone measurements at 16 weeks gestation and found that decreasing T4 concentrations were associated with a 10-fold increase in MEP. This result is not supported by our finding that MEP was not associated with T4 and that several other phthalate metabolites were positively associated with T4 early in pregnancy only. Exposure levels were generally lower than those in the present study which may be contributing to differing results. Additionally, although the median gestational ages were similar in both studies, measurements ranged from 16 to 20 weeks

in our study and 10 to 23 weeks in the study by Romano et al, further suggesting that gestational age may play a critical role in the association between phthalate exposure and maternal thyroid hormones.

We previously conducted a pilot study to analyze thyroid and sex hormones (estradiol, progesterone, SHBG) in relation to phthalate exposure among a distinct group of 106 pregnant women recruited into PROTECT[90]. The current expanded study is more robust due to a much larger sample size and thus provides more reliable results. We previously observed inverse associations between several phthalates and progesterone, SHBG and fT4. Many of the associations with SHBG remained significant in the present analysis, however many associations with progesterone and fT4 remained inverse but lost statistical significance.

Several previous studies have been conducted in Taiwan looking at gestational phthalate exposure and maternal thyroid hormones. Among 76 Taiwanese women in their second trimester, it was found that MBP was inversely associated with fT4 and T4[80], which conflicts with our finding that MBP was associated with neither fT4 nor T4. That same group later conducted a similar analysis measuring phthalates and hormones in the first trimester of pregnancy (N=97) and found that MBP was again inversely associated with T4, but the relationship between MBP and fT4 was no longer significant[81]. Median concentrations of MBP in the earlier study were almost 5 times higher than in our study, while MBP concentrations were similar between the later study and ours. Between the two Taiwanese studies in 2011, deliberate contamination with DEHP and DBP as replacements of emulsifiers in many foods and beverages occurred in Taiwan[147]. Stricter regulations put into place following the scandal may be responsible for decreased concentrations of DEHP and DBP metabolite biomarkers found in studies occurring after the scandal. Inverse associations between MBP and fT4 may have been driven by unusually high concentrations of MBP in the earlier Taiwanese population. Each of the Taiwanese studies enrolled less than 100 women, limiting their power to detect true associations.

Another study conducted in Taiwan assessed third trimester phthalate metabolites and maternal serum thyroid hormones[148]. While they found an inverse association between MBzP and TSH in fetal cord blood, they did not find any associations between phthalates and maternal serum hormones. A pilot study conducted in China reported significant positive associations between MBP and fT4 early in pregnancy (5-12 weeks gestation), but that relationship was null at 13-20 weeks[149]. Conversely, a prospective study in China found that first trimester phthalates measured around 10 weeks gestation were generally inversely associated with fT4 and T4 but positively associated with TSH[150]. Taken together, these studies suggest differential effects of phthalate exposure on maternal thyroid hormones and indicate the importance of gestational age in predicting resulting changes in associations between phthalates and maternal thyroid hormones.

Several studies have sought to determine the mechanism by which phthalates interfere with normal thyroid physiology, but results are inconsistent. Phthalates may exert thyroid-disrupting effects by altering transcription levels of thyroid hormones[151,152] or by exerting thyroid receptor antagonistic activity[153,154]. It has also been suggested that phthalates interfere with biosynthesis of thyroid hormones[70,71,155], possibly by interfering with deiodinase activity that is required for peripheral tissues to convert T4 into T3, the more bioactive hormone. Here, we observed both T3 and the ratio of T3 to T4 to be positively associated with MCNP and MCOP, and T3 was additionally positively associated with MCPP. Our results support the possibility that these DEHP metabolites may interfere with normal levels of conversion of T4 to T3 by peripheral tissues, resulting in loss of negative feedback on the thyroid and increased secretion of T3 into maternal circulation. More research including measurement of deiodinase activity needs to be conducted to better understand these relationships. Thyroid hormones play critical roles during pregnancy including direct action on the placenta to promote growth and proliferation[156], promotion of proper fetal growth and neurodevelopment[157], and placental transfer of maternal thyroid hormones upon which the fetus is totally dependent in the first trimester[158]. It has previously been shown that elevated levels of T3 are significantly associated with risk of preterm birth[159], suggesting that exposure to phthalates may increase risk for preterm birth via elevation of maternal T3.

CRH and Reproductive Hormone Discussion

Human studies of reproductive hormones have been more limited. Two previous studies have been conducted, both by the same group, looking at the relationship between urinary phthalate metabolite concentrations and maternal serum testosterone during pregnancy[83,160]. The first study took biomarker measurements late in pregnancy (98% of women were further than 20 weeks gestation), while the second study took biomarker measurements early in pregnancy (99.5% of women were less than 20 weeks gestation). Inverse associations with MBP and the sum of DEHP metabolites, and positive associations with MEP, were found with testosterone during late pregnancy but not early pregnancy. Those results are not consistent with our finding that MBP was not significantly associated with testosterone at either visit during pregnancy, or that MEP was not associated with testosterone at any point during pregnancy. Distributions of phthalate metabolite concentrations differed between the three studies, which may be driving differences in results. Additionally, the range of gestational ages used in the two previous studies may be too wide to detect the true effects of phthalates on testosterone at different points during pregnancy.

To our knowledge, no previous epidemiological studies have been conducted to evaluate the association between phthalate exposure and CRH. An *in vitro* study utilizing primary cytotrophoblast cells from term human placentas exposed cells to MEHP and quantified the subsequent protein and mRNA expression levels of CRH. They found that MEHP treatment significantly increased both CRH protein and mRNA levels. They also found that MEHP treatment significantly increased cytoplasmic-to-nuclear translocation of the RelB/p52 heterodimer, a process in the non-canonical NF-kB pathway which causes upregulation of CRH expression in the human placenta. Additionally, knockdown of NIK, a critical component of the non-canonical NF-kB pathway which induces processing of p100 into active p52 so it can heterodimerize with RelB, was found to diminish the effect of MEHP treatment on upregulation of CRH, suggesting that the effects of MEHP exposure on CRH expression is dependent on NIK activity[97]. The NF-kB signaling pathway has been implicated as a strong regulator in the process of initiating labor and thus

provides clues as to how phthalate exposure may influence CRH concentrations to affect timing of labor[161]. While we did not observe significant associations between CRH and MEHP, these results are supported by our findings that MHiBP, MCNP, MCOP, and MEP were significantly positively associated with maternal serum CRH concentrations through pregnancy. CRH concentrations are relatively low late in the second trimester and begin to exponentially increase around 20 weeks and peak at the onset of labor. Responses to higher phthalate exposures may have differential impacts on CRH concentrations beyond 26 weeks gestation as more pro-labor events begin to occur, indicating the importance of studying the associations between phthalates and CRH at both early and late stages of pregnancy. It is also important to note that concentrations of CRH binding proteins are particularly high during pregnancy[162], and our assay measured total (both bound and unbound forms) of CRH, thus reported concentrations are not necessarily indicative of bioactive concentrations.

Progesterone plays critical roles throughout pregnancy including suppression of the maternal immune system so that the fetus is not rejected, promotion of various inflammatory events at the end of pregnancy to induce labor, and helping to hold off contractions and inflammatory events until the end of the pregnancy[163]. Our results showed that exposure to MEHHTP, a metabolite of the terephthalate DEHTP, was associated with a significant decrease in maternal progesterone concentrations among the entire study population and male pregnancies specifically. Levels of terephthalate metabolites we present here are higher than those found among a convenience sample of US women prior to 2016 (median 1.1 vs. 3.65 ng/mL) in a recent study published by the CDC[33]. As phthalate replacement chemicals are used more frequently in the manufacturing of consumer products it will be increasingly important to understand the potential health threats they pose, particularly among at-risk populations such as pregnant women. To our knowledge this is the first epidemiological study to date to look at metabolites of terephthalates, and our results further indicate the need to consider these chemicals in future human health studies.

Our study has several limitations. We did not have data on maternal serum concentrations of iodine or thyroid peroxidase antibodies, both of which can impact measured concentrations of serum thyroid hormones[80,106]. Not measuring these factors limits our ability to hypothesize mechanisms of phthalate action on thyroid hormones and could have introduced bias to our study. Measuring phthalates and hormones at two time points during pregnancy that align with periods of rapid fetal growth rather than trimesters is an improvement on most published research on this topic, however two time points may not be sufficient to detect different effects of phthalates on hormones at different times through gestation. Phthalates have also been shown to have high variability within individuals, suggesting that single phthalate measurements are not typically indicative of long term exposure. However, exposure to certain phthalates may come from sources that are consumed habitually, making some of our measurements more reliable. Finally, we carried out many comparisons and thus some of our significant results may have been found by chance. Our study also has numerous strengths. Despite the risk of excess type I error from carrying out many comparisons, we were able to explore relationships that have not been well studied, particularly those between reproductive hormones and emerging phthalate replacement chemical metabolites. We present one of few studies to longitudinally assess phthalate associations with maternal hormones during pregnancy, and our sample size was greater than that of most other studies. We are the first to explore relationships between phthalates and CRH in an epidemiological study. We are also the first to explore metabolites of DEHTP, a terephthalate currently being used as a replacement for DEHP, for associations with human health measures. Our repeated measures analysis also allows us to control for intra-individual variability of measured biomarkers, enhancing our statistical power. Lastly, biomarker measurements at two different points during gestation allows for examination of possible windows of susceptibility to phthalate exposure during pregnancy.

Overall, our results suggest that gestational phthalate exposures are associated with maternal serum concentrations of CRH, testosterone and thyroid hormones through pregnancy, and that the directions of these relationships are not consistent. Sensitivity analyses indicate that timing of exposure during pregnancy and fetal sex both have significant impacts on associations with

maternal hormone levels. These results also suggest that phthalate replacement chemicals may disrupt maternal reproductive hormones during pregnancy. Future studies utilizing more frequent measurements through pregnancy and larger sample sizes for phthalate substitutes are needed to support our findings. People are rarely exposed to individual phthalate chemicals, thus studying exposures to mixtures of phthalates will be an important future step to gain a potentially fuller understanding of associations between environmental exposures and hormone levels. Future studies should also aim to assess how the impact of phthalate exposure on maternal hormones may mediate birth outcomes and child development.

Table II.1. Distributions of hormones and phthalate metabolites (raw concentrations) in the study population, through gestations and by study visits.

		N	N<LOD	Min	25th	50th	75th	90th	95th	Max	Geo. Mean	Geo. SD	P-value
CRH (pg/mL)	**Total**	1239		3.50	14.4	35.1	86.4	126	159	254	33.2	2.94	0.193
	V1	731	0	3.50	14.3	34.2	83.2	123	155	254	32.5	2.95	
	V3	508	0	3.50	14.8	37.9	88.9	131	163	249	34.4	2.94	
Estriol (ng/mL)	**Total**	1233		0.74	13.7	23.0	37.2	52.2	62.4	265	22.4	1.97	<0.001
	V1	726	0	0.74	11.3	14.9	21.6	30.2	38.3	91.9	15.3	1.73	
	V3	507	0	6.90	29.3	38.0	50.4	64.0	73.9	265	38.5	1.55	
Progesterone (ng/mL)	**Total**	1237		10.1	34.3	49.3	73.4	108	138	1037	50.8	1.79	<0.001
	V1	729	0	10.1	28.3	38.7	53.2	68.6	81.3	301	39.2	1.59	
	V3	508	0	19.4	51.0	72.5	103	142	169	1037	73.6	1.70	
Testosterone (ng/dL)	**Total**	1237		2.80	54.6	152	603	876	1041	3291	179	3.66	<0.001
	V1	729	0	2.80	51.9	159	569	809	974	2500	171	3.65	
	V3	508	0	9.20	60.3	131	652	958	1093	3291	190	3.68	
SHBG (nmol/L)	**Total**	1243		47.6	398	529	665	819	917	1461	512	1.47	<0.001
	V1	734	0	47.6	379	513	628	783	852	1461	486	1.48	
	V3	509	0	123	429	554	723	896	977	1381	552	1.45	
TSH (uIU/mL)	**Total**	1233		0.02	0.68	1.07	1.66	2.39	2.94	40.9	1.04	2.02	0.504
	V1	726	0	0.02	0.66	1.02	1.63	2.32	2.85	40.9	1.00	2.07	
	V3	507	0	0.14	0.72	1.13	1.73	2.41	3.20	25.7	1.11	1.95	
T3 (ng/mL)	**Total**	1239		0.11	1.03	1.54	2.00	2.30	2.48	8.35	1.33	1.82	0.702
	V1	731	0	0.11	0.98	1.50	1.98	2.29	2.47	8.35	1.29	1.87	
	V3	508	0	0.11	1.08	1.59	2.03	2.32	2.49	4.68	1.37	1.76	
fT4 (mg/dL)	**Total**	1241		0.35	0.86	0.98	1.1	1.2	1.27	1.72	0.97	1.20	<0.001
	V1	732	0	0.35	0.89	1.00	1.12	1.21	1.28	1.72	0.99	1.19	
	V3	509	0	0.44	0.83	0.96	1.07	1.18	1.24	1.42	0.94	1.21	
T4 (ug/dL)	**Total**	1233		5.30	10.4	11.8	13.1	14.3	15.2	19.0	11.6	1.19	0.005
	V1	726	0	6.80	10.6	11.9	13.2	14.4	15.3	19.0	11.8	1.19	
	V3	507	0	5.30	10.2	11.6	13.0	14.2	14.8	18.6	11.4	1.19	
MEHP	**Total**	1243		0.35	1.10	2.30	4.50	8.68	12.0	563	2.31	2.76	0.249
	V1	734	111	0.35	1.10	2.40	4.70	9.40	13.27	563	2.40	2.80	
	V3	509	91	0.35	1.00	2.20	4.40	8.3	10.86	64.8	2.18	2.70	
MEHHP	**Total**	1243		0.28	3.80	7.30	14.00	24.6	34.7	1040	6.99	2.86	0.043
	V1	734	3	0.28	3.95	7.60	14.7	26.0	38.2	1040	7.36	2.88	

	V3	509	1	0.28	3.50	7.00	13.4	21.7	30.7	274	6.50	2.84	
	Total	1243		0.14	3.25	6.60	11.9	21.4	28.1	690	6.14	2.83	0.387
MEOHP	**V1**	734	2	0.14	3.33	6.70	12.1	21.8	29.3	690	6.27	2.85	
	V3	509	0	0.30	3.20	6.50	11.9	20.2	27.2	231	5.95	2.80	
	Total	1243		0.60	7.05	13.2	24.2	37.8	52.2	1020	12.8	2.58	0.054
MECPP	**V1**	734	0	0.60	7.23	13.4	24.5	38.0	53.1	1020	13.2	2.57	
	V3	509	0	0.80	6.70	12.8	23.6	37.5	51.2	493	12.1	2.60	
	Total	1243		0.28	7.20	15.3	31.4	62.7	92.0	478	14.4	3.22	0.463
MBP	**V1**	734	5	0.28	7.73	15.5	31.4	63.6	91.5	285	14.9	3.13	
	V3	509	3	0.28	6.50	15.2	31.2	60.3	90.2	478	13.8	3.35	
	Total	1243		0.21	1.10	2.60	6.30	13.7	25.6	612	2.65	3.75	0.677
MBzP	**V1**	734	28	0.21	1.13	2.80	6.60	14.9	25.1	612	2.79	3.75	
	V3	509	29	0.21	1.00	2.50	5.80	12.6	25.6	298	2.45	3.74	
	Total	1243		0.40	4.65	9.60	19.2	36.2	55.8	964	9.39	2.94	0.684
MiBP	**V1**	734	4	0.40	4.90	9.60	20.0	35.2	50.7	202	9.48	2.89	
	V3	509	7	0.57	4.30	9.70	18.2	38.6	56.3	964	9.27	3.01	
	Total	895		0.28	0.60	1.40	2.90	6.06	9.10	63.2	1.38	3.00	0.408
MHBP	**V1**	542	67	0.28	0.60	1.40	2.90	5.59	8.40	45.1	1.45	2.91	
	V3	353	68	0.28	0.50	1.30	2.70	6.30	9.24	63.2	1.28	3.12	
	Total	895		0.28	1.90	3.90	8.40	15.6	23.1	68.2	3.91	2.95	0.134
MHiBP	**V1**	542	6	0.28	2.10	4.10	8.78	16.0	23.6	65.4	4.15	2.88	
	V3	353	11	0.28	1.60	3.80	7.60	15.0	20.6	68.2	3.56	3.03	
	Total	1243		0.14	0.90	1.60	2.80	5.40	8.49	146	1.65	2.57	0.237
MCNP	**V1**	734	6	0.14	1.00	1.70	2.90	5.77	8.44	59.8	1.73	2.55	
	V3	509	6	0.14	0.80	1.50	2.60	4.90	8.34	146	1.53	2.57	
	Total	1243		0.30	4.10	8.20	18.5	45.9	88.2	1230	9.25	3.48	0.135
MCOP	**V1**	734	0	0.30	4.30	8.75	19.0	47.2	97.4	1230	9.84	3.50	
	V3	509	0	0.30	3.80	7.20	16.8	41.6	77.2	890	8.46	3.42	
	Total	590		0.28	0.90	1.70	3.88	7.81	14.5	512	1.94	3.22	0.671
MONP	**V1**	359	22	0.28	0.90	1.70	4.00	7.72	13.7	512	1.95	3.07	
	V3	231	19	0.28	0.90	1.70	3.45	8.00	20.1	452	1.93	3.46	
	Total	590		0.90	9.75	20.3	44.6	141	395	4960	24.4	3.98	0.910
MECPTP	**V1**	359	0	1.40	10.4	21.3	47.3	162	592	4960	26.7	4.26	
	V3	231	0	0.90	9.45	18.2	39.8	92.6	217	2420	21.2	3.51	
	Total	590		0.28	1.60	3.65	9.28	23.1	55.9	1690	4.22	3.99	0.571
MEHHTP	**V1**	359	2	0.28	1.70	4.00	9.65	31.6	82.0	1690	4.77	4.16	
	V3	231	7	0.28	1.45	3.10	8.30	16.3	35.05	227	3.48	3.65	

	Total	1243		0.85	12.3	33.6	150	545	1207	20900	45.6	5.69	0.707
MEP	**V1**	734	2	0.85	12.6	34.3	134	524	973	20900	45.7	5.35	
	V3	509	3	0.85	11.6	31.1	195	712	1392	8930	45.6	6.21	
	Total	1243		0.14	0.60	1.30	2.50	5.00	8.39	151	1.34	3.00	0.235
MCPP	**V1**	734	77	0.14	0.70	1.30	2.60	5.40	8.87	120	1.41	2.95	
	V3	509	85	0.14	0.50	1.20	2.40	4.32	7.10	151	1.25	3.06	

P values were calculated using a paired t-test between biomarker concentrations at visit 1 and visit 3. Skewed biomarkers were ln-transformed. Phthalate concentrations are in ng/mL.

Table II.2. Results from linear mixed models showing the percent change in serum hormone concentrations corresponding to an IQR increase in urinary phthalate metabolite concentrations.

	CRH^			Estriol^			Prog^		
	N	**%Δ (95% CI)**	**P**	**N**	**%Δ (95% CI)**	**P**	**N**	**%Δ (95% CI)**	**P**
MEHP	1225	0.38 (-7.67, 9.13)	0.929	1219	-0.37 (-6.17, 5.78)	0.903	1223	0.72 (-4.36, 6.07)	0.785
MEHHP	1225	-1.07 (-8.25, 6.68)	0.781	1219	-0.68 (-6.10, 5.04)	0.811	1223	-1.23 (-5.88, 3.65)	0.616
MEOHP	1225	-3.57 (-10.75, 4.18)	0.357	1219	3.60 (-2.20, 9.73)	0.230	1223	-0.02 (-4.85, 5.06)	0.994
MECPP	1225	-0.82 (-8.50, 7.52)	0.842	1219	1.85 (-3.96, 8.01)	0.541	1223	-2.15 (-6.99, 2.94)	0.402
MBP	1225	-5.22 (-12.46, 2.62)	0.187	1219	0.36 (-5.38, 6.46)	0.904	1223	-1.22 (-6.11, 3.92)	0.635
MBzP	1225	2.63 (-5.49, 11.45)	0.538	1219	-3.45 (-8.88, 2.30)	0.235	1223	-1.48 (-6.29, 3.58)	0.560
MiBP	1225	4.86 (-3.70, 14.18)	0.275	1219	0.36 (-5.42, 6.49)	0.907	1223	2.23 (-2.87, 7.60)	0.399
MHBP	886	-5.95 (-16.39, 5.80)	0.308	883	-2.87 (-10.18, 5.03)	0.466	886	-5.44 (-11.49, 1.02)	0.099
MHiBP	**886**	**15.38 (2.12, 30.37)**	**0.023****	883	-1.02 (-8.31, 6.84)	0.792	886	-0.57 (-6.84, 6.13)	0.864
MCNP	**1225**	**6.82 (-0.02, 14.12)**	**0.051***	1219	4.52 (-0.62, 9.93)	0.086	1223	2.59 (-1.78, 7.15)	0.251
MCOP	**1225**	**14.73 (7.28, 22.69)**	**0.000****	1219	2.42 (-2.49, 7.58)	0.341	1223	0.58 (-3.61, 4.95)	0.789
MONP	583	5.42 (-4.86, 16.81)	0.315	582	4.48 (-3.02, 12.57)	0.251	582	0.10 (-6.12, 6.73)	0.976
MECPTP	583	-7.91 (-15.96, 0.90)	0.079	582	-2.85 (-8.77, 3.45)	0.369	582	-4.13 (-9.23, 1.25)	0.132
MEHHTP	583	-8.15 (-17.77, 2.60)	0.134	582	-5.32 (-12.27, 2.19)	0.162	**582**	**-7.25 (-13.18, -0.91)**	**0.027**
MEP	**1225**	**10.54 (1.96, 19.84)**	**0.016****	1219	2.78 (-2.87, 8.76)	0.343	1223	1.66 (-3.20, 6.76)	0.510
MCPP	1225	6.86 (-0.81, 15.14)	0.082	1219	1.00 (-4.33, 6.63)	0.719	1223	-2.41 (-6.89, 2.28)	0.309
	Prog/Estriol^			**Testosterone^**			**SHBG^**		
	N	**%Δ (95% CI)**	**P**	**N**	**%Δ (95% CI)**	**P**	**N**	**%Δ (95% CI)**	**P**
MEHP	1214	0.94 (-3.70, 5.81)	0.697	1223	-1.60 (-9.50, 6.99)	0.705	1229	-2.14 (-4.84, 0.63)	0.130
MEHHP	1214	-0.33 (-4.57, 4.09)	0.880	1223	-2.71 (-9.68, 4.79)	0.469	**1229**	**-4.50 (-6.85, -2.10)**	**0.000****
MEOHP	1214	-3.32 (-7.53, 1.08)	0.138	1223	1.11 (-6.31, 9.10)	0.777	**1229**	**-3.83 (-6.26, -1.33)**	**0.003****
MECPP	1214	-3.76 (-8.07, 0.76)	0.102	1223	-4.16 (-11.56, 3.86)	0.300	**1229**	**-3.71 (-6.26, -1.09)**	**0.006****
MBP	1214	-1.24 (-5.65, 3.38)	0.593	1223	6.78 (-1.26, 15.48)	0.101	**1229**	**-3.31 (-5.83, -0.72)**	**0.013****
MBzP	1214	1.97 (-2.57, 6.73)	0.402	1223	0.71 (-7.37, 9.50)	0.868	1229	-2.20 (-4.88, 0.55)	0.117

MiBP	1214	1.75 (-2.91, 6.62)	0.469	1223	-1.17 (-9.36, 7.77)	0.791	1229	-2.24 (-5.00, 0.61)	0.123
MHBP	883	-2.35 (-8.15, 3.81)	0.446	**886**	**17.03 (3.68, 32.09)**	**0.012****	887	-2.76 (-6.16, 0.76)	0.124
MHiBP	883	0.53 (-5.41, 6.84)	0.866	886	-10.73 (-21.70, 1.77)	0.091	887	-0.98 (-4.75, 2.93)	0.618
MCNP	1214	-2.00 (-5.74, 1.89)	0.311	**1223**	**-7.72 (-13.48, -1.57)**	**0.015****	1229	-1.24 (-3.38, 0.94)	0.263
MCOP	1214	-1.75 (-5.46, 2.11)	0.370	**1223**	**-9.52 (-15.43, -3.21)**	**0.004****	1229	-1.81 (-3.99, 0.42)	0.112
MONP	582	-4.62 (-10.24, 1.35)	0.129	582	7.32 (-3.26, 19.04)	0.184	583	-0.18 (-2.73, 2.43)	0.892
MECPTP	582	-1.32 (-6.32, 3.94)	0.617	582	9.36 (-0.64, 20.36)	0.069	583	-1.54 (-3.97, 0.95)	0.225
MEHHTP	582	-1.76 (-7.77, 4.63)	0.581	582	9.06 (-2.87, 22.46)	0.144	**583**	**-3.49 (-6.34, -0.56)**	**0.021****
MEP	1214	-1.21 (-5.53, 3.31)	0.595	1223	-7.26 (-14.57, 0.69)	0.073	1229	0.20 (-2.50, 2.97)	0.888
MCPP	1214	-3.12 (-7.15, 1.09)	0.145	**1223**	**-10.56 (-16.98, -3.66)**	**0.003****	1229	-1.85 (-4.27, 0.63)	0.143
		TSH^			**T3**			**fT4**	
	N	**%Δ (95% CI)**	**P**	**N**	**%Δ (95% CI)**	**P**	**N**	**%Δ (95% CI)**	**P**
MEHP	1219	2.00 (-3.45, 7.76)	0.481	1225	-0.19 (-3.45, 3.07)	0.911	1227	-0.84 (-2.28, 0.61)	0.257
MEHHP	**1219**	**5.21 (0.12, 10.56)**	**0.045***	1225	0.65 (-2.28, 3.59)	0.663	1227	-1.05 (-2.37, 0.26)	0.116
MEOHP	**1219**	**5.45 (0.22, 10.95)**	**0.042***	1225	0.42 (-2.59, 3.42)	0.787	**1227**	**-1.43 (-2.77, -0.08)**	**0.038***
MECPP	**1219**	**6.69 (1.19, 12.49)**	**0.017***	1225	1.58 (-1.56, 4.73)	0.325	1227	-1.20 (-2.60, 0.20)	0.093
MBP	1219	-0.28 (-5.36, 5.08)	0.917	1225	-2.29 (-5.37, 0.80)	0.147	1227	-0.58 (-1.96, 0.80)	0.411
MBzP	1219	2.49 (-2.91, 8.19)	0.373	1225	1.38 (-1.84, 4.61)	0.401	1227	0.34 (-1.08, 1.75)	0.639
MiBP	1219	3.46 (-2.17, 9.41)	0.235	1225	2.00 (-1.33, 5.34)	0.240	1227	0.10 (-1.36, 1.56)	0.895
MHBP	883	-0.98 (-8.10, 6.69)	0.796	**886**	**-5.85 (-10.29, -1.41)**	**0.010**	886	-0.70 (-2.53, 1.12)	0.449
MHiBP	**883**	**10.52 (2.29, 19.42)**	**0.012***	886	3.09 (-1.59, 7.77)	0.198	886	1.16 (-0.69, 3.01)	0.221
MCNP	1219	2.62 (-1.74, 7.18)	0.243	**1225**	**3.33 (0.77, 5.88)**	**0.011***	**1227**	**1.37 (0.21, 2.52)**	**0.021****
MCOP	1219	4.27 (-0.24, 8.99)	0.065	**1225**	**4.41 (1.80, 7.03)**	**0.001***	**1227**	**1.51 (0.34, 2.67)**	**0.012****
MONP	582	3.63 (-2.91, 10.61)	0.286	583	-3.05 (-7.09, 0.98)	0.140	583	-0.81 (-2.41, 0.79)	0.320
MECPTP	582	-1.00 (-6.73, 5.08)	0.741	583	-2.23 (-5.93, 1.48)	0.240	583	-0.53 (-1.91, 0.86)	0.459
MEHHTP	582	0.97 (-6.05, 8.52)	0.794	583	-1.60 (-6.08, 2.88)	0.485	583	-0.80 (-2.48, 0.88)	0.351
MEP	1219	-0.66 (-5.82, 4.77)	0.807	1225	1.03 (-2.14, 4.21)	0.525	1227	-0.04 (-1.43, 1.35)	0.957
MCPP	**1219**	**5.05 (0.03, 10.32)**	**0.050***	**1225**	**4.25 (1.34, 7.15)**	**0.004***	**1227**	**2.02 (0.74, 3.31)**	**0.002****
		T4			**T3/T4**				

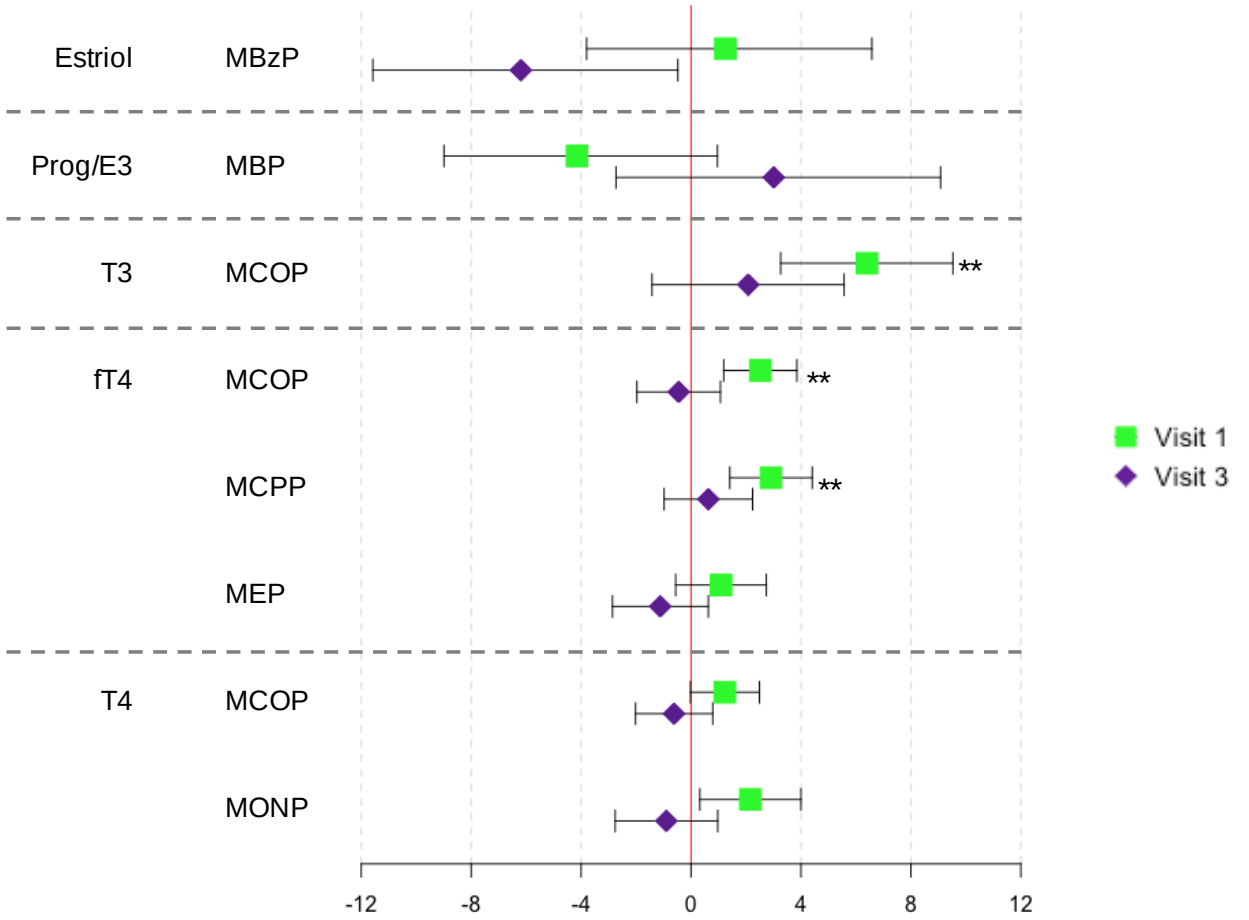
	N	%Δ (95% CI)	P	N	%Δ (95% CI)	P
MEHP	1219	-0.94 (-2.25, 0.37)	0.160	1219	0.82 (-2.58, 4.22)	0.637
MEHHP	**1219**	**-1.24 (-2.42, -0.06)**	**0.040**	1219	1.97 (-1.11, 5.04)	0.211
MEOHP	1219	-0.98 (-2.19, 0.23)	0.114	1219	1.41 (-1.74, 4.56)	0.381
MECPP	1219	-0.73 (-2.00, 0.53)	0.256	1219	2.49 (-0.79, 5.77)	0.138
MBP	1219	0.10 (-1.15, 1.35)	0.877	1219	-2.47 (-5.71, 0.76)	0.134
MBzP	1219	-0.04 (-1.34, 1.25)	0.950	1219	1.20 (-2.15, 4.55)	0.483
MiBP	1219	-0.68 (-2.02, 0.66)	0.319	1219	2.74 (-0.73, 6.20)	0.123
MHBP	883	0.41 (-1.34, 2.16)	0.646	**883**	**-6.33 (-10.92, -1.73)**	**0.007**
MHiBP	883	-0.24 (-2.07, 1.58)	0.793	883	3.61 (-1.22, 8.45)	0.144
MCNP	1219	-0.18 (-1.21, 0.85)	0.733	**1219**	**3.67 (0.99, 6.36)**	**0.008****
MCOP	1219	0.51 (-0.54, 1.57)	0.342	**1219**	**4.22 (1.48, 6.96)**	**0.003****
MONP	582	0.52 (-0.89, 1.94)	0.469	582	-3.58 (-7.63, 0.48)	0.086
MECPTP	582	0.48 (-0.82, 1.78)	0.466	582	-2.24 (-5.95, 1.48)	0.239
MEHHTP	582	0.62 (-0.95, 2.19)	0.439	582	-1.88 (-6.37, 2.60)	0.412
MEP	1219	-0.86 (-2.13, 0.42)	0.188	1219	2.00 (-1.30, 5.30)	0.235
MCPP	**1219**	**1.57 (0.41, 2.74)**	**0.009***	1219	2.73 (-0.31, 5.77)	0.079

**q<0.1, *q<0.2

^Hormone concentrations were ln-transformed for analyses.

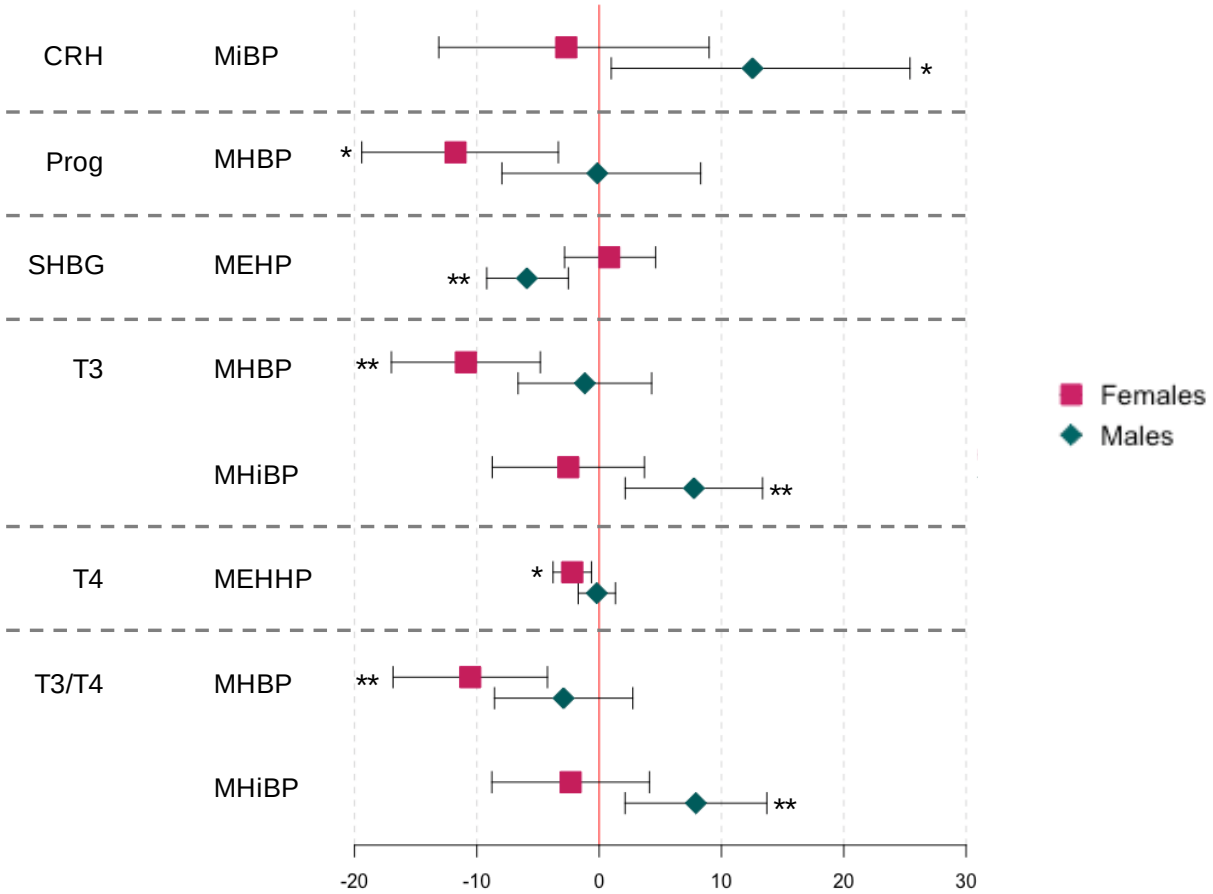
All models adjusted for categorical maternal age and education, and specific gravity.

Figure II.1. Percent changes in hormone concentrations with an IQR increase in phthalate concentrations that were significantly different between study visits.



Green squares represent effect estimates for study visit 1, purple diamonds indicate effect estimates for study visit 3, black bars represent 95% confidence intervals, and the vertical red line represents the null value.
**q-value<0.1

Figure II.2. Percent changes in hormone concentrations with an IQR increase in phthalate concentrations that were significantly different between fetal sexes.



Pink squares represent effect estimates for female fetuses, teal diamonds indicate effect estimates for male fetuses, black bars represent 95% confidence intervals, and the vertical red line represents the null value.

**q<0.1

*q<0.2

Chapter III. Fetal Sex-Dependent Associations Between Gestational Hormone Concentrations and Adverse Birth Outcomes

Abstract

Background: Adverse birth outcomes remain significant public health problems that can have long-lasting impacts on mother and child. Understanding biological mechanisms underlying these outcomes, including altered endocrine function, can inform prevention efforts.

Objective: Evaluate associations between hormones at two times points during mid-gestation and adverse birth outcomes, and explore effect modification by fetal sex.

Methods: We explored associations between repeated gestational hormone measurements (at 18 and 26 weeks) and birth outcomes among 976 women in PROTECT, a longitudinal prospective birth cohort in northern Puerto Rico, from 2011 to 2018. Birth outcomes assessed included preterm and spontaneous preterm birth (PTB), preeclampsia, gestational diabetes mellitus (GDM), small/large for gestational age (SGA, LGA), birthweight z-score, and gestational age at birth. Multivariate logistic and linear regressions were fit using visit-specific concentrations of hormones. We also conducted sensitivity analyses assessing impacts of fetal sex on observed associations. All models were adjusted for maternal age and education, and other confounders were assessed separately between birth outcomes based on *a priori* knowledge and observed associations with exposure and outcome measures.

Results: Increased odds of spontaneous PTB were observed with IQR increases in progesterone (OR: 2.12, 95% CI: 1.29, 3.47), fT4 (OR: 1.73, 95% CI: 1.04, 2.86), and the ratio of progesterone to estriol (OR: 1.63, 95% CI: 1.05, 2.54) at 26 weeks. Elevated estriol was protective against preeclampsia at 26 weeks (OR: 0.42, 95% CI: 0.17, 0.99). Increases in TSH and T3 conferred greater risk of GDM at 18 weeks. Many associations were modified by fetal sex, with hormone alterations during male pregnancies conferring greater risk of PTB, spontaneous PTB, and GDM.

Conclusions: Associations between hormones and birth outcomes vary based on timing of hormone measurement and fetal sex. Future studies are needed to understand mechanisms involved in adverse birth outcomes and fetal sex difference.

Background

Preterm birth (PTB) affects approximately 11% of live births[164] and is the leading cause of neonatal mortality worldwide[165]. Infants born preterm are at increased risk for adverse health outcomes later in life including reduced renal function[166], neurodevelopmental impairments[167], cerebral palsy[168], and reduced myocardial function[169]. Despite being a common public health problem, the causes of PTB are largely unknown. Other rare birth outcomes are also of significant concern and present safety issues for the mother and the fetus. The spontaneous subtype of preterm birth is characterized by an inflammatory uterine environment and may arise via different mechanisms than indicated PTB[17]. Preeclampsia, a hypertensive disorder of pregnancy[18], affects 6% of pregnancies globally[19] and is the leading cause of maternal mortality in the United States[20,21]. Gestational diabetes mellitus (GDM) is a disease of reduced insulin sensitivity and elevated glucose levels during gestation. High maternal glucose levels easily cross the placenta and illicit a response from the fetal pancreas. Infants born to mothers with GDM are at elevated risk for macrosomia and metabolic dysfunctions, and mothers become more likely to develop diabetes later in life[22]. Very little epidemiologic work has been done to investigate these rare adverse pregnancy outcomes and so our knowledge of the mechanisms by which they occur is limited.

The maternal and fetal endocrine milieus change and interact in unique ways at different points throughout gestation. The roles of progesterone are complex, reflected by the mixed efficacy of progesterone therapy as a preventative measure for preterm birth[170]. Estrogens are responsible for uterine maintenance and increased expression of oxytocin receptors and gap junctions that are necessary for uterine contractions to occur[171]. Through pregnancy, progesterone maintains uterine quiescence and keeps contractile effects of estrogens in check[170]. Thyroid hormones are critical early in pregnancy for proper brain and skeletal development of the fetus[75]. The maternal

supply of thyroxine (T4) is particularly important in the first half of pregnancy, before the fetal thyroid gland has matured enough to produce adequate hormones[76]. Previous studies have demonstrated associations between clinical hyper- and hypothyroidism and adverse birth outcomes[172–175], but much less is known about subclinical thyroid disruption and pregnancy outcomes.

Few large epidemiological studies exist that assess a wide array of hormone concentrations and pregnancy outcomes. The majority of existing research focuses on one hormone/class of hormones, which makes it challenging to gain a broad understanding of the endocrine pathways implicated in the onset of adverse birth outcomes[143,176,177]. Specifically, the spontaneous subtype of PTB has not been well studied, and current research on the rare outcomes of preeclampsia and GDM is sparse[178–180]. Importantly, few previous studies have investigated hormone concentrations at more than one time point during gestation, nor have they assessed the impact of fetal sex on these associations. Because of these gaps in the literature, the aim of this study was to investigate associations between various hormone concentrations, measured at two time points during gestation, and adverse birth outcomes, as well as effect modification by fetal sex. Based on previous literature, we have hypothesized that increases in CRH and estriol will be associated with increased risk of early delivery, while increases in progesterone will be associated with later delivery. Further, we expect lower thyroid hormone concentrations to be associated with smaller infant size at birth. Finally, we expect to observe more significant adverse associations among male pregnancies, given previous evidence suggesting that male pregnancies are more risky than female pregnancies[181–183].

Methods

Study Population

Pregnant women were recruited into the PROTECT birth cohort between 2011 and 2018 at 14±2 weeks' gestation from seven hospitals and prenatal clinics in northern Puerto Rico. Study design and recruitment protocols have been described elsewhere[139]. Demographic and self-reported health information was provided at the first clinic visit. This study was approved by the research

and ethics committees of the University of Michigan School of Public Health, University of Puerto Rico, Northeastern University, and participating hospitals and clinics. All study participants provided full informed consent prior to participation.

Hormone Measurements

All women provided serum samples at their first and third clinic visits, aligning with median 18 (range 16-20) and 26 (range 24-28) weeks' gestation. Serum samples were analyzed at the Central Ligand Assay Satellite Services (CLASS) laboratory in the Department of Epidemiology at the University of Michigan School of Public Health. Progesterone, sex hormone-binding globulin (SHBG), testosterone, total triiodothyronine (T3), total thyroxine (T4), free thyroxine (fT4), and thyroid-stimulating hormone (TSH) were measured using a chemiluminescence immunoassay. Estriol and corticotropin releasing hormone (CRH) were measured using an enzyme immunoassay. Some hormone concentrations were not available for all participants due to sample volume limitations. The ratios of progesterone to estriol (Prog/E3) and T3 to T4 (T3/T4) were assessed in addition to measured hormones because of previous research indicating that the ratios may be better indices of adverse pregnancy outcomes than single hormone measurements[142–144]. All hormone concentrations below the limit of detection (LOD) were replaced by the LOD divided by the square root of two.

Birth Outcome Assessment

Based on recommendations from the American College of Obstetricians and Gynecologists, self-reported date of the last menstrual period was collected at the first study visit and used in combination with early ultrasound measurements to determine gestational age at birth[184]. PTB was defined as delivery before 37 weeks' gestation. We also assessed spontaneous PTB, defined as PTB presenting with premature rupture of membranes, spontaneous preterm labor, or both[17]. Preeclampsia and GDM cases were determined based on diagnosis in the medical record by an attending physician. We calculated birthweight z-scores based on fetal sex and gestational age using widely accepted international standards[185]. Those born with a birthweight <10th percentile

and >90th percentile were considered small and large for gestational age (SGA and LGA), respectively.

Statistical Methods

Distributions of demographic, health, and pregnancy characteristics were calculated. Summary measures of gestational hormone concentrations were assessed using arithmetic means of all available concentrations for each study participant, or geometric means for log-normally distributed hormones. Distributions of hormone concentrations were also assessed individually at each study visit. Univariate linear models were used to test for significant differences between hormone concentrations at each study visit. Intraclass correlation coefficients (ICCs) were also used to assess between- and within-individual variability of hormone concentrations across study visits.

We utilized indicator variables for study visit and included interaction terms between each indicator and hormone concentration in final models to achieve effect estimates specific to each study visit. Sandwich estimators were used in these models to correct for biased standard errors due to the non-repeating nature of outcome variables. Gestational average hormone concentrations were not used in final statistical models because of the marked changes in some hormones that occur throughout gestation. We also conducted sensitivity analyses to assess effect modification by fetal sex. An additional interaction term was included between hormone concentration and a fetal sex indicator variable to achieve effect estimates specific to fetal sex within study visits.

Confounders were explored by evaluating their associations with exposure and outcome variables. All models adjusted for categorical forms of maternal age and maternal education. Further covariate adjustment differed between birth outcomes based on *a priori* knowledge, significant association with the outcome measure, and inclusion of the covariate impacting the hormone effect estimate by at least 10%. A list of covariates that were assessed and the outcome

models in which they were included, if any, is shown in Table III.1. All models assessing testosterone also included SHBG to adjust for bound testosterone.

Results

Demographics of the study population are shown in Table III.2. The majority of mothers were under the age of 30 (67.1%), had at least some college education (79%), were employed (63%), had an annual household income under $30,000 (63.1%), were married (53.1%), had never smoked (86%) or been exposed to environmental tobacco smoke (88.7%), did not drink alcohol during pregnancy (93.6%), had given birth to less than 2 previous children (86.9%), and had a pre-pregnancy BMI of less than 25 (56.1%).

Distributions of hormone concentrations are shown in Table III.3. Most hormone concentrations were significantly different at 18 and 26 weeks' gestation, with notable increases occurring with estriol (median 15.1 and 38.2 ng/mL at 18 and 26 weeks, respectively) and progesterone (median 39.3 and 73.5 ng/mL at 18 and 26 weeks, respectively). ICCs for all other hormones ranged from 0.647 (T4) to 0.856 (testosterone).

Distributions of birth outcomes are shown in Table III.4. PTB and spontaneous PTB occurred in 9.9% and 5.8% of the study population, respectively. Preeclampsia and GDM were less prevalent (2.9% and 1.9%, respectively). Occurrences of SGA and LGA births were similar (8.9% and 9.6%, respectively). Median gestational age of the study population was 39.1 weeks (IQR: 38.1-40).

Figure III.1 shows the associations between hormone concentrations and birth outcomes at each study visit (all effect estimates and p-values are shown in Table III.5). There were greater odds of spontaneous PTB with increasing progesterone concentrations at 26 weeks (OR: 2.12, 95% CI: 1.29, 3.47) and fT4 concentrations at both study visits (18wk OR: 1.60, 95% CI: 1.07, 2.39; 26wk OR: 1.73, 95% CI: 1.04, 2.86). The risk of spontaneous PTB was significantly different between study visits with an IQR increase in Prog/E3 (interaction p=0.026), a null association observed at 18 weeks and increased odds observed at 26 weeks (OR: 1.63, 95% CI: 1.05, 2.54). Reductions in

gestational age at birth were observed with increased concentrations of progesterone (β: -3.56 days, 95% CI: -6.02, -1.10), fT4 (β: -2.22 days, 95% CI: -3.84, -0.61), and T4 (β: -1.87 days, 95% CI: -3.62, -0.11) around 18 weeks, and with prog/e3 at both study visits (18wk β: -1.77 days, 95% CI: -3.36, -0.19; 26wk β: -1.98 days, 95% CI: -3.58, -0.37). Notably, the effect of progesterone was significantly different between study visits (interaction p=0.044).

Results at 18 weeks suggested that elevated progesterone and reduced estriol are associated with increased risk of having an SGA infant (E3 OR: 0.66, 95% CI: 0.45, 0.97; progesterone OR: 1.53, 95% CI: 1.09, 2.17; prog/E3 OR: 1.77, 95% CI: 1.29, 2.44). This trend remained at 26 weeks for only prog/E3 (OR: 1.53, 95% CI: 1.07, 2.17). Similarly, prog/E3 at 18 weeks was inversely associated with birthweight z-score (β: -0.12, 95% CI: -0.23, -0.02) and estriol at 26 weeks was positively associated with birthweight z-score (β: 0.21, 95% CI: 0.01, 0.41).

A protective effect against preeclampsia was observed with increases in SHBG at 18 weeks (OR: 0.55, 95% CI: 0.30, 0.99) and estriol (OR: 0.42, 95% CI: 0.17, 0.99) and SHBG (OR: 0.46, 95% CI: 0.25, 0.83) at 26 weeks. Conversely, elevated risk of preeclampsia was observed with an increase in TSH at 26 weeks (OR: 2.18, 95% CI: 1.19, 3.99). The odds of GDM increased with an IQR increase in TSH (OR: 1.67, 95% CI: 1.02, 2.72), T3 (OR: 2.83, 95% CI: 1.04, 7.68), and T3/T4 (OR: 2.97, 95% CI: 1.20, 7.35) at 18 weeks, and increased with higher estriol at 18 weeks (OR: 5.95, 95% CI: 1.27, 27.8). None of the associations with preeclampsia or GDM were significantly different between study visits.

Sensitivity analyses revealed that many associations were significantly different between male and female pregnancies (Figure III.2; all effect estimates and p-values are shown in Table III.6). The most compelling effect modification by fetal sex was observed for preterm birth; the interaction term between hormone concentration and fetal sex indicator was significant among 7 out of 11 hormones and hormone ratios assessed. SHBG was protective against PTB at 26 weeks among female (OR: 0.60, 95% CI: 0.37, 0.96), but not male, pregnancies (interaction p=0.032). Higher testosterone at both study visits was associated with increased odds of PTB among female

pregnancies and reduced odds of PTB among male pregnancies (interaction p<0.001). Notably, increased odds of PTB were observed among only male pregnancies with elevated concentrations of CRH (OR: 1.82, 95% CI: 1.09, 3.05; interaction p=0.002), estriol (OR: 1.81, 95% CI: 1.07, 3.06; interaction p=0.022), progesterone (OR: 1.88, 95% CI: 1.16, 3.04; interaction p=0.011), and fT4 (OR: 1.63, 95% CI: 1.06, 2.51; interaction p=0.115) at 18 weeks. Assessment of gestational age as a continuous variable did not provide such compelling results, but it did provide additional evidence of fetal sex modifying the association with progesterone at 18 weeks (male pregnancy β: -4.9 days, 95% CI: -2.73, -7.07 days; interaction p=0.015).

The spontaneous subtype of PTB also showed several cases of effect modification by fetal sex. An IQR increase in CRH at 18 weeks was associated with greater odds of spontaneous PTB among only male pregnancies (OR: 2.73, 95% CI: 1.38, 5.43; interaction p=0.003). Increases in testosterone at both visits were protective against spontaneous PTB among only male pregnancies (interaction p=0.001). Increases in T3 and fT4 at both study visits were associated with increased odds of spontaneous PTB among only male pregnancies, but effect modification was significant only for T3 (interaction p=0.013). Finally, higher progesterone at 26 weeks was associated with increased odds of spontaneous PTB among only male pregnancies (OR: 2.34, 95% CI: 1.36, 4.03).

Fetal sex modified the association between SGA and only the ratio prog/E3 (interaction p=0.022), which was positive among only male pregnancies at both 18 weeks (OR: 2.39, 95% CI: 1.59, 3.60) and 26 weeks (OR: 1.98, 95% CI: 1.29, 3.05). Accordingly, increased estriol resulted in increases in birthweight z-score at both 18 weeks (β: 0.19, 95% CI: 0.02, 0.36) and 26 weeks (β: 0.31, 95% CI: 0.08, 0.53) among only male pregnancies (interaction p=0.030). Fetal sex did not modify any associations between hormones and odds of LGA.

Though there was no evidence of effect modification by fetal sex on associations between hormones and preeclampsia, significant effects were observed only among female pregnancies with increases in SHBG (OR: 0.34, 95% CI: 0.14, 0.81), TSH (OR: 2.41, 95% CI: 1.11, 5.23), and fT4

(OR: 0.40, 95% CI: 0.17, 0.92) at 26 weeks. Conversely, there was significant evidence of effect modification by fetal sex on the association between various hormones and odds of GDM. Elevated thyroid hormones were observed to be protective against GDM among female pregnancies [(fT4 at 18wks OR: 0.29, 95% CI: 0.10, 0.85; interaction p=0.001), (T4 at 18wks OR: 0.32, 95% CI: 0.11, 0.90; interaction p=0.002)], but positively associated with GDM among male pregnancies [(T3 at 18wks OR: 6.04, 95% CI: 1.72, 21.3; interaction p=0.028), (fT4 at 26wks OR: 4.87, 95% CI: 1.53, 15.5), (T4 at 26wks OR: 3.05, 95% CI: 1.02, 9.13)]. A similar trend was observed for the ratio of prog/E3; there was a protective effect at 18 weeks among female pregnancies (OR: 0.25, 95% CI: 0.09, 0.71) and a positive association at 26 weeks among male pregnancies (OR: 2.93, 95% CI: 0.99, 8.69; interaction p=0.004).

Discussion

We observed a range of significant associations between gestational hormone concentrations and adverse birth outcomes in a Puerto Rican birth cohort. Alterations of progesterone, estriol, and thyroid hormones were implicated in the occurrence of most birth outcomes assessed. Though most interaction terms were not significant, we observed many associations that were unique to hormone measurements at either 18 weeks' or 26 weeks' gestation. Fetal sex differences were also observed for many associations, with most significant results observed only when the fetus was male.

PTB and Gestational Age

We observed greater odds of PTB and spontaneous PTB with increasing progesterone concentrations (when fetal sex was male), but other studies demonstrating similar significant associations are lacking. One study observed progesterone concentrations measured between 28 and 32 weeks' gestation to be higher among women who delivered preterm compared to full term[176]. We observed higher progesterone concentrations among PTB cases when fetal sex was male, but only around 18 weeks' gestation. We also observed higher progesterone concentrations around 26 weeks among women who spontaneously delivered preterm compared to women who carried to term.

Previous work has shown that a ratio favoring estriol in mid-pregnancy[143] and at delivery[186] is associated with earlier time of labor. Progesterone concentrations rise steadily during pregnancy, contributing to uterine quiescence, downregulation of prostaglandin production, and immune tolerance of the fetus[85,86]. At the onset of human labor, progesterone concentrations do not notably decrease; rather, the body's response to progesterone is dampened. It is not clear exactly how this occurs, but possibilities include reduction in progesterone receptor expression, changes in receptor isoforms, and local progesterone metabolism[89]. As term approaches, the ratio of progesterone to estriol shifts to favor estrogens, with the functional decrease in progesterone driving initiation of labor[88]. The new dominance of estrogens promotes an increase in prostaglandin and oxytocin receptors and enzymes responsible for muscle contractions, which work together to help promote labor[87]. We observed a positive association between odds of PTB and estriol concentrations (when fetal sex was male), but we also unexpectedly observed later gestational age at birth with higher concentrations of estriol at 26 weeks' gestation when the fetus was female. In contrast with previous studies, we observed that higher prog/E3 was associated with reduced gestational age and increased odds of SGA. Interestingly, among women who delivered preterm, a previous study observed lower prog/E3 among only those without premature rupture of membranes[187], possibly implicating different endocrine pathways in the occurrence of PTB with and without premature rupture of membranes.

Decreased odds of PTB have been shown with increased concentrations of fT4 in the second[177] and third[159] trimesters, which contradicts our finding that fT4 was inversely associated with gestational age at birth (among the whole study population and when the fetus was male), and increased odds of PTB (when the fetus was male) and spontaneous PTB. One prior study also found increased odds of PTB with greater T3 concentrations at 10 and 26 weeks gestation[159]. Similarly, we found that T3 was associated with spontaneous PTB when the fetus was male. Mechanisms of the association between thyroid hormones and PTB are poorly understood, but previous research has suggested that altered thyroid hormone concentrations may be involved

in other disease states or exposures for which we have evidence of associations with PTB such as oxidative stress and inflammation[188–190], or environmental exposures such as phthalates[47,78,191].

Several previous studies have observed that male fetal sex is associated with a greater risk of delivering preterm. Proposed biological explanations for this observation include a pro-inflammatory environment generated by a male fetus[181] and larger size at birth for males relative to females[182]. Increased risk of PTB when the fetus was male among only Caucasian women has also been observed, suggesting a potential interaction between race and fetal sex[183]. We observed significant associations with PTB unique to women carrying a male fetus for CRH, estriol, progesterone, and fT4, providing further evidence that the effect of fetal sex on the occurrence of PTB is complex, possibly involving diverse endocrine pathways.

Preeclampsia

Among all pregnancies, we observed reduced odds of preeclampsia with an increase in estriol at 26 weeks. In accordance with our findings, another study showed that estriol concentrations in the second trimester[192] were lower among women with preeclampsia than women with normal pregnancies. Previous studies have also found increased odds of preeclampsia with higher second trimester fT4 concentrations[177,193], and lower third trimester fT4 concentrations[194]. All associations we observed between fT4 and preeclampsia were inverse, and the inverse association at 26 weeks among female pregnancies was significant. The association between fT4 and preeclampsia has been shown to be modified by human chorionic gonadotropin (hCG) concentrations, with high fT4 positively associated with preeclampsia only when hCG is low[195]. This effect modification may be due to the known angiogenic role of hCG during early pregnancy[196].

Hormonal involvement in the etiology of preeclampsia is complex due to the angiogenic dysfunction of the affected uterus. In preeclampsia cases, proper remodeling and infiltration of blood vessels by placental extravillous trophoblasts does not occur, and this can be observed before the onset of clinical symptoms[197,198]. It is unclear whether endocrine disruption plays a

causal role in initiation of uterine dysfunction, or if uterine dysfunction triggers a maternal endocrine response in an attempt to adapt to the hypoxic state[199].

Gestational Diabetes Mellitus

A previous epidemiology study has demonstrated associations between high second trimester estriol concentrations and greater odds of GDM[179]. We also observed increased odds of GDM with estriol at 26 weeks. Testosterone concentrations were inversely associated with odds of GDM among male pregnancies in our study, which differs from previous research that showed higher testosterone concentrations among women with GDM[180] and with greater insulin resistance[200] compared to women with normal pregnancies.

Previous work has suggested that fT4 concentrations early in pregnancy are inversely associated with odds of GDM[194,201]. In accordance with those findings, the ratio of fT3 to fT4 has been observed to be positively associated with odds of GDM[202], suggesting that increased conversion of T4 to biologically active T3 may play a role in the onset of GDM. In alignment with those findings, we observed greater odds of GDM among all pregnancies with increased T3 concentrations at 18 weeks, and greater odds of GDM among male pregnancies with increased T3 at both study visits. We also observed an inverse association between fT4 and odds of GDM at 18 weeks among female pregnancies, while that association was positive among male pregnancies at 26 weeks. Previous work has shown that women with GDM have higher circulating concentrations of inflammatory cytokines such as IL-6 and TNF-alpha[203], which have been observed to be inversely associated with T3 concentrations[204]. These inflammatory markers may increase insulin resistance during pregnancy and, mediated by alterations in thyroid hormone concentrations, contribute to higher circulating glucose levels and increased odds of GDM. Several previous studies have observed greater risks for GDM among women carrying a male fetus[205–207], possibly due to poorer beta-cell function among male fetuses[208].

Birth Size

We observed that decreased birthweight among females was marginally associated with elevated T4 at 26 weeks. Previous work found similar inverse associations, but with fT4 instead of total T4[209,210]. Thyroid hormones are critical for fetal growth, possibly via their influences on fetal insulin-like growth factor, leptin, or the placenta's abilities to transfer nutrients[211]. Even in the case of nearly identical patterns of thyroid hormone concentrations throughout gestation between mothers, differences in expression of hormone transporters in the placenta and intracellular receptors in fetal tissues can result in different thyroid hormone exposure profiles for the fetus and, consequently, varying effects on fetal growth and development[77]. Assessment of thyroid hormone effects on birth outcomes in the second half of gestation is even more complex as the fetal thyroid gland begins to produce hormones and the fetus relies less on maternal supply of T4[77]. Conflicting results on the relationship between thyroid hormones and birthweight between studies may be due in part to unmeasured differences in fetal thyroid function.

Strengths and Limitations

The present study was subject to several limitations. We were not able to measure hCG or assess thyroid autoantibody status. Thus some of our results could be biased due to unmeasured confounding variables. Some critical changes in the maternal endocrine environment occur later in gestation than we were able to measure, such as the exponential increase in CRH right before the onset of labor. Although the goal of this study was to determine whether mid-pregnancy hormone levels were indicative of increased risk of adverse pregnancy outcomes, measurements at later time points could shed additional light on the various endocrine pathways implicated in adverse birth outcomes. We observed low rates of preeclampsia and GDM, which reduces the reliability of effect estimates. However, these lower rates were observed because we excluded women with preexisting conditions from our cohort to allow more precise examination of associations between hormone concentrations and birth outcomes, since preexisting conditions can influence hormone concentrations and susceptibility to adverse birth outcomes. Furthermore, excluding women with preexisting conditions may limit the generalizability of our findings. Finally, some results assessing preeclampsia and GDM may be subject to reverse

causation bias if the disease state, before clinical observation, resulted in the hormonal changes that we observed.

Despite the aforementioned limitations, this study was also strong in various ways. This is one of few studies to assess a broad panel of hormone concentrations at more than one time point during gestation to investigate relationships with various birth outcomes and different windows of susceptibility. Many epidemiological studies limit their analytical panel to either thyroid or steroid hormones, or do not assess the spontaneous subtype of PTB. We are also one of few groups to assess interactions between gestational hormone concentrations and fetal sex. Finally, our study was strengthened by a higher sample size of mothers than was seen in most previously published cohorts, which is particularly important when studying rare outcomes occurring in less than 5% of the population.

Conclusions

In conclusion, we observed a range of associations between hormones and adverse birth outcomes. We found differences based on the timing of hormone assessment, and many significant findings were unique to mothers carrying a male fetus. Future work will attempt to place these findings in the context of relevant environmental contaminants on the island of Puerto Rico by exploring possibilities of endocrine disruption as a mediator between chemical exposures and pregnancy outcomes. Additional studies are needed to more fully elucidate the role of altered hormone concentrations in the etiology of adverse birth outcomes.

Table III.1. Inclusion of covariates between different outcome models.

	PTB	Spontaneous PTB	Gestational Age	Birthweight Z-Score	SGA	LGA	Preeclampsia	GDM
Maternal Age	X	X	X	X	X	X	X	X
Maternal Education	X	X	X	X	X	X	X	X
Employment Status								
Annual Household Income								
Marital Status	X	X	X					
Smoking Status					X	X		
Environmental Tobacco Smoke Exposure	X	X	X					X
Alcohol Usage			X					X
Parity								
Pre-Pregnancy BMI			X	X			X	
Infant Sex								

Table III.2. Maternal demographic characteristics of 976 Puerto Rican mothers.

	N (%)
Maternal Age (years)	
18-24	354 (36.3%)
25-29	301 (30.8%)
30-34	206 (21.1%)
35-41	115 (11.8%)
Maternal Education	
GED or less	203 (21%)
Some College	331 (34.2%)
Bachelors or Higher	433 (44.8%)
Employment Status	
No	357 (37%)
Yes	608 (63%)
Annual Household Income	
<10k	269 (31.6%)
10k-<30k	268 (31.5%)
30k-<50k	203 (23.8%)
>=50k	112 (13.1%)
Marital Status	
Single	197 (20.4%)
Married	521 (53.9%)
Cohabitating	249 (25.7%)
Smoking Status	
Never	833 (86%)
Ever	121 (12.5%)
Current	15 (1.55%)
Daily Environmental Tobacco Smoke Exposure	
Never	808 (88.7%)
1 Hour or less	40 (4.39%)
>1 Hour	63 (6.92%)
Alcohol Use	
Never	504 (52.2%)
Yes, before Pregnancy	400 (41.4%)
Yes, currently	62 (6.42%)
Number of Previous Children	
0	355 (42.7%)

1	367 (44.2%)
2 to 5	109 (13.1%)
Pre-Pregnancy BMI	
[0,25]	520 (56.1%)
(25, 30]	240 (25.9%)
Above 30	167 (18%)
Fetal Sex	
Female	464 (48%)
Male	502 (52%)

Table III.3. Distributions of gestational average (GA)[a] and visit-specific hormone concentrations among 976 Puerto Rican mothers.

		N	min	25th	50th	75th	90th	95th	Max	Geometric Mean	Geometric SD	IQR	Visit P value[b]	ICC (95% CI)
CRH (pg/mL)	GA	976	3.50	15.4	43.2	86.3	118	148	243	35.7	2.77	70.9	0.914	0.71 (0.66, 0.74)
	V1	818	3.50	15.1	37.6	84.3	121	156	254	34.4	2.89	69.2		
	V2	602	3.50	14.7	39.3	88.2	130	159	249	34.2	2.95	73.4		
Estriol (mg/mL)	GA	971	0.74	15.6	23.1	33.0	44.7	57.5	265	22.7	1.80	17.4	**0.000**	-0.22 (-0.35, -0.11)
	V1	812	0.74	11.3	15.1	22.2	31.8	41.5	108	15.8	1.75	10.9		
	V2	600	6.90	29.3	38.2	50.5	64.4	74.6	265	38.7	1.55	21.2		
SHBG (pg/mL)	GA	976	47.6	413	538	668	818	895	1404	522	1.45	254	**0.000**	0.76 (0.72, 0.79)
	V1	820	47.6	389	516	630	775	850	1461	491	1.47	241		
	V2	602	123	434	566	723	898	979	1428	558	1.45	289		
Prog. (ng/mL)	GA	973	10.1	36.6	50.4	71.0	99.4	124	1037	51.8	1.68	34.5	**0.000**	0.07 (-0.04, 0.17)
	V1	815	10.1	29.2	39.3	54.5	71.9	85.0	301	40.1	1.59	25.3		
	V2	601	19.4	51.2	73.5	104	146	179	1037	74.4	1.70	53.2		
TSH (uIU/mL)	GA	971	0.03	0.71	1.10	1.72	2.38	2.99	32.4	1.08	1.96	1.02	**0.031**	0.72 (0.67, 0.75)
	V1	812	0.02	0.67	1.05	1.66	2.38	2.88	40.9	1.03	2.06	0.99		
	V2	600	0.11	0.72	1.15	1.75	2.43	3.23	25.7	1.12	1.96	1.03		
fT4 (ng/dL)	GA	976	0.11	1.09	1.62	2.02	2.32	2.50	8.35	1.41	1.68	0.93	0.452	0.75 (0.71, 0.79)
	V1	818	0.11	1.03	1.57	2.01	2.30	2.48	8.35	1.34	1.84	0.98		
	V2	602	0.11	1.10	1.61	2.03	2.33	2.49	4.68	1.39	1.75	0.93		
T4 (ug/dL)	GA	975	0.35	0.89	1.00	1.10	1.21	1.28	1.72	0.99	1.19	0.21	**0.000**	0.65 (0.59, 0.69)
	V1	818	0.35	0.90	1.01	1.12	1.21	1.28	1.72	1.00	1.19	0.22		
	V2	602	0.44	0.83	0.96	1.08	1.19	1.23	1.43	0.94	1.21	0.25		
T3 (mg/mL)	GA	971	6.20	10.5	11.8	13.2	14.4	15.2	19.0	11.7	1.18	2.70	**0.008**	0.72 (0.67, 0.75)
	V1	812	6.80	10.6	11.9	13.3	14.4	15.3	19.0	11.8	1.19	2.70		
	V2	600	5.30	10.3	11.6	13.0	14.2	14.9	20.6	11.5	1.19	2.75		
	GA	973	2.80	53.0	107	557	819	992	2868	160	3.55	504	**0.012**	0.86 (0.83, 0.88)

Test. (pg/mL)	V1	815	1.10	50.1	105	544	789	952	2500	156	3.66	493
	V2	601	9.20	59.3	121	650	933	1092	3291	185	3.64	591

[a]Gestational average values were calculated as arithmetic means for normally distributed hormones and geometric means for log-normally distributed hormones.

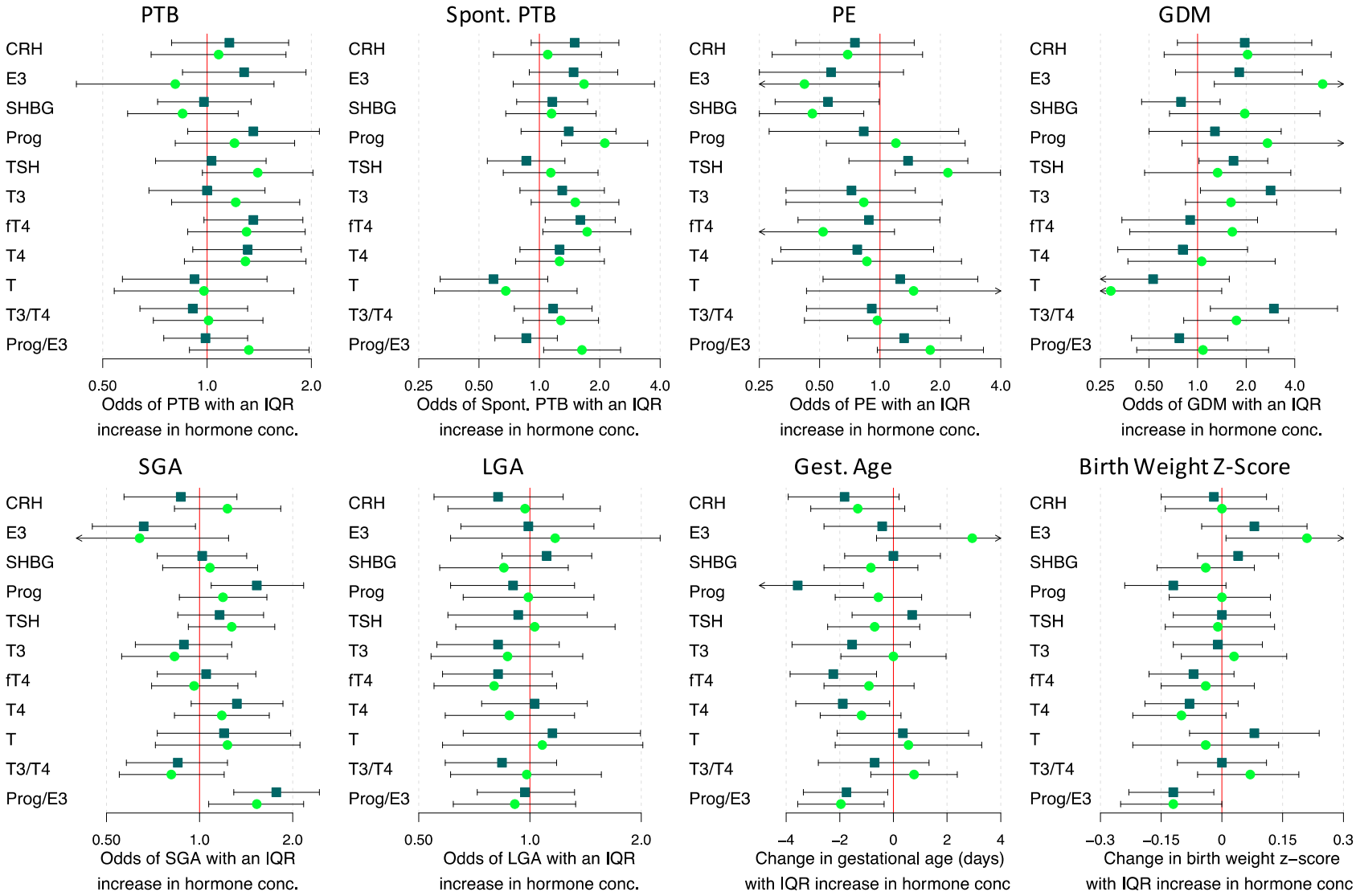
[b]P-value from a univariate linear model for association between hormone concentrations and study visit. Boldface p-values are <0.05.

Table III.4. Distributions of continuous and binary birth outcomes among 976 Puerto Rican mothers.

	Min	10th	25th	50th	75th	90th	Max
Gestational Age (weeks)	20.3	36.7	38.1	39.1	40	40.7	42.7
Birth Weight Z-Score (ounces)	-5.34 (19.0)	-1.19 (91.0)	-0.571 (102)	-0.00005 (113)	0.707 (123)	1.25 (133)	9.70 (224)

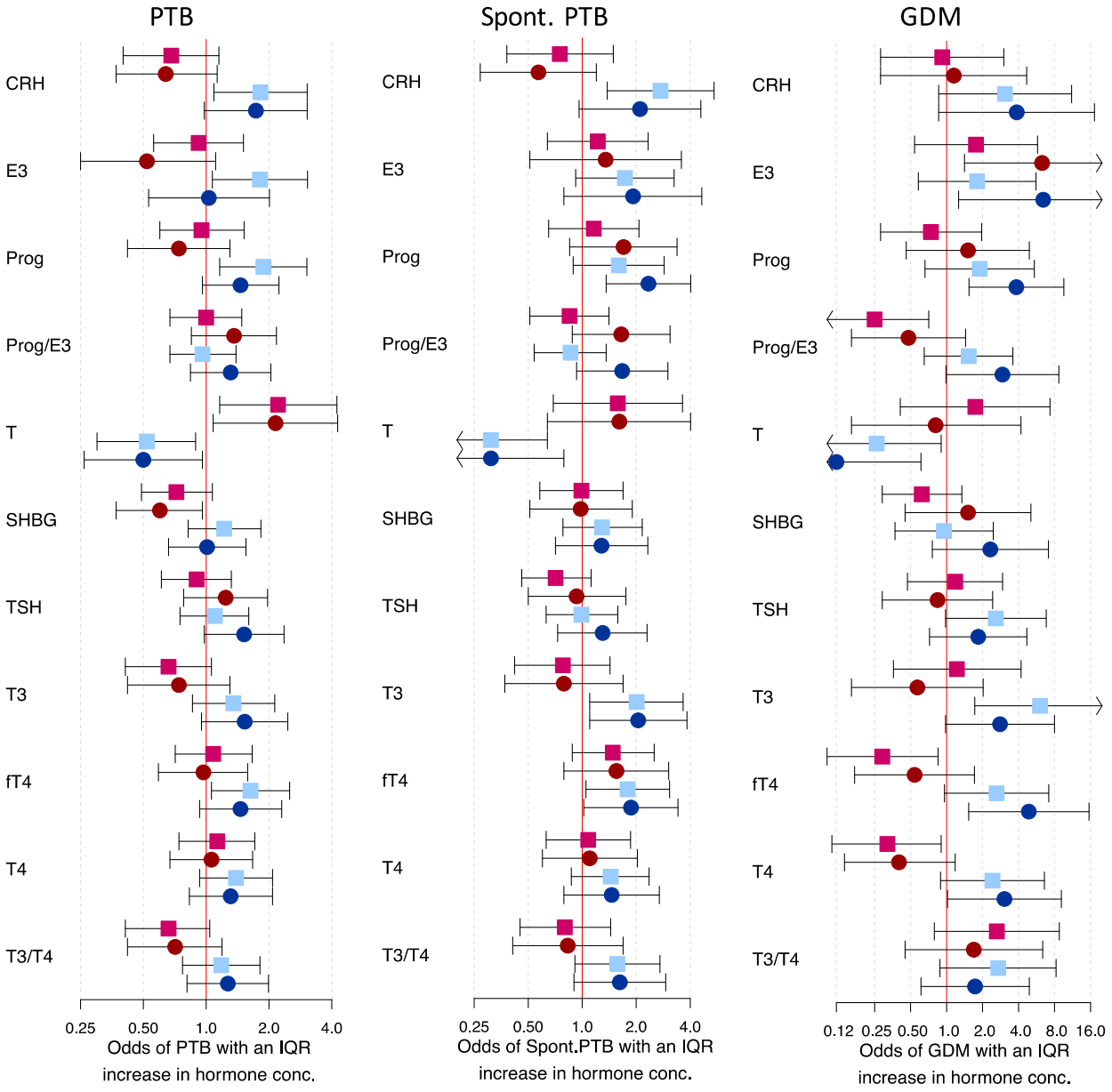
	N (%)
Preterm Birth	
No	867 (90.1%)
Yes	95 (9.88%)
Spontaneous Preterm Birth	
No	883 (94.2%)
Yes	54 (5.76%)
Preeclampsia	
No	947 (97.1%)
Yes	28 (2.87%)
Gestational Diabetes	
No	900 (98.1%)
Yes	17 (1.85%)
Small for Gestational Age	
No	842 (91.1%)
Yes	82 (8.87%)
Large for Gestational Age	
No	835 (90.4%)
Yes	89 (9.63%)

Figure III.1. Differential associations between hormones and birth outcomes measured at 18 and 26 weeks' gestation.



Dark green boxes represent effect estimates for hormones measured around 18 weeks, light green circles represent effect estimates for hormones measured around 26 weeks, black bars represent 95% confidence intervals, and vertical red lines represent the null value. IQR: Interquartile range.

Figure III.2. Differential associations between hormones and birth outcomes at 18 and 26 weeks based on fetal sex.



Pink boxes represent the effect estimates for hormones measured at 18 weeks among female pregnancies, red circles represent the effect estimates for hormones measured at 26 weeks among female pregnancies, light blue boxes represent the effect estimates for hormones measured at 18 weeks among male pregnancies, dark blue circles represent the effect estimates for hormones measured at 26 weeks among male pregnancies, and black bars represent the 95% confidence interval. The vertical red line represents the null value. IQR: Interquartile range.

Table III.5. Associations between birth outcomes and hormones measured at 18 and 26 weeks' gestation.

	Preterm Birth			Spontaneous Preterm Birth		
	18 weeks OR (95% CI)	Int P	26 weeks OR (95% CI)	18 weeks OR (95% CI)	Int P	26 weeks OR (95% CI)
CRH	1.16 (0.79, 1.72)	0.800	1.08 (0.69, 1.69)	1.50 (0.91, 2.49)	0.443	1.10 (0.59, 2.04)
Estriol	1.28 (0.85, 1.93)	0.241	0.81 (0.42, 1.56)	1.48 (0.89, 2.46)	0.804	1.67 (0.74, 3.75)
SHBG	0.98 (0.72, 1.34)	0.561	0.85 (0.59, 1.23)	1.16 (0.77, 1.74)	0.970	1.15 (0.68, 1.92)
Progesterone	1.36 (0.88, 2.11)	0.689	1.20 (0.81, 1.79)	1.40 (0.81, 2.41)	0.269	2.12 (1.29, 3.47)
TSH	1.03 (0.71, 1.48)	0.250	1.40 (0.97, 2.02)	0.86 (0.55, 1.34)	0.437	1.14 (0.66, 1.96)
T3	1.00 (0.68, 1.47)	0.519	1.21 (0.79, 1.85)	1.30 (0.80, 2.11)	0.679	1.51 (0.91, 2.49)
fT4	1.36 (0.98, 1.89)	0.855	1.30 (0.88, 1.92)	1.60 (1.07, 2.39)	0.810	1.73 (1.04, 2.86)
T4	1.31 (0.91, 1.87)	0.964	1.29 (0.86, 1.93)	1.26 (0.80, 2.00)	1.000	1.26 (0.76, 2.11)
Testosterone	0.92 (0.57, 1.49)	0.872	0.98 (0.54, 1.78)	0.59 (0.32, 1.10)	0.772	0.68 (0.30, 1.54)
T3/T4	0.91 (0.64, 1.31)	0.711	1.01 (0.70, 1.45)	1.17 (0.75, 1.83)	0.780	1.28 (0.83, 1.97)
Prog/E3	0.99 (0.75, 1.31)	0.243	1.32 (0.89, 1.97)	0.86 (0.60, 1.23)	0.026	1.63 (1.05, 2.54)
	Gestational Age at Birth (weeks)			**Birthweight Z-Score**		
	18 weeks β (95% CI)	Int P	26 weeks β (95% CI)	18 weeks β (95% CI)	Int P	26 weeks β (95% CI)
CRH	-0.26 (-0.56, 0.03)	0.702	-0.19 (-0.44, 0.06)	-0.02 (-0.15, 0.11)	0.783	0.00 (-0.14, 0.14)
Estriol	-0.06 (-0.37, 0.25)	0.113	0.42 (-0.09, 0.93)	0.08 (-0.05, 0.21)	0.292	0.21 (0.01, 0.41)
SHBG	0.00 (-0.26, 0.25)	0.512	-0.12 (-0.37, 0.13)	0.04 (-0.06, 0.14)	0.341	-0.04 (-0.16, 0.08)
Progesterone	-0.51 (-0.86,-0.16)	0.044	-0.08 (-0.31, 0.15)	-0.12 (-0.24, 0.01)	0.201	0.00 (-0.13, 0.12)
TSH	0.10 (-0.22, 0.41)	0.323	-0.10 (-0.35, 0.14)	0.00 (-0.12, 0.12)	0.939	-0.01 (-0.14, 0.13)
T3	-0.22 (-0.54, 0.09)	0.302	0.00 (-0.28, 0.28)	-0.01 (-0.12, 0.10)	0.641	0.03 (-0.10, 0.16)
fT4	-0.32 (-0.55 -0.09)	0.278	-0.13 (-0.37, 0.11)	-0.07 (-0.18, 0.03)	0.638	-0.04 (-0.15, 0.08)
T4	-0.27 (-0.52 -0.02)	0.577	-0.17 (-0.39, 0.04)	-0.08 (-0.19, 0.04)	0.744	-0.10 (-0.22, 0.01)
Testosterone	0.05 (-0.30, 0.40)	0.895	0.08 (-0.31, 0.47)	0.08 (-0.08, 0.24)	0.306	-0.04 (-0.22, 0.14)
T3/T4	-0.10 (-0.40, 0.19)	0.269	0.11 (-0.12, 0.34)	0.00 (-0.11, 0.11)	0.448	0.07 (-0.06, 0.19)
Prog/E3	-0.25 (-0.48,-0.03)	0.856	-0.28 (-0.51,-0.05)	-0.12 (-0.23,-0.02)	0.985	-0.12 (-0.25, 0.00)
	Small for Gestational Age			**Large for Gestational Age**		
	18 weeks OR (95% CI)	Int P	26 weeks OR (95% CI)	18 weeks OR (95% CI)	Int P	26 weeks OR (95% CI)

CRH	0.87 (0.57, 1.32)	0.232	1.23 (0.83, 1.83)	0.82 (0.55, 1.23)	0.602	0.97 (0.60, 1.55)
Estriol	0.66 (0.45, 0.97)	0.940	0.64 (0.33, 1.24)	0.99 (0.65, 1.49)	0.662	1.17 (0.61, 2.25)
SHBG	1.02 (0.73, 1.42)	0.798	1.08 (0.76, 1.54)	1.11 (0.84, 1.47)	0.274	0.85 (0.57, 1.27)
Progesterone	1.53 (1.09, 2.17)	0.295	1.19 (0.86, 1.65)	0.90 (0.61, 1.32)	0.715	0.99 (0.66, 1.49)
TSH	1.16 (0.85, 1.61)	0.706	1.27 (0.92, 1.75)	0.93 (0.60, 1.43)	0.756	1.03 (0.63, 1.70)
T3	0.89 (0.62, 1.27)	0.794	0.83 (0.56, 1.23)	0.82 (0.56, 1.20)	0.844	0.87 (0.54, 1.39)
fT4	1.05 (0.73, 1.52)	0.727	0.96 (0.70, 1.33)	0.82 (0.58, 1.15)	0.951	0.80 (0.55, 1.18)
T4	1.32 (0.94, 1.86)	0.643	1.18 (0.83, 1.68)	1.03 (0.74, 1.43)	0.568	0.88 (0.59, 1.32)
Testosterone	1.20 (0.73, 1.97)	0.944	1.23 (0.72, 2.11)	1.15 (0.66, 1.99)	0.880	1.08 (0.58, 2.02)
T3/T4	0.85 (0.58, 1.23)	0.886	0.81 (0.55, 1.20)	0.84 (0.59, 1.18)	0.597	0.98 (0.61, 1.56)
Prog/E3	1.77 (1.29, 2.44)	0.537	1.53 (1.07, 2.17)	0.97 (0.72, 1.32)	0.772	0.91 (0.62, 1.33)
	Preeclampsia			**Gestational Diabetes**		
	18 weeks OR (95% CI)	**Int P**	**26 weeks OR (95% CI)**	**18 weeks OR (95% CI)**	**Int P**	**26 weeks OR (95% CI)**
CRH	0.75 (0.38, 1.48)	0.871	0.69 (0.29, 1.63)	1.96 (0.75, 5.09)	0.953	2.04 (0.62, 6.72)
Estriol	0.57 (0.25, 1.31)	0.604	0.42 (0.17, 0.99)	1.81 (0.73, 4.45)	0.184	5.95 (1.27, 27.8)
SHBG	0.55 (0.30, 0.99)	0.638	0.46 (0.25, 0.83)	0.79 (0.45, 1.38)	0.137	1.96 (0.67, 5.72)
Progesterone	0.83 (0.28, 2.47)	0.593	1.20 (0.54, 2.66)	1.28 (0.50, 3.29)	0.324	2.71 (0.80, 9.12)
TSH	1.38 (0.70, 2.74)	0.335	2.18 (1.19, 3.99)	1.67 (1.02, 2.72)	0.697	1.33 (0.47, 3.78)
T3	0.72 (0.34, 1.50)	0.807	0.83 (0.34, 2.04)	2.83 (1.04, 7.68)	0.335	1.61 (0.84, 3.09)
fT4	0.88 (0.39, 1.99)	0.372	0.52 (0.22, 1.18)	0.90 (0.34, 2.35)	0.486	1.64 (0.38, 7.20)
T4	0.77 (0.32, 1.85)	0.875	0.86 (0.29, 2.55)	0.81 (0.32, 2.04)	0.697	1.06 (0.37, 3.02)
Testosterone	1.26 (0.52, 3.07)	0.839	1.47 (0.43, 5.00)	0.53 (0.18, 1.57)	0.500	0.29 (0.06, 1.41)
T3/T4	0.91 (0.43, 1.93)	0.917	0.97 (0.42, 2.22)	2.97 (1.20, 7.35)	0.352	1.74 (0.82, 3.67)
Prog/E3	1.32 (0.69, 2.54)	0.514	1.78 (0.97, 3.28)	0.77 (0.39, 1.54)	0.566	1.08 (0.42, 2.75)

CRH, estriol, SHBG, progesterone, TSH, testosterone, and prog/E3 were natural log transformed for analyses.
Effect estimates refer to an interquartile range increase in hormone concentration.
Int P indicates significance of effect modification by study visit – i.e. the p-value for the interaction term between hormone concentration and study visit.

Table III.6. Differential associations between birth outcomes and hormones measured at 18 and 26 weeks by fetal sex.

	Preterm Birth				
	Female pregnancies			Male pregnancies	
	18 weeks	26 weeks		18 weeks	26 weeks
	OR (95% CI)	OR (95% CI)	Int P	OR (95% CI)	OR (95% CI)
CRH	0.68 (0.40, 1.15)	0.64 (0.37, 1.13)	0.002	1.82 (1.09, 3.05)	1.73 (0.98, 3.05)
Estriol	0.92 (0.56, 1.51)	0.52 (0.25, 1.11)	0.022	1.81 (1.07, 3.06)	1.03 (0.53, 2.01)
SHBG	0.72 (0.49, 1.07)	0.60 (0.37, 0.96)	0.032	1.22 (0.82, 1.83)	1.01 (0.66, 1.55)
Progesterone	0.95 (0.60, 1.52)	0.74 (0.42, 1.30)	0.011	1.88 (1.16, 3.04)	1.46 (0.96, 2.23)
TSH	0.90 (0.61, 1.32)	1.24 (0.78, 1.97)	0.397	1.10 (0.75, 1.60)	1.52 (0.98, 2.36)
T3	0.66 (0.41, 1.06)	0.74 (0.42, 1.30)	0.013	1.35 (0.86, 2.13)	1.53 (0.95, 2.46)
fT4	1.08 (0.71, 1.66)	0.97 (0.59, 1.58)	0.115	1.63 (1.06, 2.51)	1.46 (0.93, 2.30)
T4	1.13 (0.74, 1.71)	1.06 (0.67, 1.67)	0.397	1.39 (0.93, 2.08)	1.31 (0.83, 2.08)
Testosterone	2.21 (1.16, 4.23)	2.15 (1.08, 4.27)	0.000	0.52 (0.30, 0.89)	0.50 (0.26, 0.96)
T3/T4	0.66 (0.41, 1.04)	0.71 (0.42, 1.19)	0.032	1.18 (0.77, 1.81)	1.27 (0.81, 1.99)
Prog/E3	1.00 (0.67, 1.48)	1.36 (0.85, 2.17)	0.880	0.96 (0.67, 1.39)	1.31 (0.84, 2.04)
	Spontaneous Preterm Birth				
	Female pregnancies			Male pregnancies	
	18 weeks	26 weeks		18 weeks	26 weeks
	OR (95% CI)	OR (95% CI)	Int P	OR (95% CI)	OR (95% CI)
CRH	0.75 (0.38, 1.49)	0.57 (0.27, 1.20)	0.003	2.73 (1.38, 5.43)	2.10 (0.96, 4.58)
Estriol	1.22 (0.64, 2.33)	1.35 (0.51, 3.57)	0.340	1.73 (0.92, 3.25)	1.92 (0.79, 4.64)
SHBG	0.99 (0.58, 1.69)	0.98 (0.51, 1.90)	0.418	1.29 (0.78, 2.16)	1.28 (0.71, 2.32)
Progesterone	1.16 (0.65, 2.07)	1.70 (0.85, 3.38)	0.303	1.60 (0.89, 2.86)	2.34 (1.36, 4.03)
TSH	0.71 (0.46, 1.12)	0.93 (0.50, 1.75)	0.265	0.99 (0.63, 1.58)	1.30 (0.73, 2.30)
T3	0.78 (0.42, 1.43)	0.79 (0.37, 1.69)	0.013	2.01 (1.10, 3.65)	2.05 (1.10, 3.84)
fT4	1.48 (0.88, 2.52)	1.55 (0.79, 3.03)	0.577	1.79 (1.05, 3.07)	1.87 (1.02, 3.42)
T4	1.08 (0.63, 1.86)	1.10 (0.60, 2.03)	0.386	1.44 (0.87, 2.36)	1.46 (0.79, 2.69)
Testosterone	1.58 (0.69, 3.62)	1.61 (0.64, 4.02)	0.001	0.31 (0.15, 0.64)	0.31 (0.13, 0.79)
T3/T4	0.80 (0.45, 1.44)	0.83 (0.41, 1.69)	0.063	1.57 (0.91, 2.71)	1.62 (0.90, 2.92)
Prog/E3	0.85 (0.51, 1.41)	1.65 (0.88, 3.09)	0.979	0.86 (0.54, 1.36)	1.67 (0.93, 3.00)
	Gestational Age at Birth				
	Female pregnancies			Male pregnancies	

	18 weeks	26 weeks		18 weeks	26 weeks
	OR (95% CI)	OR (95% CI)	Int P	OR (95% CI)	OR (95% CI)
CRH	-0.18 (-0.53, 0.16)	-0.10 (-0.46, 0.27)	0.319	-0.39 (-0.71, -0.06)	-0.30 (-0.66, 0.06)
Estriol	0.01 (-0.32, 0.33)	0.56 (0.11, 1.01)	0.125	-0.28 (-0.63, 0.07)	0.28 (-0.17, 0.74)
SHBG	-0.12 (-0.39, 0.15)	-0.16 (-0.47, 0.15)	0.510	-0.01 (-0.28, 0.25)	-0.05 (-0.35, 0.24)
Progesterone	-0.30 (-0.61, 0.02)	0.16 (-0.17, 0.49)	0.015	-0.70 (-1.01, -0.39)	-0.24 (-0.54, 0.06)
TSH	0.07 (-0.18, 0.33)	-0.08 (-0.38, 0.21)	0.914	0.06 (-0.20, 0.31)	-0.10 (-0.40, 0.19)
T3	-0.06 (-0.37, 0.26)	0.12 (-0.24, 0.48)	0.281	-0.26 (-0.56, 0.04)	-0.08 (-0.41, 0.24)
fT4	-0.19 (-0.46, 0.08)	-0.01 (-0.33, 0.30)	0.245	-0.39 (-0.68, -0.10)	-0.21 (-0.51, 0.09)
T4	-0.15 (-0.42, 0.13)	-0.13 (-0.42, 0.17)	0.653	-0.22 (-0.50, 0.06)	-0.20 (-0.51, 0.11)
Testosterone	-0.07 (-0.47, 0.33)	-0.11 (-0.55, 0.32)	0.121	0.29 (-0.09, 0.66)	0.24 (-0.18, 0.66)
T3/T4	0.01 (-0.29, 0.32)	0.22 (-0.12, 0.56)	0.260	-0.19 (-0.48, 0.10)	0.02 (-0.29, 0.33)
Prog/E3	-0.16 (-0.40, 0.09)	-0.23 (-0.53, 0.07)	0.542	-0.25 (-0.50, -0.01)	-0.32 (-0.61, -0.04)

Birthweight Z-Score

	Female pregnancies			Male pregnancies	
	18 weeks	26 weeks		18 weeks	26 weeks
	OR (95% CI)	OR (95% CI)	Int P	OR (95% CI)	OR (95% CI)
CRH	-0.01 (-0.17, 0.16)	0.02 (-0.16, 0.19)	0.763	-0.04 (-0.20, 0.12)	-0.01 (-0.19, 0.16)
Estriol	0.00 (-0.17, 0.16)	0.11 (-0.11, 0.34)	0.030	0.19 (0.02, 0.36)	0.31 (0.08, 0.53)
SHBG	0.05 (-0.08, 0.19)	-0.02 (-0.17, 0.13)	0.646	0.02 (-0.11, 0.15)	-0.05 (-0.20, 0.09)
Progesterone	-0.14 (-0.30, 0.02)	-0.03 (-0.20, 0.13)	0.505	-0.09 (-0.24, 0.07)	0.02 (-0.13, 0.17)
TSH	-0.03 (-0.16, 0.10)	-0.04 (-0.18, 0.11)	0.495	0.02 (-0.10, 0.15)	0.02 (-0.13, 0.16)
T3	-0.04 (-0.20, 0.11)	-0.01 (-0.18, 0.17)	0.527	0.01 (-0.12, 0.15)	0.05 (-0.11, 0.21)
fT4	-0.10 (-0.23, 0.04)	-0.06 (-0.22, 0.09)	0.545	-0.05 (-0.19, 0.10)	-0.01 (-0.16, 0.13)
T4	-0.12 (-0.26, 0.01)	-0.15 (-0.29, 0.00)	0.279	-0.03 (-0.17, 0.10)	-0.06 (-0.21, 0.09)
Testosterone	0.14 (-0.05, 0.34)	0.02 (-0.19, 0.24)	0.299	0.02 (-0.16, 0.21)	-0.09 (-0.30, 0.11)
T3/T4	0.00 (-0.14, 0.15)	0.06 (-0.10, 0.23)	0.993	0.00 (-0.13, 0.13)	0.06 (-0.08, 0.21)
Prog/E3	-0.06 (-0.18, 0.06)	-0.05 (-0.20, 0.10)	0.086	-0.19 (-0.31, -0.07)	-0.18 (-0.33, -0.04)

Small for Gestational Age

	Female pregnancies			Male pregnancies	
	18 weeks	26 weeks		18 weeks	26 weeks
	OR (95% CI)	OR (95% CI)	Int P	OR (95% CI)	OR (95% CI)
CRH	0.90 (0.53, 1.55)	1.28 (0.75, 2.19)	0.821	0.84 (0.51, 1.40)	1.19 (0.70, 2.02)
Estriol	0.76 (0.45, 1.30)	0.76 (0.40, 1.45)	0.216	0.55 (0.32, 0.94)	0.54 (0.28, 1.05)

SHBG	1.17 (0.76, 1.80)	1.25 (0.81, 1.93)	0.287	0.90 (0.60, 1.35)	0.96 (0.64, 1.45)
Progesterone	1.42 (0.87, 2.32)	1.09 (0.67, 1.77)	0.564	1.64 (1.00, 2.68)	1.26 (0.83, 1.92)
TSH	1.01 (0.66, 1.54)	1.10 (0.71, 1.71)	0.265	1.33 (0.88, 2.03)	1.46 (0.95, 2.24)
T3	1.03 (0.63, 1.70)	0.98 (0.58, 1.66)	0.344	0.79 (0.50, 1.25)	0.75 (0.47, 1.18)
fT4	1.17 (0.76, 1.80)	1.10 (0.69, 1.76)	0.383	0.93 (0.59, 1.48)	0.87 (0.57, 1.34)
T4	1.32 (0.85, 2.04)	1.17 (0.76, 1.81)	0.964	1.34 (0.87, 2.05)	1.19 (0.76, 1.84)
Testosterone	1.11 (0.59, 2.10)	1.14 (0.60, 2.18)	0.696	1.28 (0.70, 2.32)	1.31 (0.71, 2.41)
T3/T4	0.93 (0.57, 1.51)	0.90 (0.55, 1.48)	0.541	0.78 (0.50, 1.22)	0.76 (0.49, 1.19)
Prog/E3	1.39 (0.94, 2.06)	1.15 (0.75, 1.76)	0.022	2.39 (1.59, 3.60)	1.98 (1.29, 3.05)

Large for Gestational Age

	Female pregnancies			**Male pregnancies**	
	18 weeks	**26 weeks**		**18 weeks**	**26 weeks**
	OR (95% CI)	**OR (95% CI)**	**Int P**	**OR (95% CI)**	**OR (95% CI)**
CRH	0.81 (0.49, 1.34)	0.96 (0.54, 1.69)	0.936	0.83 (0.51, 1.36)	0.98 (0.55, 1.74)
Estriol	0.94 (0.58, 1.54)	1.11 (0.53, 2.33)	0.707	1.05 (0.62, 1.76)	1.23 (0.59, 2.58)
SHBG	1.22 (0.80, 1.84)	0.94 (0.58, 1.52)	0.488	1.02 (0.68, 1.52)	0.79 (0.50, 1.24)
Progesterone	0.91 (0.56, 1.48)	1.01 (0.59, 1.73)	0.898	0.88 (0.54, 1.42)	0.98 (0.60, 1.61)
TSH	0.80 (0.54, 1.20)	0.89 (0.55, 1.44)	0.265	1.06 (0.72, 1.58)	1.18 (0.74, 1.89)
T3	0.78 (0.49, 1.25)	0.83 (0.47, 1.46)	0.772	0.85 (0.55, 1.31)	0.90 (0.54, 1.51)
fT4	0.81 (0.53, 1.23)	0.80 (0.48, 1.32)	0.944	0.83 (0.53, 1.29)	0.81 (0.50, 1.32)
T4	0.97 (0.63, 1.47)	0.83 (0.52, 1.34)	0.635	1.09 (0.72, 1.65)	0.94 (0.58, 1.53)
Testosterone	1.40 (0.76, 2.59)	1.32 (0.65, 2.65)	0.306	0.97 (0.54, 1.72)	0.91 (0.46, 1.79)
T3/T4	0.78 (0.50, 1.24)	0.92 (0.54, 1.55)	0.682	0.88 (0.58, 1.33)	1.03 (0.63, 1.66)
Prog/E3	1.03 (0.71, 1.50)	0.96 (0.59, 1.58)	0.638	0.92 (0.63, 1.35)	0.86 (0.53, 1.39)

Preeclampsia

	Female pregnancies			**Male pregnancies**	
	18 weeks	**26 weeks**		**18 weeks**	**26 weeks**
	OR (95% CI)	**OR (95% CI)**	**Int P**	**OR (95% CI)**	**OR (95% CI)**
CRH	0.77 (0.30, 1.97)	0.65 (0.25, 1.73)	0.808	0.67 (0.24, 1.85)	0.56 (0.19, 1.66)
Estriol	0.68 (0.27, 1.70)	0.38 (0.11, 1.31)	0.669	0.87 (0.28, 2.66)	0.48 (0.13, 1.71)
SHBG	0.53 (0.27, 1.03)	0.34 (0.14, 0.81)	0.360	0.83 (0.35, 1.93)	0.52 (0.21, 1.28)
Progesterone	0.75 (0.32, 1.77)	0.62 (0.23, 1.70)	0.136	1.57 (0.61, 4.05)	1.30 (0.59, 2.85)
TSH	1.69 (0.82, 3.48)	2.41 (1.11, 5.23)	0.760	1.46 (0.62, 3.42)	2.08 (0.82, 5.27)
T3	0.67 (0.28, 1.62)	0.75 (0.29, 1.95)	0.742	0.80 (0.32, 2.01)	0.90 (0.33, 2.43)

fT4	0.71 (0.33, 1.54)	0.40 (0.17, 0.92)	0.730	0.84 (0.35, 2.04)	0.47 (0.19, 1.16)
T4	0.72 (0.32, 1.60)	0.69 (0.30, 1.61)	0.537	0.99 (0.41, 2.37)	0.95 (0.36, 2.48)
Testosterone	1.90 (0.58, 6.29)	2.49 (0.72, 8.67)	0.472	1.15 (0.35, 3.76)	1.50 (0.39, 5.85)
T3/T4	0.91 (0.39, 2.12)	1.01 (0.41, 2.50)	0.971	0.90 (0.38, 2.12)	0.99 (0.40, 2.47)
Prog/E3	1.02 (0.52, 2.02)	1.25 (0.61, 2.53)	0.232	1.74 (0.79, 3.84)	2.12 (0.95, 4.71)

Gestational Diabetes

	Female pregnancies			**Male pregnancies**	
	18 weeks	**26 weeks**		**18 weeks**	**26 weeks**
	OR (95% CI)	**OR (95% CI)**	**Int P**	**OR (95% CI)**	**OR (95% CI)**
CRH	0.92 (0.28, 3.01)	1.15 (0.28, 4.67)	0.122	3.08 (0.86, 11.10)	3.86 (0.86, 17.27)
Estriol	1.76 (0.54, 5.77)	6.29 (1.41, 28.00)	0.970	1.80 (0.58, 5.59)	6.43 (1.26, 32.78)
SHBG	0.62 (0.29, 1.34)	1.51 (0.45, 5.07)	0.429	0.95 (0.37, 2.46)	2.32 (0.76, 7.12)
Progesterone	0.74 (0.28, 1.96)	1.51 (0.46, 4.92)	0.081	1.89 (0.66, 5.42)	3.83 (1.54, 9.57)
TSH	1.18 (0.47, 2.94)	0.84 (0.29, 2.43)	0.179	2.58 (0.98, 6.81)	1.84 (0.72, 4.69)
T3	1.22 (0.36, 4.20)	0.57 (0.16, 2.02)	0.028	6.04 (1.72, 21.26)	2.80 (0.98, 7.98)
fT4	0.29 (0.10, 0.85)	0.54 (0.17, 1.71)	0.001	2.62 (0.96, 7.15)	4.87 (1.53, 15.52)
T4	0.32 (0.11, 0.90)	0.40 (0.14, 1.18)	0.002	2.42 (0.89, 6.57)	3.05 (1.02, 9.13)
Testosterone	1.74 (0.41, 7.32)	0.81 (0.16, 4.18)	0.025	0.26 (0.08, 0.90)	0.12 (0.02, 0.61)
T3/T4	2.63 (0.79, 8.76)	1.69 (0.45, 6.36)	0.972	2.70 (0.88, 8.24)	1.73 (0.61, 4.92)
Prog/E3	0.25 (0.09, 0.71)	0.48 (0.16, 1.44)	0.004	1.53 (0.65, 3.58)	2.93 (0.99, 8.69)

CRH, estriol, SHBG, progesterone, TSH, testosterone, and prog/E3 were natural log transformed for analyses.
Effect estimates refer to an interquartile range increase in hormone concentration.
Int P indicates significance of effect modification by fetal sex – i.e. the p-value for the interaction term between hormone concentration and fetal sex indicator.

Chapter IV. Longitudinal Mediation by Hormone Concentrations on the Associations Between Exposure to Phthalate Mixtures and Adverse Birth Outcomes Among Male Pregnancies

ABSTRACT

Background: Phthalates are used in the manufacturing of a myriad of consumer products, resulting in ubiquitous human exposure to a mixture of phthalate compounds. Previous work has suggested that phthalates display endocrine disrupting capabilities, and associations with adverse birth outcomes including preterm birth.

Objectives: Given the importance of hormone regulation during pregnancy, we hypothesized that phthalates may affect pregnancy outcomes via disruption of hormone concentrations. This work therefore aimed to assess the mediating effects of hormone concentrations on the associations between phthalate mixtures and adverse birth outcomes.

Methods: Repeated urinary phthalate metabolite (N=13) and serum hormone (N=9) measurements were taken at 16-20, 20-24 (urine only), and 24-28 weeks gestation among 1011 women in the PROTECT (Puerto Rico Testsite for Exploring Contamination Threats) longitudinal birth cohort. We utilized ridge regression to create phthalate environmental risk scores (ERS) at each study visit and specific to phthalates of high versus low molecular weight (LMW, HMW), which represent a weighted sum of each individual's exposure to the mixture of metabolites. Causal mediation analyses were then conducted on a subset of 705 women for whom hormone data was available. All analyses were conducted separately by study visit and fetal sex.

Results: Though total effects did not reach statistical significance, various hormones including CRH, progesterone, testosterone, and TSH showed suggestive evidence of mediating the association between exposure to LMW phthalates and risk of early delivery. Changes in TSH were important at 24-28 weeks, while changes on the other hormones were important earlier in

pregnancy at 16-20 weeks. Interestingly, there was no evidence of mediation by hormones on the associations between exposure to HMW phthalates and risk of early delivery among pregnancies with a male fetus, nor was there evidence of mediation by hormones with exposure to any phthalates among pregnancies with a female fetus.

Discussion: These results provide introductory evidence of hormone disruption on the causal pathway between phthalate exposure and preterm birth. Larger overlap of phthalate exposure and hormone mediator measurements, as well as higher case number, are necessary to validate these findings.

Introduction

Humans are exposed to a myriad of environmental contaminants from diverse sources on a daily basis. The result is a consistent body burden of a mixture of many different toxicants which have unknown effects on human physiology. Many epidemiology and toxicology studies have explored health effects of single pollutants, but very few have attempted to understand the biological effects of complex mixtures. Pregnant women are especially susceptible to adverse health outcomes resulting from environmental exposures, particularly those with endocrine disrupting capabilities. Hormone concentrations through pregnancy are important for proper fetal development, maintenance of the uterine wall, and initiation of pro-labor events[170–175]. Understanding how exposures to environmental chemical mixtures may interfere with hormone regulation in pregnant women is critically important for protection of this vulnerable population.

Phthalates are synthetic plasticizers used in production of many consumer products such as vinyl flooring, plastic food packaging, and personal care products[212]. Humans are never exposed to single phthalate compounds; exposure rather occurs in complex mixtures which differ based on an individual's use of consumer products, socioeconomic status, and diet[49]. Each parent phthalate compound is metabolized into a bioactive form within the body, and sometimes several different metabolites result from one parent compound[213], furthering the need to study mixtures of phthalates rather than individual metabolites. Phthalate metabolites are often highly

correlated with one another, and so methods which accommodate issues of multicollinearity are preferred over those which assess associations with many individual metabolites.

Previous research has shown phthalate metabolites to be associated with preterm and spontaneous preterm birth, as well as earlier gestational age at delivery[47,57–60,101,136,214]. Phthalates are also known endocrine disruptors, and greater exposures to phthalates have been associated with altered concentrations of various hormones that are important for pregnancy such as corticotropin releasing hormone (CRH), estriol, progesterone, thyroid hormones, and testosterone[78,79,83,160,191]. Given the hormonal activity of phthalates and their association with early delivery, we have hypothesized that phthalate exposure may lead to adverse pregnancy outcomes via disruption of hormone concentrations throughout pregnancy.

To test this hypothesis, we utilize a novel analysis pipeline which incorporates repeated measures of phthalate mixture exposure and hormone concentrations, in addition to causal mediation analyses. We use ridge regression to construct environmental risk scores (ERS), which are weighted sums of one's overall exposure to a mixture of phthalate metabolites, to assess exposure to high and low molecular weight phthalate mixtures at an individual level over multiple time points during gestation. ERS were then used in causal mediation analysis to determine the mediating effect of hormone concentrations on the associations between phthalate mixtures and adverse birth outcomes.

Methods

Study Population

Data for the present study was obtained from the PROTECT (Puerto Rico Testsite for Exploring Contamination Threats) cohort, a longitudinal birth cohort in the northern karst region of Puerto Rico designed to investigate environmental contaminants in relation to adverse pregnancy outcomes. Details of the study design and recruitment protocols have been previously described[139]. Briefly, women were recruited at 14$\pm$2 weeks gestation and were eligible to participate if they were between the ages of 18 and 40 years, participated in their first clinic visit

before their 20th week of pregnancy, had not taken oral contraceptives within 3 months of getting pregnant, had not used *in vitro* fertilization to get pregnant, and had no known preexisting medical or obstetric conditions. This study was approved by the research and ethics committees of the University of Michigan School of Public Health, University of Puerto Rico, Northeastern University, and participating hospitals and clinics. All study participants provided full informed consent prior to participation.

Phthalate Exposure Assessment

All spot urine samples were frozen at -80°C and shipped over night on dry ice to the CDC for analysis. All samples were analyzed for 13 phthalate metabolites: mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), monobenzyl phthalate (MBzP), mono-isobutyl phthalate (MiBP), mono-hydroxyisobutyl phthalate (MHiBP), mono-3-carboxypropyl phthalate (MCPP), mono carboxyisononyl phthalate (MCNP), mono carboxyisooctyl phthalate (MCOP), and mono-hydroxybutyl phthalate (MHBP). Urine samples were analyzed using solid phase extraction high-performance liquid chromatography-isotope dilution tandem mass spectrometry, the details of which are described elsewhere[140]. Values detected below the limit of detection (LOD) were assigned a value of the LOD divided by the square root of two[141].

Hormone Measurement

All women provided serum samples at their first and third clinic visits, aligning with median 18 (16-20) and 26 (24-28) weeks' gestation. Serum samples were analyzed at the Central Ligand Assay Satellite Services (CLASS) laboratory in the Department of Epidemiology at the University of Michigan School of Public Health. Progesterone, sex hormone-binding globulin (SHBG), testosterone, total triiodothyronine (T3), total thyroxine (T4), free thyroxine (fT4) and thyroid-stimulating hormone (TSH) were measured using a chemiluminescence immunoassay. Estriol (E3) and corticotropin releasing hormone (CRH) were measured using an enzyme immunoassay. Some hormone concentrations were not available for all participants due to sample volume limitations.

The ratios of progesterone to estriol (Prog/E3) and T3 to T4 (T3/T4) were assessed in addition to measured hormones because of previous research indicating that the ratios may be better indices of adverse pregnancy outcomes than single hormone measurements[142–144]. All hormone concentrations below the limit of detection (LOD) were replaced by the LOD divided by the square root of two.

Birth Outcome Assessment

Self-reported date of the last menstrual period was collected at the first study visit and used in combination with early ultrasound measurements to determine gestational age at birth, based on recommendations from the American College of Obstetricians and Gynecologists[184]. PTB was defined as delivery before 37 weeks gestation. We also assessed spontaneous PTB, defined as PTB presenting with premature rupture of membranes, spontaneous preterm labor, or both[17].

Statistical Analyses

Distributions of demographic characteristics and other relevant health information were tabulated. Environmental risk scores were calculated for all women in the study sample for whom we had full exposure data and data on at least one birth outcome (N=1011). Mediation analyses were conducted on a subset of those women for whom we also had mediator data (N=705).

Calculation of Phthalate ERS

Study participant's exposures to mixtures of phthalates were estimated utilizing ridge regression to calculate environmental risk scores (ERS), which represent a weighted sum of each individual's overall phthalate exposure profile. Ridge employs two tuning parameters, lambda and alpha, which shrink the coefficients of unimportant predictors towards zero (but never to zero) and stabilize selection in the presence of highly correlated predictors. Five-fold cross validation and optimization of prediction errors were used to estimate lambda. Ridge returns a vector of coefficients which represent the relative importance of each predictor for the outcome of interest. These coefficients were then multiplied by each study participant's measured phthalate metabolite concentrations, giving weighted concentrations of each metabolite. Weighted

concentrations were then summed to arrive at the ERS. Effects of high versus low molecular weight phthalates were assessed by running ridge analysis on metabolite mixtures separated into high versus low molecular weight groups, and then constructing a high molecular weight (HMW) ERS and a low molecular weight (LMW) ERS.

Ridge analysis and ERS calculation were conducted utilizing a cumulative average approach over up to 3 study visits. ERS at visit 1 were derived from only phthalate concentrations measured at study visit 1. ERS at visit 2 were derived using the geometric mean of phthalate concentrations at the first and second study visits, and ERS at visit 3 were derived using the geometric mean of phthalate concentrations measured at all 3 study visits. Analyses were conducted for each birth outcome, and separately for women carrying male versus female fetuses. All analyses included maternal age and maternal education as unpenalized covariates. All phthalate concentrations were adjusted for specific gravity to account for differences in urinary dilution between study subjects. Ridge regression was conducted utilizing the *glmnet* package in R (version 3.5.1).

Causal Mediation Analyses

In the causal mediation framework, the relationship between exposures and outcomes can be framed in several ways. The mediated effect, also known as the natural indirect effect (NIE), is the change in outcome when the exposure is held constant and the mediator is changed to the level it would have been with an increase in exposure. The natural direct effect (NDE) corresponds to the change in the outcome in association with a change in exposure while keeping the mediator at the level it would have been at the original exposure level. Finally, the total effect (TE) corresponds to a change in the outcome associated with a change in exposure without any consideration or adjustment for the mediator. The TE is also equal to the sum of the NDE and NIE. We can then calculate the proportion of mediation by dividing the NIE by the TE.

These effects can be estimated using this method only if the following assumptions hold true: 1) there is no unmeasured confounding for the relationship between the exposure and outcome, 2) there is no unmeasured confounding for the relationship between the mediator and outcome,

after controlling for the exposure, 3) there is no unmeasured confounding on the relationship between the exposure and the mediator, and 4) there is no downstream effect of the exposure which confounds the relationship between the mediator and the outcome. The temporal ordering assumption must also be met, such that the exposure precedes the mediator, which precedes the outcome. A causal diagram depicting these relationships is shown in Figure IV.1. If all of these assumptions are met, the following statistical models can be used to estimate mediating effects:

$$Model\ 1: logit[P(Y = 1|a, m, c)] = \beta_{y0} + \beta_{ya}\overline{a_t} + \beta_{ym}m_t + \beta_{yc}^T c$$

$$Model\ 2: E[M|a, c] = \beta_{m0} + \beta_{ma}\overline{a_t} + \beta_{mc}^T c$$

where $\overline{a_t}$ represents the phthalate ERS calculated from the cumulative average approach at study visit t, corrected for specific gravity; m_t represents the observed hormone concentrations at study visit t; c represents observed values of covariates which are constant over time; and Y represents the outcome.

Mediation methods applied in the present analysis were adapted from those described in Aung et al[215]. Visit-specific phthalate ERS were used as exposure variables, and visit-specific hormone concentrations were used as mediators, in causal mediation analyses. Using ERS provides an advantage over individual phthalate metabolites because it reduces the potential for bias due to correlation between metabolites, and it allows for risk assessment and ascertainment of the biological pathways implicated with exposure to a whole class of environmental contaminants. All models adjusted for continuous maternal age and categorical maternal education. All mediation analyses were conducted using the *mediation* package in R (version 3.5.1).

Results

Characteristics of the study population are shown in Table IV.1. Preterm and spontaneous preterm birth occurred in about 9% and 5% of the cohort, respectively. Pregnancies were about 53% male and 46% female. Most women were under the age of 30, had at least some college education, were employed, lived in a home earning less than $30k per year, were either married or cohabitating, did not smoke and reported never being exposed to environmental tobacco

smoke, did not consume alcohol during pregnancy, had given less than two previous live births, and had a pre-pregnancy BMI below 30 kg/m^2. Pregnancy and demographic characteristics did not differ appreciably between the full population and the mediation subset.

Weights derived from ridge regression for each birth outcome are shown in Figure IV.2. For PTB, the strongest weights were assigned to metabolites of DBP and DiBP, and weights were particularly strong at visit 2 among pregnancies with a male fetus. Interestingly, for both DBP and DiBP, the weight for one metabolite was positive (MBP and MHiBP) while the other was inverse (MHBP and MiBP). Weights were similar for spontaneous PTB, except that DBP and DiBP metabolite weights were also very strong at visit 3 among pregnancies with a male fetus. Weights for gestational age at birth were generally weaker than those for PTB and spontaneous PTB, but DBP and DiBP metabolites still had the strongest weights. Finally, weights for SGA, LGA and birth weight z-score were very weak and are not displayed.

Associations between phthalate ERS and birth outcomes across the study period, subset to mothers with mediator data, are shown in Table IV.2. Among pregnancies with a female fetus, all 3 study visits showed a positive association between odds of PTB and LMW phthalate ERS (v1 OR: 1.87, 95% CI: 1.01, 3.46; v2 OR: 2.96, 95% CI: 1.35, 6.52; v3 OR: 2.78, 95% CI: 1.25, 6.18), while HMW phthalate ERS was associated with odds of PTB only at the first (OR: 2.02, 95% CI: 1.14, 3.58) and second study visits (OR: 2.46, 95% CI: 1.29, 4.66). Increased risk of spontaneous PTB was observed at visit 1 with increases in both LMW phthalate ERS (OR: 2.23, 95% CI: 1.02, 4.90) and HMW phthalate ERS (OR: 1.98, 95% CI: 1.07, 3.65). Increased LMW phthalate ERS was associated with reduced gestational age at birth at the second (β: -0.45 weeks, 95% CI: -0.85, -0.06) and third study visits (β: -0.52 weeks, 95% CI: -0.91, -0.13), while the HMW phthalate ERS was associated with reduced gestational age at birth at all three study visits (v1 β: -0.64 weeks, 95% CI: -1.01, -0.27; v2 β: -0.42 weeks, 95% CI: -0.77, -0.08; v3 β: -0.39 weeks, 95% CI: -0.74, -0.05).

Among pregnancies with a male fetus, risk of PTB was associated with HMW phthalate ERS at the first study visit (OR: 2.30, 95% CI: 1.19, 4.42) and LMW phthalate ERS at the second study visit (OR: 1.82, 95% CI: 1.01, 3.31). Odds of spontaneous PTB were associated with LMW phthalate ERS at the second (OR: 4.40, 95% CI: 1.50, 12.9) and third study visit (OR: 2.74, 95% CI: 1.23, 6.13), and with HMW phthalate ERS at the first study visit (OR: 2.48, 95% CI: 1.14, 5.40). Finally, reductions in gestational age at birth were observed at the first study visit with increasing HMW phthalate ERS (β: -0.39 weeks, 95% CI: -0.75, -0.03) and at the second study visit with increasing LMW phthalate ERS (β: -0.43 weeks, 95% CI: -0.69, -0.16).

Estimations of natural indirect effects and percent mediated across the study for PTB, spontaneous PTB, and gestational age at birth among male pregnancies are shown in Tables IV.3 (LMW phthalate ERS) and IV.4 (HMW phthalate ERS). Corresponding p-values for natural indirect effects are depicted in Figure IV.3. The mediating effect of TSH on the association between visit 3 LMW phthalate ERS and PTB was marginally significant, resulting in a 0.008 increase (95% CI: -0.001, 0.020) in probability of PTB. Testosterone and the ratio of testosterone to SHBG had significant mediating effects on the association between visit 1 LMW phthalate ERS and spontaneous PTB, resulting in a 0.010 increase (95% CI: 0.002, 0.023) and 0.011 increase (95% CI: 0.002, 0.024) in probability of spontaneous PTB, respectively, and mediated about 29% of the total association. CRH and progesterone also had marginally significant mediating effects on the association between visit 1 LMW phthalate ERS and spontaneous PTB, resulting in a 0.010 increase (95% CI: -0.001, 0.025) and 0.006 increase (95% CI: -0.001, 0.016) in probability of spontaneous PTB, respectively. The mediating effect of testosterone on the association between visit 1 LMW phthalate ERS and gestational age at birth was marginally significant, resulting in a 0.049 week reduction in gestational age at birth (95% CI: -0.129, 0.003). Numerous mediating effects on the associations between HMW phthalate ERS and birth outcomes were significant, but most were in the opposite directions as the corresponding total effects. There was one exception; the mediating effect of CRH on the association between visit 3 HMW phthalate ERS and gestational age at birth was significant, resulting in a 0.098 week reduction in gestational age at birth (95% CI: -0.226, -0.007) and mediating about 35% of the total association.

Estimations of natural indirect effects and percent mediated across the study for PTB, spontaneous PTB, and gestational age at birth among female pregnancies are shown in Tables IV.5 (LMW phthalate ERS) and IV.6 (HMW phthalate ERS). Corresponding p-values for natural indirect effects are depicted in Figure IV.4. There were no significant mediating effects observed on the associations between LMW phthalate ERS and birth outcomes. Though numerous significant mediating effects were observed on the associations between HMW phthalate ERS and birth outcomes, all mediating effects were in the opposite direction as their corresponding total effects, and so these results do not present evidence of mediation.

Discussion

In this novel analysis, we explored the mediating effects of hormone concentrations on the associations between gestational exposure to a mixture of phthalates and adverse birth outcomes. This work builds upon previously published research by combining novel mixtures methods[215] with repeated measures analyses to provide the first causal mediation analysis using repeated biomarker data within an exposure mixtures framework. We provide evidence that significant associations exist between gestational exposure to a mixture of phthalates and increased odds of PTB and spontaneous PTB, and gestational age at birth, and that these associations differ by molecular weights of phthalates, fetal sex, and gestational age at exposure assessment. We also provide introductory evidence of mediation by various hormones on the associations between phthalate mixtures and these adverse birth outcomes.

We observed suggestive evidence of mediation by TSH, CRH, progesterone, and testosterone on the associations between exposure to LMW phthalate metabolites and metrics of early delivery among pregnancies with a male fetus. Previous work has shown some of these hormones to be important for regulation of the timing of labor. Concentrations of CRH exponentially increase at the end of gestation, possibly acting as a major influence on the timing of labor[94]. This physiological role, coupled with past observations of significant positive associations with phthalate exposure[191], suggests that CRH could in fact mediate the association between

phthalates and preterm delivery. Additionally, it has been postulated that CRH may signal to the fetal zone of the fetal adrenal gland to stimulate production of DHEA-S, a precursor of androgens and estrogens, to activate pro-labor events[96].

Mediation by progesterone on the association between phthalate exposures and early delivery is also biologically plausible. During the first 9 weeks of pregnancy, the corpus luteum is responsible for secreting the necessary progesterone for maintenance of the fetus. After that, the placenta becomes the main source of progesterone. A previous *in silico* study found strong binding affinity between phthalate metabolites and the progesterone receptor[216]. Accordingly, another *in vitro* study found that treatment of human placental cells with phthalate metabolites resulted in an inhibition of the progesterone receptor gene via negative feedback from an increase in progesterone concentrations[217]. Thus, phthalate exposure at this time could stimulate progesterone production by the placenta via interaction with the progesterone receptor. Elevated circulating progesterone could then inhibit the progesterone receptor gene, which could result in reduced expression of the progesterone receptor gene and thus reduced progesterone function. Taking all of this information together, maternal exposure to mixtures of phthalates during mid gestation could result in increased production of progesterone by the placenta, which then participates in a negative feedback loop with the progesterone receptor, resulting in a reduction of the anti-labor effects of progesterone on the pregnancy, possibly contributing to increased risk of preterm birth.

Finally, there is a biological basis for the proposed mediating effect of testosterone on the association between phthalate exposures and preterm delivery. Despite existing evidence that phthalates possess anti-androgenic biological effects, previous work has shown a positive association between testosterone concentrations during pregnancy and exposure to LMW phthalates[191]. Higher circulating concentrations of testosterone may act on the endometrium to produce lower levels of PP14, an endometrial secretory protein which has been shown to be inversely associated with risk of preterm birth as early as 6-18 weeks' gestation[218]. Decreased production of PP14 is associated with abnormal development of the endometrium and greater

likelihood of downstream pregnancy complications[82,219,220]. Therefore, gestational exposure to LMW phthalates may result in elevated testosterone production, which could then adversely affect the endometrium to produce less PP14 and cause endometrial dysfunction leading to elevated risk of preterm delivery.

This study was subject to several limitations. Some phthalate metabolite weights from ridge analysis were strongest at the second study visit, at which time we did not have access to hormone measurements, and so we may have missed important associations at that time point. We also detected some significant mediating effects which did not correspond to significant total effects. Detection of significant mediation signals could have been an artifact of strong associations between our exposure and mediator measures, to which the total effect would be robust. However, despite our large sample size, the small number of PTB and spontaneous PTB cases could also be interfering with our ability to detect truly significant total effects. We did not have access to measurements for thyroid autoantibody status, which could confound associations with thyroid hormones. Some critical changes in the maternal endocrine environment occur earlier or later in gestation than we were able to measure, which could shed additional light on the various endocrine pathways implicated in adverse birth outcomes. Women with preexisting conditions were excluded from the analysis, which may limit the generalizability of our findings. It is likely that all models with ERS are overfit because we did not use separate training and testing data sets for creating the ERS and running subsequent mediation analyses. Finally, the mediation analyses implemented here cannot accommodate situations where mediators confound one another, so it is possible that our results are biased if multiple mediators are operating on the same causal pathway. Future work will attempt to better understand the endocrine pathways implicated with phthalate exposures in order to create mediator risk scores that are reflective of entire pathways.

Despite these limitations, this study was also strong in many ways. This is the first study to utilize this analysis pipeline with repeated exposure and mediator data, and our sample size was higher than many other epidemiology studies which assessed only single pollutant associations. We

included a wide panel of hormone measurements to test a variety of endocrine pathways, and we add to a very limited body of epidemiology literature supporting a role for CRH in adverse birth outcomes. Exclusion of women with preexisting conditions, though it limited our generalizability as stated previously, allowed us to better understand biological effects related only to environmental exposures and not confounded by other health conditions. We assessed the more rare and homogenous spontaneous subtype of preterm birth, which may help in understanding the physiological pathways that make this subtype unique. We also provide novel evidence of differential toxicity pathways of high versus low molecular weight phthalate compounds, and that molecular weight may influence the gestational age at which exposure confers the greatest toxicity. Lastly, we added to a growing body of evidence suggesting differential biological pathways and risks associated with adverse birth outcomes between male and female pregnancies.

In conclusion, we provide novel suggestive evidence of various hormone concentrations mediating the association between gestational exposure to a mixture of phthalates and elevated risk for preterm delivery among male pregnancies. Importantly, we add to a limited body of evidence suggesting that environmental exposures and subsequent risk for adverse pregnancy outcomes are not equitable between male and female pregnancies. Future work will aim to increase statistical power with more cases of adverse pregnancy outcomes, and to better understand the true physiological implications of altered hormone concentrations during pregnancy.

Acknowledgements

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Table IV.1. Maternal demographic and birth characteristics of 1011 Puerto Rican mothers.

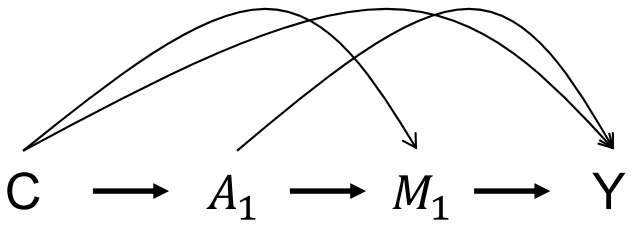
		Median (IQR)	
		ERS Population	**Mediation Population***
Gestational Age at Delivery (weeks)		39.3 (1.79)	39.3 (1.86)
		N (%)	
		ERS Population	**Mediation Population***
Preterm Birth			
	Yes	89 (8.8%)	65 (9.2%)
	No	911 (90.1%)	632 (89.6%)
	Missing	11 (1.1%)	8 (1.1%)
Spontaneous PTB			
	Yes	52 (5.1%)	39 (5.5%)
	No	921 (91.1%)	640 (90.8%)
	Missing	38 (3.8%)	26 (3.7%)
Maternal Age (years)			
	18-24	357 (35.3%)	247 (35.0%)
	25-29	309 (30.6%)	215 (30.5%)
	30-34	214 (21.2%)	150 (21.3%)
	35-41	131 (13.0%)	93 (13.2%)
	Missing	0 (0.0%)	0 (0.0%)
Maternal Education			
	GED or less	195 (19.3%)	147 (20.9%)
	Some College	337 (33.3%)	236 (33.5%)
	Bachelors or Higher	479 (47.4%)	322 (45.7%)
	Missing	0 (0.0%)	0 (0.0%)
Employment Status			
	No	344 (34.0%)	243 (34.5%)
	Yes	662 (65.5%)	458 (65.0%)
	Missing	5 (0.5%)	4 (0.6%)
Annual Household Income			
	<10k	255 (25.2%)	195 (27.7%)
	10k-<30k	293 (29.0%)	196 (27.8%)
	30k-<50k	223 (22.1%)	157 (22.3%)
	>=50k	126 (12.5%)	77 (10.9%)
	Missing	114 (11.3%)	80 (11.3%)
Marital Status			
	Single	168 (16.6%)	128 (18.2%)
	Married	553 (54.7%)	371 (52.6%)

Cohabitating	286 (28.3%)	202 (28.7%)
Missing	4 (0.4%)	4 (0.6%)
Smoking Status		
Never	873 (86.4%)	611 (86.7%)
Ever	118 (11.7%)	80 (11.3%)
Current	17 (1.7%)	12 (1.7%)
Missing	3 (0.3%)	2 (0.3%)
Daily Environmental Tobacco Smoke Exposure		
Never	848 (83.9%)	590 (83.7%)
1 Hour or less	37 (3.7%)	21 (3.0%)
>1 Hour	42 (4.2%)	35 (5.0%)
Missing	84 (8.3%)	59 (8.4%)
Alcohol Use		
Never	520 (51.4%)	358 (50.8%)
Yes, before Pregnancy	429 (42.4%)	303 (43.0%)
Yes, currently	58 (5.7%)	42 (6.0%)
Missing	4 (0.4%)	2 (0.3%)
Number of Previous Children		
0	327 (32.3%)	233 (33.0%)
1	375 (37.1%)	260 (36.9%)
2 to 5	117 (11.6%)	73 (10.4%)
Missing	192 (19.0%)	139 (19.7%)
Pre-Pregnancy BMI		
[0,25]	515 (50.9%)	360 (51.1%)
(25, 30]	269 (26.6%)	177 (25.1%)
Above 30	178 (17.6%)	128 (18.2%)
Missing	49 (4.8%)	40 (5.7%)
Fetal Sex		
Female	462 (45.7%)	331 (47.0%)
Male	540 (53.4%)	369 (52.3%)
Missing	9 (0.9%)	5 (0.7%)

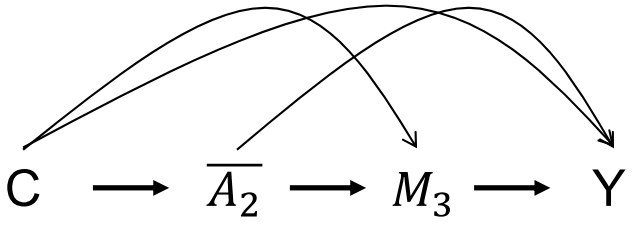
*Subset includes all women with mediator data (N=705).

Figure IV.1. Causal diagrams for mediation analyses in the counterfactual framework with a) exposures at visit 1 (A_1) and mediators at visit 1 (M_1), b) the average of exposures at visits 1 and 2 ($\overline{\mathrm{A}_2}$) and mediators at visit 3 (M_3), and c) the average of exposures at all 3 visits ($\overline{\mathrm{A}_3}$) and mediators at visit 3 (M_3), with confounders (C) and outcomes (Y) that do not vary with time.

a)



b)



c)

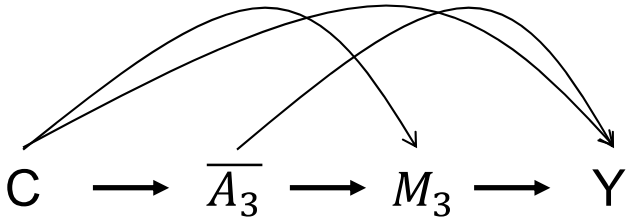


Figure IV.2. Weights assigned from ridge regression depicting the relative importance of each phthalate metabolites for predicting birth outcomes.

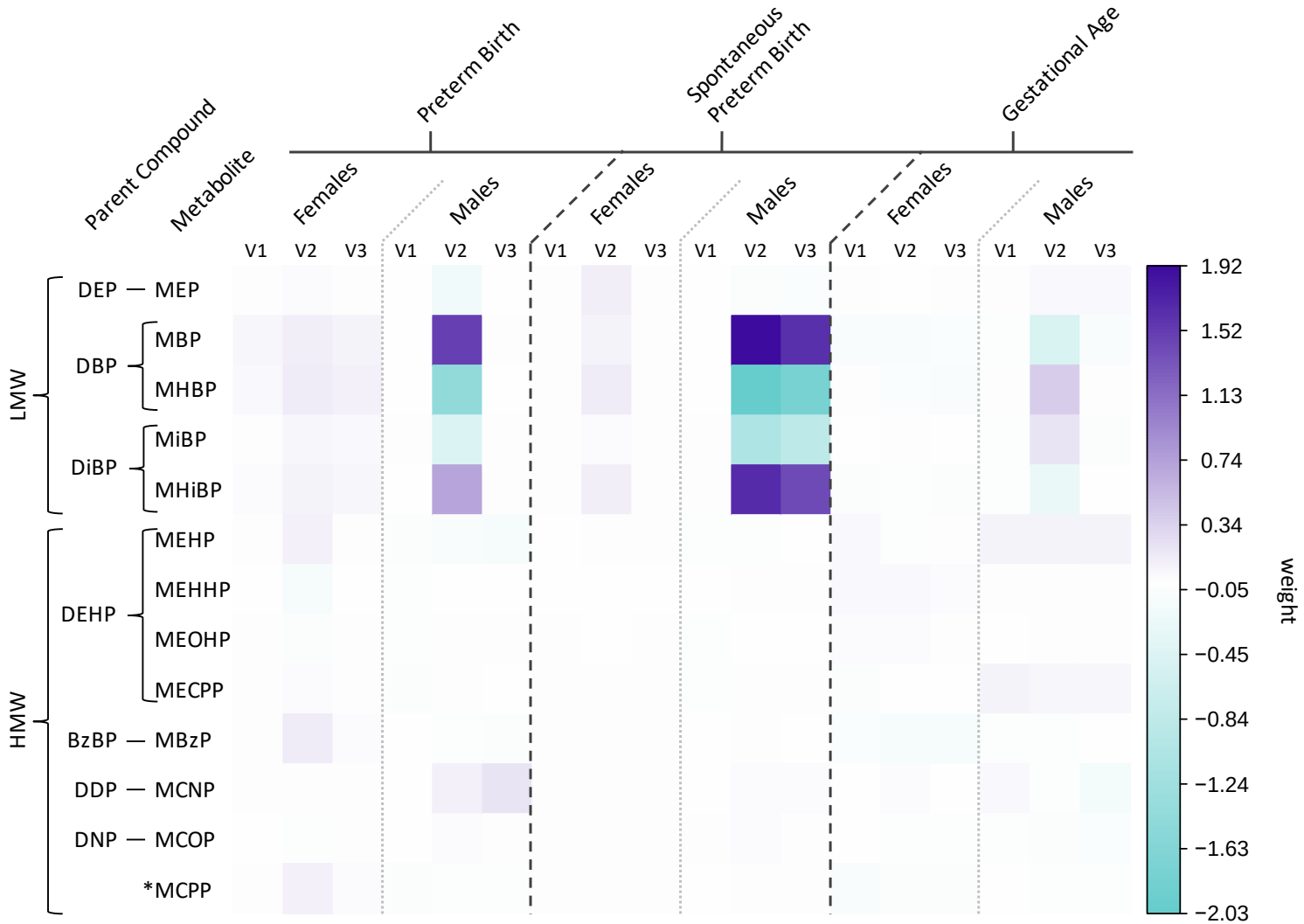


Table IV.2. Associations between phthalate ERS and birth outcomes across the study period between male and female fetuses, among women with mediator data.

	Visit 1				Visit 2				Visit 3			
	LMW		HMW		LMW		HMW		LMW		HMW	
	N	Est (95% CI)	N	Est (95% CI)	N	Est (95% CI)	N	Est (95% CI)	N	Est (95% CI)	N	Est (95% CI)
Female Fetuses												
Preterm Birth	244	**1.87 (1.01, 3.46)**	244	**2.02 (1.14, 3.58)**	195	**2.96 (1.35, 6.52)**	195	**2.46 (1.29, 4.66)**	206	**2.78 (1.25, 6.18)**	206	1.42 (0.72, 2.77)
Spont. Preterm Birth	237	**2.23 (1.02, 4.90)**	237	**1.98 (1.07, 3.65)**	189	3.16 (0.95, 10.54)	189	2.20 (0.80, 6.03)	200	2.05 (0.62, 6.83)	200	1.35 (0.47, 3.90)
Gest. Age (weeks)	245	-0.30 (-0.67, 0.07)	245	**-0.64 (-1.01, -0.27)**	195	**-0.45 (-0.85, -0.06)**	195	**-0.42 (-0.77, -0.08)**	206	**-0.52 (-0.91, -0.13)**	206	**-0.39 (-0.74, -0.05)**
Male Fetuses												
Preterm Birth	282	1.36 (0.78, 2.38)	282	**2.30 (1.19, 4.42)**	210	**1.82 (1.01, 3.31)**	210	1.30 (0.70, 2.43)	218	1.11 (0.64, 1.95)	218	1.40 (0.80, 2.42)
Spont. Preterm Birth	274	1.67 (0.89, 3.11)	274	**2.48 (1.14, 5.40)**	203	**4.40 (1.50, 12.9)**	203	0.81 (0.27, 2.48)	211	**2.74 (1.23, 6.13)**	211	1.00 (0.41, 2.41)
Gest. Age (weeks)	286	-0.20 (-0.56, 0.16)	286	**-0.39 (-0.75, -0.03)**	210	**-0.43 (-0.69, -0.16)**	210	-0.02 (-0.33, 0.29)	218	-0.29 (-0.62, 0.04)	218	-0.10 (-0.45, 0.25)

Effect estimates refer to the odds of binary birth outcomes, or unit changes in continuous outcomes, with an interquartile range increase in phthalate ERS. ERS were calculated using a cumulative average approach; visit 2 was comprised of the geometric means of phthalate concentrations at visits 1 and 2, and visit 3 was comprised of the geometric means of phthalate concentrations from all 3 visits. All models adjust for continuous maternal age and categorical maternal education, and birth weight models further adjusted for categorical maternal pre-pregnancy BMI.

Boldface text denotes significant findings with $p \leq 0.05$. LMW: low molecular weight; HMW: high molecular weight.

Table IV.3. Natural indirect effect estimates and percent mediated with an interquartile range increase in low molecular weight phthalate ERS over the study period, among mothers carrying a male fetus.

		$ERS_{v1} \rightarrow Hormones_{v1}$		$ERS_{v2} \rightarrow Hormones_{v3}$		$ERS_{v3} \rightarrow Hormones_{v3}$	
Outcome	**Mediator**	**NIE (95% CI)**	**Percent Mediated[a]**	**NIE (95% CI)**	**Percent Mediated[a]**	**NIE (95% CI)**	**Percent Mediated[a]**
PTB	CRH	0.000 (-0.006, 0.007)	0.81%	0.005 (-0.002, 0.016)	8.64%	0.006 (-0.002, 0.017)	10.5%
	Estriol	0.003 (-0.008, 0.014)	7.84%	-0.001 (-0.008, 0.003)	NA	-0.003 (-0.015, 0.006)	NA
	Prog.	0.006 (-0.005, 0.019)	16.2%	0.002 (-0.004, 0.010)	2.35%	0.003 (-0.003, 0.011)	2.12%
	Prog/E3	0.000 (-0.004, 0.003)	NA	0.003 (-0.002, 0.012)	4.89%	-0.002 (-0.011, 0.005)	NA
	Test.	0.000 (-0.008, 0.007)	0.33%	0.002 (-0.004, 0.011)	3.48%	0.005 (-0.003, 0.016)	8.03%
	Test./SHBG	0.001 (-0.005, 0.009)	2.57%	0.002 (-0.005, 0.011)	2.61%	0.004 (-0.005, 0.015)	5.43%
	SHBG	0.003 (-0.002, 0.012)	6.95%	0.000 (-0.006, 0.005)	NA	-0.001 (-0.008, 0.005)	NA
	TSH	-0.002 (-0.010, 0.003)	NA	0.007 (-0.002, 0.019)	10.8%	**0.008 (-0.001, 0.020)**	17.3%
	T3	0.000 (-0.005, 0.004)	NA	0.000 (-0.003, 0.005)	0.20%	0.002 (-0.008, 0.013)	2.59%
	fT4	0.001 (-0.005, 0.008)	2.35%	0.004 (-0.002, 0.013)	5.42%	0.005 (-0.002, 0.016)	7.39%
	T4	0.001 (-0.004, 0.007)	1.16%	0.000 (-0.005, 0.004)	NA	0.001 (-0.004, 0.007)	0.51%
	T3/T4	0.000 (-0.005, 0.003)	NA	0.000 (-0.004, 0.004)	0.02%	0.000 (-0.009, 0.009)	0.32%
Spont. PTB	CRH	**0.010 (-0.001, 0.025)**	28.18%	0.006 (-0.002, 0.017)	9.64%	0.007 (-0.003, 0.018)	11.80%
	Estriol	-0.002 (-0.012, 0.005)	NA	-0.001 (-0.005, 0.002)	NA	-0.001 (-0.007, 0.003)	NA
	Prog.	**0.006 (-0.001, 0.016)**	18.17%	0.002 (-0.002, 0.008)	2.63%	0.003 (-0.001, 0.010)	5.06%
	Prog/E3	-0.001 (-0.008, 0.004)	NA	0.003 (-0.002, 0.012)	4.96%	0.002 (-0.004, 0.011)	3.72%
	Test.	**0.010 (0.002, 0.023)**	28.53%	0.005 (-0.004, 0.016)	7.65%	0.008 (-0.005, 0.023)	15.68%
	Test./SHBG	**0.011 (0.002, 0.024)**	29.58%	0.005 (-0.004, 0.015)	7.60%	0.007 (-0.004, 0.021)	13.66%
	SHBG	0.003 (-0.003, 0.011)	6.76%	0.002 (-0.003, 0.008)	2.02%	0.001 (-0.003, 0.006)	0.64%
	TSH	0.003 (-0.002, 0.010)	6.33%	0.003 (-0.006, 0.014)	6.31%	0.005 (-0.005, 0.018)	10.26%
	T3	0.003 (-0.001, 0.009)	7.75%	0.002 (-0.004, 0.009)	2.92%	0.003 (-0.007, 0.014)	5.79%
	fT4	0.002 (-0.003, 0.009)	5.17%	0.003 (-0.002, 0.011)	5.02%	0.004 (-0.006, 0.015)	6.72%
	T4	0.000 (-0.003, 0.003)	NA	0.000 (-0.004, 0.003)	NA	0.000 (-0.004, 0.004)	NA
	T3/T4	0.003 (-0.002, 0.010)	7.71%	0.002 (-0.004, 0.008)	2.22%	0.002 (-0.006, 0.012)	4.07%
Gest. Age	CRH	-0.032 (-0.107, 0.015)	9.32%	-0.030 (-0.094, 0.009)	6.01%	-0.020 (-0.089, 0.032)	4.89%
	Estriol	0.035 (-0.027, 0.113)	NA	0.000 (-0.027, 0.026)	0.00%	-0.002 (-0.063, 0.058)	0.25%
	Prog.	-0.010 (-0.108, 0.083)	4.96%	-0.027 (-0.086, 0.010)	5.20%	-0.032 (-0.107, 0.017)	8.90%

	Prog/E3	-0.016 (-0.071, 0.017)	3.59%	-0.027 (-0.090, 0.014)	5.46%	0.012 (-0.051, 0.084)	NA
	Test.	**-0.049 (-0.129, 0.003)**	16.07%	-0.012 (-0.056, 0.018)	1.88%	-0.011 (-0.063, 0.023)	2.38%
	Test./SHBG	-0.040 (-0.112, 0.005)	12.41%	-0.011 (-0.056, 0.019)	1.81%	-0.013 (-0.062, 0.019)	2.70%
	SHBG	-0.001 (-0.029, 0.023)	0.05%	-0.003 (-0.033, 0.020)	0.21%	-0.002 (-0.041, 0.030)	0.25%
	TSH	-0.021 (-0.083, 0.017)	5.49%	-0.025 (-0.092, 0.028)	4.95%	-0.036 (-0.114, 0.012)	9.18%
	T3	-0.004 (-0.048, 0.031)	0.39%	0.001 (-0.022, 0.028)	NA	0.005 (-0.040, 0.050)	NA
	fT4	-0.033 (-0.110, 0.023)	10.54%	-0.012 (-0.057, 0.016)	1.85%	-0.017 (-0.077, 0.018)	3.72%
	T4	-0.002 (-0.053, 0.046)	0.47%	0.002 (-0.020, 0.032)	NA	-0.005 (-0.048, 0.028)	0.54%
	T3/T4	0.002 (-0.038, 0.042)	NA	0.001 (-0.023, 0.030)	NA	0.006 (-0.025, 0.048)	NA

[a]Indication is NA when the TE and NIE are in different directions, rendering the percent mediated uninterpretable. Estimates refer to the increase in probability of experiencing binary outcomes, or the unit change in continuous outcomes, due to the resulting change in the mediator with an interquartile range increase in exposure, while holding the exposure constant. Boldface text indicates a p-value < 0.1.

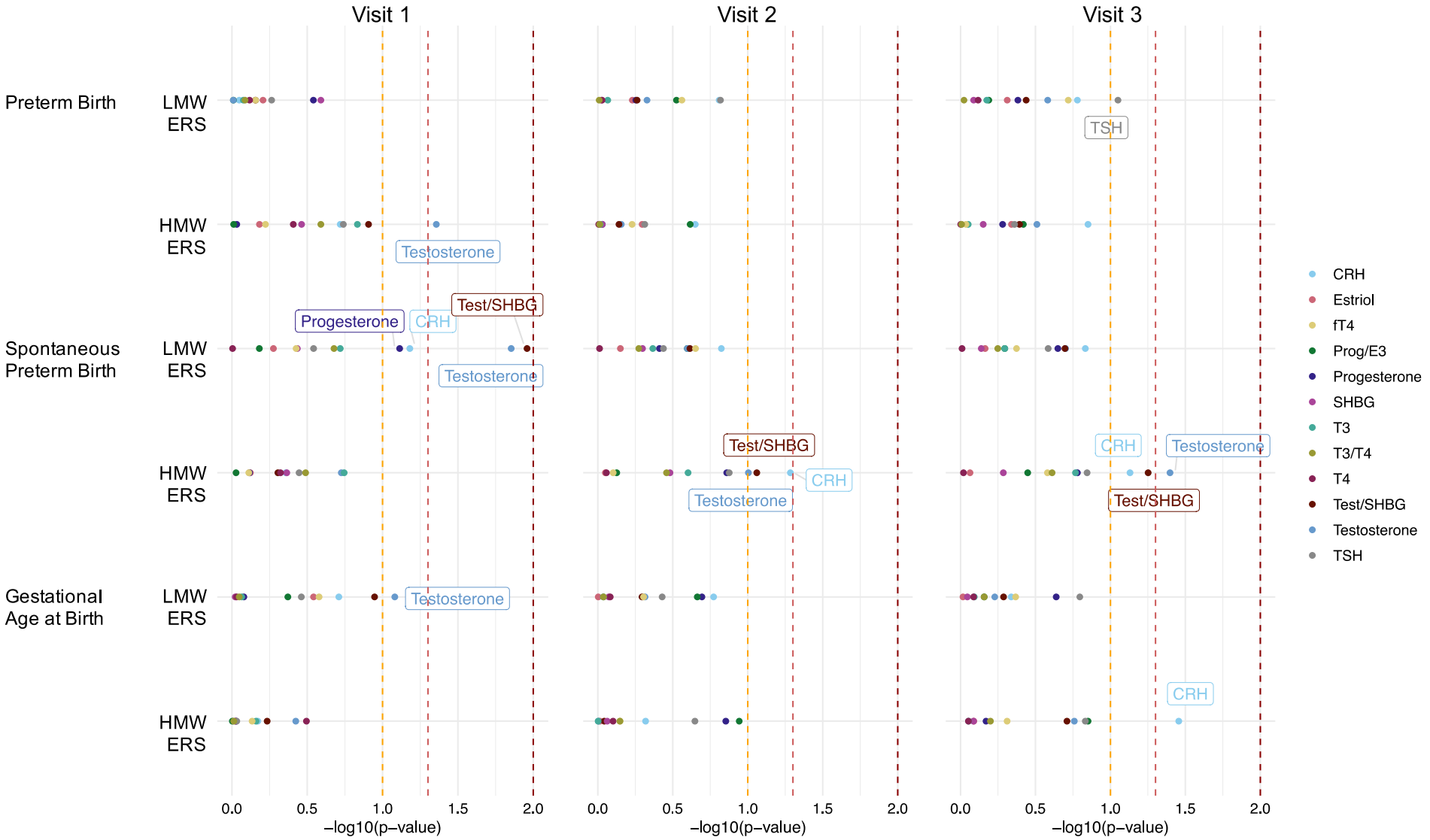
Table IV.4. Natural indirect effect estimates and percent mediated with an interquartile range increase in high molecular weight phthalate ERS over the study period, among mothers carrying a male fetus.

		$ERS_{v1} \rightarrow Hormones_{v1}$		$ERS_{v2} \rightarrow Hormones_{v3}$		$ERS_{v3} \rightarrow Hormones_{v3}$	
Outcome	**Mediator**	**NIE (95% CI)**	**Percent Mediated[a]**	**NIE (95% CI)**	**Percent Mediated[a]**	**NIE (95% CI)**	**Percent Mediated[a]**
PTB	CRH	-0.005 (-0.016, 0.001)	NA	0.005 (-0.002, 0.017)	9.80%	0.008 (-0.003, 0.023)	19.46%
	Estriol	0.003 (-0.009, 0.015)	3.29%	-0.003 (-0.013, 0.005)	NA	-0.003 (-0.015, 0.006)	NA
	Prog.	-0.001 (-0.014, 0.013)	NA	0.000 (-0.006, 0.005)	NA	0.002 (-0.003, 0.010)	2.85%
	Prog/E3	0.000 (-0.005, 0.004)	-0.02%	-0.005 (-0.017, 0.002)	NA	-0.004 (-0.014, 0.003)	NA
	Test.	**-0.009 (-0.023, 0.000)**	NA	0.001 (-0.004, 0.009)	1.06%	0.004 (-0.004, 0.015)	7.86%
	Test./SHBG	-0.006 (-0.019, 0.002)	NA	0.001 (-0.004, 0.009)	0.69%	0.003 (-0.005, 0.014)	6.80%
	SHBG	0.003 (-0.003, 0.012)	3.14%	0.000 (-0.008, 0.006)	NA	-0.002 (-0.012, 0.008)	NA
	TSH	-0.006 (-0.018, 0.002)	NA	0.003 (-0.004, 0.012)	5.06%	0.003 (-0.004, 0.013)	6.05%
	T3	-0.005 (-0.015, 0.001)	NA	0.000 (-0.004, 0.005)	0.04%	0.000 (-0.004, 0.005)	0.23%
	fT4	-0.002 (-0.011, 0.005)	NA	-0.002 (-0.013, 0.006)	NA	0.000 (-0.007, 0.008)	0.60%
	T4	-0.003 (-0.012, 0.003)	NA	0.000 (-0.005, 0.005)	0.03%	0.000 (-0.005, 0.005)	0.03%
	T3/T4	-0.003 (-0.012, 0.002)	NA	0.000 (-0.005, 0.004)	NA	0.000 (-0.005, 0.004)	0.01%
Spont. PTB	CRH	-0.003 (-0.012, 0.004)	NA	**0.014 (0.000, 0.035)**	NA	**0.015 (-0.002, 0.037)**	NA
	Estriol	0.001 (-0.008, 0.011)	2.19%	0.001 (-0.012, 0.014)	NA	0.001 (-0.011, 0.014)	NA
	Prog.	-0.001 (-0.011, 0.008)	NA	0.005 (-0.001, 0.017)	NA	0.005 (-0.001, 0.015)	1.57%
	Prog/E3	0.000 (-0.003, 0.004)	0.05%	-0.002 (-0.013, 0.008)	4.12%	-0.005 (-0.017, 0.004)	5.32%
	Test.	-0.006 (-0.018, 0.003)	NA	**0.009 (-0.001, 0.025)**	NA	**0.014 (0.001, 0.032)**	NA
	Test./SHBG	-0.003 (-0.013, 0.005)	NA	**0.009 (-0.001, 0.026)**	NA	**0.013 (0.000, 0.032)**	NA
	SHBG	0.002 (-0.003, 0.011)	3.08%	0.004 (-0.004, 0.016)	NA	0.003 (-0.006, 0.014)	NA
	TSH	-0.003 (-0.012, 0.002)	NA	0.006 (-0.002, 0.019)	NA	0.006 (-0.001, 0.018)	NA
	T3	-0.003 (-0.011, 0.001)	NA	0.004 (-0.004, 0.015)	NA	0.006 (-0.003, 0.018)	NA
	fT4	-0.001 (-0.006, 0.003)	NA	0.001 (-0.006, 0.009)	NA	0.004 (-0.002, 0.014)	NA
	T4	-0.002 (-0.009, 0.003)	NA	0.000 (-0.007, 0.005)	0.16%	0.000 (-0.004, 0.005)	0.04%
	T3/T4	-0.002 (-0.009, 0.002)	NA	0.004 (-0.005, 0.015)	NA	0.005 (-0.003, 0.016)	NA
Gest. Age	CRH	0.008 (-0.033, 0.058)	NA	-0.020 (-0.088, 0.034)	3.12%	**-0.098 (-0.226, -0.007)**	34.64%
	Estriol	0.002 (-0.068, 0.073)	NA	0.001 (-0.027, 0.031)	0.10%	-0.007 (-0.076, 0.057)	1.32%
	Prog.	-0.004 (-0.104, 0.092)	0.95%	0.039 (-0.008, 0.117)	NA	-0.013 (-0.085, 0.047)	3.74%

	Prog/E3	-0.001 (-0.041, 0.037)	0.03%	0.045 (-0.007, 0.127)	NA	0.048 (-0.013, 0.138)	NA
	Test.	0.022 (-0.025, 0.084)	NA	0.000 (-0.037, 0.035)	0.46%	-0.047 (-0.134, 0.018)	15.20%
	Test./SHBG	0.013 (-0.032, 0.066)	NA	0.002 (-0.031, 0.038)	0.27%	-0.044 (-0.133, 0.024)	13.04%
	SHBG	-0.002 (-0.037, 0.028)	0.12%	0.003 (-0.026, 0.037)	0.03%	-0.006 (-0.065, 0.048)	1.20%
	TSH	0.001 (-0.038, 0.041)	NA	-0.028 (-0.097, 0.013)	0.39%	-0.040 (-0.122, 0.009)	9.67%
	T3	0.008 (-0.028, 0.055)	NA	0.000 (-0.027, 0.027)	0.10%	0.003 (-0.049, 0.054)	NA
	fT4	-0.011 (-0.082, 0.053)	2.10%	0.007 (-0.033, 0.055)	0.14%	-0.016 (-0.076, 0.022)	2.90%
	T4	0.024 (-0.021, 0.090)	NA	0.005 (-0.028, 0.046)	0.02%	0.002 (-0.029, 0.036)	NA
	T3/T4	0.001 (-0.034, 0.038)	NA	0.006 (-0.022, 0.045)	NA	0.011 (-0.039, 0.071)	NA

[a]Indication is NA when the TE and NIE are in different directions, rendering the percent mediated uninterpretable. Estimates refer to the increase in probability of experiencing binary outcomes, or the unit change in continuous outcomes, due to the resulting change in the mediator with an interquartile range increase in exposure, while holding the exposure constant. Boldface text indicates a p-value < 0.1.

Figure IV.3. Estimated -$\log_{10}$(p-values) of mediating effects by hormone concentrations on the associations between phthalate ERS and birth outcomes, among mothers carrying a male fetus.



From left to right within each panel, the vertical dashed lines represent p-values of 0.1, 0.05, and 0.01. All models were adjusted for continuous maternal age and categorical education.

Table IV.5. Natural indirect effect estimates and percent mediated with an interquartile range increase in low molecular weight phthalate ERS over the study period, among mothers carrying a female fetus.

		$ERS_{v1} \rightarrow Hormones_{v1}$		$ERS_{v2} \rightarrow Hormones_{v3}$		$ERS_{v3} \rightarrow Hormones_{v3}$	
Outcome	**Mediator**	**NIE (95% CI)**	**Percent Mediated[a]**	**NIE (95% CI)**	**Percent Mediated[a]**	**NIE (95% CI)**	**Percent Mediated[a]**
PTB	CRH	0.000 (-0.005, 0.005)	0.04%	-0.001 (-0.010, 0.007)	NA	0.001 (-0.006, 0.010)	1.03%
	Estriol	0.000 (-0.003, 0.004)	0.12%	-0.003 (-0.015, 0.003)	NA	0.001 (-0.007, 0.009)	0.50%
	Prog.	0.002 (-0.003, 0.009)	2.06%	0.000 (-0.007, 0.004)	NA	0.000 (-0.004, 0.006)	0.09%
	Prog/E3	0.000 (-0.004, 0.005)	0.09%	-0.001 (-0.009, 0.004)	NA	0.000 (-0.007, 0.006)	NA
	Test.	0.000 (-0.006, 0.006)	0.20%	0.000 (-0.010, 0.011)	0.35%	0.005 (-0.005, 0.018)	6.04%
	Test./SHBG	0.000 (-0.006, 0.006)	NA	-0.002 (-0.015, 0.009)	NA	0.003 (-0.007, 0.016)	4.35%
	SHBG	-0.001 (-0.005, 0.003)	NA	-0.007 (-0.022, 0.002)	NA	-0.003 (-0.015, 0.005)	NA
	TSH	0.000 (-0.005, 0.003)	NA	0.000 (-0.004, 0.004)	0.00%	0.000 (-0.006, 0.005)	NA
	T3	0.000 (-0.004, 0.006)	0.41%	0.000 (-0.005, 0.004)	NA	-0.001 (-0.008, 0.004)	NA
	fT4	0.001 (-0.004, 0.007)	0.86%	0.000 (-0.006, 0.005)	NA	0.000 (-0.005, 0.004)	0.01%
	T4	0.001 (-0.005, 0.008)	1.26%	0.000 (-0.007, 0.006)	NA	-0.002 (-0.010, 0.005)	NA
	T3/T4	0.000 (-0.005, 0.007)	0.51%	0.000 (-0.005, 0.004)	0.00%	0.000 (-0.005, 0.006)	0.10%
Spont. PTB	CRH	0.000 (-0.004, 0.003)	NA	-0.001 (-0.010, 0.004)	NA	0.000 (-0.007, 0.007)	0.46%
	Estriol	0.000 (-0.004, 0.003)	NA	0.002 (-0.003, 0.008)	3.80%	0.000 (-0.004, 0.005)	0.21%
	Prog.	0.000 (-0.003, 0.004)	0.12%	0.001 (-0.002, 0.007)	2.57%	0.000 (-0.004, 0.005)	0.17%
	Prog/E3	0.000 (-0.004, 0.003)	NA	0.000 (-0.004, 0.003)	NA	0.000 (-0.004, 0.003)	0.02%
	Test.	0.000 (-0.004, 0.003)	NA	-0.001 (-0.008, 0.004)	NA	0.001 (-0.005, 0.007)	1.06%
	Test./SHBG	0.000 (-0.004, 0.004)	NA	-0.002 (-0.010, 0.002)	NA	0.000 (-0.007, 0.005)	NA
	SHBG	0.000 (-0.004, 0.005)	0.20%	-0.003 (-0.014, 0.005)	NA	-0.001 (-0.009, 0.004)	NA
	TSH	-0.001 (-0.008, 0.002)	NA	-0.001 (-0.009, 0.004)	NA	-0.002 (-0.012, 0.003)	NA
	T3	0.000 (-0.005, 0.003)	NA	0.000 (-0.003, 0.004)	0.24%	0.000 (-0.004, 0.003)	NA
	fT4	0.003 (-0.002, 0.010)	5.24%	0.001 (-0.004, 0.006)	0.84%	0.000 (-0.004, 0.004)	0.11%
	T4	0.001 (-0.003, 0.007)	1.39%	0.000 (-0.003, 0.005)	0.33%	-0.001 (-0.006, 0.003)	NA
	T3/T4	-0.001 (-0.006, 0.004)	NA	0.000 (-0.005, 0.003)	NA	0.000 (-0.004, 0.004)	0.01%
Gest. Age	CRH	0.002 (-0.028, 0.038)	NA	-0.001 (-0.042, 0.038)	0.09%	-0.009 (-0.062, 0.025)	0.93%
	Estriol	0.000 (-0.027, 0.028)	0.00%	0.029 (-0.056, 0.126)	NA	-0.014 (-0.106, 0.066)	2.16%
	Prog.	0.002 (-0.046, 0.052)	NA	-0.001 (-0.040, 0.035)	0.05%	-0.006 (-0.053, 0.027)	0.44%

	Prog/E3	-0.002 (-0.038, 0.033)	0.17%	0.028 (-0.039, 0.116)	NA	0.011 (-0.056, 0.087)	NA
	Test.	0.000 (-0.027, 0.030)	0.00%	-0.009 (-0.078, 0.047)	1.07%	-0.029 (-0.112, 0.020)	4.28%
	Test./SHBG	0.001 (-0.027, 0.030)	NA	-0.002 (-0.065, 0.060)	0.12%	-0.023 (-0.099, 0.028)	3.06%
	SHBG	0.000 (-0.031, 0.032)	0.01%	0.012 (-0.027, 0.074)	NA	0.006 (-0.031, 0.060)	NA
	TSH	0.003 (-0.027, 0.042)	NA	0.000 (-0.034, 0.032)	0.02%	0.002 (-0.036, 0.040)	NA
	T3	0.004 (-0.025, 0.044)	NA	-0.001 (-0.034, 0.030)	0.07%	-0.004 (-0.052, 0.039)	0.18%
	fT4	0.000 (-0.042, 0.043)	0.01%	0.002 (-0.033, 0.042)	NA	-0.003 (-0.045, 0.031)	0.19%
	T4	-0.006 (-0.052, 0.030)	0.68%	0.002 (-0.051, 0.058)	NA	0.010 (-0.039, 0.072)	NA
	T3/T4	0.000 (-0.028, 0.030)	0.01%	-0.006 (-0.052, 0.032)	0.52%	-0.014 (-0.074, 0.023)	1.53%

[a]Indication is NA when the TE and NIE are in different directions, rendering the percent mediated uninterpretable. Estimates refer to the increase in probability of experiencing binary outcomes, or the unit change in continuous outcomes, due to the resulting change in the mediator with an interquartile range increase in exposure, while holding the exposure constant. Boldface text indicates a p-value < 0.1.

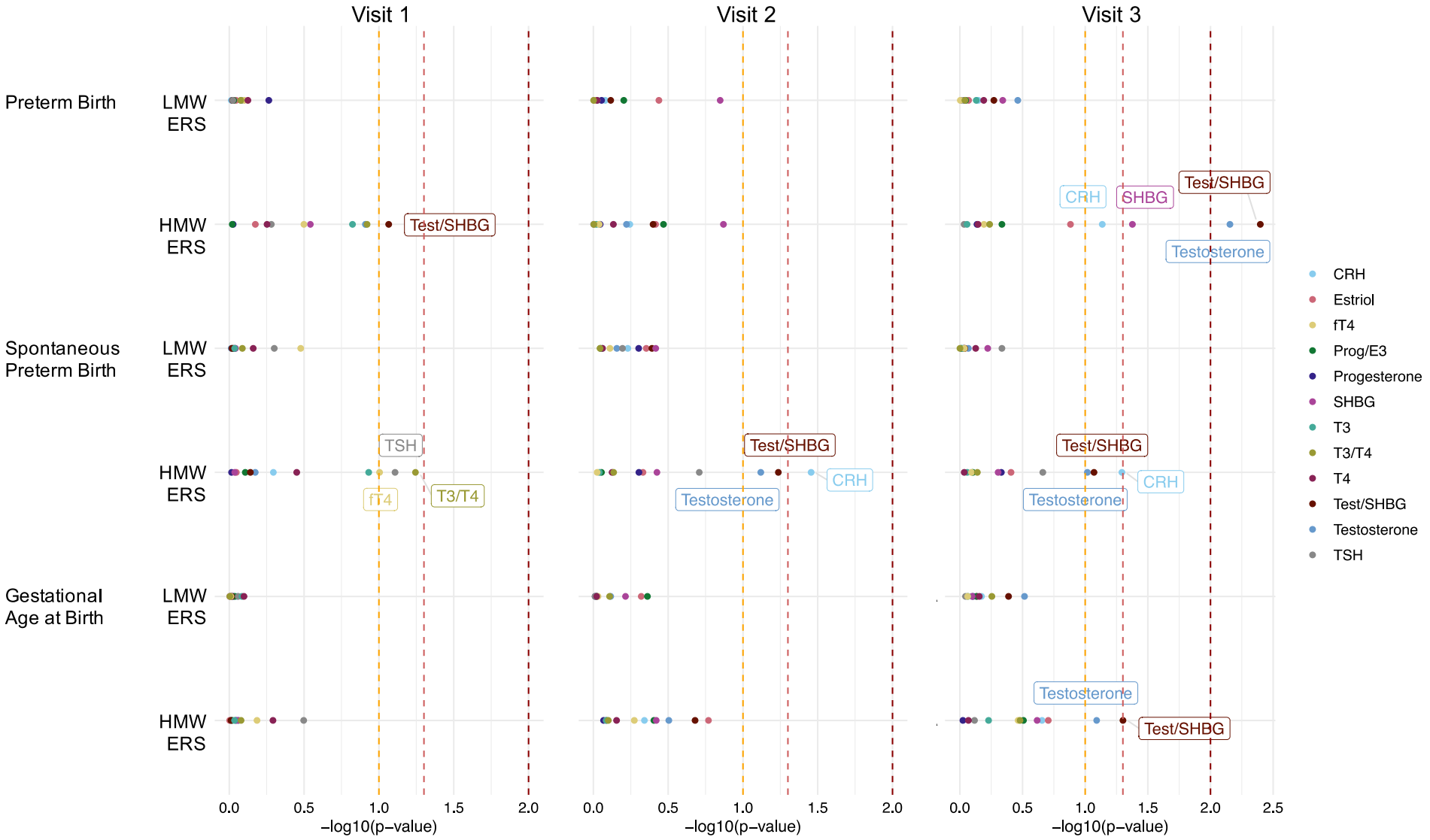
Table IV.6. Natural indirect effect estimates and percent mediated with an interquartile range increase in high molecular weight phthalate ERS over the study period, among mothers carrying a female fetus.

		$ERS_{v1} \rightarrow Hormones_{v1}$		$ERS_{v2} \rightarrow Hormones_{v3}$		$ERS_{v3} \rightarrow Hormones_{v3}$	
Outcome	**Mediator**	**NIE (95% CI)**	**Percent Mediated[a]**	**NIE (95% CI)**	**Percent Mediated[a]**	**NIE (95% CI)**	**Percent Mediated[a]**
PTB	CRH	-0.008 (-0.023, 0.002)	NA	-0.002 (-0.012, 0.005)	NA	**-0.010 (-0.027, 0.001)**	NA
	Estriol	-0.001 (-0.008, 0.003)	NA	-0.003 (-0.014, 0.003)	NA	-0.007 (-0.020, 0.001)	NA
	Prog.	0.000 (-0.005, 0.006)	0.09%	0.000 (-0.005, 0.006)	0.12%	-0.001 (-0.009, 0.004)	NA
	Prog/E3	0.000 (-0.006, 0.006)	0.10%	-0.003 (-0.013, 0.002)	NA	-0.002 (-0.011, 0.003)	NA
	Test.	-0.006 (-0.017, 0.001)	NA	-0.003 (-0.015, 0.007)	NA	**-0.017 (-0.037, -0.003)**	NA
	Test./SHBG	**-0.007 (-0.019, 0.001)**	NA	-0.005 (-0.018, 0.005)	NA	**-0.020 (-0.042, -0.005)**	NA
	SHBG	-0.004 (-0.014, 0.003)	NA	-0.008 (-0.023, 0.002)	NA	**-0.012 (-0.030, 0.000)**	NA
	TSH	-0.002 (-0.011, 0.004)	NA	0.000 (-0.006, 0.005)	0.00%	0.000 (-0.009, 0.007)	NA
	T3	-0.005 (-0.018, 0.002)	NA	0.000 (-0.004, 0.005)	0.05%	0.001 (-0.009, 0.010)	1.16%
	fT4	0.004 (-0.004, 0.014)	6.06%	0.000 (-0.006, 0.004)	NA	-0.002 (-0.013, 0.007)	NA
	T4	0.002 (-0.004, 0.009)	2.05%	0.001 (-0.004, 0.008)	0.78%	-0.001 (-0.008, 0.005)	NA
	T3/T4	-0.006 (-0.018, 0.001)	NA	0.000 (-0.005, 0.004)	0.00%	-0.002 (-0.013, 0.006)	NA
Spont. PTB	CRH	-0.003 (-0.014, 0.006)	NA	**-0.013 (-0.036, -0.001)**	NA	**-0.010 (-0.029, 0.000)**	NA
	Estriol	0.000 (-0.004, 0.004)	0.19%	0.003 (-0.005, 0.014)	7.66%	0.003 (-0.005, 0.014)	6.68%
	Prog.	0.000 (-0.003, 0.003)	NA	0.002 (-0.004, 0.010)	4.63%	0.002 (-0.003, 0.008)	2.19%
	Prog/E3	0.000 (-0.003, 0.005)	0.52%	0.000 (-0.004, 0.003)	NA	-0.001 (-0.006, 0.003)	NA
	Test.	-0.002 (-0.011, 0.006)	NA	**-0.010 (-0.028, 0.001)**	NA	**-0.011 (-0.030, 0.002)**	NA
	Test./SHBG	-0.001 (-0.010, 0.006)	NA	**-0.011 (-0.029, 0.000)**	NA	**-0.011 (-0.030, 0.002)**	NA
	SHBG	0.000 (-0.005, 0.006)	0.40%	-0.004 (-0.016, 0.005)	NA	-0.003 (-0.013, 0.006)	NA
	TSH	**-0.005 (-0.015, 0.000)**	NA	-0.005 (-0.018, 0.002)	NA	-0.004 (-0.015, 0.002)	NA
	T3	-0.006 (-0.018, 0.001)	NA	0.000 (-0.010, 0.010)	1.51%	0.001 (-0.009, 0.010)	1.86%
	fT4	**0.005 (-0.001, 0.015)**	16.10%	0.000 (-0.008, 0.009)	0.59%	0.001 (-0.008, 0.010)	1.74%
	T4	0.002 (-0.002, 0.008)	4.52%	0.001 (-0.003, 0.006)	0.82%	0.000 (-0.004, 0.004)	0.12%
	T3/T4	**-0.006 (-0.018, 0.000)**	NA	-0.001 (-0.011, 0.006)	NA	-0.001 (-0.011, 0.007)	NA
Gest. Age	CRH	-0.001 (-0.080, 0.071)	0.13%	0.016 (-0.022, 0.075)	NA	0.033 (-0.016, 0.108)	NA
	Estriol	-0.001 (-0.034, 0.031)	0.00%	0.052 (-0.023, 0.148)	NA	0.049 (-0.027, 0.143)	NA
	Prog.	0.003 (-0.037, 0.049)	NA	0.003 (-0.028, 0.045)	NA	0.002 (-0.032, 0.040)	NA

	Prog/E3	0.002 (-0.045, 0.053)	NA	0.026 (-0.032, 0.103)	NA	0.030 (-0.024, 0.107)	NA
	Test.	0.004 (-0.045, 0.060)	NA	0.028 (-0.021, 0.104)	NA	**0.051 (-0.005, 0.141)**	NA
	Test./SHBG	0.001 (-0.051, 0.052)	NA	0.035 (-0.013, 0.116)	NA	**0.060 (0.000, 0.157)**	NA
	SHBG	-0.003 (-0.043, 0.026)	0.14%	0.022 (-0.025, 0.095)	NA	0.034 (-0.023, 0.117)	NA
	TSH	0.026 (-0.021, 0.095)	NA	0.004 (-0.038, 0.049)	NA	0.006 (-0.040, 0.058)	NA
	T3	-0.004 (-0.076, 0.060)	0.32%	0.004 (-0.031, 0.044)	NA	0.011 (-0.033, 0.065)	NA
	fT4	-0.022 (-0.125, 0.071)	3.10%	0.014 (-0.029, 0.071)	NA	0.025 (-0.026, 0.094)	NA
	T4	-0.017 (-0.086, 0.034)	1.84%	-0.008 (-0.062, 0.030)	0.97%	0.004 (-0.041, 0.054)	NA
	T3/T4	0.004 (-0.045, 0.056)	NA	0.005 (-0.029, 0.050)	NA	0.022 (-0.017, 0.085)	NA

[a]Indication is NA when the TE and NIE are in different directions, rendering the percent mediated uninterpretable. Estimates refer to the increase in probability of experiencing binary outcomes, or the unit change in continuous outcomes, due to the resulting change in the mediator with an interquartile range increase in exposure, while holding the exposure constant. Boldface text indicates a p-value < 0.10.

Figure IV.4. Estimated -$\log_{10}$(p-values) of mediating effects by hormone concentrations on the associations between phthalate ERS and birth outcomes, among mothers carrying a female fetus.



From left to right within each panel, the vertical dashed lines represent p-values of 0.1, 0.05, and 0.01. All models were adjusted for continuous maternal age and categorical education.

Chapter V. Conclusions

Environmental contamination is extensive on the island of Puerto Rico and the karst aquifer system on the island, which distributes drinking water throughout Puerto Rico, allows contaminated water to travel large distances with ease. Preterm birth rates are also disproportionately high in Puerto Rico, so researchers suspect the high levels of environmental pollution may be playing a causal role in the elevated risk of early delivery. Environmental exposures, particularly phthalates, have the potential to disrupt the maternal endocrine system, regulation of which is essential for maintenance of a healthy pregnancy. Many studies have established the endocrine disrupting capacity of phthalates and the critical roles of hormones during pregnancy, but few studies have investigated phthalate endocrine disruption in the context of human pregnancy, nor have many studies assessed the possible endocrine mechanisms by which phthalates may illicit their effects on adverse birth outcomes. This dissertation adds significant knowledge to the pregnancy health literature by advancing our understanding of how exposures to phthalate mixtures affect hormone concentrations and downstream risk of adverse birth outcomes.

Summary of findings

This dissertation combined three aims which evaluated the endocrine disrupting effects of phthalate metabolites during pregnancy and the resulting impacts on risk of adverse birth outcomes. Aim 1 tested for associations between repeated measures of urinary phthalate metabolites and serum hormones over two time points during pregnancy in the PROTECT prospective birth cohort. We observed diverse phthalate metabolite associations with CRH, thyroid, and reproductive hormones. In alignment with our hypothesis, many observed associations were specific to certain fetal sexes or developmental windows. Additionally, the

direction of many associations within classes of hormones tended to depend on the molecular weight of the phthalate metabolite. CRH positive associations were observed with both HMW and LMW metabolites, most of which were stronger at study visit 1 and among male pregnancies. Most significant findings with HMW phthalates involved metabolites of DNP (MCNP and MCOP) with which positive associations were observed for thyroid hormones and inverse associations were observed with testosterone and SHBG. Conversely, most significant findings with LMW phthalates involved metabolites of DBP (MHBP) and DiBP (MHiBP) with which positive associations were observed for testosterone and inverse associations were observed for thyroid hormones. Given the anti-androgenic effects of phthalates previously reported in the epidemiology and toxicology literature, the inverse associations we observed with HMW phthalates were in line with our hypothesis. We did not expect to observe positive associations with any phthalates and testosterone, which was in fact observed with MHBP. We were also surprised to find minimal significant associations between phthalate metabolites and concentrations of progesterone and estriol in repeated measures analyses. However, sensitivity analyses did uncover additional significant relationships. MBzP and MHBP were inversely associated with estriol later in pregnancy, while MCNP and MCOP were positively associated with estriol earlier in pregnancy. Progesterone concentrations significantly decreased with increasing MHBP exposure among female pregnancies only, and inverse associations with MEHHTP were present among male pregnancies and in repeated measures analyses.

Various previous studies investigating phthalate associations with thyroid hormones during pregnancy report results that do not align with ours. One study reporting various inverse associations with T3[146] that we did not observe was conducted in a different time frame and among a population which has been shown to have distinct consumer product usage patterns to those seen in Puerto Rico, likely contributing to differing results. Another study which reported a positive association between MEP and T4[79], which we did not observe here, showed lower exposure levels than those in our study. Finally, studies from Taiwan and China likely found associations distinct from ours due to significant differences in exposure distributions[80] and gestational age at exposure and outcome assessment[148–150]. Similarly, previous studies assessing

testosterone associations with phthalate metabolites reported different exposure distributions than those in our study, and wide ranges of gestational ages at exposure assessment were used, likely driving inconsistencies between previous results and ours[83,160]. We have added new evidence of associations between phthalate metabolites and CRH and progesterone that have not been previously explored and thus need to be substantiated by more extensive research.

In aim 2 we evaluated associations between hormone concentrations over two time points during pregnancy and adverse birth outcomes in the PROTECT cohort. We observed significant increases in the risk of various adverse birth outcomes with changes in progesterone, estriol, and thyroid hormone concentrations. Upon fetal sex specific analyses, a large number of observed associations remained significant only among male pregnancies. Significant increases in the odds of spontaneous preterm birth among male pregnancies were observed with increases in CRH, progesterone, T3, and fT4, and with a decrease in testosterone, while all of those associations were null among female pregnancies. Very similar results were found for gestational age at birth. Progesterone and the ratio of progesterone to estriol were positively associated with odds of having a small for gestational age infant among all pregnancies and also among male pregnancies specifically. The progesterone to estriol ratio was also marginally associated with increased odds of preeclampsia, however case numbers for preeclampsia, as well as gestational diabetes, were low (less than 5% of study participants) and so confidence in those findings is relatively weak.

Our observed associations between timing of delivery and progesterone are somewhat supported by the literature, but also surprising. Previous work has shown higher concentrations of progesterone around 30 weeks among women who delivered preterm compared to term[176]. However, given the anti-labor functions of progesterone during pregnancy, we also expected to observe inverse associations between preterm birth and progesterone concentrations, particularly at later time points in pregnancy. Our observations of the ratio of progesterone to estriol being associated with reduced gestational age and risk of having a small for gestational age infant have not been substantiated in the previous literature. The existing research on thyroid hormone associations with preterm birth is heavily mixed; our findings for fT4 do not align with

any previously reported results, but our findings for T3 do align with previous results showing increased odds of preterm birth with elevated T3 concentrations at 10 weeks and 26 weeks gestation[159]. Our findings largely suggest that hormonal influences on birth outcomes differ between fetal sexes, an observation that has not been well studied in the past. Previous research does support the notion that male fetuses confer more risky pregnancies than female fetuses[181–183], further suggesting the need to study environmental exposures during pregnancy within a fetal sex-dependent framework.

Aim 3 provided a novel analysis which combined emerging statistical methods for evaluating environmental mixtures and causal mediation pathways. Specifically, we assessed the total effects of exposure to a mixture of phthalate metabolites, quantified as a phthalate environmental risk score (ERS), on adverse birth outcomes, and the mediating effects of hormone concentrations on those relationships. In alignment with our hypothesis, we observed significant mediation by progesterone on the association between phthalate ERS and odds of preterm birth among male pregnancies. We also hypothesized that we would observe significant mediation by CRH and estriol. While we did observe suggestive evidence of mediation by CRH and estriol on the association between phthalate ERS and spontaneous preterm birth, these findings were not robust and must be validated in future studies. Previous work led us to expect a larger number of significant results among male pregnancies relative to female pregnancies. However, we did not expect to observe entirely null mediating effects among female pregnancies. This aim provided novel results which lay the ground work for future epidemiology studies targeted at determining biological mechanisms of environmental contaminants within a mixtures framework.

No previous studies have assessed the mediating effects of hormone concentrations on associations between phthalate mixtures exposure and adverse birth outcomes. Previous studies do, however, provide evidence of the biological basis for progesterone mediating the association between phthalates and preterm birth. Those studies have shown disruption of the progesterone receptor by phthalate metabolites[216,217], which could result in elevated circulating progesterone

concentrations, which then participate in a negative feedback loop with the progesterone receptor gene to cause a reduction in expression of the progesterone receptor and thus reduced progesterone function.

Integration of findings

Findings across three aims have shown significant evidence of associations between phthalate metabolites and hormones which are critical for progression of a healthy pregnancy. Many observed associations between phthalates, hormones, and birth outcomes are supported by results from previous studies, but some findings are contradictory or novel. We provide introductory evidence of significant mediating effects of hormones, and it is critical that future studies work to substantiate our findings. Taken together, the three aims of this dissertation provided several insights:

Importance of phthalate metabolites for predicting changes in hormone concentrations and risk of adverse birth outcomes follows trends based on molecular weight.

The tendency of many previous studies to focus on a small number of phthalate metabolites, or even metabolites from only one parent phthalate compound, have impeded our abilities to understand differential threats posed by each phthalate. Here we have depicted that phthalate metabolites coming from high versus low molecular weight metabolites show differing associations with hormones and have differential predictive capacities for adverse birth outcomes. In aim 1, we showed that LMW phthalates were associated with increases in testosterone and decreases in thyroid hormones, while associations in the opposite directions were observed with HMW phthalates. Further, aim 3 showed that phthalate metabolites contributing most significantly to risk scores for preterm and spontaneous preterm birth were largely LMW metabolites, with one exception of MCNP. LMW metabolites, particularly those of DBP and DiBP, have been previously shown to be important for prediction of preterm birth in the PROTECT cohort[47], and these findings together call attention to the need for epidemiological and toxicological assessments beyond HMW phthalates, particularly DEHP, that are so commonly reported in the current literature.

Fetal sex is important for determination of true associations.

Findings from all three aims strongly suggest that any future epidemiology studies during pregnancy should assess differences by fetal sex. We have provided evidence that hormone concentrations during pregnancy are heavily influenced by fetal sex, manifested in differential associations between phthalates and hormones, between hormones and birth outcomes, and differential mediating effects of hormone concentrations on associations between phthalate mixtures and birth outcomes. In aim 1, increasing phthalate exposures were associated with significant decreases in progesterone, T3 and T4 among female pregnancies, while increasing phthalate exposures were associated with increasing CRH and T3 among male fetuses. In aim 2, results among female pregnancies were mostly null, while results among male pregnancies showed increased odds of multiple birth outcomes with changing hormone concentrations. Finally in aim 3, despite established associations between phthalate exposure and hormone alterations among both fetal sexes in aim 1, we showed that significant mediation by hormone concentrations on associations between phthalate ERS and adverse birth outcomes could only be observed among male pregnancies. These findings do not discount the importance of phthalate endocrine disruption during female pregnancies, but rather they point to a mechanism of phthalate action on pregnancy via endocrine disruption that is particularly significant during male pregnancy.

On the pathway from phthalate exposure to adverse birth outcomes, CHR and reproductive hormones may be more important than thyroid hormones.

While significant findings for thyroid hormones were observed, and previous research clearly indicate the importance of thyroid hormones during pregnancy, our aggregate results do not suggest that phthalate disruption of thyroid hormones plays a significant role in the risk of experiencing adverse birth outcomes. As previously mentioned, LMW phthalates appear to be largely responsible for predicting adverse birth outcomes relative to HMW phthalates. The majority of significant associations observed between phthalates and thyroid hormones in aim 1 involved HMW metabolites, particularly when study visit and fetal sex effects were

being assessed. Further, fetal sex-specific assessments of hormones in aim 2 revealed the most compelling results for CRH, estriol, and progesterone conferring differential risk of preterm and spontaneous preterm birth, while thyroid hormones showed largely null results for those outcomes. Finally, not even suggestive evidence of mediation by thyroid hormones on relationships between phthalate ERS and birth outcomes was observed, while both significant and suggestive evidence of mediation by CRH and reproductive hormones was observed. All together, these results indicate that CRH and reproductive hormones should be interrogated in future research as potentially playing a role in the causal pathway between phthalate exposures and adverse birth outcomes.

Directions of future research

Despite the novel and significant findings of this dissertation, future work should still seek to substantiate and improve upon results reported here. First and foremost, as the PROTECT cohort grows and we obtain larger numbers of cases of these adverse pregnancy outcomes, it is critical to continuously reevaluate observed associations. This is particularly important for rare birth outcomes such as preeclampsia and gestational diabetes which occur in less than 5% of the PROTECT population. Additionally, testing for associations with phthalate concentrations presents a unique set of challenges due to the rapid clearance of metabolites from the body. While we were able to assess urinary phthalate concentrations at more than one time point during pregnancy, a larger number of measurements during mid-pregnancy and in earlier and later stages of pregnancy will help to educate us on phthalate effects during different developmental windows of susceptibility. Further to this point, the PROTECT cohort has urinary phthalate data at one time point in addition to those assessed in this dissertation, however we do not have serum hormone data at that additional time point, preventing us from assessing relationships at that time. Future phthalate epidemiology studies should also strive to include assessments of phthalate replacement chemicals and both high and low molecular weight phthalate metabolites, given the heavy emphasis on DEHP metabolites in the present literature and the importance of LMW metabolites evidenced in this dissertation.

Assessing hormone concentrations during pregnancy also presents challenges to investigators that must be considered. Some hormones are present at differing concentrations depending on the stage of pregnancy and so it is important to understand how environmental toxicants may impact hormone concentrations differently during each of these gestational stages. Some hormones should also be assessed with the status of other health conditions in mind. For example, individuals with thyroid autoimmunity disorders possess antibodies against their own thyroid hormones and so knowledge of thyroid autoimmunity status is necessary to truly understand associations. We did not have access to thyroid autoantibody measurements for these analyses, but future work should seek to include those measures. Hormone concentrations in maternal circulation may not indicate actual physiological changes that occur in response to endocrine disruption. As previously discussed in regards to progesterone, increasing concentrations may result from reduced expression of hormone receptors, and so the body's response to progesterone is lowered, despite elevated hormone concentrations. Future epidemiologic work should seek to evaluate other measures of endocrine disruption in addition to circulating serum concentrations of hormones. Finally, and very importantly, the current pregnancy literature does not consistently assess differences in associations between fetal sexes. Some previous findings, in addition to those reported in this dissertation, point to significant differences between male and female fetal sexes in the risk for experiencing adverse birth outcomes and for the endocrine disrupting abilities of gestational phthalate exposures. Particularly when trying to determine mechanisms by which these phthalates act, it is important that studies attempt to disentangle relationships that are different between fetal sexes.

Overall conclusions

In conclusion, this dissertation provides significant and novel information regarding the endocrine disrupting capabilities of gestational phthalate exposures and the resulting implications for the health of human pregnancy. The results reported here add to an existing body of literature demonstrating the hormone disrupting capacity of phthalates and add new evidence of differential associations based on molecular weight of the phthalate and sex of the fetus. We also add evidence to existing literature that changes in hormone concentrations have

significant impacts on the risk of experiencing adverse birth outcomes, and that this risk is significantly different between male and female pregnancies. Finally, we add novel mechanistic information to the reproductive epidemiology literature suggesting that mixtures of phthalate metabolites interfere with progesterone concentrations to confer greater risk of preterm delivery among only male pregnancies. Results from this dissertation further our efforts to understand increased rates of preterm birth observed on the island of Puerto Rico, and provide additional tools that can be used to predict at-risk pregnancies and better protect this highly vulnerable population.

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