Environmental Phthalate Exposure, Maternal Thyroid Function, and Birth Outcomes

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Environmental Health Sciences)
in the University of Michigan
2017

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ACKNOWLEDGEMENTS

I am extremely grateful to have attended and completed both my master's and doctoral training at University of Michigan's School of Public Health. My time in Ann Arbor has been extremely gratifying both academically as well as personally, and I will miss it dearly. I would especially like to thank my adviser, Dr. John Meeker, for his unwavering support and expertise he has provided me throughout my time in the doctoral program. His encouragement to pursue new ideas as well as academic and professional experiences have allowed to me gain skills well beyond what I thought was possible. I would also like to thank my amazing committee members, Drs. Thomas McElrath, Bhramar Mukherjee, and Rita Loch-Caruso, for their mentorship, guidance, and enthusiasm. I appreciate your good humor and insight at every stage of my research, and I am incredibly grateful to have you as life-long mentors. I would also like to thank Dr. Kelly Ferguson for her mentorship and guidance throughout the tenure of my doctoral research. I am appreciative of my funding sources including University of Michigan's Rackham Pre-Doctoral Fellowship; National Institute of Environmental Health Sciences' (NIEHS) Environmental Toxicology and Epidemiology Training Grant (T32ES007062); as well as NIEHS grants P42ES017198, R01ES018872, P30ES017885, and P01ES022844. I am indebted to my family and friends both near and far for their emotional support and willingness to provide invaluable distraction. A special thank you to my mom and dad as well as my sisters, Jenny and Sarah, for their love, guidance, and friendly reminders that I need to graduate. I couldn't have completed this without you! Finally, I would like to thank my friends and colleagues in the Department of Environmental Health Sciences, especially the Meeker Lab Research Group for their wonderful support.

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ABSTRACT

Preterm birth (< 37 completed weeks of gestation) and impaired fetal growth are among the most frequent causes of perinatal morality worldwide, and are associated with numerous long-term health consequences among surviving infants. While the causes of preterm birth and its consequences are complex and likely interrelated, characterization of potentially modifiable risk factors –such as those posed by environmental exposure to phthalates – may help diminish the substantial public health burden associated with these adverse pregnancy outcomes. Increasing scientific evidence suggests that exposure to phthalates during pregnancy may be associated with increased risks of deleterious birth outcomes such as preterm birth. Maternal endocrine disruption in gestation may be one pathway mediating some of these relationships. This dissertation research focuses on subclinical maternal thyroid hormone disruption as a potential biological pathway by which prenatal phthalate exposure influences adverse birth outcomes because (1) maintaining homeostatic maternal thyroid hormone levels throughout pregnancy is crucial for normal fetal growth and development; (2) few data are available on the direct effects of subclinical thyroid hormone changes in gestation on the risk of preterm birth; and (3) phthalate-associated thyroidal disturbances in pregnancy is a largely understudied area of environmental and reproductive epidemiology.

In the first aim of this dissertation research, we observed that repeated measures of urinary phthalate metabolites were associated with altered maternal thyroid hormone levels in two qualitatively disparate populations of pregnant women in Northern Puerto Rico and Boston, MA. The second and third aims were conducted among the main dissertation cohort (a nested case-control study of preterm birth in Boston, MA), and explored the associations between subclinical changes in maternal thyroid function parameters and preterm birth as well as ultrasound and delivery indices of fetal growth. Our findings from Aim 2 suggest that subclinical alterations in individual maternal thyroid hormones, specifically free T4 (FT4) and total T3 (T3), influence the risk of preterm birth and the strength of these associations vary by gestational age.

Additionally, we observed differences in the temporal hormonal patterns across gestation between women who delivered preterm and those who delivered at term, and these variations were most evident in the first trimester of pregnancy. Finally, our results from Aim 3 support findings from previous studies showing inverse associations between subclinical changes in FT4 and fetal growth indices including birth weight in generally euthyroid pregnant women. Overall, the public health implications of this research need to be determined. Additional human health and animal studies are required to confirm these observed relationships and to determine the potential biological mechanisms that underlie the effects of phthalate-induced thyroidal disturbances on pregnancy and birth outcomes.

CHAPTER I. Introduction

BACKGROUND

Preterm birth (< 37 completed weeks of gestation) and impaired fetal growth are among the most frequent causes of perinatal morality.[1-5] In 2015, the rate of preterm birth in the United States was 9.62%, a marginal increase from the 9.57% rate recorded in 2014.[6] This marks the first rise in the preterm birth rate since at least 2007, the earliest year for which national data were available for the National Center for Health Statistics' new measure for estimating gestational age of a newborn (named the obstetric estimate of gestation at delivery).[6,7] The rate of babies born with low birth weight (< 2,500 grams), a potential consequence of both preterm delivery as well as fetal growth restriction (birth weight < 10th percentile for gestational age), has followed a similar temporal trend.[5,6] Subsequent to the decreasing rates observed between 2007 and 2014, the low birth weight rate increased slightly to 8.07% in 2015 from the 8.00% rate documented in 2014.[6] Although birth outcomes in the United States are arguably better than those observed in many developing countries, the United States still has one of the highest rates of infant mortality and preterm birth of developed nations.[8] In 2010, the preterm birth rate in the United States (excluding births at less than 24 weeks) was 40% higher than England and Wales and over 69% higher than Finland, Ireland, and Sweden; and ranked last out of the 19 European countries studied.[9]

Although recent medical advances have improved survival among both preterm and growth-restricted infants, the long-term health consequences associated with these adverse birth outcomes are significant.[1,2] Babies born preterm or growth-restricted are at an elevated risk for lasting physical and neurodevelopmental complications such as hearing and vision impairment, cerebral palsy, mental retardation, learning difficulties, and non-communicable disease.[2,10-13] These life-long complications, coupled with neonatal and maternal care at delivery, have a

profound societal economic impact.[10] In 2007, the Institute of Medicine (IOM) estimated that the annual economic cost associated with preterm birth in the United States was at least $26 billion.[11] As a result of the persistent and deleterious consequences of preterm birth over the last two decades, the IOM assembled a committee to identify research gaps and priorities needed to address the risk factors contributing to the considerable preterm birth rate in the United States.[11] In its 2007 report, the IOM committee noted that the dearth of scientific knowledge pertaining to the environmental etiologies of preterm birth presented "a potentially significant shortcoming for the design of public health prevention strategies".[11] While the causes of preterm birth and fetal growth restriction are complex and interrelated,[5,11,12] characterization of potentially modifiable risk factors, such as those posed by exposure to environmental contaminants, may help diminish the substantial public health and economic burdens associated with these adverse birth outcomes.

Phthalate uses, metabolic patterns, and exposure assessment. The phthalate metabolites measured in this current dissertation research as well as their parent compounds are listed in **Table I.1**. Phthalate diesters are commonly used as plasticizers in industrial applications.[12] Additionally, many personal care products and cosmetics, such as fragrances, skin lotions, nail polish, and eye shadows, may contain some types of phthalates (e.g., diethyl phthalate [DEP], di-n-butyl phthalate [DBP], diisobutyl phthalate [DiBP]) as a solvent, fixative or alcohol denaturant.[13-17] DEP, DiBP and di(2-ethylhexyl) phthalate (DEHP) have been used as a component of food and pharmaceutical packaging.[14,16,18-21] Other uses of phthalates have included vinyl flooring (butylbenzyl phthalate [BBzP]) and medical tubing and devices (DEHP). Importantly, over the last decade, alternative chemicals have been increasingly substituted for polyvinyl chloride and/or certain phthalates (e.g., DEHP and DBP) in some consumer products (e.g., cosmetics and children's toys) and medical devices (e.g., blood storage bags).[16,22,23] Thus, the above descriptions may not be entirely representative of the current phthalate content of these products.

Following exposure and uptake, phthalates are rapidly metabolized and excreted in urine and feces.[16] The biological half-lives of phthalate metabolites have been estimated to be between approximately 3 to 18 hours.[16] Phthalates typically undergo phase I hydrolysis followed by phase II conjugation, but metabolism patterns can differ by phthalate [16,24]. In phase I the phthalate diester is hydrolyzed into the potentially more bioactive monoester metabolite by lipases and

esterases in the intestinal epithelium, liver, blood and other tissues, and systemically distributed [16,25,26]. The monoester metabolites then: 1) undergo phase II biotransformation, catalyzed by UGTs (uridine 5'-diphosphate glucuronosyltransferases), to form glucuronide-conjugated monoesters that are excreted in the urine [27,28]; 2) go through phase I biotransformation reactions (e.g., oxidation) to form more hydrophilic (and likely less bioactive) secondary oxidized metabolites prior to glucuronidation [29,30]; and/or 3) a portion of the unconjugated (free) monoester and/or secondary metabolites may also be directly excreted in urine.[16] The extent to which hydrolytic monoesters are further oxidized to secondary metabolites depends on the alkyl chain length of the parent compound. For the shorter chained phthalates (DEP, DBP, DiBP, butyl benzyl phthalate [BBzP]), approximately 70-80% of an oral dose is excreted as the simple monoester metabolite in urine compared to less than 10% and 2% of long-chained phthalates DEHP and di-isononyl phthalate (DiNP), respectively. [16,27,31-36]

In epidemiology and human biomonitoring studies, urine is the most common biological matrix used to assess environmental exposure to phthalates. Although many different biological specimens have been used for assessing environmental exposure to chemicals in humans – such as blood (serum and plasma), saliva, sweat, semen, breast milk, amniotic fluid, and umbilical cord blood – urine offers several advantages in exposure assessment.[16] These advantages include: ease of sample collection, larger sample volumes, greater concentrations of the metabolites, and reduced potential for contamination by the parent diester and subsequent formation of metabolites by enzymes present in blood.[16,36] However, because phthalates are metabolized and excreted rapidly, concentrations in a single urine sample reflect exposure to the parent compound or the metabolite itself in the preceding hours or days depending on the phthalate.[16] Thus, there is some concern as to whether a single urine sample can accurately capture longer term exposures (such as weeks to months). Additional urine samples collected serially over time, as was utilized in the present dissertation research, can help mitigate the potential exposure misclassification over longer periods of time.

Environmental phthalate exposure is widespread. Due to their ubiquitous use, human exposure to phthalates is widespread.[37] Because phthalates are not chemically bound to the plastics and other products that contain them, they can easily migrate into household dust, food and water

sources, and ambient air.[12,38,39] Consequently, human exposure to phthalates can occur via ingestion, inhalation, and/or dermal absorption.[12,40]

Biomonitoring studies have documented pervasive exposure to phthalates among the United States general population in the last decade.[41,42] Urinary concentrations of monobenzyl phthalate (MBzP), monobutyl phthalate (MBP), and monoethyl phthalate (MEP) – metabolites of BBzP, DBP, and DEP, respectively – were detected in at least 98% of participants in each cycle of the National Health and Nutrition Examination Survey (NHANES) from 2001 to 2010.[42] Urinary concentrations of the oxidized metabolites of DEHP (e.g., mono(2-ethyl-5-oxohexyl) phthalate [MEOHP], mono(2-ethyl-5-hydroxyhexyl) phthalate [MEHHP], and mono(2-ethyl-5-carboxypentyl) phthalate [MECPP]) were also detected in nearly all participants.[42] Notably, females had consistently greater concentrations of certain urinary phthalate metabolites than males across the 10-year study.[41,42]

Extensive exposure to phthalates has also been reported in pregnant women in the United States[43,44] and Puerto Rico.[45] The detection of several phthalates in amniotic fluid and cord blood indicate that phthalates or their metabolites can cross the placental barrier, resulting in fetal exposure.[46-49] Given the reproductive and developmental toxicities of phthalates reported in animal studies, exposure to these chemicals during particularly vulnerable periods of development may pose significant health risks for pregnant women and their unborn babies.[50,51]

Phthalate exposure and birth outcomes. Growing evidence suggests that environmental contaminants may influence gestational age at delivery as well as fetal growth.[11,52-54] Until recently, epidemiology studies investigating the environmental factors associated preterm birth and fetal growth have focused predominately on prenatal exposures to organochlorine and organophosphate pesticides, lead, tobacco smoke, and air pollution.[11,55] The contribution of low-dose exposures to ubiquitous environmental contaminants such as phthalates is less understood.

Animal studies have shown phthalate-induced adverse effects on pregnancy outcomes at exposure levels greater than those experienced by humans. In particular, rodents exposed *in utero* to phthalates had increased pregnancy loss, elevated fetal resorption and death, reduced fetal weight, and decreased gestational length.[56-60] Results from available human studies

assessing the associations between prenatal phthalate exposure and adverse birth outcomes (including preterm birth) and fetal growth have been less conclusive.

Pertaining to preterm birth, a nested case-control study conducted among Mexican mother-infant pairs found higher mean third-trimester urinary concentrations of MECPP, MBP, and mono(3-carboxypropyl) phthalate ([MCPP]; an oxidized metabolite of both DBP and di-n-octyl phthalate [DOP]) in women delivering preterm (<37 weeks of gestation) compared with women delivering at term (≥37 weeks of gestation).[61] Additionally, phthalates detected in cord blood samples collected from Chinese pregnant women were associated with an elevated odds of preterm delivery.[62] In contrast to these findings, increased levels of urinary DEHP metabolites (e.g., mono(2-ethylhexyl)phthalate [MEHP], MEOHP, and MEHHP) in the latter half of pregnancy were associated with reduced odds of preterm birth among pregnant women participating in a multicenter birth cohort in the United States.[63] Moreover, a subsequent investigation among pregnant women in the Netherlands reported a null association between maternal occupational exposure to phthalates and preterm birth.[64] Several studies have investigated the role of prenatal exposure to phthalates in the length of gestation.[47,62,65-68] However, the results of these studies have been inconsistent, showing both positive, negative, and null associations. These discrepant findings related to preterm birth and gestational age at delivery may be consequent to study limitations related to exposure assessment (i.e., single spot urine samples collected at varying time points in pregnancy, self-reports of occupational exposure, and blood measurements of phthalate exposure), small sample sizes, ascertainment of outcomes (i.e., gestational age based on participant recall of last menstrual period), and/or residual confounding.

In a case-control study nested within an ongoing prospective birth cohort in Boston – the main study population of the this dissertation research – urinary concentrations of MEHP, MECPP, and molar sum of DEHP metabolites were associated with significantly elevated odds of preterm birth compared with controls.[69] In contrast to many of the previous aforementioned epidemiological investigations that assessed phthalate exposure via a single spot urine sample in late pregnancy, which may be an unreliable indicator of prenatal exposure,[70] Ferguson and colleagues[69] measured urinary phthalate metabolite concentrations using multiple urine samples collected longitudinally across pregnancy. To reduce potential outcome misclassification, the

authors used clinically and first trimester ultrasound validated gestational dates (in lieu of self-reported dates of last menstrual period). While unequivocally additional epidemiological investigations are required to affirm these findings in qualitatively disparate populations of pregnant women, Ferguson and colleagues' study[69] nonetheless provides compelling evidence for a potential role of *in utero* phthalate exposure in the risk of preterm birth.

A similar inconsistent pattern of results, likely due to the same study limitations explicated above for preterm birth, exists in the body of literature pertaining to prenatal phthalate exposure and ultrasound and delivery indices of fetal growth. In a multiethnic cohort of mother-infant pairs in New York City, higher third trimester urinary concentrations of summed low molecular weight phthalates were significantly associated with increased head circumference at birth.[67] In a subsequent nested case-control study in China, *in utero* DBP and DEHP exposure (assessed via parent compound concentrations in cord blood and metabolite concentrations in meconium) were significantly associated with low birth weight and shorter birth length among term babies, respectively.[71] Similar findings were observed in a population-based cohort in the Netherlands, wherein occupational exposure to phthalates was associated with decreased fetal length and weight.[72] However, positive associations have also been reported between phthalate metabolites and birth weight,[73,74] and analyses among other, large birth cohorts have produced generally null associations between maternal urinary concentrations of phthalates and fetal growth parameters during pregnancy and at birth [54,65,75] and between maternal occupational exposure to phthalates and decreased weight at birth (< 3000 grams).[64]

Ferguson and colleagues recently performed an additional analysis within the same nested case-control study as that mentioned previously for their work on preterm birth (i.e., the population of pregnant women included in the current dissertation research) wherein the authors investigated the extent to which maternal exposure to phthalates was associated with various indices of fetal growth.[76] In their longitudinal analysis, utilizing repeated biomarker and ultrasound measurements in pregnancy, the authors observed inverse associations between urinary DEHP metabolites and head and abdominal circumferences, femur length, and estimated fetal weight.[76] Results from this study provide suggestive evidence that maternal exposure to phthalates, specifically DEHP, in pregnancy may be associated with reduced fetal growth. Building upon these and previous findings related to preterm birth, the present study seeks to

augment the current mechanistic understanding of phthalate-induced adverse birth outcomes in this cohort of pregnant women.

Thyroid hormone disruption as a biological mechanism. The biological mechanisms through which phthalates act to influence downstream adverse birth outcomes may involve inflammation, oxidative stress, and endocrine disruption. There is a well-established link between strong inflammatory responses in pregnancy (e.g., as a result of maternal infection), particularly those mediated by cytokine and chemokine activation, and preterm labor and preterm premature rupture of the membranes (PPROM).[11,77-82] Research has also shown that elevated levels of oxidative stress in pregnancy, caused by an imbalance of free radical production and availability of antioxidants necessary for detoxification, is associated with various adverse pregnancy outcomes such as fetal growth restriction, preterm birth (involving intact membranes), and PPROM.[83] Animal and cellular studies have shown that phthalates, in particular DEHP and its hydrolytic monoester (MEHP), may induce pro-inflammatory and oxidative stress processes.[84-87] Human studies have corroborated these findings in adult men and non-pregnant women.[88-90] Among pregnant women, a recent analysis performed among this study's cohort in Boston showed that increases in urinary phthalate metabolites were significantly associated with elevated levels of urinary biomarkers of oxidative stress (8-hydroxydeoxyguanosine and 8-isoprostane), each measured at multiple times points across gestation.[91] Similar findings were reported in a subsequent longitudinal analysis conducted among Puerto Rican pregnant women, an additional prospective birth cohort among whom the present dissertation analyses were performed.[92]

Phthalates may alter the hormonal milieu of pregnancy to induce downstream adverse birth outcomes as well. To date, much of the scientific literature has highlighted phthalate-induced hormonal disruptions along the hypothalamic-pituitary-gonadal axes. Less is known about the role of phthalates in altering thyroidal function in particularly susceptible populations such as pregnant women.

Thyroid hormones are essential for metabolism as well as growth and development.[93] Production of thyroid hormones are tightly regulated by the negative feed-back system that involves hypothalamus, pituitary, and thyroid gland (**Figure I.1**).[94] Thyroid hormones (T3 and

T4) remain within narrow ranges due to the release of hypothalamic thyrotropin releasing hormone (TRH) and pituitary thyroid stimulating hormone (TSH).[95] T4 is exclusively produced by the thyroid gland whereas ~80% of T3 is produced in extrathyroidal tissues through the conversion of T4 to T3 by deiodinase enzymes.[96] The vast majority of thyroid hormones in circulation are bound to serum proteins; over 99.95% of T4 and 99.5% of T3 are bound to transport proteins.[94,96] Although both free T4 (FT4) and free T3 (FT3) are unbound in circulation and are available for cellular uptake, FT3 binds more readily to nuclear receptors in thyroid-responsive tissues (including placenta) where its binding initiates transcriptional regulation of target genes.[94-96] Thus, FT3 is considered the primary bioactive hormone and FT4 is largely a prohormone. [94-96] In clinical practice, TSH has been recommended as the main screening test for thyroid dysfunction and often is tested in combination with FT4 to mitigate potential misdiagnosis. [95]

In pregnancy, the hormonal and metabolic demands increase and induce profound alterations in maternal thyroid physiology.[97] During the first half of pregnancy, total T4 and T3 may increase by as much as 50% due to estrogen-stimulated increases in thyroxine-binding globulin (TBG), the major blood transport protein of thyroid hormones.[98,99] Levels of T4 and T3 eventually level off and the increased thyroidal output is maintained until term, owing in part to transplacental passage of maternal hormones and increased deiodinase activity of the placenta, which regulate circulating levels of thyroid hormones by metabolizing T4 to T3 and/or deactivating T4 to reverse T3 (rT3).[98] A second major change of maternal thyroid parameters in pregnancy involves a transient decrease in maternal serum TSH during the first trimester.[97,99,100] The characteristic dip in TSH in early pregnancy is a result of the weak thyroid-stimulating activity of human chorionic gonadotropin (hCG), whose secretion by the placenta increases during this period of gestation, and the subsequent negative feedback on the thyroid gland by increases in total hormone levels [97,99,100] Also contributing to the elevated thyroidal activity during pregnancy is the increased renal clearance of iodine due to an increased glomerular filtration rate.[98] Finally, in iodine sufficient pregnancies, free hormone levels in pregnant women remain within the non-pregnant reference ranges, although slight reductions in free T4 (FT4) and free T3 (FT3) concentrations occur during the latter half of pregnancy.[98]

Maintaining maternal euthyroidism throughout pregnancy is essential for embryogenesis as well as fetal growth and development, especially neurodevelopment.[43,101-105] Thyroid hormones play an integral role in early placental development as well as fetal tissue accretion and differentiation.[106,107] During the first trimester, the fetus relies solely on maternal thyroid hormones until the fetal thyroid gland becomes fully functional after 18 weeks of gestation.[91,92] In later pregnancy, maternal thyroid hormones are essential for fetal thyroid homeostasis.[102]

Epidemiological investigations have shown that overt thyroid disease in early and late gestation is associated with various pregnancy complications such as preterm birth, low birth weight, and fetal growth impairment.[108-112] Maternal subclinical thyroid disease, specifically subclinical hypothyroidism (elevated TSH with normal FT4), has also been linked to similar deleterious birth outcomes such as preterm birth and fetal growth impairment, although these data are not as conclusive.[109] For example, several studies have shown associations between subclinical hypothyroidism and an increased risk of preterm birth in cohorts in the United States [113,114] and China,[112] whereas other investigations have reported null associations.[115-117] The observed dissimilarities in the results of these studies may be due to limitations associated with a small number of cases consequent to a lack of sample size, differences in laboratory methods used for hormone measurements, variations in the classification of thyroid disease, and/or inconsistent ascertainment of outcome measurements (e.g., gestational age based on self-reported last menstrual period vs. ultrasound measurements). Additionally, each of the above studies assessed subclinical thyroid disease using a single blood sample taken in the first half of pregnancy. There is a lack of data pertaining to the effects of trimester-specific subclinical alterations in individual thyroid hormone parameters, especially in late gestation, on the risk of preterm birth. Furthermore, the extent to which these relationships vary by clinical presentation of preterm birth remains unknown.

Inconsistent findings, likely for similar reasons as explicated above for preterm birth, have also been observed across studies investigating the effects of subclinical hypothyroidism on various indices of fetal growth: both positive [113,118,119] and null associations [115-117,120] have been reported. Additionally, in a prospective birth cohort study in the Netherlands, higher normal-range FT4 concentrations were associated with low birth weight and an increased risk of small for gestational age at birth; no associations were observed for TSH.[121] This inverse relationship

between higher FT4 concentrations within generally euthyroid pregnant women and birth weight have been corroborated by other studies.[122-124] Notably, the majority of these studies restricted assessment of thyroid function to early pregnancy. While the fetus is unable to independently synthesize thyroid hormones until the second trimester, there is evidence that the fetus may rely on maternal thyroid hormones for growth and development even in late gestation.[123,125] To our knowledge, only one longitudinal study has investigated the impact of subclinical alterations in thyroid hormone parameters across trimesters on fetal growth. Specifically, Nishioka and colleagues[126] reported an inverse association between an increase in maternal TSH concentrations between the first and third trimester and low birth weight. This study's small sample size (N=163 neonates) and thus limited number of low birth weight babies (N=10), precluded trimester-specific analyses (in lieu of examining absolute changes across the three trimesters) and likely contributed to the lack of variation in free hormone concentrations observed across the study population. In contrast to Nishioka and coauthors' study,[126] the present dissertation research includes nearly three times as many participants, permitting both longitudinal and cross-analyses that utilize thyroid hormone measurements and standardized growth measurements collected at multiple time points in pregnancy.

Various biological mechanisms have been proposed through which phthalates may exert their action on thyroid function. It has been suggested that phthalates may bind to thyroid hormone receptors, consequently activating or inhibiting thyroid hormone action,[127-129] although data overtly demonstrating the binding of phthalates to thyroid receptors are lacking. Available experimental studies have provided some evidence for these potential mechanisms of thyroid disruption. *In vitro* studies have shown that phthalates may alter the sodium/iodide symporter-mediated uptake of iodide by thyroid follicular cells[130,131],[113,114] exhibit thyroid receptor antagonist activities,[132-134] or displace thyroid hormones (e.g., T3) from distributor proteins.[135] Additionally, phthalates were found to alter the transcription of genes involved in the hypothalamic-pituitary-thyroid axis as well as the whole-body content of thyroid hormones in zebrafish.[136]

Limited human health studies have examined the potential thyroid-altering effects of phthalates. In a cross-sectional study of men recruited from a U.S. fertility clinic, the urinary concentration of MEHP was inversely associated with FT4 and total T3.[137] Urinary

concentrations of DEHP metabolites were also inversely associated with total T3 and total and free T4, and positively associated with TSH in a representative sample of U.S. adults (non-pregnant women and men) participating in NHANES.[138] Similar inverse relationships between urinary DEHP metabolites and total and free T3 were reported in a cross-sectional study of Danish children.[139] While pervasive exposure to phthalates has been documented among pregnant women worldwide,[48,62,123-126] human health studies investigating the effects of phthalate exposure during pregnancy on maternal thyroid function are scarce. Huang and colleagues [140] reported an inverse association between urinary concentrations of MBP, and both free and total T4 in the second trimester among 76 Taiwanese pregnant women undergoing amniocentesis. In a more recent cross-sectional analysis conducted among a separate cohort of Taiwanese pregnant women (N=148), Kuo and colleagues[141] observed significant inverse unadjusted associations between several urinary phthalate metabolites (MEOHP, MEHHP, and MBzP) and serum TSH in the third trimester. While these findings provide suggestive evidence for phthalate-induced thyroidal disturbances during pregnancy, these studies are limited by their small sample size and cross-sectional study designs.

In conclusion, this dissertation research focuses on subclinical maternal thyroid hormone disruption as a potential biological pathway by which prenatal phthalate exposure influences adverse birth outcomes because (1) maintaining homeostatic maternal thyroid hormone levels throughout pregnancy is crucial for normal fetal growth and development; (2) few data are available on the direct effects of subclinical thyroid hormone changes in gestation on the risk of preterm birth; and (3) phthalate-associated thyroidal disturbances in pregnancy is a largely understudied area of environmental and reproductive epidemiology.

SPECIFIC AIMS

The present dissertation will focus on the effects of maternal subclinical thyroid hormone disruption in potentially mediating the relationships between maternal phthalate exposure and preterm birth and fetal growth.

Aim 1. *Examine the extent to which maternal urinary phthalate metabolite levels are associated with maternal thyroid hormones concentrations (including TSH, free and total T4, and/or free and total T3), each measured at multiple time points during pregnancy.*

Phthalate-associated thyroidal disturbances in pregnancy is a largely understudied area of environmental and reproductive epidemiology. These associations were initially explored in a relatively small, prospective cohort of pregnant women from Northern Puerto Rico, and were subsequently repeated among the main dissertation cohort consisting of pregnant women who planned to deliver at Brigham and Women's Hospital in Boston, MA.

I hypothesized that (1) urinary phthalate metabolites will be inversely associated with maternal thyroid hormone concentrations, including free and total T4 and T3, and (2) the relationships between urinary phthalate metabolite levels and plasma thyroid hormone concentrations vary by time point of sample collection during gestation, with stronger associations observed in the latter half of pregnancy.

Aim 2. *Assess the extent to which maternal thyroid hormone concentrations measured at multiple time points in pregnancy are associated with preterm birth, and to explore differences in the patterns of these hormones across pregnancy between women who delivered preterm and at term.*

Maternal thyroid hormones are essential for early placental development and for initiation and maintenance of the pregnancy state. Few data are available on are available on the direct effects of subclinical changes in maternal thyroid hormones in gestation on the risk of preterm birth, and no studies have investigated differences in hormonal patterns across pregnancy in women delivering preterm vs. term.

I hypothesized that (1) maternal plasma thyroid hormone associated are associated with an elevated odds of preterm birth, and (2) these associations vary by clinical presentation of preterm delivery (spontaneous preterm birth vs. preterm birth resulting from aberrant placentation) and by the time point of sample collection during gestation.

Aim 3. *Determine the extent to which maternal thyroid hormone concentrations are associated with ultrasound and delivery indices of fetal growth.*

Results from available human health studies investigating the associations between subclinical thyroid dysfunction in pregnancy and fetal growth are conflicting. The majority of these studies assess fetal growth via birth weight or other anthropometric measurements at delivery. Few analyses have explored these associations using repeated measures of maternal thyroid hormone concentrations and fetal growth collected across pregnancy.

I hypothesized that maternal plasma thyroid hormone concentrations are inversely associated with fetal growth measurements, including birth weight.

STUDY POPULATIONS

PROTECT cohort [Aim 1]. The Puerto Rico Testsite for Exploring Contamination Threats (PROTECT) project is an ongoing prospective birth cohort in the Northern Karst Region of Puerto Rico designed to investigate the relationship between phthalates and other environmental contaminants and adverse pregnancy outcomes such as preterm birth. The study population for the present dissertation research included data collected from the first 106 pregnant women with urinary phthalate metabolites and serum thyroid and sex hormones measures completed as of November 2012. Biomarker measurements were available at up to two time points in pregnancy. Additional information regarding subject recruitment and eligibility as well as data collection is provided in the subsequent dissertation data chapters.

LifeCodes cohort [Aims 1-3]. Participants were part of a nested case-control study of preterm birth drawn from the LifeCodes cohort, a prospective birth cohort of pregnant women who planned to deliver at Brigham and Women's Hospital in Boston, MA. Women were followed until delivery, and provided urine and blood samples for biomarker measurements and underwent ultrasound scans at up to four time points in pregnancy. Additional information regarding subject recruitment and eligibility as well as data collection is provided in the subsequent dissertation data chapters. From the prospective LifeCodes cohort, 130 women who delivered preterm (< 37 weeks) and 352 randomly selected controls were included in the nested case-control study. For the current dissertation research, an additional 41 women with self-reported pre-existing or gestational thyroid disease/conditions (e.g., hyper- or hypothyroidism, Graves' disease, or thyroid cancer) based on answers to medical questionnaires administered at each of the study visits were additionally excluded. Two women who did not provide plasma samples at any study visit were also excluded. The final study population included 116 cases of preterm birth and 323 controls.

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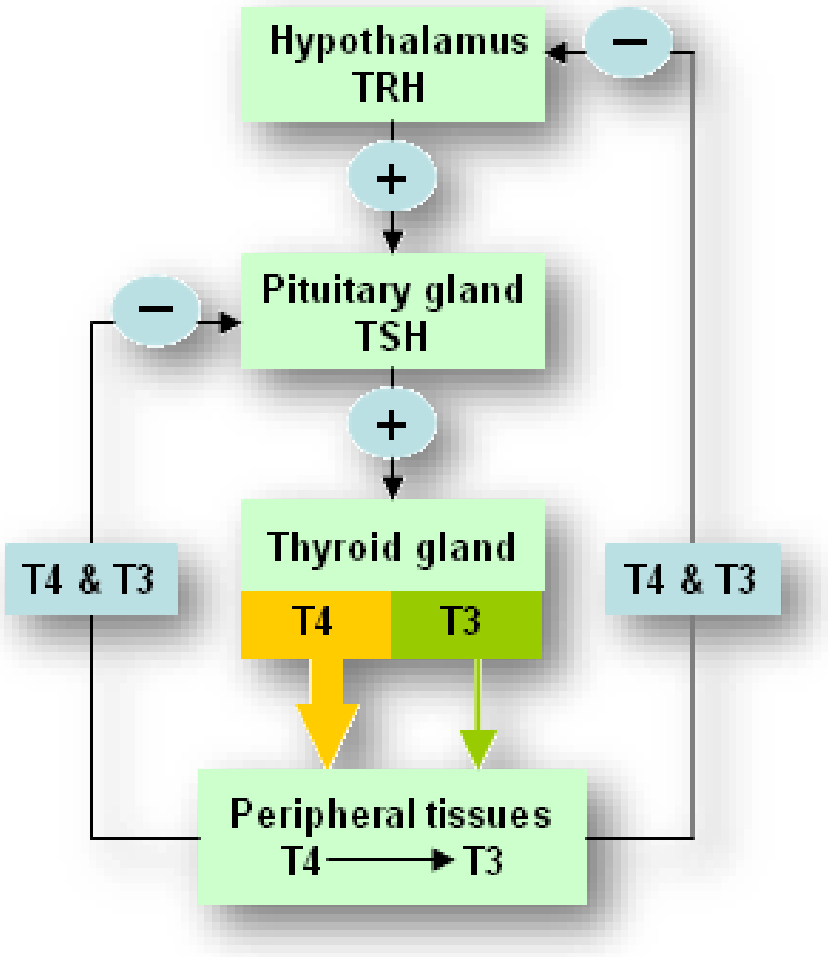
TABLES AND FIGURES

Table I.1. Phthalate parent compounds and their metabolites

Parent Compound, Abbreviation(s)		**Major Metabolite(s), Abbreviation(s)**	
Diethyl phthalate	DEP	Mono-ethyl phthalate	MEP
Dibutyl phthalate	DBP	Monobutyl phthalate	MBP
		Mono(3-carboxypropyl) phthalate	MCPP *(minor metabolite)*
Di-isobutyl phthalate	DiBP	Mono-isobutyl phthalate	MiBP
Butyl benzyl phthalate	BBzP	Mono-benzyl phthalate	MBzP
Di(2-ethylhexyl) phthalate	DEHP	Mono(2-ethylhexyl) phthalate	MEHP
		Mono(2-ethyl-5-hydroxyhexyl) phthalate	MEHHP MEOHP
		Mono(2-ethyl-5-oxohexyl) phthalate	MECPP
		Mono(2-ethyl-5-carboxypentyl) phthalate	
Di-isononyl phthalate	DiNP	Mono(carboxy-isooctyl) phthalate	MCOP
Diisodecyl phthalate	DiDP	Monocarboxyisononyl phthalate	MCNP
Di-n-octyl phthalate	DOP	Mono(3-carboxypropyl) phthalate	MCPP

Figure I.1. The hypothalamic-pituitary-thyroid negative feedback loop[142]



CHAPTER II. Urinary Phthalate Metabolites in Relation to Maternal Serum Thyroid and Sex Hormone Concentrations during Pregnancy: A Longitudinal Analysis (PROTECT Cohort)

ABSTRACT

Background: Increasing scientific evidence suggests that exposure to phthalates during pregnancy may be associated with an elevated risk of adverse reproductive outcomes such as preterm birth. Maternal endocrine disruption across pregnancy may be one pathway mediating some of these relationships. We investigated whether urinary phthalate metabolites were associated with maternal serum thyroid (free thyroxine [FT4], free triiodothyronine [FT3], and thyroid-stimulating hormone [TSH]), and sex (estradiol, progesterone, and sex hormone-binding globulin [SHBG]) hormone levels at multiple time points during pregnancy.

Methods: Preliminary data (n=106) were obtained from an ongoing prospective birth cohort in Northern Puerto Rico. We collected urine and serum sample at the first and third study visits that occurred at 18 +/- 2 and 26 +/-2 weeks of gestation, respectively. To explore the longitudinal relationships between urinary phthalate metabolites and serum thyroid and sex hormone concentrations, we used linear mixed models (LMMs) adjusted for prepregnancy body mass index (BMI) and maternal age. An interaction term was added to each LMM to test whether the effect of urinary phthalate metabolites on serum thyroid and sex hormone levels varied by study visit. In cross-sectional analyses, we stratified BMI- and age-adjusted linear regression models by study visit.

Results: In adjusted LMMs, we observed significant inverse associations between mono-3-carboxypropyl phthalate (MCPP) and FT3 and between mono-ethyl phthalate (MEP) and progesterone. In cross-sectional analyses by study visit, we detected stronger and statistically significant inverse associations at the third study visit between FT3 and MCPP as well as mono-carboxyisooctyl phthalate (MCOP); also at the third study visit, significant inverse associations were observed between FT4 and metabolites of di-(2-ethylhexyl) phthalate (DEHP). The inverse association between MEP and progesterone was consistent across study visits.

Conclusions: In this group of pregnant women, urinary phthalate metabolites may be associated with altered maternal serum thyroid and sex hormone levels, and the magnitude of these effects may depend on the timing of exposure during gestation.

INTRODUCTION

Phthalate diesters are commonly used as plasticizers in industrial applications.[1] Additionally, many household and consumer products, including flooring and wall coverings, food packaging, and cosmetics such as lotions and fragrances, contain phthalates. [2-4] Due to their ubiquitous use, human exposure to phthalates is widespread.[5] Because phthalates are not chemically bound to the plastics and other products that contain them, they can easily leach into household dust, food and water sources, and ambient air.[1,6,7] Consequently, human exposure to phthalates can occur through ingestion, inhalation, or dermal absorption.[1,3]

Growing evidence suggests that urinary concentrations of phthalate metabolites during pregnancy are associated with adverse reproductive outcomes including preterm birth and pregnancy loss.[8,9] Hormonal production and regulation are critical for pregnancy maintenance and fetal growth and neurodevelopment; maternal endocrine disruption during pregnancy may be one pathway mediating some of these relationships.[10-13]

Limited human health studies have examined the potential thyroid-altering effects of phthalates. In a cross-sectional study of men recruited from a U.S. fertility clinic, the urinary concentration of mono-(2-ethylhexyl) phthalate (MEHP), a metabolite of di-(2-ethylhexyl) phthalate (DEHP), was inversely associated with free thyroxine (3, 3', 5, 5'-tetraiodo-L-

thyronine, FT4) and total triiodothyronine (3, 3', 5-triiodo-L-thyronine, T3).[14] Urinary concentrations of DEHP metabolites were also inversely associated with total T3 and T4, and positively associated with thyroid-stimulating hormone (thyrotropin, TSH) in a representative sample of U.S. adults (non-pregnant women and men) participating in the National Health and Nutritional Examination Survey (NHANES).[15] Similar inverse relationships between urinary DEHP metabolites and total and free T3 (FT3) were reported in a cross-sectional study of Danish children.[16] While pervasive exposure to phthalates has been documented among pregnant women worldwide,[17-22] human health studies investigating the effects of phthalate exposure during pregnancy on maternal thyroid function are scarce. Huang and colleagues[23] reported an inverse association between urinary concentrations of monobutyl phthalate (MBP), the metabolite of dibutyl phthalate (DBP), and both free and total T4 in the second trimester of Taiwanese pregnant women.

Animal and *in vitro* studies have also shown that phthalates can interfere with sex hormone concentrations, signaling, and/or function,[24,25] which may profoundly affect implantation, fetal development, and parturition.[10,26] However, human data pertaining to the relationships between phthalates and sex hormones are limited. In men recruited through a U.S. fertility clinic, urinary MEHP was inversely associated with serum estradiol levels.[27] A positive association between urinary MEHP and sex hormone-binding globulin (SHBG) was also reported in separate cohort of fertile U.S. men.[28] In a recent study conducted among pregnant women in the U.S., an inverse association was found between urinary DEHP metabolite concentrations and serum testosterone concentrations, while no statistically significant relationship was observed for estradiol.[29] Additionally, Hart and coauthors[30] reported significantly inverse correlations between serum MEHP and SHBG concentrations at both 18 weeks and 36 weeks of gestation among pregnant women in Australia.

We are aware of no published investigations that longitudinally evaluate the potential thyroid-disrupting effects of environmental phthalate exposure among pregnant women. Furthermore, whether alterations in thyroid and sex hormone levels vary by time point of exposure during gestation remains largely undetermined and may have important influences on downstream hormone-mediated reproductive outcomes. In this preliminary analysis, we investigated the relationship between urinary phthalate metabolites and maternal serum thyroid

(FT3, FT4, and TSH) and sex (estradiol, progesterone, and SHBG) hormone levels measured in samples collected at two time points in pregnancy from women participating in a prospective birth cohort in Puerto Rico.

METHODS

Study Population

The Puerto Rico Testsite for Exploring Contamination Threats (PROTECT) project is an ongoing prospective birth cohort in the Northern Karst Region of Puerto Rico designed to investigate the relationship between phthalates and other environmental contaminants and adverse pregnancy outcomes such as preterm birth. A previous study within this project reported greater concentrations of some urinary phthalate metabolites measured in Puerto Rican participants compared to women of reproductive age in the contiguous U.S.[31] The current analysis included data collected from the first 106 pregnant women participating in the PROTECT project with urinary phthalate metabolites and serum thyroid and sex hormones measures completed as of November 2012. Study participants, aged 18 to 40 years, were recruited around 14 ± 2 weeks gestation from 7 prenatal clinics and hospitals throughout Northern Puerto Rico from 2010 to 2012. Participant recruitment and eligibility criteria as well as sample collection and processing are described in detail elsewhere.[31] Demographic information was obtained from questionnaires administered at the initial study visit. Spot urine samples were collected from each participant at three separate study visits (visit 1: 18 ± 2 weeks, visit 2: 22 ± 2 weeks, visit 3: 26±2 weeks of gestation). Only urine samples from visits 1 and 3 were utilized in the present analyses because blood samples were not collected at visit 2. All participants in the present analysis provided urine and serum samples for at least one visit (visits 1 and/or 3). Upon collection and processing (e.g., aliquoting, centrifugation, and separation of blood into plasma and serum components), all urine and blood samples were frozen at -80˚C until shipped overnight on dry ice to the analytical laboratories where samples were again stored at -80˚C until analysis.

The study protocols were approved by the ethics and research committees of the participating institutions. The involvement of the Centers for Disease Control and Prevention

(CDC) laboratory was determined not to constitute engagement in human subjects research. The study was described in detail to all participating women and all study participants gave informed consent.

Measurement of Phthalate Metabolites

Available urine samples (N=196 samples, N=106 participants) were analyzed by the Centers for Disease Control and Prevention (CDC) laboratories, using protocols developed for NHANES, to measure urinary concentrations (free plus glucuronidated) of 11 phthalate metabolites: MEHP, MBP, mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), mono-3-carboxypropyl phthalate (MCPP), mono-carboxyisooctyl phthalate (MCOP), mono-carboxyisononyl phthalate (MCNP), mono-benzyl phthalate (MBzP), mono-iso-butyl phthalate (MiBP), and mono-ethyl phthalate (MEP). The analytical method involved enzymatic deconjugation of the metabolites from their glucuronidated form, solid-phase extraction, separation by high-performance liquid chromatography, and detection by isotope-dilution tandem mass spectrometry.[32] The limits of detection (LOD) were in the low nanogram per milliliter range.[31] Concentrations below the LOD were assigned a value of LOD divided by the square root of 2.[33] To account for urine dilution, urinary specific gravity (SG) was measured using a digital handheld refractometer (Atago Co., Ltd., Tokyo, Japan). For descriptive analyses, metabolite concentrations were standardized using the following formula: $P_{SG}=P[(1.019-1)/(SG-1)]$, where P_{SG} is the specific gravity-adjusted phthalate metabolite concentration (ng/mL), P is the observed phthalate metabolite concentration, 1.019 was the specific gravity population median, and SG is the specific gravity of the urine sample.

Measurement of Thyroid Hormones

For 106 subjects, serum samples from visits 1 (N= 106) and 3 (N= 89) were available for measurement of FT3, FT4, and TSH at the Bioanalytical Core Laboratory of Georgetown University (Washington, DC). Limitations in sample volume contributed to differences in the number of samples per study visit available for analysis. Following an ultrafiltration step to separate the free hormones, FT3 and FT4 were measured using isotope dilution liquid

chromatography tandem mass spectrometry per the methods described in detail previously.[34-37] TSH concentrations were measured using a solid-phase immunochemiluminometric assay (DPC Immulite, Diagnostic Products Corporation) according to the manufacturer's instructions.

Measurement of Sex Hormones

For 104 subjects, serum samples were analyzed for estradiol (visit 1: N=103 samples; visit 3: N=86 samples) and progesterone (visit 1: N=104; visit 3: N=89) using a chemiluminescence immunoassay (DPC Immulite, Diagnostic Products Corporation). Serum levels of SHBG from samples collected from 99 subjects (visit 1: N=88; visit 3: N=69) were determined using the same procedures. Similar to the thyroid hormone analyses, limitations in sample volume contributed to differences in available samples for each sex hormone/study visit. These analyses were also performed by the Bioanalytical Core Laboratory at Georgetown University (Washington, DC).

Statistical Analysis

Serum concentrations of FT4, FT3, estradiol, and SHBG closely approximated normality and were untransformed in statistical analyses. TSH, progesterone, and urinary phthalate metabolite concentrations were positively skewed and were logarithmically transformed prior to analyses. Because MEHP, MEOHP, MEHHP, and MECPP share a single parent compound, the sum of concentrations of the DEHP metabolites (ΣDEHP) was calculated from the molar sum (nmol/mL) of these four metabolites and log-transformed in statistical analyses. Means and standard deviations as well as selected percentiles were used to examine the distributions of the urinary phthalate metabolites, thyroid hormones, and sex hormones. Geometric means and standard deviations were calculated for non-normally distributed variables.

Pearson correlations were calculated to assess the relationships between continuous variables. To test the differences in the mean concentrations of sex and thyroid hormones by visit, we used linear mixed models (LMMs) with serum hormones levels regressed on study visit and included a random intercept for subject ID to account for intra-individual correlation of repeated measurements over time. Urinary phthalate metabolites and sex and thyroid hormones

were tested for associations with demographic variables to examine potential confounding. We used LMMs with one phthalate metabolite concentration measure (as exposure) and one outcome per model to explore the longitudinal relationships between urinary phthalate metabolites and sex and thyroid hormone concentrations. Crude models were adjusted for urinary specific gravity and study visit. Full models additionally included maternal prepregnancy body mass index (BMI) and age, both measured at the initial study visit. BMI was modeled as a categorical variable ($\leq$25 kg/m^2, >25 and $\leq$30 kg/m^2, >30 kg/m^2) and age was modeled as a continuous variable. BMI and age were included as covariates because of their potential influence on urinary phthalate metabolite concentrations[38] and sex and thyroid hormone levels.[39-41] A likelihood ratio test for fixed effects was used to identify additional potential covariates in full models. An interaction term was added to each full LMM to test whether the effect of urinary phthalate metabolites on thyroid and sex hormone serum levels varied by study visit.

In a secondary analysis, we used linear regression models with one phthalate metabolite concentration and one outcome per model to investigate the cross-sectional relationships between urinary phthalate metabolites and sex and thyroid hormone serum concentrations at each study visit (visits 1 and 3). These models were also adjusted for maternal BMI, age, and urinary specific gravity. To enhance interpretability, all regression coefficients and associated 95% confidence intervals (CIs) generated from the LMMs and linear regression models were expressed as the percent change in hormone serum levels for an interquartile range (IQR) increase in urinary phthalate metabolite concentrations. Associations were considered statistically significant at the 5% level and marginally significant at the 10% level. All statistical analyses were conducted using SAS version 9.2 (SAS Institute Inc., Cary, NC).

RESULTS

Table II.1 shows the distribution of sociodemographic characteristics of the 106 pregnant women included in the present analysis. The study participants were generally highly educated (79.2% had at least a college education), and did not smoke during pregnancy (94.3%). Approximately half (55.7%) of the women had a prepregnancy BMI $\leq$25 kg/m^2. No statistically significant differences were observed between the demographic characteristics of study participants with measurable thyroid and sex hormone concentrations at visit 1 vs. those with

measurable thyroid and sex hormone concentrations at visit 3 (chi-square test p-values > 0.05). Based on the eligibility criteria, no participants used oral contraceptives within three months prior to pregnancy or underwent *in vitro* fertilization as a method of assisted reproductive technology, and all participants were free of known medical or obstetric complications.

The distributions of the 11 urinary phthalate metabolite concentrations across pregnancy have been previously investigated in this cohort.[31] Greater than 90% of the measured concentrations of all 11 urinary phthalate metabolites were detectable.[31] Concentrations for a majority of the metabolites measured were greater in the Puerto Rican participants when compared to women of reproductive age in the contiguous U.S.[31] For example, the geometric mean concentration of urinary MEHP (unadjusted for urinary dilution) was more than twice as high in the Puerto Rican cohort than the corresponding concentration found in women of reproductive age participating in the 2009-2010 NHANES study (3.3 vs. 1.6 ng/mL, respectively).[31] Spearman correlations between the majority of urinary metabolites were modest (R= 0.23-0.45), while strong correlations were observed between all urinary metabolites of DEHP (MEHP, MEHHP, MEOHP, and MECPP; R>0.83).[31] With the exception of MCOP, no statistically significant differences were observed in the geometric mean concentrations between study visits for any of the other urinary phthalate metabolites (**Table II.S1**).

The distributions of the thyroid and sex hormones are presented in **Table II.2**. All of the measured concentrations of the hormones were detectable. We found a significantly positive Pearson correlation between estradiol and progesterone (R=0.66, p<0.001). Weak but significant positive correlations were observed between FT4 and FT3 (R=0.17, p= 0.02), and between SHBG and both progesterone (R= 0.23, p= 0.004) and estradiol (R=0.29, p= 0.0002). Age was inversely correlated with thyroid and sex hormone levels (**Table II.S2**). With the exception of TSH, LMMs showed that thyroid hormone levels differed significantly by study visit (**Table II.3**). We found significantly higher mean levels of serum FT3 and FT4 at visit 1 compared to corresponding levels at visit 3. Conversely, the mean serum levels of all three sex hormones were significantly lower at visit 1 compared to visit 3.

Table II.4 shows the fully adjusted longitudinal associations between urinary phthalate metabolite concentrations and serum hormones concentrations from LMMs. Each model contained random intercepts only for subject ID to account for intra-individual correlation, as addition of random slopes did not improve the model fit. A likelihood ratio test for fixed effects indicated that maternal education did not have a significant effect on serum hormone levels. Thus, prepregnancy BMI, maternal age, study visit, and urinary specific gravity were the only covariates retained in the final models. The crude regression results were similar to the adjusted results (not shown). We detected a significant inverse relationship between MCPP and FT3, where an IQR increase in MCPP was associated with a 2.89% decrease in FT3 (95% CI= -5.65 to -0.02, p=0.049). We also observed a significant inverse association between MEP and progesterone. An IQR increase in MEP was associated with a 10.6% decrease in progesterone (95% CI= -17.6 to -2.84, p=0.01). We detected no significant associations between serum hormones levels and ΣDEHP urinary metabolites in longitudinal analyses. Similarly, null results were observed for individual DEHP metabolites, with the exception of a marginally significant inverse association found between MEHHP and SHBG (percent change in outcome with IQR increase [%Δ] =-4.62, 95%CI= -10.0 to 0.75, p=0.09). Most urinary phthalate metabolites were positively associated with TSH, although none of these relationships were statistically significant. We also found no statistically significant associations for FT4 or estradiol in the longitudinal analyses.

In our examination of the potential interaction between study visit and urinary phthalate metabolite concentrations in longitudinal analyses, we observed that study visit significantly modified the relationships between FT3 and MBzP (p=0.03) and MCPP (p=0.03), between FT4 and MiBP (p=0.01) and ΣDEHP metabolites (p=0.048), and between TSH and MBP (p=0.04). We found no statistically significant interactions between study visit and urinary phthalate metabolites for any of the sex hormones examined (data not shown).

Table II.5 presents the results from cross-sectional analyses by study visit for associations between urinary phthalate metabolites and serum thyroid hormone concentrations. At visit 1, we observed a statistically significant positive association between MiBP and FT4 (%Δ =4.95, 95%CI= 0.27 to 9.28, p=0.04). MBzP and ΣDEHP metabolites were also positively, though not significantly, associated with FT4 at visit 1. At visit 3, urinary phthalate metabolites

were generally inversely associated with FT4. At this visit, we found a statistically significant inverse association between ΣDEHP and FT4 (%Δ =-8.02, 95%CI= -15.3 to -0.80, p=0.03). Urinary phthalate metabolites were generally positively associated with TSH at each study visit, and suggestive associations were observed with MBzP at visit 1 and MEP at visit 3. While FT3 was inversely associated with both MCPP and MCOP at visit 1, these effects were greater and statistically significant at visit 3.

The results of cross-sectional analyses for the relationships between urinary phthalate metabolites and sex hormone concentrations are presented in **Table II.6**. We observed a marginally significant inverse relationship between MEP and SHBG at visit one (%Δ =-10.1, 95%CI= -21.8 to 1.65, p=0.09), and a null association at visit 3. The inverse association between MEP and progesterone observed in the longitudinal analyses appeared consistent across study visits.

DISCUSSION

To our knowledge the present study is the first to examine the relationships between urinary phthalate metabolites and serum thyroid hormone levels longitudinally across pregnancy. Few associations were observed in longitudinal analyses, although we did find statistically significant inverse associations between FT3 and MCPP, a non-specific metabolite of several high molecular weight phthalate plasticizers, and also between progesterone and MEP, the main metabolite of diethyl phthalate commonly used in personal care products. We also found that study visit of sample collection significantly modified the relationships between certain urinary phthalate metabolites and thyroid hormone levels. Our cross-sectional analyses corroborated our findings from the interaction analyses, and showed that associations between certain urinary phthalate metabolites and FT3 and FT4 hormone levels differed markedly (in terms of both magnitude and significance) based on visit of sample collection. For visit 1, associations with FT3 and FT4 were generally inverse, but at visit 3 these inverse relationships were stronger. Taken together, these results suggest that phthalate exposure during pregnancy may alter maternal thyroid and sex hormone levels. They also suggest that the magnitude of the potential endocrine-disrupting effect of phthalates may depend on the timing of exposure during gestation.

Of the thyroid hormones, we observed that FT4 was inversely associated with certain urinary phthalate metabolites at visit 3 (26 ± 2 weeks of gestation). These results are in contrast to a previous cross-sectional investigation conducted among 76 Taiwanese pregnant women with urinary phthalate metabolites and hormone levels measured in the second trimester (mean time of sample collection = 27.9 ± 2.3 weeks of gestation).[23] Huang and colleagues reported a statistically significant inverse association between urinary MBP and serum FT4 after adjusting for similar covariates in their regression model. We did not observe an analogous association at visit 3 (a comparable gestational age at sample collection) in the present study. FT3 was not measured in this previous investigation and regression results for TSH were not presented by the authors, thereby precluding additional comparisons with findings from our analyses. Differences between the results of our study and the Taiwanese study may be due to dissimilarities in study design, participant demographic characteristics, exclusion/inclusion criteria and/or exposures. For example, the median concentration (unadjusted for urinary dilution) of MBP was approximately four times higher in the Taiwanese pregnant women (81.8 ng/mL)[23] compared to the corresponding concentration found in women in the PROTECT cohort (20.1 ng/mL).[31]

Our findings for urinary metabolites of DEHP and serum FT4 and TSH are consistent with a previous study of adults (including non-pregnant women and men) participating in NHANES that reported significant inverse associations between certain urinary DEHP metabolites and FT4, although that larger study also reported positive relationships between these metabolites and TSH which were not significant in the present study.[15] Our results for FT3 and FT4 are also in agreement with the limited animal studies in which rats fed DEHP-contaminated diets, at doses orders of magnitude higher than those experienced by the PROTECT participants, had lower plasma T4 levels compared to controls and plasma T3 levels remained unchanged.[42-45]

Various biological mechanisms have been proposed through which environmental chemicals may exert their action on thyroid function. Thyroid-disrupting chemicals may target the hypothalamic-pituitary-thyroid axis at multiple levels and may disrupt thyroid hormone homeostasis by interfering with the synthesis and regulation by the hypothalamic-pituitary thyroid hormones (i.e., thyrotropin-releasing hormone [TRH] and TSH), binding of thyroid hormones to distributor proteins, cellular uptake mechanisms of thyroid hormones, metabolism

of thyroid hormones by iodothyronine deiodinases, transcriptional activity of thyroid hormone receptors and/or receptor activation.[46,47] It has been suggested that phthalates may bind to thyroid hormone receptors, consequently activating or inhibiting thyroid hormone action,[46-48] although data overtly demonstrating the binding of phthalates to thyroid receptors are lacking. Available experimental studies have provided some evidence for these potential mechanisms of thyroid disruption. *In vitro* studies have shown that phthalates may alter the sodium/iodide symporter-mediated uptake of iodide by thyroid follicular cells,[49,50] exhibit thyroid receptor antagonist activities,[24,49,50] or displace thyroid hormones (e.g., T3) from distributor proteins.[51] Additionally, phthalates were found to alter the transcription of genes involved in the hypothalamic-pituitary-thyroid axis as well as the whole-body content of thyroid hormones in zebrafish.[52]

Of the sex hormones, we observed a consistent inverse relationship between urinary MEP and serum progesterone across the two time points in pregnancy. In contrast to these findings, the previously described study by Huang et al.[23] reported no relationship between urinary MEP or other phthalate metabolites and progesterone during approximately the same sampling period in pregnancy as visit 3 in the current study. At visit 1 (18 ± 2 weeks of gestation), we observed a marginally significant inverse relationship between urinary MEP and SHBG, although no association was observed at visit 3. These results are in agreement with a previous study conducted among 1,377 pregnant Australian women, in which an inverse relationship between serum MEP and SHBG concentrations was observed early in pregnancy (18 weeks of gestation).[30] Animal studies investigating the potential toxicity of phthalates on the female reproductive system are scarce, and have primarily examined effects of DEHP and DBP. These phthalates have been shown to reduce progesterone production in female rats *in vivo* and in rat granulosa cells *in vitro*.[53-55] However, we did not observe these relationships with metabolites of DEHP and DBP in the present analysis.

Our null findings for estradiol were comparable to those of previous epidemiologic studies conducted among pregnant women.[23,29] However, available toxicological data have shown that the ovary, in particular the granulosa cells of preovulatory follicles, may be the primary target site for DEHP.[56] Specifically, *in vivo* and *in vitro* studies of DEHP-treated female cycling rats have demonstrated that suppressed estradiol production may be the principal functional modification by DEHP.[56-58] These toxicology studies suggest that phthalates have the

potential to induce alterations in steroidogenesis in female animals, although further research is needed to fully characterize the specific modes of action. Examining this association in a larger sample of pregnant women may enable identification of subtler relationships.

While the timing of exposure to endocrine disrupting chemicals during pregnancy has been shown to influence both the severity and onset of adverse developmental and reproductive outcomes,[59] we are aware of no epidemiologic studies that have attempted to identify periods of susceptibility to phthalate-induced alterations in thyroid and sex steroid hormone levels in pregnant women. During the first trimester, the fetus relies solely on maternal T3 and T4 until the fetal thyroid gland fully develops at approximately 10 weeks of gestation.[60] Thus, maintaining maternal euthyroidism during the first trimester is critical – even slight alterations in maternal thyroid hormones during this period of gestation has been associated with deleterious neurodevelopmental and reproductive outcomes.[61-63] In later pregnancy, maternal thyroid hormones are essential for fetal thyroid homeostasis.[64] Although we detected a significant inverse relationship between urinary MiBP and serum FT4 in the first trimester (i.e., at visit 1), the strongest findings for FT4 were observed in the second half of pregnancy (i.e., at visit 3). Despite epidemiological investigations that have shown the potential adverse reproductive consequences of overt thyroid disease in early and late gestation,[65,66] the impact of trimester-specific subclinical alterations in maternal thyroid function on pregnancy outcomes remains largely understudied. Furthermore, we observed a consistent inverse association between urinary MEP and progesterone at each study visit, and this effect was greatest earlier in pregnancy (i.e., visit 1). Because progesterone plays an essential physiological role in the establishment and maintenance of pregnancy, insufficient concentrations of this hormone throughout gestation may lead to pregnancy loss or preterm birth, depending on the timing of hormonal disruption.[12,67,68]

While our findings may have important public health implications, our study has several limitations. Characteristic of most preliminary analyses in an ongoing prospective cohort, our study was limited by a small sample size. We expected that the availability of multiple measurements per subject would increase the power to detect associations, but analysis revealed that the effects of phthalates on hormone disruption may be different depending on timing of exposure. Furthermore, given the large number of statistical tests performed in the present analyses, there is possibility of type-1 error. Another potential limitation is our timing of data

collection during mid-pregnancy, which may potentially bias our results since women who spontaneously aborted in the first trimester are not captured in our analyses. We do not have biological data from these women pertaining to the exposure and outcomes of interest to assess the direction of the potential bias. Our study may also have been limited by our evaluation of circulating thyroid hormones as a sole indicator of thyroid toxicity.[69] Measurements of peripheral thyroid hormones may not fully capture the phthalate-induced effects on thyroid homeostasis.[64] That is, blood levels of thyroid hormones may not correspond with actions at the receptor, such as regulation of gene expression and the developmental processes in which they are involved.[69,70] However, given the limited amount of data on this subject and the infeasibility of collecting more specific markers during pregnancy, levels of circulating thyroid hormone measurements may serve as the most appropriate biomarker of thyroid disruption in pregnant women. Additional studies are necessary to address potential phthalate-induced alterations in thyroid-hormone responsive genes relevant to pregnancy outcomes. It may also be possible that the serum measurements included in our analyses represented transient changes in thyroid and sex hormone levels that may not have persisted outside of the two measurement time points. However, even temporary and/or subclinical alterations in maternal hormone levels may be biologically relevant and may induce permanent effects on parturition and/or the developing fetus.[64] Our study was also limited by the lack of information concerning the iodine and selenium status of our study participants, which may be important because deficiencies in these trace elements can impair normal thyroid hormone function.[71] However, we have no reason to expect that these would be associated with phthalate exposure, so any deficiencies in these substances would affect the precision of the effect estimates rather than the effect estimates themselves.[72] Finally, our study was conducted among pregnant women in Northern Puerto Rico, which may have implications for the generalizability of results.

Despite these limitations, our study had several strengths. The collection of biomarker measurements at two time points during pregnancy enabled us to utilize mixed modeling techniques to more powerfully detect associations among repeated measurements and also evaluate potential periods of gestation during which phthalates may have a more profound impact on maternal hormone levels. Also, as pregnancy is characterized by a dynamic interplay between maternal endocrine hormones, our measurements of hormone concentrations from two

disparate endocrine axes is advantageous. Each set of hormones may represent distinct mechanisms through which phthalates may influence downstream reproductive health outcomes. Furthermore, we used novel and precise analytical techniques to measure serum levels of FT3 and FT4. Especially pertinent to a population of pregnant women, this method has advantages over traditional immunoassays because it is more specific, does not cross-react with other analytes, and is not influenced by serum binding proteins.[35,73,74] Notably, thyroxine binding proteins may increase as much as 50 percent during pregnancy.[74]

CONCLUSIONS

The results of our study provide suggestive evidence for phthalate-induced endocrinal disturbances during pregnancy, and may augment mechanistic understanding of the impact of phthalates on reproductive health outcomes. Future research on the specific pathways through which phthalates may alter thyroid and sex hormone concentrations are required for targeted interventions aimed at preventing downstream hormone-mediated adverse reproductive health outcomes in pregnant women.

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TABLES AND FIGURES

Table II.1. Sample characteristics of 106 pregnant women participating in PROTECT Project (2010-2012)

Variable	mean ± SD or n (%)
Maternal age at enrollment (years)	27.1 ± 4.8
Maternal education	
Missing	3 (2.8)
<High school	12 (11.3)
High school/equivalent	7 (6.6)
College	84 (79.2)
Annual household income (US$)	
Missing	17 (16.0)
<$20,000	45 (42.4)
≥$20,000 to <$50,000	27 (25.5)
≥$50,000	17 (16.0)
Race	
Missing	3 (2.8)
White	52 (49.1)
Mixed	39 (36.8)
Other	12 (11.3)
Prepregnancy BMI (kg m^{-2})	
Missing	4 (3.8)
≤25	59 (55.7)
>25 to ≤30	31 (29.2)
>30	12 (11.3)
Smoked during pregnancy	
Missing	5 (4.7)
yes	1 (0.9)
no	100 (94.3)
Alcohol use at first visit during pregnancy	
Missing	4 (3.8)
yes	12 (11.3)
no	90 (84.9)

Abbreviations: PROTECT, Puerto Rico Testsite for Exploring Contamination Threats, SD, standard deviation, BMI, body mass index.

Table II.2. Distributions of serum hormone concentrations

Biomarker	N	Mean (SD)	Selected Percentiles				
			25th	50th	75th	95th	Max.
Thyroid hormones							
TSH (uIU/mL)†	195	1.13 (1.62)	0.81	1.10	1.51	2.83	4.29
Free T3 (pg/mL)	195	3.94 (0.60)	3.50	3.90	4.40	4.90	5.70
Free T4 (ng/dL)	195	1.39 (0.32)	1.20	1.40	1.50	1.90	3.10
Sex hormones*							
Progesterone (ng/mL)†	193	62.8 (1.60)	45.7	57.3	82.0	140	374
Estradiol (ng/mL)	189	8.16 (4.06)	4.79	7.58	11.8	15.0	15.0
SHBG (nmol/L)	157	368 (109)	293	352	428	563	736

SD, standard deviation, TSH, thyroid-stimulating hormone, SHBG, sex hormone-binding globulin.
† Geometric mean and geometric standard deviation reported.
* Limitations in sample volume contributed to differences in available samples for each sex hormone.

Table II.3. Mean serum hormone concentrations (SD) by visit during gestation

Biomarker	**Visit 1 (14-20 weeks)**	**Visit 3 (24-28 weeks)**	**p-value***
Thyroid Hormones			
TSH (uIU/mL)†	1.10 (1.60)	1.17 (1.65)	0.25
Free T3 (pg/mL)	4.01 (0.61)	3.86 (0.59)	0.02
Free T4 (ng/dL)	1.45 (0.31)	1.32 (0.32)	0.001
Sex Hormones			
Progesterone (ng/mL)†	46.9 (1.37)	88.4 (1.48)	<0.001
Estradiol (ng/mL)	5.70 (2.48)	11.1 (3.60)	<0.001
SHBG (nmol/L)	349 (108)	391 (106)	0.002

Abbreviations: SD, standard deviation, TSH, thyroid-stimulating hormone, SHBG, sex hormone-binding globulin.
*Test for fixed effects from linear mixed models with random intercepts.
† Geometric mean and geometric standard deviation reported.

Table II.4. Longitudinal Analysis: Percent change (95% CIs) in thyroid and sex hormone concentrations in relation to interquartile range increase in urinary phthalate metabolite concentration

Analyte	% Change (95% CI)	p-value	% Change (95% CI)	p-value	% Change (95% CI)	p-value
	Free T3 (n=181 observations)		**Free T4 (n=181 observations)**		**ln-TSH (n=181 observations)**	
MBzP	0.18 (-3.42, 3.77)	0.92	0.58 (-4.45, 5.56)	0.82	5.51 (-4.40, 16.5)	0.28
MBP	1.33 (-1.90, 4.67)	0.42	0.63 (-4.00, 5.21)	0.79	3.28 (-5.58, 12.8)	0.48
MiBP	0.19 (-2.86, 3.14)	0.90	1.14 (-3.06, 5.42)	0.59	4.64 (-3.73, 13.0)	0.28
MEP	1.11 (-2.74, 4.96)	0.57	2.68 (-2.68, 7.83)	0.33	0.05 (-10.1, 11.3)	0.99
MCPP	-2.89 (-5.65, -0.02)	0.049*	-2.01 (-6.12, 2.10)	0.34	1.60 (-6.05, 9.88)	0.68
MCOP	-2.03 (-5.08, 0.98)	0.18	-1.11 (-5.26, 3.13)	0.61	-2.52 (-10.3, 6.13)	0.56
MCNP	0.48 (-2.03, 2.94)	0.71	-1.64 (-5.28, 1.93)	0.36	3.50 (-2.98, 10.4)	0.30
ΣDEHP	-0.28 (-3.63, 0.30)	0.87	-0.93 (-5.56, 3.79)	0.70	7.21 (-1.87, 16.6)	0.12
	Estradiol (n=175 observations)		**SHBG (n=147 observations)**		**ln-Progesterone (n=179 observations)**	
MBzP	0.86 (-8.52, 10.3)	0.86	4.89 (-1.52, 11.3)	0.13	3.16 (-4.56, 11.5)	0.67
MBP	2.65 (-5.90, 11.2)	0.54	-0.65 (-6.29, 4.98)	0.82	3.71 (-3.44, 11.2)	0.31
MiBP	1.56 (-6.27, 9.39)	0.69	1.19 (-4.25, 6.61)	0.66	2.48 (-3.96, 9.49)	0.46
MEP	1.60 (-8.49, 11.7)	0.75	-2.24 (-9.43, 4.97)	0.54	-10.6 (-17.6, -2.84)	0.01*
MCPP	-3.86 (-11.5, 3.80)	0.32	-1.27 (-6.40, 3.86)	0.62	-4.31 (-10.2, 1.98)	0.17
MCOP	-3.59 (-11.4, 4.24)	0.36	-0.93 (-6.44, 4.58)	0.74	-4.83 (-11.0, 1.57)	0.13
MCNP	-2.03 (-8.50, 4.41)	0.53	-0.66 (-4.85, 3.46)	0.75	-2.06 (-7.22, 3.28)	0.43
ΣDEHP	-0.56 (-9.17, 8.06)	0.90	-4.11 (-9.83, 1.62)	0.16	1.79 (-5.17, 9.39)	0.62

CI, confidence interval, TSH, thyroid-stimulating hormone, SHBG, sex hormone-binding globulin. Linear mixed models include a random intercept for subject ID and are adjusted for age at enrollment, prepregnancy body mass index, as well as urinary specific gravity and study visit.
*P<0.05

Table II.5. Cross-Sectional Analysis: Percent change (95% CIs) in thyroid hormone concentrations in relation to interquartile range increase in urinary phthalate metabolite concentration by visit during gestation

	Visit 1 (16-20 weeks)					
Analyte	**% Change (95% CI)**	**p-value**	**% Change (95% CI)**	**p-value**	**% Change (95% CI)**	**p-value**
	Free T3 (n=100 observations)		**Free T4 (n=100 observations)**		**ln-TSH (n=100 observations)**	
MBzP	1.87 (-2.55, 6.37)	0.40	2.44 (-2.77, 7.65)	0.36	12.9 (-0.93, 28.2)	0.07†
MBP	-0.72 (-4.94, 3.29)	0.73	0.26 (-4.58, 5.04)	0.92	1.29 (-10.2, 13.7)	0.83
MiBP	-1.64 (-5.55, 2.42)	0.43	4.95 (0.27, 9.28)	0.04*	1.09 (-10.3, 13.9)	0.86
MEP	-0.20 (-5.37, 5.02)	0.94	-0.05 (-6.10, 6.10)	0.99	0.77 (-13.6, 17.7)	0.92
MCPP	-1.04 (-4.54, 2.62)	0.57	1.41 (-2.83, 5.80)	0.50	-2.57 (-12.7, 8.69)	0.64
MCOP	-0.50 (-4.11, 2.93)	0.78	-0.44 (-4.58, 3.76)	0.83	-0.39 (-10.3, 10.7)	0.94
MCNP	1.05 (-2.27, 4.49)	0.53	0.43 (-3.47, 4.37)	0.83	-2.68 (-11.9, 7.35)	0.58
ΣDEHP	-1.62 (-6.05, 2.85)	0.47	3.09 (-2.17, 8.27)	0.25	7.14 (-6.12, 22.3)	0.30
	Visit 3 (24-28 weeks)					
	Free T3 (n=81 observations)		**Free T4 (n=81 observations)**		**ln-TSH (n=81 observations)**	
MBzP	-2.67 (-8.3, 2.86)	0.34	-2.97 (-12.1, 6.07)	0.51	-4.62 (-20.4, 13.5)	0.58
MBP	-0.57 (-5.68, 4.34)	0.82	-1.46 (-9.67, 6.74)	0.72	9.57 (-6.27, 27.3)	0.25
MiBP	-2.51 (-6.81, 1.70)	0.24	-4.70 (-11.8, 2.08)	0.17	2.74 (-9.94, 17.9)	0.68
MEP	2.76 (-2.68, 8.44)	0.32	4.93 (-3.81, 13.7)	0.27	18.1 (0.00, 37.8)	0.05†
MCPP	-5.93 (-10.2, -1.75)	0.01*	-5.11 (-12.5, 2.22)	0.17	2.41 (-11.1, 17.7)	0.74
MCOP	-5.83 (-10.8, -0.58)	0.03*	-3.02 (-11.5, 5.38)	0.47	-3.35 (-17.1, 13.6)	0.67
MCNP	-1.44 (-5.92, 3.10)	0.54	-6.34 (-14.0, 0.99)	0.09†	5.39 (-8.50, 21.3)	0.46
ΣDEHP	0.05 (-4.49, 4.49)	0.98	-8.02 (-15.3, -0.80)	0.03*	2.79 (-10.8, 18.6)	0.70

Abbreviations: CI, confidence interval, TSH, thyroid-stimulating hormone. Linear regression models adjusted for age at enrollment, prepregnancy body mass index, and urinary specific gravity.

*P<0.05

†P<0.10

Table II.6. Cross-Sectional Analysis: Percent change (95% CIs) in sex hormone concentrations in relation to interquartile range increase in urinary phthalate metabolite concentration by visit during gestation

	Visit 1 (16-20 weeks)					
Analyte	**% Change (95% CI)**	**p-value**	**% Change (95% CI)**	**p-value**	**% Change (95% CI)**	**p-value**
	Estradiol (n=97 observations)		**SHBG (n=83 observations)**		**ln-Progesterone (n=98 observations)**	
MBzP	-5.65 (-18.9, 7.57)	0.40	4.99 (-4.43, 14.4)	0.29	1.88 (-6.60, 11.3)	0.66
MBP	0.20 (-10.3, 14.2)	0.75	4.13 (-3.66, 11.9)	0.29	5.68 (-2.41, 13.7)	0.18
MiBP	-0.99 (-13.1, 11.1)	0.87	-0.92 (-9.77, 7.28)	0.77	5.56 (-2.46, 13.9)	0.18
MEP	0.03 (-15.5, 15.6)	1.00	-10.1 (-21.8, 1.65)	0.09†	-12.15 (-20.5, -2.99)	0.01*
MCPP	-2.93 (-14.4, 8.54)	0.61	-2.66 (-10.6, 5.27)	0.51	-0.42 (-7.61, 7.34)	0.91
MCOP	0.70 (-9.86, 11.3)	0.90	0.86 (-6.74, 8.48)	0.82	-0.57 (-7.27, 6.62)	0.87
MCNP	2.43 (-7.42, 12.3)	0.63	-0.65 (-7.75, 6.45)	0.86	-0.58 (-6.85, 6.11)	0.86
ΣDEHP	-7.50 (-20.9, 5.92)	0.27	-5.93 (-15.4, 3.54)	0.22	2.37 (-6.23, 11.9)	0.59
	Visit 3 (24-28 weeks)					
	Estradiol (n=78 observations)		**SHBG (n=64 observations)**		**ln-Progesterone (n=81 observations)**	
MBzP	4.38 (-7.12, 15.9)	0.45	6.66 (-4.98, 18.3)	0.26	8.02 (-5.12, 23.4)	0.24
MBP	6.55 (-3.60, 16.7)	0.20	5.22 (-5.18, 15.7)	0.32	8.19 (-3.61, 21.0)	0.18
MiBP	2.42 (-5.96, 10.8)	0.57	3.46 (-4.89, 11.8)	0.41	2.86 (-6.81, 13.8)	0.58
MEP	1.36 (-9.57, 12.3)	0.81	1.00 (-10.4, 12.4)	0.86	-9.43 (-20.1, 2.51)	0.11
MCPP	-0.48 (-9.45, 8.48)	0.91	4.76 (-3.69, 13.2)	0.26	-3.81 (-11.8, 6.87)	0.47
MCOP	-2.17 (-12.4, 8.09)	0.67	3.93 (-5.77, 13.6)	0.42	-5.15 (-15.7, 7.06)	0.39
MCNP	-2.64 (-11.7, 6.45)	0.57	2.70 (-6.31, 11.7)	0.55	-3.99 (-13.9, 6.75)	0.45
ΣDEHP	2.87 (-6.17, 11.9)	0.53	1.14 (-8.61, 10.9)	0.82	5.85 (-4.81, 17.6)	0.29

Abbreviations: CI, confidence interval, SHBG, sex hormone-binding globulin. Linear regression models adjusted for age at enrollment, prepregnancy body mass index, and urinary specific gravity.

*P<0.05

†P<0.10

SUPPLEMENTAL MATERIAL

Table II.S1. Urinary phthalate metabolite concentrations (ng/mL) in pregnant women from Puerto Rico†

			Percentiles				
	GM (GSD)	**p-value***	**25th**	**50th**	**75th**	**95th**	**Max**
MEHP							
Visit 1	3.22 (2.90)	0.98	1.61	3.14	6.36	13.5	50.9
Visit 3	3.24 (2.79)		1.69	3.08	6.73	19.7	32.8
MEHHP							
Visit 1	10.7 (2.61)	0.98	6.14	10.5	19.9	37.9	290
Visit 3	10.8 (2.27)		7.28	11.1	16.9	42.0	88.2
MEOHP							
Visit 1	9.09 (2.49)	0.95	5.57	8.33	16.5	29.0	259
Visit 3	9.41 (2.26)		6.22	9.86	14.8	38.6	64.7
MECPP							
Visit 1	20.4 (2.27)	0.97	12.7	20.8	31.4	61.0	712
Visit 3	20.2 (1.96)		13.4	20.8	29.3	69.7	121
MBzP							
Visit 1	3.62 (3.16)	0.54	1.74	3.73	7.65	27.0	59.0
Visit 3	3.60 (3.19)		1.49	3.22	6.99	29.8	108
MBP							
Visit 1	19.1 (2.99)	0.81	10.8	19.3	35.3	112	278
Visit 3	19.1 (2.48)		10.4	18.6	36	86.6	182
MiBP							
Visit 1	10.1 (2.47)	0.46	5.97	10.3	17.9	35.2	157
Visit 3	11.5 (2.61)		6.33	10.9	16.9	61.6	654
MEP							
Visit 1	96.9 (5.39)	0.95	24.2	97.7	365	1860	6910
Visit 3	106.1 (6.36)		24.7	83.6	354	2410	7640
MCPP							
Visit 1	1.98 (2.52)	0.09	1.04	2.08	3.02	8.00	82.1
Visit 3	2.27 (2.70)		1.24	1.86	3.52	14.8	64.8
MCOP							
Visit 1	14.9 (2.93)	0.02	7.96	13.4	24.8	89.8	1060
Visit 3	18.5 (3.32)		8.23	15.8	36.0	214	939
MCNP							
Visit 1	2.42 (2.56)	0.13	1.33	2.12	3.73	14.9	51.6
Visit 3	2.24 (2.24)		1.34	2.06	3.23	11.3	33.6

Abbreviations: GM, geometric mean, GSD, geometric standard deviation.
†All concentrations adjusted for specific gravity.
*Test for fixed effects from linear mixed models with random intercepts.

Table II.S2. Pearson correlations between serum sex hormones, serum thyroid hormones, and maternal age in pregnant women from Puerto Rico

	Progesterone †	Estradiol	SHBG	TSH †	Free T3	Free T4	Age
Progesterone †	1.00	0.66*	0.23*	0.05	-0.14*	-0.17*	-0.12
Estradiol		1.00	0.29*	0.10	-0.08	-0.27*	-0.13
SHBG			1.00	0.06	-0.10	-0.11	-0.09
TSH †				1.00	0.06	-0.01	-0.17*
Free T3					1.00	0.17*	-0.11
Free T4						1.00	-0.10
Age							1.00

Abbreviations: TSH, thyroid-stimulating hormone, SHBG, sex hormone-binding globulin.
† Log-transformed in statistical analysis.
*P<0.05

CHAPTER III. Associations between Repeated Measures of Maternal Urinary Phthalate Metabolites and Thyroid Hormone Parameters during Pregnancy (LifeCodes Cohort)

ABSTRACT

Background: Maintaining thyroid homeostasis during pregnancy is essential for normal fetal growth and development. Growing evidence suggests that phthalates interfere with normal thyroid function. Few human studies have investigated the degree to which phthalates may affect thyroid hormone levels in particularly susceptible populations such as pregnant women.

Objectives: We examined the associations between repeated measures of urinary phthalate metabolites and plasma thyroid hormone levels in samples collected at up to four time points per subject in pregnancy. Additionally, we investigated the potential windows of susceptibility to thyroid hormone disturbances related to study visit of sample collection.

Methods: Data were obtained from pregnant women (N=439) participating in a nested case-control study of preterm birth with 116 cases and 323 controls. We measured 9 phthalate metabolite concentrations in urine samples collected at up to four study visits per subject during pregnancy (median= 10, 18, 26, and 35 weeks of gestation, respectively). We also measured a panel of thyroid function markers in plasma collected at the same four time points per subject during pregnancy.

Results: While our results were generally null, in repeated measures analyses we observed that phthalate metabolites were largely inversely associated with thyrotropin (TSH) and positively associated with free and total thyroid hormones. Cross-sectional analyses by study visit revealed

that the magnitude and/or direction of these relationships varied by timing of exposure during gestation.

Conclusions: These results support previous reports showing the potential for environmental phthalate exposure to alter circulating levels of thyroid hormones in pregnant women.

INTRODUCTION

Maintaining thyroid homeostasis during pregnancy is essential for normal fetal growth and development, and especially for early fetal neurodevelopment.[1-3] Human health studies have shown that both overt and subclinical maternal thyroid disease (hyper- and hypothyroidism) may be associated with adverse birth outcomes such as preterm birth,[4-6] low birth weight[4,7-9] and impaired fetal growth,[4,7,10] although similar associations have not been observed for maternal subclinical hyperthyroidism.[10] Notably, these birth outcomes are associated with lasting physical and neurodevelopmental complications among surviving infants.[11]

Phthalate diesters have been commonly used as plasticizers and solvents in a variety of consumer and industrial products.[12,13] Due to their extensive use, phthalate metabolites have been consistently detected in humans, and more specifically in pregnant women worldwide.[14-17] Growing scientific evidence suggests that this group of environmental chemicals may interfere with normal thyroid function.[18,19]

Animal and *in vitro* studies suggest that phthalates may be capable of disrupting circulating thyroid hormone levels, although the exact biological mechanism(s) of action remain unclear.[20-22] Additionally, a limited number of epidemiological studies have shown that phthalates may alter thyroid hormone levels in adult men and non-pregnant women as well as children.[23-25] Less is known about the degree to which phthalates may affect thyroid function in other vulnerable populations such as pregnant women.

To date, three epidemiological investigations have assessed the relationships between phthalate exposure and thyroid hormone levels in pregnant women.[26-28] While the findings reported in these investigations provide suggestive evidence for the potential thyroid-disrupting effects of phthalates during pregnancy, these studies are limited by study design and/or sample size. The current analyses build upon this existing research on the possible role of phthalates in

disturbing thyroid hormone levels in pregnant women by investigating similar associations in a large nested case-control study. Here, we examined the associations between repeated measures of urinary phthalate metabolites and plasma thyroid hormone levels in samples collected at up to four time points per subject in pregnancy. Additionally, we investigated the potential windows of susceptibility to phthalate exposure related to study visit of sample collection.

METHODS

Study Population

This was a secondary analysis of data from a nested case-control study with the primary aim of investigating the effects of environmental phthalate exposure on the risk of preterm birth.[29] The study population includes a subset of pregnant women participating in the ongoing LifeCodes prospective birth cohort. All pregnant women who planned to deliver at the Brigham and Women's Hospital in Boston, who were older than 18 years old, and whose initial visit was before 15 weeks of gestation were eligible to participate and were recruited between 2006 and 2008. The only exclusion criterion was higher-order multiple gestations (e.g., triplets or greater).[30] Additional information regarding recruitment as well as sample collection and processing are described in detail elsewhere.[29-31] Briefly, at the initial study visit (median: 9.71 weeks of gestation; range: 4.71 to 19.1 weeks), participants completed a questionnaire to collect sociodemographic information (e.g., race/ethnicity, income, health insurance provider, etc.) and relevant health information (e.g., tobacco and alcohol use, family health history, etc.), and provided urine and blood samples for biomarker analysis. Participants were followed until delivery, and provided relevant health information (e.g., body mass index [BMI] and blood pressure) as well as urine and blood samples at three additional study visits: visit 2 (median: 17.9 weeks of gestation; range: 14.9 to 32.1 weeks), visit 3 (median: 26.0 weeks of gestation; range: 22.9 to 36.3 weeks), and visit 4 (median: 35.1 weeks of gestation; range: 33.1 to 38.3 weeks).

Approximately 1,600 women were enrolled in the original cohort at the Brigham and Women's Hospital, and 1,181 were followed until delivery and had a singleton live birth. In 2011, 130 women who delivered a preterm singleton infant (<37 completed weeks of gestation) and 352 randomly selected women who delivered singletons at or after 37 weeks of gestation were included in the nested case-control study. In the current analysis, we additionally excluded

women diagnosed with thyroid disease based on medical records (e.g., diagnosed hyper- or hypothyroidism, Grave's disease, or thyroid cancer) (N=41) and those who did not provide blood samples at any study visit during follow-up (N=2). The final study population (N=439) included 116 preterm birth cases and 323 controls. The study protocols were approved by the ethics and research committees of the participating institutions and all study participants gave written informed consent.

Thyroid Hormone Measurements

We assayed plasma samples (N=439 participants; N=1,445 total samples) collected up to four time points in pregnancy at the Clinical Ligand Assay Service Satellite (CLASS) Lab at the University of Michigan (Ann Arbor, MI). Samples were analyzed for thyrotropin (TSH) as well as total triiodothyronine (T3) and thyroxine (T4) using an automated chemiluminescence immunoassay according to manufacturer's instructions (Bayer ADVIA Centaur, Siemens Health Care Diagnostics, Inc.). We measured free T4 using direct equilibrium dialysis followed by radioimmunoassay (IVD Technologies). The manufacturer did not provide trimester-specific reference ranges for TSH. In their absence, the American Thyroid Association recommends the following for TSH: first trimester, 0.1-2.5 μIU/mL; second trimester, 0.2-3.0 μIU/mL; third trimester, 0.3-3.0 μIU/mL.[32] The free T4 pregnancy reference ranges provided by the laboratory were: first trimester, 0.7-2.0 ng/dL; second trimester, 0.5-1.6 ng/dL; third trimester, 0.5-1.6 ng/dL. The limits of detection (LOD) were 0.01 μIU/mL for TSH, 10 ng/dL for total T3, 0.3 μg/dL for total T4, and 0.1 ng/dL for free T4. Thyroid hormone concentrations less than the LOD were assigned a value of LOD divided by the square root of 2.[33]

In addition to exploring individual thyroid hormone parameters, we calculated the ratio of T3 to T4 (T3/T4) from the respective total hormone concentrations. The T3/T4 ratio is an index of thyroid homeostasis and reflects the action of thyroid hormones on peripheral tissues.[34,35]

Phthalate Metabolite Measurements

NSF International (Ann Arbor, MI) analyzed available urine samples (N=439 participants; N=1,443 samples), also collected up to four times in pregnancy, for phthalate

metabolites using a method developed by the Center for Disease Control (CDC) described elsewhere.[36,37] Briefly, the analytical technique involved enzymatic deconjugation of metabolites from their glucuronidated form, solid-phase extraction, separation by high-performance liquid chromatography, and detection by tandem mass spectrometry. The following nine metabolites were measured in urine samples: MEHP, MBP, mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), mono-benzyl phthalate (MBzP), mono-iso-butyl phthalate (MiBP), and mono-ethyl phthalate (MEP), and mono-3-carboxypropyl phthalate (MCPP). LOD for individual metabolites were in the low microgram per liter range.[31] As with the hormones, phthalate metabolite concentrations below the LOD were assigned a value of LOD divided by the square root of 2 [33]. In addition to examining individual phthalate metabolites, we created a variable for the molar sum (μmol/L) of the four measured DEHP metabolites (MEHP, MEHHP, MEOHP, and MECPP; ΣDEHP).[17]

To correct for urinary dilution in univariate analyses, we standardized phthalate metabolite concentrations using specific gravity (SG) according to the following equation: PSG=P[(1.015-1)/(SG-1)], where PSG is the specific gravity-adjusted phthalate metabolite concentration (μg/L), P is the observed phthalate metabolite concentration, 1.015 is the specific gravity population median, and SG is the specific gravity of the urine sample [17]. Unadjusted phthalate metabolite concentrations were used in multivariate analyses with SG added as a separate covariate since modeling corrected metabolite levels may introduce bias.[38]

Statistical Analyses

To make our study population more representative of the original cohort from which the case-control sample arose, we applied inverse probability weighting to all analyses considering association between secondary variables measured under case-control sampling. Specifically, we corrected for over-representation of preterm birth cases by applying study-specific weights related to the inverse probability of inclusion of controls so that the relative weights of cases and controls in the present study population were similar to what would be observed in the overall LifeCodes cohort.[39]

The empirical histogram of total T3 as well as free and total T4 approximately resembled a normal distribution. The distributions of TSH as well as all 9 phthalate metabolites and ΣDEHP were right-skewed; thus, we used the natural log transformation of these variables for statistical analyses. We tabulated means and percentiles for all urinary phthalate metabolites and plasma thyroid hormones. We calculated geometric means and geometric standard deviations for log-normally distributed variables. We examined the distribution of thyroid hormone parameters by study visit of sample collection and demographic characteristics. We calculated Spearman correlations between phthalate metabolites using SG-corrected values. We used linear mixed models (LMMs) with subject-specific random intercepts and slopes for gestational age at sample collection to test the differences in repeated measures of thyroid hormone levels by each categorical covariate that were introduced as predictors in the mixed model regression.

In repeated measures analyses, we explored the associations between urinary phthalate metabolites and plasma thyroid hormone concentrations across pregnancy using LMMs with one hormone regressed on one phthalate metabolite per model, with each model including a subject-specific random intercept and slope for gestational age at sample collection. Crude models included fixed effects terms for gestational age at sample collection and urinary SG. Full models were additionally adjusted for maternal age at enrollment, body mass index (BMI) at time of sample collection, and health insurance provider. We chose maternal age and BMI *a priori* as covariates in full models because of their known associations with thyroid hormone concentrations and urinary phthalate metabolite levels.[40-42] We identified additional covariates based on ≥ 10% change in the main effect estimates when added to the models.

In our secondary analyses, we investigated the cross-sectional relationships between urinary phthalate metabolites and plasma thyroid hormone concentrations at each study visit (visits 1 through 4) using linear regression models with one phthalate metabolite and one outcome variable per model. We adjusted these models for maternal age at enrollment, BMI at time of sample collection, health insurance provider, and urinary specific gravity. To enhance the interpretation of statistical models containing log-transformed exposure and/or outcome variables, we expressed all regression coefficients and associated 95% confidence intervals (CIs) as the percent change in thyroid hormone levels for an interquartile range (IQR) increase in urinary phthalate metabolite concentrations. We considered associations statistically significant

at the 5% level. We performed all data analyses using SAS version 9.3 (SAS Institute Inc., Cary, North Carolina).

RESULTS

Population characteristics of the case-control study population as well as the distributions of the phthalate metabolites by study visit have been previously reported.[29,43] Bivariate analyses showed that thyroid hormone concentrations significantly varied by certain demographic characteristics (**Table III.1**). Specifically, TSH concentrations were significantly lower among pregnant women who identified as African-American or Other race/ethnicity compared to White, and who had public health insurance compared to private. Women who reported no alcohol use during pregnancy had higher concentrations of TSH compared to those who reported drinking alcohol. For free T4, concentrations were significantly lower among women who graduated from technical school compared to those with a high school diploma or the equivalent, and among women who were obese (>30 kg/m^2) compared to those with a BMI <25 kg/m^2.

All thyroid hormone parameters were detected in most samples in this study population (% detected for total T4 and T3=100%, TSH=99.5%, and free T4=98%), and measurable concentrations of the 9 urinary phthalate metabolites were detected in at least 95% of urine samples.[29,43] Correlations between phthalate metabolites were strongest for DEHP metabolites (Spearman r= 0.74-0.93), were moderate between MBzP, MBP, and MiBP (r= 0.43-0.61) and between DEHP metabolites and MCPP (r= 0.35-0.44), and were weak between all other metabolites (r=-0.03-0.29). Weighted geometric mean concentrations of urinary and plasma biomarkers varied by study visit of sample collection (**Table III.2**). Compared to visit 1, we observed significantly decreased levels of all DEHP metabolites and MCPP at visit 3. We detected significantly increased concentrations of MBzP, MBP, MiBP, and MEP at visit 4. For the hormones, compared to visit 1, we found significantly increased levels of TSH at visits 2-4 whereas free T4 levels were significantly lower at these three subsequent study visits.

Associations from repeated measures analyses using fully adjusted LMMs were similar to those observed in crude unadjusted models (data not shown). We detected a significant inverse relationship between MEHP and TSH, where an IQR increase in MEHP was associated with a

5.31% (95% CI: -10.1, -0.23) decrease in TSH (**Table III.3**). We also observed significant inverse associations between MiBP (percent change in outcome for an IQR increase in exposure [%Δ] = -9.51; 95% CI: -16.4, -2.01) and MCPP (%Δ= -6.63; 95% CI: -11.6, -1.41). We detected generally positive associations between each metabolite and free T4, with a significant relationship observed for MCPP (%Δ=6.91; 95% CI: 1.70, 12.1). Finally, we observed significant positive associations between MEP and both total T3 (%Δ=2.24; 95% CI: 0.32, 4.17) and the T3/T4 ratio (%Δ=2.87; 95% CI: 1.27, 4.47) as well as between MEHP and total T4 (%Δ=1.29; 95% CI: 0.26, 2.32).

To explore potential windows of susceptibility, we stratified linear regression analyses by time of sample collection in pregnancy (see **Table III.S1**). Similar to repeated measures analyses, phthalate metabolites were generally inversely associated with TSH at each of the four study visits, although statistically significant associations were observed only in visits 1 and 2 (**Figure III.1**). We detected significant positive associations between several urinary phthalate metabolites and free T4 at visits 1 and 4. In contrast to repeated measures analyses, phthalate metabolites were inversely related to free T4 at visit 3, although these associations were not statistically significant (**Figure III.2**). For total hormones, ΣDEHP was significantly positively associated with T3 and T4 at visit 1, and with T4 at visit 4. We observed null associations between metabolites and the T3/T4 ratio at visits 1-3, with inverse associations for ΣDEHP, including several individual DEHP metabolites, at visit 4.

DISCUSSION

In the largest cohort study conducted on this topic to date, we report significant associations between several phthalate metabolites and thyroid hormone parameters in samples collected at up to four time points in pregnancy. In repeated measures analyses, we observed that phthalate metabolites were largely inversely associated with TSH and positively associated with free and total thyroid hormones. Cross-sectional analyses by study visit revealed that the magnitude and/or direction of these relationships varied by time point of exposure during gestation. We detected inverse relationships between several metabolites (particularly DEHP metabolites) and TSH at visits 1 and 2, while no significant associations were observed in the latter half of pregnancy. For free T4, we observed generally positive associations at all study

visits with the exception of visit 3 (median 26 weeks of gestation), where these associations became inverse in direction. These results suggest that environmental phthalate exposure may alter thyroid hormone parameters in pregnant women. Moreover, our findings indicate that the timing of phthalate exposure during gestation may be important for a pregnant woman's susceptibility to thyroidal disruption.

Three epidemiological studies have previously investigated the potential phthalate-associated alterations in thyroid hormone parameters among pregnant women.[26-28] Notably, only one of these investigations, which we conducted using pilot data, assessed the relationships using biomarker measurements collected at multiple time points in pregnancy (Johns et al. 2015). In that population of pregnant women in Puerto Rico, no statistically significant associations were observed between urinary phthalate metabolites and serum concentrations of TSH or free T4 in repeated measures analyses using data from two study visits in pregnancy. However, in cross-sectional analyses, we previously observed a significant positive association between MiBP and free T4 at median 18 weeks of gestation as well as inverse associations between several phthalate metabolites, including ΣDEHP, and free T4 at median 26 weeks of gestation.[27] While these associations were similar in direction to the corresponding results reported at visit 2 (median 18 weeks of gestation) and visit 3 (median 26 weeks of gestation) in the current study, here we did not report statistically significant associations for free T4 at either visit. The discrepant results observed between these two studies may be due to differences in: population size, number of serial biological samples available as well as the timing of sample collection in pregnancy, phthalate exposure levels, laboratory methods used to measure free hormones, and/or population demographic characteristics.

Our results for free and total T4 also differ from those reported in a prior cross-sectional study conducted among a cohort of Taiwanese pregnant women undergoing amniocentesis (N=76).[26] Huang and colleagues[26] observed significant inverse associations between urinary MBP and both plasma free and total T4 at mean 27.9 weeks of gestation. In contrast, we observed largely null and in some cases positive associations between phthalate metabolites, including MBP, and free or total T4 in both repeated measures and cross-sectional analyses. In a more recent cross-sectional analysis conducted among a separate cohort of Taiwanese pregnant women (N=148), Kuo and colleagues[28] observed significant inverse unadjusted associations

between several urinary phthalate metabolites (MEOHP, MEHHP, and MBzP) and serum TSH in the third trimester. Likewise, we generally found inverse relationships between phthalate metabolites and TSH in both repeated measures and cross-sectional analyses, although these were specific to visits early in gestation.

The pattern of results reported in the current study, specifically those observed for urinary DEHP metabolites, conflict with findings from previous human health studies conducted among adult men and non-pregnant women. In a cross-sectional study of men recruited from a fertility clinic, urinary concentrations of MEHP were inversely associated with free T4 and total T3.[24] Urinary concentrations of DEHP metabolites were also inversely associated with total T3 and total and free T4 in a representative sample of U.S. adults.[25] No significant associations were observed for TSH in either study. It is possible that differences in exposure levels and/or in the physiological state of participants (i.e., pregnancy) may have contributed to discrepancies in the results between these studies and the present study.

Various biological mechanisms have been proposed through which phthalates may act to alter thyroid function. Phthalates may exert thyroid-disrupting action at multiple points along the hypothalamic-pituitary-thyroid axis. It has been suggested that phthalates may bind to thyroid hormone receptors and alter their signaling, although evidence for overt binding is lacking.[18,44,45] Additionally, limited *in vitro* studies have shown that phthalates may have thyroid hormone receptor antagonist activity.[46,47] Several studies have also demonstrated potential phthalate actions on thyroid hormone biosynthesis and biotransport.[21,22,48-50]

Phthalates may also impact the peripheral metabolism of thyroid hormones. To our knowledge, this is the first study to investigate the effects of phthalates on thyroid homeostasis using the T3/T4 ratio. This ratio has been used as an index of the peripheral conversion of T4 to T3 (the more biologically active hormone) by deiodinase enzymes, and can be high or low in certain thyroid disease states.[34,35] Here, we observed a statistically significant positive association between urinary MEP and the T3/T4 ratio in repeated measures analyses. Cross-sectional analyses by study visit revealed significant inverse associations with several phthalate metabolites, including DEHP metabolites, at visit 4. While we did not directly measure deiodinase activity in tissues, these results suggest that phthalates may influence circulating levels of thyroid hormones in pregnant women by altering the peripheral metabolism of thyroid

hormones. Indeed, limited animal studies have shown that certain phthalates and/or their metabolites may influence the gene expression of deiodinase enzymes.[21,22] However, additional research is required to examine the influences of phthalates on extrathyroidal regulation of thyroid hormone production in humans, particularly in tissues relevant to pregnancy (e.g., the placenta).

Since each organ system develops at different time points in pregnancy and because any disturbances in the normal growth and maturation of these systems may have lasting consequences on the developing fetus, the health effects of *in utero* exposures depend not only on the structure and dose of the chemical but also on the timing of exposure in gestation.[51] In humans, the fetus relies exclusively on maternal thyroid hormones in the first trimester until the fetal thyroid gland becomes fully functional after 18 weeks of gestation.[52,53] In later pregnancy, maternal thyroid hormones are essential for fetal thyroid homeostasis.[1] Even mild alterations in circulating thyroid hormones in pregnancy may have important implications for fetal health. In pregnant women with normal range free T4 and TSH levels, increases in free T4 in the first trimester were associated with lower birth weight and an increased risk of small for gestational age.[54] Notably, we observed significant phthalate-associated increases in free T4 levels at study visit 1 (first trimester) in the current study.

Our study was limited by the lack of iodine status of our study participants, which is a trace element essential for normal thyroid function.[55] While recent population-based studies have shown that the pregnant women in the U.S. may have less than adequate median urinary iodine levels,[56] it is unlikely that this would be a confounder in the phthalate-thyroid hormone associations. Although some studies have observed correlations between urinary iodine and phthalate concentrations, it is unclear whether an individual's phthalate exposure directly influences iodine status or whether both are simply found in the same dietary source. Moreover, in a study conducted among a representative sample of U.S. adult men and women, iodine excretion had a negligible impact on the significant relationships observed between phthalate metabolites and thyroid hormone levels.[57] An additional limitation is that we did not assess the thyroid autoimmunity of the study participants. It is possible that the associations observed in our study may differ by level of anti-thyroid antibodies, which may be present in approximately 10-20% of pregnant women.[58,59] Finally, we performed a number of comparisons, and there is the

potential that some of the observed associations may have been due to chance. We did not correct for multiple comparisons because available methods (e.g., Bonferroni adjustments) are often too conservative due to underlying assumptions of independence and increase the probability of type II errors, thereby potentially masking truly important differences.[60] Despite these limitations, our study has many strengths. We have investigated the effects of environmental phthalate exposure on maternal thyroid hormone levels in the largest longitudinal study to date. The collection of biomarker measurements at multiple time points in pregnancy allows for the use of statistical modeling techniques to more powerfully detect associations among repeated measurements. Furthermore, our analytical method for measuring free T4 is advantageous over traditional immunoassays since it is specific and not influenced by serum binding proteins, which change dramatically over normal pregnancy.[61,62]

CONCLUSIONS

Overall, the results from our analyses support previous reports showing the potential for environmental phthalate exposure to disturb circulating levels of thyroid hormones in pregnant women. Additional human health and animal studies are required to resolve the direction of the specific relationships, to further elucidate periods of vulnerability in pregnancy to phthalate exposure, and to reveal the specific biological mechanisms involved at phthalate levels comparable to those to which humans (and more specifically, pregnant women) are environmentally exposed. Furthermore, the implications of these findings to maternal and fetal health need to be determined.

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TABLES AND FIGURES

Table III.1. Thyroid hormone measurements (weighted median [25th, 75th percentiles]) by demographic characteristics in all samples measured (N=439 participants, 1,443 plasma samples).

Population Characteristics		%Total Pop.[a]	TSH (uIU/mL)	Free T4 (ng/dL)	Total T3 (ng/dL)	Total T4 (ug/dL)	T3/T4 Ratio[b]
Age	18-24 years old [ref]	13	1.04 (0.70, 1.60)	1.12 (0.91, 1.36)	179 (155, 209)	11.2 (10.1, 12.3)	16.2 (13.6, 18.8)
	25-29 years old	21	1.20 (0.79, 1.64)	1.13 (0.87, 1.35)	157 (130, 186)*	10.4 (9.30, 11.6)	15.1 (13.2, 17.4)*
	30-34 years old	40	1.25 (0.81, 1.75)	1.09 (0.86, 1.35)	149 (127, 182)*	10.0 (8.90, 11.3)*	14.8 (12.7, 17.6)*
	35+ years old	26	1.33 (0.96, 1.83)	1.11 (0.87, 1.37)	149 (124, 184)*	10.0 (8.90, 11.2)*	14.8 (12.5, 17.7)*
Race/Ethnicity	White [ref]	56	1.36 (0.97, 1.88)	1.09 (0.86, 1.34)	148 (127, 182)	10.0 (8.90, 11.1)	14.8 (12.6, 17.6)
	African-American	17	0.96 (0.70, 1.35)*	1.11 (0.90, 1.32)	169 (145, 198)*	10.9 (9.60, 12.4)*	15.5 (13.6, 17.9)*
	Other	27	1.12 (0.70, 1.66)*	1.16 (0.88, 1.41)	162 (130, 192)*	10.4 (9.20, 11.9) *	15.4 (12.8, 18.2)
Education	High School [ref]	15	1.13 (0.69, 1.60)	1.14 (0.93, 1.43)	171 (147, 200)	11.1 (9.90, 12.3)	15.6 (13.3, 18.4)
	Technical School	17	1.09 (0.76, 1.64)	1.09 (0.86, 1.31)*	164 (136, 195)	10.3 (9.00, 11.6)*	16.2 (13.6, 18.6)
	Junior College or some college	29	1.30 (0.90, 1.82)	1.10 (0.84, 1.35)	152 (132, 183)*	10.0 (8.80, 11.3)*	14.9 (13.0, 17.6)*
	College graduate	39	1.30 (0.89, 1.83)	1.09 (0.87, 1.37)	147 (124, 178)*	10.1 (9.10, 11.2) *	14.6 (12.5, 17.0)*
Health Insurance	Private/HMO/Self-pay [ref]	80	1.27 (0.85, 1.79)	1.10 (0.87, 1.35)	150 (127, 182)	10.0 (8.90, 11.3)	14.8 (12.7, 17.6)
	Medicaid/SSI/MassHealth	20	1.06 (0.72, 1.60)*	1.13 (0.90, 1.36)	182 (153, 214)*	11.1 (10.0, 12.4)*	16.2 (13.8, 18.6)*
BMI at Initial Visit	<25 kg/m^2 [ref]	53	1.25 (0.82, 1.79)	1.15 (0.89, 1.42)	144 (122, 169)	10.1 (9.00, 11.4)	14.0 (12.0, 16.5)
	25-30 kg/m^2	26	1.28 (0.84, 1.78)	1.11 (0.87, 1.35)	168 (142, 194)*	10.4 (9.30, 11.6)	16.1 (13.8, 18.7)*
	>30 kg/m^2	21	1.17 (0.78, 1.66)	1.05 (0.83, 1.25)*	181 (142, 208)*	10.3 (8.90, 11.6)	17.1 (14.3, 19.6)*
Tobacco Use	Smoked during pregnancy [ref]	7	1.23 (0.85, 1.60)	1.13 (0.85, 1.35)	171 (145, 209)	10.2 (9.10, 11.1)	16.8 (13.9, 20.7)
	No smoking during pregnancy	93	1.25 (0.81, 1.76)	1.10 (0.87, 1.35)	154 (129, 185)*	10.3 (9.10, 11.6)	15.0 (12.8, 17.6)*
Alcohol Use	Alcohol use during pregnancy [ref]	5	0.93 (0.66, 1.34)	1.10 (0.96, 1.34)	154 (118, 182)	9.60 (8.10, 10.8)	16.5 (14.0, 19.4)
	No alcohol use during pregnancy	95	1.25 (0.82, 1.76)*	1.11 (0.87, 1.35)	156 (130, 186)	10.3 (9.10, 11.6)*	15.0 (12.9, 17.7)
Fetal sex	Male [ref]	46	1.28 (0.85, 1.73)	1.09 (0.88, 1.33)	157 (130, 187)	10.3 (9.00, 11.6)	15.4 (13.3, 18.0)
	Female	54	1.22 (0.80, 1.77)	1.12 (0.89, 1.38)	154 (129, 185)	10.2 (9.10, 11.5)	14.7 (12.5, 17.6)

Abbreviations: Pop., Population BMI, Body Mass Index; HMO, Health Maintenance Organization; SSI, Supplemental Security Income.
[a] Weighted by case-control sampling probabilities to represent the general sampling population.
[b] Total T3 expressed in ng/dL and Total T4 in µg/dL.
*Significant difference ($p<0.05$) in thyroid hormone concentration in the category compared to reference (first category listed) using linear mixed models with a random intercept and slope for each subject.

Table III.2: Weighted distributions of urinary and plasma biomarkers by study visit of sample collection in pregnancy

Biomarker	# Samples[c]	Geometric Mean (Geometric Standard Deviation)			
		Visit 1 (median 10 weeks gestation)	Visit 2 (median 18 weeks gestation)	Visit 3 (median 26 weeks gestation)	Visit 4 (median 35 weeks gestation)
Phthalate Metabolites[a]					
MEHP (µg/L)	1,541	10.6 (3.52)	10.9 (3.39)	9.46 (3.28)*	9.83 (3.52)*
MEHHP (µg/L)	1,541	34.7 (3.37)	34.8 (3.10)	27.2 (3.21)*	36.6 (3.33)
MEOHP (µg/L)	1,541	18.6 (3.28)	18.3 (3.03)	15.6 (3.19)*	20.9 (3.22)
MECPP (µg/L)	1,541	44.4 (3.35)	42.6 (3.25)*	36.8 (3.31)*	49.3 (3.35)
ΣDEHP (µmol/L)	1,541	0.39 (3.16)	0.38 (3.01)	0.32 (3.04)*	0.42 (3.18)
MBzP (µg/L)	1,541	7.36 (3.07)	7.34 (3.15)	7.05 (2.93)	8.03 (2.94)*
MBP (µg/L)	1,541	18.3 (2.39)	18.4 (2.53)	17.3 (2.50)	19.7 (2.11)*
MiBP (µg/L)	1,541	7.66 (2.29)	7.14 (2.38)	7.45 (2.32)	9.05 (2.17)*
MEP (µg/L)	1,541	145 (4.66)	144 (4.84)	141 (4.48)	156 (4.99)*
MCPP (µg/L)	1,541	2.11 (3.09)	2.25 (3.26)*	1.94 (2.89)*	2.04 (2.77)
Thyroid Hormones					
TSH (µIU/mL)	1,210	1.13 (2.11)	1.30 (1.90)*	1.26 (1.67)*	1.31 (1.71)*
Free T4 (ng/dL)[b]	1,435	1.49 (0.87)	1.16 (0.63)*	1.08 (0.81)*	0.99 (0.49)*
Total T3 (ng/dL)[b]	1,130	140 (39.9)	166 (38.9)*	170 (39.8)*	171 (41.6)*
Total T4 (µg/dL)[b]	1,391	10.2 (2.03)	10.7 (1.73)*	10.5 (1.97)*	10.2 (2.04)
T3/T4 Ratio[b]	1,120	13.8 (2.67)	15.5 (3.43)*	16.5 (4.00)*	17.1 (4.46)*

[a] Urinary phthalate concentrations corrected for specific gravity.
[b] Arithmetic mean and standard deviation reported.
[c] Number of plasma samples per hormone varied due to limitations in sample volume.
*Significant difference ($p<0.05$) in urinary phthalate metabolite concentration or thyroid hormone compared to Visit 1 (reference) using linear mixed models with a random intercept for each subject.

Table III.3. Repeated measures analysis: percent change (95% CIs) in thyroid hormone concentrations in relation to interquartile range increase in urinary phthalate metabolite concentrations

Analyte	ln-TSH		Free T4		Total T3		Total T4		T3/T4 Ratio	
	%Δ (95%CI)	p-value	%Δ (95%CI)	p-value	%Δ (95%CI)	p-value	%Δ (95%CI)	p-value	%Δ (95%CI)	p-value
MEHP	-5.31 (-10.1, -0.23)	0.04*	4.15 (-0.87, 9.16)	0.10	0.28 (-1.29, 1.85)	0.72	1.29 (0.26, 2.32)	0.01*	-1.05 (-2.33, 0.23)	0.11
MEHHP	-3.95 (-8.67, 1.01)	0.11	2.67 (-2.27, 7.62)	0.29	0.97 (-0.55, 2.50)	0.21	0.66 (-0.34, 1.66)	0.19	-0.06 (-1.31, 1.19)	0.93
MEOHP	-3.74 (-8.38, 1.15)	0.13	3.89 (-0.99, 8.77)	0.12	1.08 (-0.41, 2.58)	0.16	0.86 (-0.13, 1.84)	0.09	-0.15 (-1.38, 1.08)	0.81
MECPP	-3.98 (-9.17, 1.51)	0.15	4.89 (-0.52, 10.3)	0.08	0.86 (-0.83, 2.54)	0.32	0.86 (-0.25, 1.97)	0.13	-0.23 (-1.61, 1.15)	0.74
ΣDEHP	-4.33 (-9.23, 0.84)	0.10	4.09 (-1.12, 9.29)	0.12	0.82 (-0.77, 2.41)	0.31	0.87 (-0.17, 1.91)	0.10	-0.29 (-1.59, 1.01)	0.66
MBzP	-4.5 (-11.26, 2.78)	0.22	2.57 (-3.89, 9.03)	0.43	0.47 (-1.79, 2.74)	0.68	1.04 (-0.47, 2.55)	0.18	-0.60 (-2.47, 1.26)	0.52
MBP	-2.66 (-8.95, 4.07)	0.43	2.83 (-3.37, 9.04)	0.37	1.10 (-0.92, 3.13)	0.29	0.24 (-1.14, 1.62)	0.73	0.85 (-0.82, 2.53)	0.32
MiBP	-9.51 (-16.4, -2.01)	0.01*	3.61 (-3.48, 10.7)	0.32	0.99 (-1.51, 3.49)	0.44	0.47 (-1.23, 2.17)	0.56	0.54 (-1.58, 2.66)	0.62
MEP	-4.56 (-10.4, 1.70)	0.15	-0.48 (-6.33, 5.38)	0.87	2.24 (0.32, 4.17)	0.02*	-0.48 (-1.79, 0.82)	0.47	2.87 (1.27, 4.47)	0.00*
MCPP	-6.63 (-11.6, -1.41)	0.01*	6.91 (1.70, 12.1)	0.01*	1.55 (-0.11, 3.21)	0.07	0.14 (-0.96, 1.23)	0.81	1.30 (-0.05, 2.64)	0.06

Linear mixed models include random intercept and slope for each subject and were adjusted for urinary specific gravity, gestational age at time of sample collection, maternal age at enrollment, body mass index (BMI) at time of sample collection, and health insurance provider.
*p<0.05

Figure III.1. Cross-sectional analysis: percent change in TSH concentrations in relation to an interquartile range increase in urinary phthalate metabolite concentrations (*p<0.05)

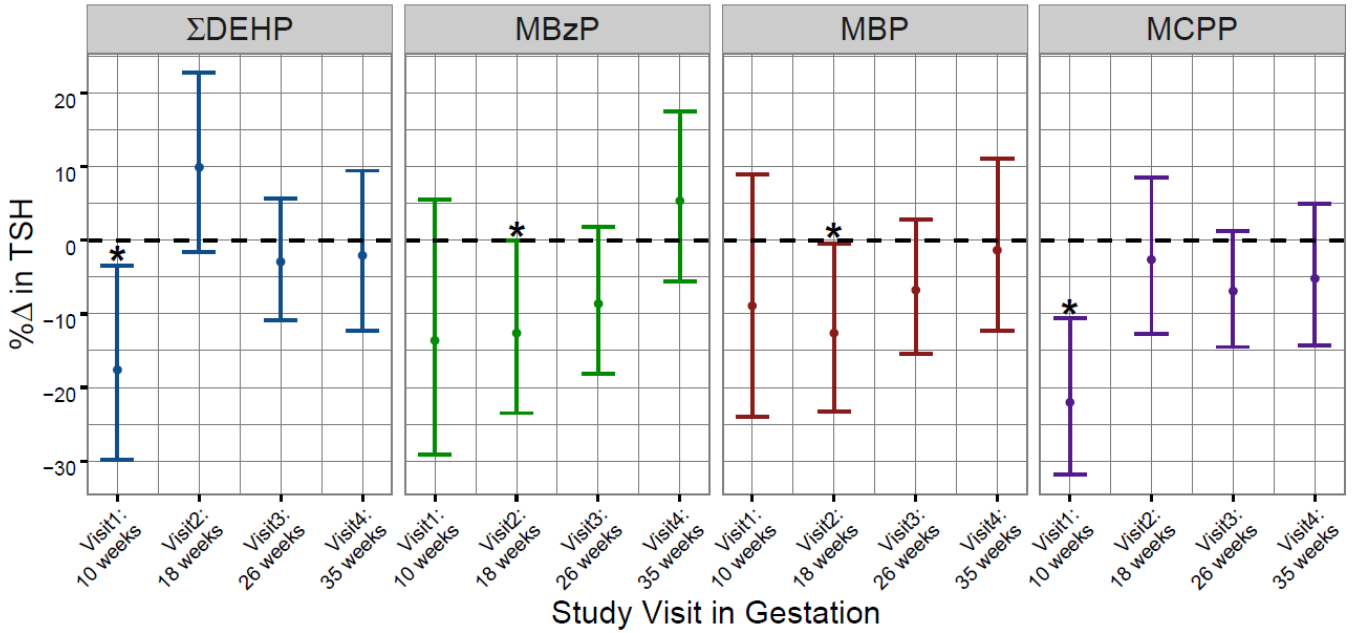
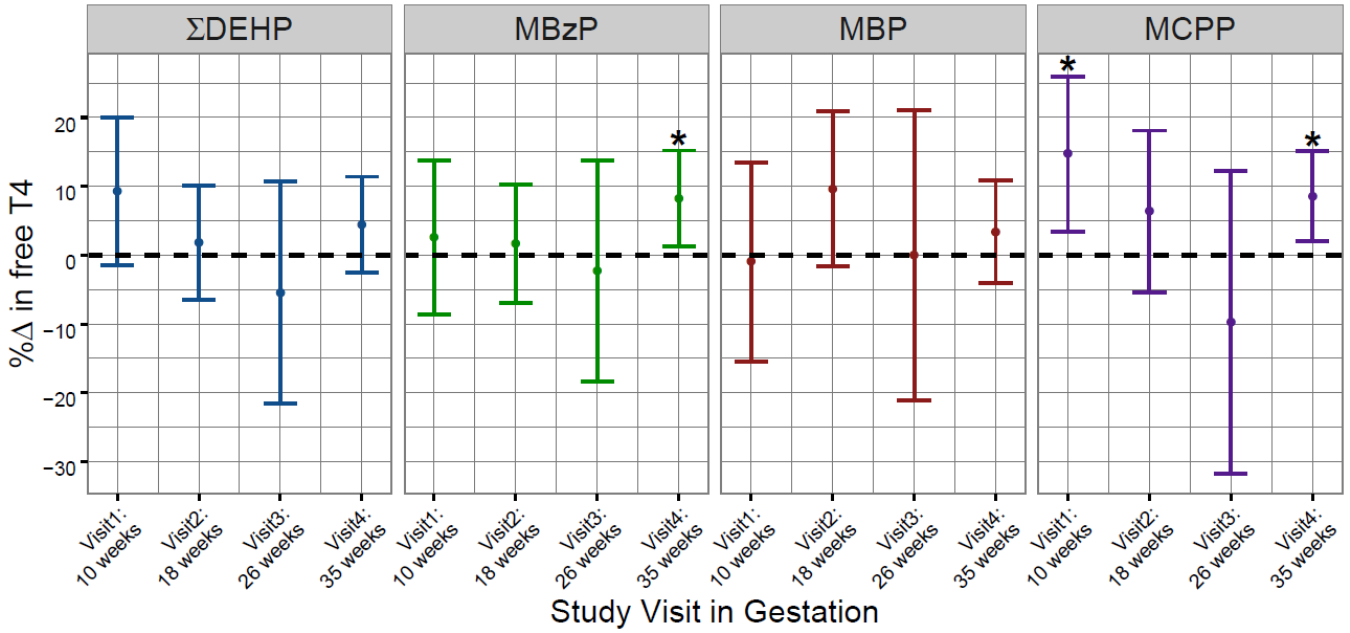


Figure III.2. Cross-sectional analysis: percent change in free T4 concentrations in relation to an interquartile range increase in urinary phthalate metabolite concentrations (*p<0.05)



SUPPLEMENTAL MATERIAL

Table III.S1. Cross-sectional analysis: percent change (95% CIs) in thyroid hormone concentrations in relation to interquartile range increase in urinary phthalate metabolite concentrations by study visit during gestation

Visit 1 : median 10 weeks of gestation										
Analyte	%Δ (95%CI)	p-value	%Δ (95%CI)	p-value	%Δ (95%CI)	p-value	%Δ (95%CI)	p-value	%Δ (95%CI)	p-value
	ln-TSH		**Free T4**		**Total T3**		**Total T4**		**T3/T4 Ratio**	
MEHP	-16.0 (-27.6, -2.55)	0.02*	8.24 (-1.06, 17.5)	0.08	-0.11 (-III.98, 3.77)	0.96	0.20 (-2.45, 2.85)	0.88	-0.26 (-2.89, 2.36)	0.84
MEHHP	-14.9 (-26.1, -1.90)	0.03*	3.22 (-6.00, 12.4)	0.49	0.76 (-3.22, 4.74)	0.71	0.04 (-2.72, 2.80)	0.98	1.00 (-1.70, 3.71)	0.46
MEOHP	-15.7 (-27.5, -2.01)	0.03*	5.84 (-2.94, 14.6)	0.19	1.19 (-2.62, 4.99)	0.54	0.42 (-2.21, 3.06)	0.75	0.87 (-1.72, 3.46)	0.51
MECPP	-17.8 (-30.6, -2.52)	0.02*	10.5 (0.86, 20.1)	0.03*	0.57 (-3.55, 4.70)	0.78	0.67 (-2.12, 3.45)	0.64	0.13 (-2.66, 2.91)	0.93
ΣDEHP	-17.7 (-29.8, -3.46)	0.02*	9.30 (-1.42, 20.0)	0.09	0.68 (-3.87, 5.23)	0.77	0.51 (-2.61, 3.62)	0.75	0.44 (-2.64, 3.52)	0.78
MBzP	-13.6 (-29.2, 5.57)	0.15	2.60 (-8.56, 13.8)	0.65	-0.14 (-4.94, 4.66)	0.95	0.62 (-2.59, 3.82)	0.71	-1.64 (-4.87, 1.59)	0.32
MBP	-8.99 (-24.0, 8.93)	0.30	-0.99 (-15.4, 13.4)	0.89	1.66 (-4.34, 7.66)	0.59	0.96 (-3.24, 5.17)	0.65	-0.06 (-4.17, 4.05)	0.98
MiBP	-11.0 (-27.9, 9.79)	0.27	-1.67 (-13.8, 10.5)	0.79	3.00 (-2.28, 8.27)	0.26	2.43 (-1.17, 6.04)	0.18	0.04 (-3.55, 3.64)	0.98
MEP	3.82 (-11.8, 22.1)	0.65	-3.68 (-10.9, 3.54)	0.32	2.36 (-0.76, 5.49)	0.14	0.51 (-1.59, 2.61)	0.63	1.18 (-0.94, 3.29)	0.27
MCPP	-22.0 (-31.9, -10.6)	0.00*	14.7 (3.41, 26.0)	0.01*	2.14 (-2.78, 7.05)	0.39	-0.24 (-3.57, 3.08)	0.89	2.14 (-1.16, 5.45)	0.20
Visit 2 : median 18 weeks of gestation										
	ln-TSH		**Free T4**		**Total T3**		**Total T4**		**T3/T4 Ratio**	
MEHP	3.77 (-6.10, 14.7)	0.47	4.42 (-2.7, 11.5)	0.22	1.79 (-1.56, 5.15)	0.29	1.81 (-0.52, 4.14)	0.13	-0.43 (-3.56, 2.69)	0.79
MEHHP	6.44 (-4.38, 18.5)	0.26	1.36 (-6.23, 8.94)	0.73	4.45 (0.90, 7.99)	0.01*	1.31 (-1.16, 3.77)	0.30	3.25 (-0.05, 6.55)	0.05
MEOHP	8.55 (-2.69, 21.1)	0.14	1.53 (-6.30, 9.37)	0.70	4.31 (0.65, 7.97)	0.02*	2.39 (-0.16, 4.93)	0.07	1.75 (-1.68, 5.18)	0.32
MECPP	11.3 (-0.03, 24.0)	0.05	0.89 (-6.50, 8.28)	0.81	3.41 (-0.05, 6.87)	0.05	3.27 (0.87, 5.67)	0.01*	0.01 (-3.24, 3.26)	0.99
ΣDEHP	9.93 (-1.62, 22.8)	0.09	1.79 (-6.54, 10.1)	0.67	4.11 (0.20, 8.02)	0.04*	2.89 (0.18, 5.60)	0.04*	1.04 (-2.63, 4.70)	0.58
MBzP	-12.6 (-23.5, -0.05)	0.049*	1.63 (-6.94, 10.2)	0.71	3.91 (-0.08, 7.90)	0.06	5.18 (2.42, 7.94)	0.00*	-0.6 (-4.32, 3.12)	0.75
MBP	-12.6 (-23.3, -0.37)	0.04*	9.62 (-1.61, 20.9)	0.09	3.42 (-1.60, 8.44)	0.18	2.49 (-1.37, 6.35)	0.21	1.65 (-3.27, 6.58)	0.51
MiBP	-15.1 (-26.9, -1.51)	0.03*	1.77 (-8.43, 12.0)	0.73	3.05 (-1.57, 7.68)	0.19	4.18 (0.68, 7.68)	0.02*	1.29 (-3.26, 5.85)	0.58
MEP	-4.99 (-18.0, 5.90)	0.28	0.95 (-5.32, 7.22)	0.77	1.44 (-1.46, 4.34)	0.33	0.89 (-1.17, 2.95)	0.40	1.54 (-1.16, 4.25)	0.26
MCPP	-2.65 (-12.7, 8.56)	0.63	6.34 (-5.39, 18.1)	0.29	3.05 (-2.38, 8.48)	0.27	-0.25 (-4.12, 3.63)	0.91	3.44 (-1.66, 8.55)	0.19

Table III.S1. Continued

	Visit 3 : median 26 weeks of gestation									
	ln-TSH		**Free T4**		**Total T3**		**Total T4**		**T3/T4 Ratio**	
MEHP	-6.31 (-13.9, 1.93)	0.13	-2.48 (-16.6, 11.7)	0.73	-0.13 (-4.02, 3.75)	0.95	0.22 (-2.83, 3.28)	0.89	0.65 (-3.37, 4.67)	0.75
MEHHP	-1.14 (-9.30, 7.74)	0.79	-7.35 (-20.8, 6.14)	0.28	-0.07 (-3.73, 3.59)	0.97	-0.17 (-3.04, 2.70)	0.91	0.82 (-2.97, 4.61)	0.67
MEOHP	-2.58 (-10.5, 6.08)	0.55	-4.18 (-18.8, 10.4)	0.57	0.65 (-3.28, 4.58)	0.75	1.46 (-1.64, 4.57)	0.35	0.46 (-3.62, 4.53)	0.83
MECPP	-2.92 (-11.1, 5.97)	0.51	-3.27 (-17.4, 10.9)	0.65	1.27 (-2.58, 5.13)	0.52	2.67 (-0.30, 5.63)	0.08	0.59 (-3.41, 4.58)	0.77
ΣDEHP	-2.91 (-10.8, 5.73)	0.50	-5.44 (-21.6, 10.7)	0.51	0.92 (-3.48, 5.33)	0.68	2.08 (-1.35, 5.51)	0.23	0.74 (-3.82, 5.30)	0.75
MBzP	-8.68 (-18.2, 1.88)	0.10	-2.26 (-18.3, 13.8)	0.78	1.08 (-3.31, 5.47)	0.63	3.75 (0.36, 7.14)	0.03*	-2.69 (-7.23, 1.85)	0.24
MBP	-6.71 (-15.4, 2.81)	0.16	-0.02 (-21.1, 21.1)	1.00	1.42 (-4.07, 6.90)	0.61	0.50 (-3.81, 4.80)	0.82	0.76 (-4.93, 6.45)	0.79
MiBP	-8.29 (-17.5, 1.91)	0.11	-2.98 (-21.0, 15.0)	0.74	-1.53 (-6.41, 3.36)	0.54	0.86 (-2.89, 4.60)	0.65	-0.27 (-5.35, 4.82)	0.92
MEP	-4.11 (-12.3, 4.85)	0.36	-2.07 (-12.5, 8.33)	0.70	2.12 (-0.56, 4.81)	0.12	0.74 (-1.38, 2.86)	0.49	1.83 (-0.96, 4.62)	0.20
MCPP	-6.92 (-14.5, 1.31)	0.10	-9.80 (-31.8, 12.2)	0.38	0.74 (-5.19, 6.67)	0.81	1.24 (-3.31, 5.78)	0.59	0.46 (-5.70, 6.62)	0.88
	Visit 4 : median 35 weeks of gestation									
	ln-TSH		**Free T4**		**Total T3**		**Total T4**		**T3/T4 Ratio**	
MEHP	-6.31 (-14.7, 2.86)	0.17	2.98 (-2.84, 8.80)	0.31	0.31 (-4.31, 4.93)	0.90	4.03 (1.19, 6.88)	0.01*	-6.18 (-10.9, -1.47)	0.01*
MEHHP	0.17 (-9.36, 10.7)	0.97	2.95 (-3.32, 9.21)	0.36	1.20 (-3.74, 6.13)	0.63	1.90 (-1.20, 4.99)	0.23	-4.30 (-9.39, 0.78)	0.10
MEOHP	-1.12 (-10.9, 9.78)	0.83	3.59 (3.00, 10.17)	0.28	1.34 (-3.81, 6.49)	0.61	3.01 (-0.23, 6.25)	0.07	-5.76 (-11.0, -0.48)	0.03*
MECPP	-0.74 (-11.5, 11.4)	0.90	4.32 (-2.94, 11.6)	0.24	1.68 (-3.86, 7.23)	0.55	4.23 (0.65, 7.80)	0.02*	-5.37 (-11.1, 0.33)	0.06
ΣDEHP	-2.03 (-12.3, 9.49)	0.72	4.43 (-2.55, 11.4)	0.21	1.71 (-3.75, 7.17)	0.54	3.99 (0.56, 7.42)	0.02*	-5.88 (-11.5, -0.28)	0.04*
MBzP	5.29 (-5.63, 17.5)	0.35	8.26 (1.34, 15.2)	0.02*	-2.84 (-8.03, 2.35)	0.28	0.42 (-3.03, 3.87)	0.81	-6.09 (-11.4, -0.77)	0.03*
MBP	-1.29 (-12.3, 11.1)	0.83	3.42 (-4.07, 10.9)	0.37	-0.51 (-6.00, 4.98)	0.85	0.02 (-3.67, 3.71)	0.99	-3.60 (-9.26, 2.06)	0.21
MiBP	-2.44 (-13.4, 9.96)	0.68	3.45 (-4.35, 11.2)	0.39	-1.23 (-6.94, 4.48)	0.67	0.37 (-3.56, 4.29)	0.85	-1.58 (-7.74, 4.58)	0.61
MEP	-2.04 (-11.8, 8.79)	0.70	2.97 (-3.82, 9.77)	0.39	3.34 (-1.59, 8.27)	0.18	0.61 (-2.78, 3.99)	0.73	3.01 (-2.19, 8.22)	0.26
MCPP	-5.15 (-14.3, 5.01)	0.31	8.55 (2.05, 15.1)	0.01*	1.64 (-3.11, 6.38)	0.50	0.51 (-2.74, 3.75)	0.76	-1.26 (-6.18, 3.66)	0.61

Linear regression models adjusted for urinary specific gravity, gestational age at time of sample collection, maternal age at enrollment, body mass index (BMI) at time of sample collection, and health insurance provider.
*p<0.05

CHAPTER IV. Longitudinal Profiles of Thyroid Hormone Parameters in Pregnancy and Associations with Preterm Birth

ABSTRACT

Background: Overt thyroid disease in pregnancy is associated with numerous maternal and neonatal complications including preterm birth. Less is known about the contribution of trimester-specific subclinical alterations in individual thyroid hormones, especially in late gestation, on the risk of preterm birth. Herein, we examined the associations between subclinical changes in maternal thyroid hormone concentrations (TSH, total T3, free and total T4), measured at multiple time points in pregnancy, and the odds of preterm birth in pregnant women without clinical thyroid disease.

Methods: Data were obtained from pregnant women participating in a nested case-control study of preterm birth within on ongoing birth cohort study at Brigham and Women's Hospital in Boston, MA (N=439; 116 cases and 323 controls). We measured thyroid hormones in plasma collected at up to four time points in pregnancy (median= 10, 18, 26, and 35 weeks). We used multivariate logistic regression models stratified by study visit of sample collection to examine associations. To reveal potential biological pathways, we also explored these relationships by obstetric presentation of preterm birth (e.g., spontaneous preterm delivery) that have been previously hypothesized to share common underlying mechanisms.

Results: In samples collected at median 10 and 26 weeks of gestation, we found inverse associations between FT4 and the odds of overall preterm birth (odds ratio [OR] = 0.57, 95% confidence interval (CI)=0.33, 1.00; and OR=0.53, 95% CI=0.34, 0.84, respectively). Positive

associations were detected for total T3 at these same time points (OR=2.52, 95% CI=1.20, 5.31; and OR=3.40, 95% CI=1.56, 7.40, respectively). These effect estimates were stronger for spontaneous preterm birth.

Conclusions: Our results suggest that subclinical alterations in individual maternal thyroid hormones may influence the risk of preterm birth, and the strength of these associations vary by gestational age.

INTRODUCTION

Preterm birth is among the most frequent causes of global infant and neonatal mortality.[1] While recent medical advances have improved survival among preterm infants, the long-term health and economic consequences associated with prematurity are substantial.[1,2] Prevention of preterm birth is a challenge owing to the complexity of its causes, many of which are poorly understood.[3]

Maternal thyroid hormones are crucial for normal fetal growth and development, especially neurodevelopment.[4] This is particularly true in the first trimester when the fetus is entirely dependent on the transplacental passage of maternal thyroid hormones.[5,6] Maternal thyroid hormones also play a physiological role in early placental development by regulating human trophoblast proliferation and invasion.[6-10] Inadequate trophoblast cell invasion may result in abnormal placentation, which notably is a risk factor for preterm delivery.[9,11]

Research has shown that overt hyper- and hypothyroidism in pregnancy are associated with poor maternal and neonatal outcomes.[12-16] However, data on the consequences of milder forms of maternal thyroid dysfunction on the risk of preterm birth in particular have been less conclusive. Subclinical hypothyroidism or elevated thyrotropin (TSH) has been associated with preterm delivery in some studies[17-20] but not in others.[13,21] There has also been suggestive evidence that hypothyroxinemia (normal TSH concentrations with low free thyroxine [FT4]) in early pregnancy may increase the risk of prematurity.[19] Notably, these studies are limited by single biomarker measurements from the first or second trimester.

Currently, there are a lack of data on the effects of trimester-specific subclinical alterations in individual parameters of thyroid function, especially in late gestation, on the risk of preterm birth. The purpose of this study was to examine the associations between subclinical fluctuations in biochemical markers of thyroid function, measured at up to four time points in pregnancy, and the risk of preterm birth in a nested case-control study of pregnant women without clinical thyroid disease.

METHODS

Study Population

Participants were part of a nested case-control study of preterm birth drawn from a prospective birth cohort (the LIFECODES cohort) of pregnant women recruited early in gestation (<15 weeks) at Brigham and Women's Hospital in Boston, MA. Additional information regarding recruitment and eligibility criteria are described in detail elsewhere.[22,23] The nested case-control study includes 130 women who delivered preterm (<37 weeks) and 352 randomly selected controls. We additionally excluded from the study, women who reported pre-existing or gestational thyroid disease/conditions based on answers to medical questionnaires administered at each of the study visits (N=41; e.g., hyper- or hypothyroidism, Graves' disease, or thyroid cancer) and those who did not provide plasma samples at any study visit (N=2). The final study population for the current analyses included 116 cases of preterm birth and 323 controls. The proportion of women delivering preterm did not significantly differ between women included vs. excluded from the current study (χ^2=0.75, p=0.39). The study protocols were approved by the ethics review board at Brigham and Women's Hospital (Partners Health Research Committee) and all study participants gave written informed consent.

Gestational age at individual study visits and at delivery were calculated based on last menstrual period and confirmed by first trimester ultrasound.[22] Overall preterm birth was defined as delivery before 37 weeks postmenstrual gestation.[24] To create more homogenous subpopulations, outcome measures were additionally stratified by clinical presentation of preterm birth that were found to share common placental features.[25] Based on the findings by McElrath and colleagues[25] and to remain consistent with previous studies conducted within this

cohort[22,24], preterm birth was additionally classified as: (1) spontaneous preterm birth (defined by preterm labor or preterm premature rupture of the membranes [PPROM]; N=49); and (2) preterm birth resulting from aberrant placentation or placental preterm birth (defined by preeclampsia or intrauterine growth restriction [IUGR]; N=33). Deliveries for non-medical indications (i.e. prior intrauterine fetal death or prior classical cesarean section) were not analyzed separately in this study as these cases have not been found to share common underlying biological processes.[25]

Thyroid Hormone Measurements

Plasma samples were collected at up to four study visits: visit 1 (median 10.0 weeks; range: 4.7 to 19.1 weeks), visit 2 (median 17.9 weeks; range: 14.9 to 32.1 weeks), visit 3 (median 26.0 weeks; range: 22.9 to 36.3 weeks), and visit 4 (median 35.2 weeks; range: 33.1 to 38.3 weeks). A total of 1,443 plasma samples were assayed at the Clinical Ligand Assay Service Satellite (CLASS) Lab at University of Michigan (Ann Arbor) for TSH, total and free thyroxine (T4 and FT4, respectively), and total triiodothyronine (T3). TSH and total hormones (T3 and T4) were assayed via automated chemiluminescence immunoassay (Bayer ADVIA Centaur, Siemens Health Care Diagnostics, Inc.). FT4 was measured using direct equilibrium dialysis followed by radioimmunoassay (IVD Technologies).

The manufacturer did not provide trimester-specific reference ranges for TSH only a non-pregnant normal range of 0.35-5.50 uIU/mL. In their absence, the American Thyroid Association (ATA) recommended in 2011 the following TSH reference ranges: first trimester, 0.1-2.5 μIU/mL; second trimester, 0.2-3.0 μIU/mL; and third trimester, 0.3-3.0 μIU/mL.[26] However, the ATA has recently proposed changing the upper limit of these intervals to the non-pregnancy upper limit (~ 4.0 mU/l).[27,28] The FT4 pregnancy reference ranges provided by the laboratory were: first trimester, 0.7-2.0 ng/dL; second trimester, 0.5-1.6 ng/dL; and third trimester, 0.5-1.6 ng/dL. The limits of detection (LOD) were 0.01 μIU/mL for TSH, 0.1 ng/mL for T3, 0.3 μg/dL for T4, and 0.1 ng/dL for FT4. The inter-assay coefficients of variation (CV) for all hormones ranged from 2.3% (for total T3) to 10.4% (for FT4) and the intra-assay CVs ranged from 1.2% (for total T3) to 12.3% (for FT4). Thyroid hormone concentrations less than the LOD were assigned a value of LOD divided by the square root of 2.[29]

Free T3 (FT3) was not measured in this study due to sample volume constraints. Since unbound T3 is the principal bioactive hormone and potentially relevant to the mechanisms involved in preterm birth [30], we estimated its concentration using total T3 and the ratio of free vs. bound T4 concentrations assayed for each individual at visits 1-3. We determined the fraction of T4 that was unbound by dividing the concentration of FT4 by the concentration of T4 (both in µg/dL). Since approximately 10 times more T3 is unbound compared to T4 (~0.05% of T4 is in free form vs. ~0.5% of T3),[31] we multiplied each FT4/T4 fraction by 10 to obtain the approximate proportion of unbound T3 per sample. We then applied this proportion to the measured total T3 concentrations to estimate the fractional concentration of FT3 (in pg/mL) per sample collected from each individual at visits 1-3. While our estimations are potentially limited by differences between T3 and T4 in binding ratios of free to total hormones and in intracellular versus extracellular concentrations of unbound hormones, we used the estimated concentrations of FT3 to explore our hypothesis about its potential association with preterm birth and included this estimation in only the regression models assessing the odds of overall preterm birth.

Statistical Methods

Analyses were performed using SAS version 9.3 (SAS Institute Inc.) and R version 3.1.1 (R Foundation for Statistical Computing). The empirical histograms of total T3 and T4 approximated a normal distribution. The distributions of TSH and FT4 were right-skewed so we used the natural log transformation (ln) of these variables in statistical analyses. We used a chi-square statistic to test the differences in demographic characteristics between cases and controls.

We assessed the variability of the assayed thyroid hormones (TSH, free and total T4, and total T3) across pregnancy for the overall population as well as separately for cases and controls using several methods. First, we tabulated the median and interquartile range (25^{th} and 75^{th} percentiles) for each hormone, and evaluated differences in visits 2-4 compared to visit 1 using linear mixed models (LMMs) with a subject-specific random intercept. We also calculated the intraclass correlation coefficient (ICC) and associated 95% confidence intervals to examine the temporal variability in hormones for each subject. ICC measures the reproducibility of repeated measures from the same subject and is the ratio of between-subject variance to total variance

(between- plus within-subject variance).[32] ICC ranges from 0 to 1, with the latter indicating no within-subject variability.[32]

In our third variability analysis, we examined the patterns of each hormone across pregnancy by fitting generalized additive mixed effects models (GAMM) using the R *mgcv* package. For each model, we used repeated measures of individual thyroid hormones and regressed them on a penalized spline of gestational age to assess potential nonlinear associations. We accounted for the correlation of repeated measures taken from the same subject by including subject-specific random intercepts and slopes. Predicted thyroid hormone concentrations were plotted in relation to gestational age at time of sample collection to examine patterns across pregnancy. To test whether the observed patterns varied by case-control status, we included an interaction term between preterm birth and gestational age.

We used logistic regression to explore associations between increases in individual thyroid hormones and the odds of preterm birth. Crude models included gestational age at time of sample collection. Full models were additionally adjusted for maternal age at enrollment, body mass index (BMI) at time of sample collection, parity, health insurance provider, and educational attainment. We chose age, BMI, and parity as covariates *a priori* based on their biological relevance to maternal thyroid hormone concentrations and preterm birth.[33-37] We identified the additional covariates based on ≥ 10% change in the main effect estimates when added to the models in a forward stepwise procedure.

We stratified logistic regression models by study visit of sample collection. We excluded data collected at visit 4 from all regression analyses in order to avoid potential bias resulting from a disproportionate number of controls compared to cases providing plasma samples at this study visit. To reveal potential biological pathways, we explored these relationships by obstetric presentation of preterm birth that have been previously hypothesized to share common underlying mechanisms.[25] Specifically, we repeated these stratified analyses for each subtype of preterm birth (spontaneous and placental preterm birth), adjusting logistic regression analyses for gestational age at time of sample collection, maternal age at enrollment, and maternal race. Since the gestational age ranges varied considerably for each study visit of sample collection, we also explored narrower windows of susceptibility in a separate sensitivity analysis by stratifying

regression models for overall preterm birth by five-week intervals of gestational age (e.g., 5-10 weeks, 10-15 weeks, etc.).

RESULTS

The demographic characteristics of the study population by preterm birth status are presented in **Table IV.1** and are consistent with our prior publications.[24] Overall, the study population was predominately white, highly educated, and non-smokers. The majority of women were giving birth for the first time and a greater proportion of women delivering preterm were obese (>30 kg/m^2) compared to controls (31% of cases vs. 20% of controls).

Variability in hormones across pregnancy

The distributions of thyroid hormones by study visit of sample collection are reported in **Table IV.2** for the overall population and by preterm birth status. Results from linear mixed models (LMMs) indicated that thyroid hormone concentrations varied by study visit of sample collection for both cases and controls. Intraclass correlation coefficients (ICCs) showed the lowest temporal reliability for FT4 and the highest reliability for total hormones (T3 and T4).

Smoothed plots of predicted thyroid hormone concentrations in association with gestational age at sample collection in cases and controls are presented in **Figure IV.1**. The observed trajectories of each hormone across gestation were similar to the pattern of results reported in **Table IV.2.** Interaction terms from generalized additive mixed effects models (GAMMs) indicated that trends in hormone concentrations across pregnancy were significantly different between cases and controls ($p<0.001$ for all hormones). The smoothed plot for TSH showed that concentrations were greater in controls in early pregnancy and subsequently decreased to lower concentrations than those observed in cases in the latter half of the first trimester. Predicted values of FT4 in controls were also higher in samples taken in early pregnancy, but converged to similar concentrations as those observed in cases as pregnancy progressed. Total thyroid hormone concentrations (T4 and T3) increased across gestation in both cases and controls, with a greater increase observed between approximately 5 and 15 weeks of gestation in controls.

Gestational age-stratified analyses

Adjusted odds ratios (OR) of overall preterm birth in relation to a unit increase in thyroid hormone concentrations are presented in **Table IV.3** by study visit of sample collection. ORs from fully adjusted logistic regression models were similar to those observed in crude models. At visits 1 and 3, a one ng/dL decrease in ln-transformed FT4 was associated with approximately two times the odds of preterm birth. Similar to results detected for FT4, ORs for overall preterm birth were reduced for estimated FT3 at visits 1 and 3, although these associations were not statistically significant (data not shown).

For total hormones, total T4 concentrations were suggestively associated with an increase in odds of overall preterm birth (p=0.07-0.16). A unit increase of total T3 was associated with a two- to threefold increase in odds of overall preterm birth at all study visits with the exception of visit 2. Associations for TSH were null at all time points.

Narrower windows of gestational age were explored by stratifying ORs by five-week intervals of gestational age at time of sample collection (**Table IV.S1**). Results from this sensitivity analysis were similar to those reported in **Table IV.3** by study visit of sample collection. Significant elevated ORs were observed for total T3 measured in samples taken in early (5-10 weeks) and mid- to late pregnancy (20-25 weeks and 25-30 weeks). At these same time points, reduced albeit nonsignificant, ORs were detected for FT4.

Figures IV.2 and **IV.3** show visit-specific associations between measured thyroid hormone concentrations and odds of spontaneous and placental preterm birth, respectively (data reported in **Tables IV.S2** and **IV.S3**). For spontaneous preterm birth, reduced ORs for FT4 and estimated FT3 at visits 1 and 3 were similar in direction to those observed for overall preterm birth but were stronger and statistically significant for this subtype. The results for total T3 were in the opposite directions as those observed for FT4, and were significantly elevated at study visits 1 and 2. For placental preterm birth, no significant associations were observed for any of the hormones.

DISCUSSION

In this nested case-control study drawn from a large prospective birth cohort, we characterized the temporal patterns of thyroid function parameters across gestation. We explored windows of vulnerability for the risk of preterm birth using plasma samples collected at up to four time points in pregnancy. Additionally, we evaluated whether the effects of these subclinical hormonal deviations on the risk of preterm birth varied by clinical presentation.

Profiles of hormones across pregnancy

This is the first study to evaluate the differences in the variability and trajectories of maternal thyroid hormone concentrations across pregnancy between women delivering preterm and at term. Various physiological changes that accompany the normal pregnancy state increase the demands of the maternal thyroid gland.[4,38] In response to the estrogen-stimulated rise in the transport protein, thyroxine-binding globulin (TBG), there is a concomitant rise in total T3 and T4 in the first half of pregnancy until a new steady state is reached.[4] Also in the first trimester, there is a transient lowering of circulating TSH that coincides with peak human chorionic gonadotropin (hCG) concentrations.[4] Due to the structural homology between hCG and TSH molecules, hCG binds to the TSH receptor and exerts a stimulatory effect – the increased hormonal output of FT4 results in the lowering of TSH levels via the negative feedback system.[4,39] Following the initial increase in FT4 between approximately 6 and 10 weeks of gestation as a result of the high placental production of hCG during this time period,[40] FT4 subsequently decreases over pregnancy.[4] In the present study, the trajectories of thyroid hormone parameters in women delivering at term were consistent with what has been reported in the medical literature. Differences in the temporal hormonal patterns between cases and controls were most evident in the first trimester of pregnancy.

We found that total T3 and T4 concentrations were lower in controls in early pregnancy, and rose to similar or slightly greater concentrations as cases by the end of the first trimester (**Figure IV.1**). An upward trend in total hormones was observed in both groups until approximately 20 to 25 weeks of gestation, when a more stable concentration was reached. For TSH, concentrations were greater in controls than in cases in the earlier half of the first trimester.

In controls, we observed the characteristic sharp decrease in TSH in the first trimester followed by an increase in concentrations until approximately 20 to 25 weeks of gestation (**Figure IV.1).** Whereas TSH concentrations fluctuated across gestation in controls and exhibited patterns similar to those reported in the literature, there was a constant upward slope in concentrations across pregnancy in women delivering preterm. However, it is uncertain whether this somewhat linear trend is a consequence of a smaller sample size in cases or if this pattern represents the true trajectory of TSH in women delivering preterm. Finally, we detected downward trends in FT4 across gestation in both cases and controls, with greater concentrations of FT4 observed in controls in early pregnancy (**Figure IV.1**). In response to peak hCG production in normal pregnancy, TSH decreases to its lowest concentration and FT4 to its highest concentration between approximately 9 and 12 weeks of gestation.[40] The temporal differences in the trends of TSH and FT4 between cases and controls that we observed in early pregnancy – specifically, the higher concentrations of TSH and lower concentrations of FT4 in cases compared to controls around approximately 10 weeks of gestation – may indicate a lack of thyroidal response to hCG in women delivering preterm [41]. In their recent study, Korevaar et al. observed an impaired thyroidal response to hCG in thyroperoxidase antibody (TPOAb)-positive pregnant women.[41] While TPOAb positivity is a risk factor for premature delivery,[19] we did not assess thyroid autoimmunity in our study participants and therefore cannot examine the extent to which thyroid autoimmunity modifies the relationships between subclinical changes in thyroid function parameters and the risk of preterm birth in this study.

Associations by gestational age

In the present study, we found that a unit decrease in FT4 was associated with an approximate twofold increase in the odds of overall preterm birth at median 10 weeks of gestation. These findings are in agreement with a previous study showing associations between low FT4 concentrations (<2.5th percentile) at median 13 weeks of pregnancy and an increased risk of preterm delivery.[19] However, null associations have been reported by other studies for low FT4 in early pregnancy[42-45] and for continuous measures of FT4 sampled in the first half of gestation.[46,47] Currently, there are no published data on the relationship of total T3 concentrations with preterm birth. However, our nonsignificant findings for estimated concentrations of FT3

and overall preterm birth are compatible with an earlier birth cohort study in which a lack of association was observed in early pregnancy.[46]

Our null findings for TSH contrast with the results reported in studies showing associations between preterm birth and elevated TSH concentrations in early pregnancy[20,48] and maternal subclinical hypothyroidism (defined as elevated TSH with normal FT4). [42,49,50] However, these results were not confirmed by other studies.[13,17,45,46,51]

The observed dissimilarities between our analyses and findings reported previously may be due to differences in assay methods used to measure FT4 (electrochemiluminescence immunoassay vs. direct equilibrium dialysis followed be radioimmunoassay), variability in the classification of subclinical thyroid dysfunction (e.g., differing statistical cutoff points to define elevated TSH or low FT4), inconsistent ascertainment of preterm birth (e.g., gestational age based on self-reported last menstrual period vs. first trimester ultrasound-validated measurements), and/or the proportion of spontaneous versus iatrogenic preterm birth cases. Furthermore, no other studies assessed additional time points outside of the first or second trimester.

One of the strengths of our study was our analyses by subtype of preterm birth. Only one previous study has examined associations between thyroid hormone concentrations and preterm birth with attention to presentation at delivery.[52] In that study, women with spontaneous preterm delivery (delivery <34 weeks) had significantly reduced concentrations of FT4 (within the normal range) measured in the first trimester compared to women delivering at term, although no differences were observed for TSH between the two groups. In our analysis we observed odds ratios that were greater in magnitude and more precise in models of spontaneous preterm birth alone, specifically for FT4 and total T3. While these findings may be due to differences in sample size between the two stratified analyses, our results for spontaneous preterm birth suggests that changes in these hormone concentrations during gestation may have particular consequences for spontaneous preterm labor and/or PPROM. For placental preterm birth, we did not observe any significant associations. Specifically, our generally null results for TSH are in contrast to studies showing associations between abnormally elevated TSH concentrations and an increased risk of preeclampsia[53] and IUGR,[54] which are characteristics of placental preterm

birth.[25] Additional studies with larger sample sizes are required to disentangle the relationships between subclinical maternal thyroid dysfunction and subtype of preterm birth, and to identify the underlying biological mechanisms potentially driving these associations.

It is possible that the observed fluctuations in thyroid function parameters are a result of other underlying physiological processes that ultimately lead to preterm birth. Spontaneous preterm birth is strongly associated with inflammation at the maternal-fetal interface.[55,56] We previously demonstrated that the pro-inflammatory cytokine, interleukin-6 (IL-6), is a strong predictor of spontaneous preterm delivery in the current study population.[57] Mild thyroid hormone dysfunction at various time points in pregnancy may contribute to the inflammatory processes involved in the pathogenesis of spontaneous preterm birth, or vice versa. Indeed, human health studies have shown increased pro-inflammatory markers, including IL-6, in overt and subclinical hypothyroid patients.[58,59] Consistent with this hypothesis, we found strong and highly significant inverse relationships between free hormones and spontaneous preterm birth in early and/or late pregnancy.

Strengths and Limitations

The primary strengths of our study was our repeated measures of thyroid function parameters collected in each trimester of pregnancy and our accurately defined clinical outcomes. Our longitudinal study design permitted an assessment of the variability in individual parameters across gestation in cases and controls as well as time points in pregnancy during which subclinical thyroidal disturbances may have a more profound effect on the risk of preterm birth. Additionally, our assay method for measuring FT4 using equilibrium dialysis is considered analytically accurate and is preferred over traditional immunoassays since measurements are not affected by thyroid hormone binding protein concentrations, which increase in pregnancy.[60,61] Despite these strengths, our study was limited by the lack of assessment of the thyroid autoimmunity of our study participants due to biological sample volume constraints. As mentioned previously, the presence of thyroid anti-thyroid antibodies have been found to modify the relationships between circulating thyroid hormone concentrations and adverse birth outcomes.[19,26]

CONCLUSIONS

In conclusion, our results support previous studies showing the potential for subclinical changes in thyroid hormone concentrations in pregnancy to influence the risk of preterm birth. Our stratified analyses showed that these effects may vary by gestational age and clinical presentation of preterm birth. Additional human health and animal studies should take these findings into account when trying to elucidate the mechanism(s) of subclinical thyroid dysfunction in the pathogenesis of preterm birth.

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TABLES AND FIGURES

Table IV.1. Population demographic characteristics by cases (N=116) and controls (N=323)

Population Characteristics		Cases	Controls
		N (%)	N (%)
Age	18-24 years old	10 (9)	44 (14)
	25-29 years old	25 (22)	67 (21)
	30-34 years old	49 (42)	127 (39)
	35+ years old	32 (28)	85 (26)
Race	White	65 (56)	182 (56)
	African-American	21 (18)	54 (17)
	Other	30 (26)	87 (27)
Education	High School	21 (18)	46 (15)
	Technical School	25 (22)	51 (16)
	Junior College or some college	34 (30)	93 (30)
	College graduate	35 (30)	124 (39)
Health Insurance Provider	Private	94 (82)	250 (80)
	Public	20 (18)	63 (20)
BMI at Initial Visit	<25 kg/m^2	51 (44)	176 (54)*
	25-30 kg/m^2	29 (25)	84 (26)
	>30 kg/m^2	36 (31)	63 (20)
Tobacco Use	Smoked during pregnancy	11 (9)	20 (6)
	No smoking during pregnancy	105 (91)	297 (94)
Alcohol Use	Alcohol use during pregnancy	1 (1)	12 (4)
	No alcohol use during pregnancy	113 (99)	299 (96)
Fetal sex	Male	50 (43)	148 (46)
	Female	66 (57)	175 (54)
Parity	Nulliparous	50 (43)	147 (45)
	Primiparous	32 (28)	112 (35)
	Multiparous	34 (29)	64 (20)

Abbreviations: BMI, Body Mass Index

* $p<0.05$ for chi-square test

Table IV.2. Median (25th-75th) concentrations and intraclass correlation coefficient (ICCs) of thyroid hormone parameters by case-control status and study visit of sample collection.

Study Visit	TSH (μIU/mL)	FT4 (ng/dL)	T4 (μg/dL)	T3 (ng/mL)
All Samples (N=1756 observations)				
visit 1 [ref]	0.92 (0.54, 1.50)	1.37 (1.15, 1.62)	10.1 (8.78, 11.4)	1.32 (1.13, 1.62)
visit 2	1.34 (0.97, 1.90)*	1.13 (0.90, 1.30)*	10.6 (9.60, 11.9)*	1.61 (1.35, 1.92)*
visit 3	1.28 (0.93, 1.70)*	1.00 (0.81, 1.18)*	10.4 (9.20, 11.5)*	1.66 (1.38, 1.96)*
visit 4	1.39 (0.97, 1.93)*	0.96 (0.77, 1.17)*	10.0 (9.00, 11.5)	1.66 (1.41, 2.02)*
ICC (95%CI)	**0.51 (0.46, 0.57)†**	**0.18 (0.13, 0.24)†**	**0.67 (0.63, 0.71)**	**0.62 (0.57, 0.67)**
Cases (N=116; 464 observations)				
visit 1 [ref]	0.94 (0.51, 1.40)	1.35 (1.08, 1.55)	10.4 (8.93, 11.6)	1.38 (1.25, 1.68)
visit 2	1.24 (0.89, 1.83)*	1.13 (0.83, 1.30)*	10.6 (9.85, 12.1)*	1.70 (1.32, 2.09)*
visit 3	1.24 (0.96, 1.73)*	0.95 (0.79, 1.15)*	10.5 (9.45, 11.8)*	1.83 (1.55, 2.19)*
visit 4	1.54 (1.00, 2.02)*	0.96 (0.75, 1.24)*	10.7 (9.18, 11.9)	1.92 (1.45, 2.13)*
ICC (95%CI)	**0.47 (0.35, 0.58)†**	**0.32 (0.20, 0.43)†**	**0.59 (0.49, 0.68)**	**0.62 (0.51, 0.71)**
Controls (N= 323; 1292 observations)				
visit 1 [ref]	0.91 (0.55, 1.52)	1.39 (1.17, 1.65)	10.0 (8.70, 11.2)	1.30 (1.11, 1.60)
visit 2	1.39 (0.99, 1.91)*	1.14 (0.94, 1.30)*	10.6 (9.43, 11.8)*	1.60 (1.37, 1.87)*
visit 3	1.30 (0.90, 1.70)*	1.00 (0.82, 1.20)*	10.3 (9.10, 11.3)*	1.60 (1.35, 1.87)*
visit 4	1.34 (0.97, 1.92)*	0.96 (0.77, 1.17)*	10.0 (8.90, 11.4)	1.65 (1.39, 1.99)*
ICC (95%CI)	**0.53 (0.46, 0.59)†**	**0.15 (0.09, 0.22)†**	**0.69 (0.65, 0.74)**	**0.61 (0.55, 0.67)**

Abbreviations: CI, confidence interval; ICC, intraclass correlation coefficient.

* Indicates significant difference ($p<0.05$) in thyroid hormone concentration at the study visit compared to the reference (visit=1) using linear mixed models with a subject-specific random intercept.

† ICCs calculated using ln-transformed concentrations.

Table IV.3 Adjusted odds ratios (95% CI) of overall preterm birth (N=116 cases) associated with a unit increase in thyroid hormone parameters.

	Visit 1 (median 10 weeks of gestation)			Visit 2 (median 18 weeks of gestation)			Visit 3 (median 26 weeks of gestation		
Thyroid Hormone	**N (cases, controls)**	**OR (95%CI)**	**p-value**	**N (cases, controls)**	**OR (95%CI)**	**p-value**	**N (cases, controls)**	**OR (95%CI)**	**p-value**
ln-TSH	81, 220	0.91 (0.71, 1.16)	0.43	86, 221	0.88 (0.61, 1.28)	0.52	75, 219	1.43 (0.85, 2.46)	0.19
ln-FT4	98, 257	0.57 (0.33, 1.00)	0.05	96, 260	0.97 (0.60, 1.54)	0.89	88, 247	0.53 (0.34, 0.84)	<0.01*
T4	100, 246	1.12 (0.99, 1.27)	0.07	92, 253	1.11 (0.96, 1.28)	0.16	85, 235	1.13 (0.99, 1.29)	0.08
T3	76, 212	2.52 (1.20, 5.31)	0.01*	82, 209	1.71 (0.81, 3.60)	0.16	70, 204	3.40 (1.56, 7.40)	<0.01*

Adjusted models include gestational age at time of sample collection, maternal age at enrollment, body mass index (BMI) at time of sample collection, parity, health insurance provider, and educational attainment.

* $p<0.05$

Figure IV.1. Predicted thyroid hormone concentrations across pregnancy by case-control status.

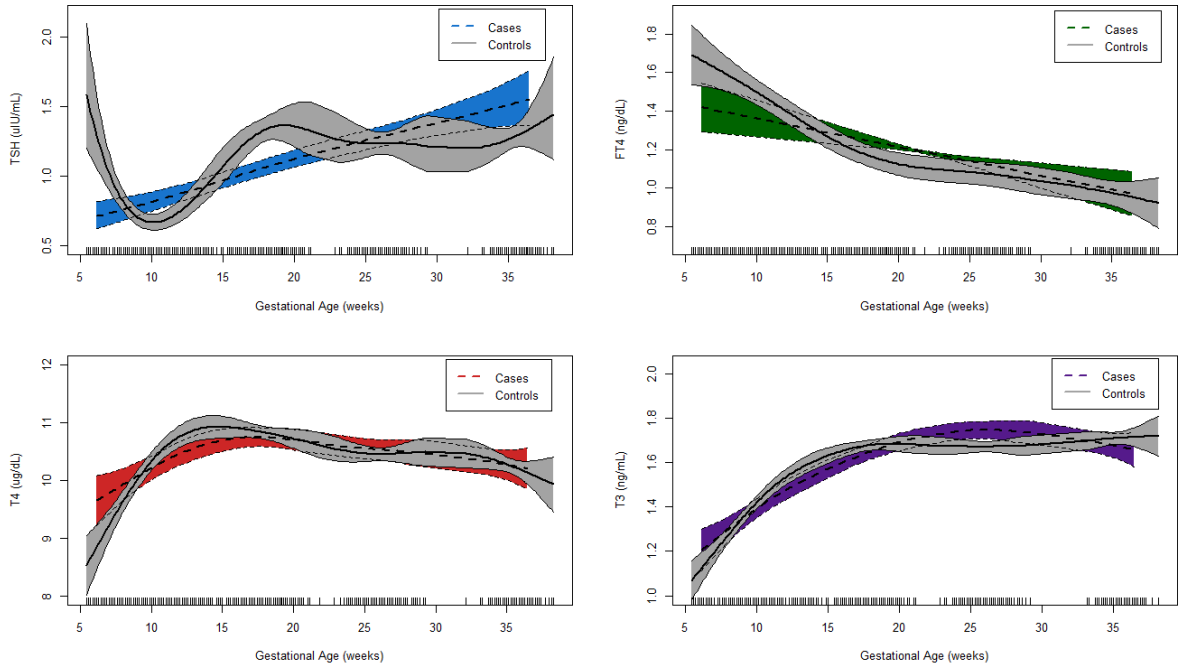


Figure IV.2. Adjusted odds ratios of spontaneous preterm birth (N=49 cases) associated with a unit increase in thyroid hormone concentrations (*p<0.05).

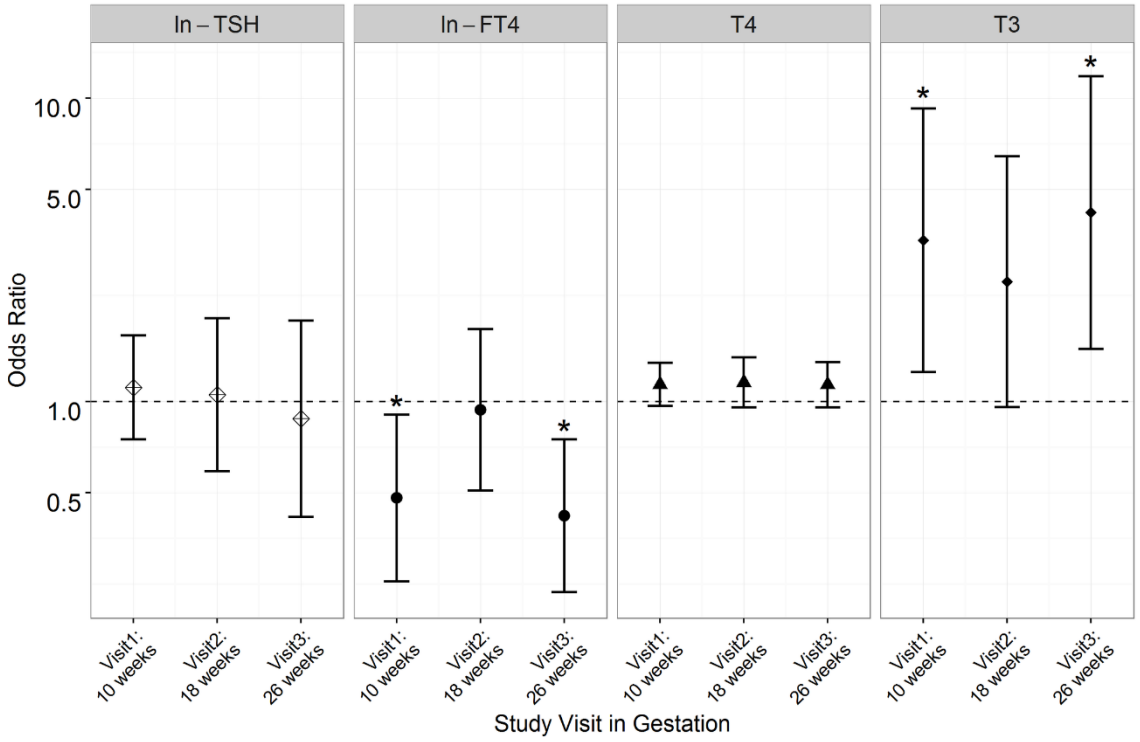
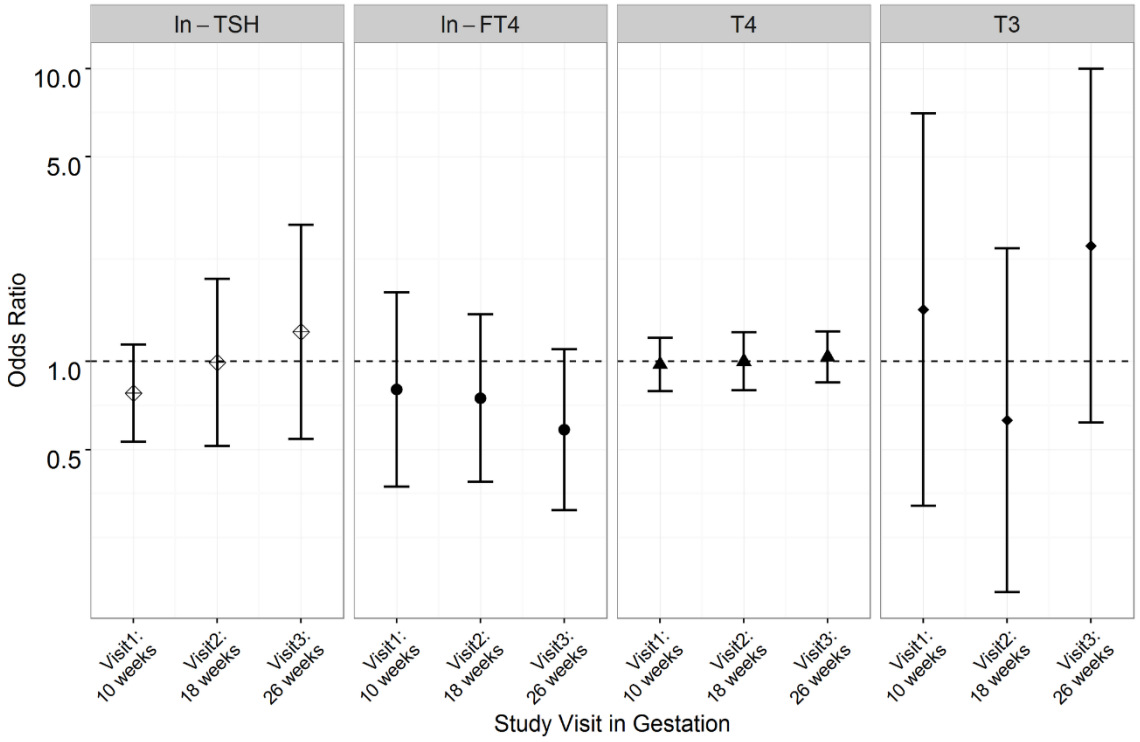


Figure IV.3. Adjusted odds ratios of placental preterm birth (N=33 cases) associated with a unit increase in thyroid hormone concentrations (*p<0.05)



SUPPLEMENTAL MATERIAL

Table IV.S1. Adjusted odds ratios (95% CI) of overall preterm birth (N=116 cases) associated unit increase in thyroid hormone concentrations: results stratified by gestational age.

Gestational Age (weeks)	**ln-TSH**			**ln-FT4**			**T4**			**T3**		
	N (cases, controls)	OR (95%CI)	p-value	N (cases, controls)	OR (95%CI)	p-value	N (cases, controls)	OR (95%CI)	p-value	N (cases, controls)	OR (95%CI)	p-value
5-10	44, 122	0.74 (0.52, 1.07)	0.11	53, 143	0.44 (0.18, 1.11)	0.08	54, 137	1.12 (0.93, 1.35)	0.22	42, 118	**3.05 (0.98, 9.50)**	**0.05**
10-15	36, 98	1.18 (0.77, 1.81)	0.46	44, 114	0.63 (0.29, 1.34)	0.23	45, 109	1.11 (0.93, 1.34)	0.25	34, 95	2.52 (0.86, 7.39)	0.09
15-20	84, 211	0.83 (0.58, 1.17)	0.29	94, 249	0.98 (0.60, 1.62)	0.95	90, 243	1.14 (0.98, 1.31)	0.09	79, 201	1.80 (0.83, 3.88)	0.14
20-25	31, 48	1.46 (0.49, 4.34)	0.49	33, 58	0.44 (0.18, 1.05)	0.06	32, 52	1.11 (0.82, 1.50)	0.49	28, 46	**4.52 (1.05, 19.4)**	**0.04**
25-30	47, 178	1.49 (0.77, 2.89)	0.23	58, 197	0.61 (0.36, 1.01)	0.06	56, 190	1.07 (0.92, 1.25)	0.38	45, 164	**2.59 (1.03, 6.51)**	**0.04**

Adjusted models include gestational age at time of sample collection, maternal age at enrollment, body mass index (BMI) at enrollment, parity, health insurance provider, and educational attainment.

Table IV. S2. Adjusted odds ratios (95% CI) of spontaneous preterm birth associated with a unit increase in thyroid hormone parameters.

Thyroid Hormone	Visit 1 (median 10 weeks of gestation)			Visit 2 (median 18 weeks of gestation)			Visit 3 (median 26 weeks of gestation)		
	N (cases, controls)	OR (95% CI)	p-value	N (cases, controls)	OR (95% CI)	p-value	N (cases, controls)	OR (95% CI)	p-value
ln-TSH	36, 233	1.11 (0.75, 1.65)	0.60	38, 229	1.05 (0.59, 1.88)	0.86	32, 228	0.88 (0.42, 1.85)	0.73
ln-FT4	42, 272	**0.48 (0.25, 0.91)**	**0.02**	45, 269	0.94 (0.51, 1.74)	0.84	38, 257	**0.42 (0.24, 0.75)**	**<0.01**
T4	43, 260	1.14 (0.97, 1.34)	0.12	42, 262	1.16 (0.96, 1.40)	0.14	36, 235	1.14 (0.96, 1.35)	0.15
T3	34, 225	**3.40 (1.25, 9.25)**	**0.02**	36, 217	2.48 (0.96, 6.42)	0.06	29, 213	**4.20 (1.49, 11.8)**	**<0.01**

Logistic regression models adjusted for gestational age at time of sample collection, maternal age at enrollment, and maternal race.

Table IV.S3. Adjusted odds ratios (95% CI) of placental preterm birth associated with a unit increase in thyroid hormone parameters.

Thyroid Hormone	Visit 1 (median 10 weeks of gestation)			Visit 2 (median 18 weeks of gestation)			Visit 3 (median 26 weeks of gestation)		
	N (cases, controls)	OR (95%CI)	p-value	N (cases, controls)	OR (95%CI)	p-value	N (cases, controls)	OR (95%CI)	p-value
ln-TSH	21, 233	0.78 (0.53, 1.14)	0.20	27, 229	0.99 (0.51, 1.91)	0.98	24, 228	1.26 (0.54, 2.93)	0.59
ln-FT4	29, 272	0.80 (0.37, 1.72)	0.57	29, 269	0.75 (0.39, 1.45)	0.39	29, 257	0.58 (0.31, 1.10)	0.10
T4	29, 260	0.98 (0.79, 1.20)	0.82	28, 262	1.00 (0.80, 1.26)	1.00	26, 244	1.04 (0.85, 1.26)	0.73
T3	18, 225	1.50 (0.32, 7.03)	0.60	25, 217	0.63 (0.16, 2.44)	0.50	23, 213	2.48 (0.62, 9.90)	0.20

Logistic regression models adjusted for gestational age at time of sample collection, maternal age at enrollment, and maternal race.

CHAPTER V. Subclinical Changes in Maternal Thyroid Function Parameters in Pregnancy are Associated with Fetal Growth

ABSTRACT

Introduction: Thyroid hormones play a crucial role in placental development and intrauterine growth. While overt thyroid disease in pregnancy is a known risk factor for abnormal fetal growth and development, data on the effects of milder forms of variation in maternal thyroid function on intrauterine growth are less well examined.

Methods: Data were obtained from 439 pregnant women without diagnosed thyroid disease who were participants in a nested case-control study of preterm birth within an ongoing prospective birth cohort in Boston, MA. Thyroid function parameters were measured and ultrasound scans were performed at up to four time points in pregnancy. Birth weight was recorded at delivery. All fetal growth indicators were standardized to those measured in a larger population.

Results: At median 10, 18, and 26 weeks of gestation, we observed significant inverse associations between FT4 and birth weight z-scores, with the greatest association detected at median 10 weeks. At this time point, a unit increase in ln-transformed FT4 was associated with almost a half of a z-score decrease (approximately 175 grams) in birth weight (β = -0.41; 95% confidence interval [CI] = -0.64, -0.18). FT4 was also inversely associated with repeated fetal growth measurements, with significant estimates found for estimated fetal weight, head circumference, and abdominal circumference. Birth weight did not significantly differ between women with subclinical hypothyroidism (N=10) and those without (N=426), although the lack of an association may be due to the limited sample size. We observed weaker inverse associations

between total T4 and fetal growth indices, and a positive relationship between total T3 at median 26 weeks of gestation and birth weight. We did not observe any associations for TSH.

Conclusions: In pregnant women without overt thyroid disease, subclinical changes in thyroid function parameters may influence fetal growth.

INTRODUCTION

Impaired fetal growth is a major predictor of neonatal mortality and morbidity, and may increase the risk of long-term health complications such as diabetes and cardiovascular disease in adulthood.[1,2] Thyroid hormones play a crucial physiological role in early placental development as well as intrauterine growth and fetal tissue accretion and differentiation.[3,4] Overt thyroid dysfunction in pregnancy (hyper- and hypothyroidism) has been consistently linked to abnormal fetal growth and development, including neurodevelopment.[5-8]

Investigations of the effects of milder forms of thyroid dysfunction on fetal growth are less conclusive. Subclinical hypothyroidism (high thyroid-stimulating hormone [TSH] with normal free thyroxine [FT4]) and hypothyroxinemia (normal TSH with low FT4) have been associated with a smaller head circumference and birth length,[8] intrauterine growth restriction (IUGR),[9] low birth weight[9,10] and/or an increased risk for small for gestational age (SGA) neonates.[8,11] However, similar studies on these relationships have reported conflicting results[12-14] or null associations.[15-18] The more consistent findings have been in generally euthyroid pregnant women in whom increases in FT4 have been associated with lower birth weight[14,19-21] and an increased risk for SGA newborns.[20]

Few longitudinal studies have been performed with repeated measures of maternal thyroid hormones in pregnant women.[22-25] To our knowledge, only one study has utilized serial measurements of thyroid function parameters to explore potential associations with fetal growth.[26] Specifically, Nishioka et al.[26] reported that an increase in maternal TSH concentrations between the first and third trimesters was associated with low birth weight (< 2500 g). This study's relatively small sample size, and thus limited number of low birth weight babies (N=10),

precluded trimester-specific analyses and likely contributed to the lack of variation in free hormones between women who delivered low birth weight babies and controls.

Given the heterogeneous results in the available literature as well as the lack of longitudinally collected data across pregnancy, we investigated the extent to which thyroid function parameters, collected at up to four times points in pregnancy, were associated with birth weight and repeated measurements of fetal growth in 439 pregnant women without clinical thyroid disease.

METHODS

Study Population

The present study is a secondary analysis of data from a nested case-control study of preterm birth drawn from the LIFECODES cohort – a prospective birth cohort of pregnant women in Boston, MA. Women ages ≥ 18 years old were recruited early in pregnancy (< 15 weeks of gestation) between 2006 and 2008 and were eligible for participation if they were carrying a singleton, non-anomalous fetus and planned to deliver at Brigham and Women's Hospital. Details regarding recruitment and eligibility criteria are described in detail elsewhere.[27,28] At the initial study visit (median = 10 weeks of gestation), women completed a medical questionnaire that collected sociodemographic and health-related information (e.g., personal and family health history). Participants were followed until delivery, and provided relevant health information as well as blood samples at three additional study visits: visit 2 (median = 18 weeks of gestation), visit 3 (median = 26 weeks of gestation), and visit 4 (median = 35 weeks of gestation). Gestational age at individual study visits and at delivery were calculated based on last menstrual period and confirmed by first trimester ultrasound or based entirely upon first trimester ultrasound. Birth weight was recorded at delivery.

From the prospective birth cohort, we selected 130 women who delivered preterm (< 37 weeks) and 352 randomly selected controls for inclusion in the nested case-control study. For the current analysis, we additionally excluded 41 women with self-reported pre-existing or gestational thyroid disease/conditions (e.g., hyper- or hypothyroidism, Graves' disease, or

thyroid cancer) based on answers to medical questionnaires administered at each of the study visits. Two women who did not provide plasma samples at any study visit were also excluded. Our final study population included 116 cases of preterm birth and 323 controls. The study protocols were approved by the ethics review board at Brigham and Women's Hospital (Partners Health Research Committee) and all study participants gave written informed consent.

Ultrasound Measurements

The American College of Obstetricians and Gynecologists' (ACOG) guidelines for perinatal care recommend that all women undergo evaluations for aneuploidy in the first trimester and again in the second trimester to examine fetal anatomy.[29] Thus, in our study population all women had ultrasound scans that provide crown rump length at visit 1 and fetal morphology at visit 2. Ultrasound scans at visits 3 and 4 were not routine, but were obtained frequently for a variety of indications including maternal gestational diabetes or suspected restricted fetal growth).[30] All ultrasound measurements were conducted by experienced faculty sonologists with active Society of Maternal-Fetal Medicine certification.

In the current analysis, we used the following ultrasound measurements from visits 2 through 4: head circumference (HC), abdominal circumference (AC), and femur length (FL). We calculated estimated fetal weight (EFW) at each of the study visits using the Hadlock formula that combines biparietal diameter, abdominal circumference, and femur length.[31] In order to combine and compare fetal growth measurements across different time points, we standardized raw ultrasound measurements using z-scores based on the mean and standard deviation of ultrasound measurements available for 18,904 non-anomalous singleton pregnancies delivered between 2006 and 2012 at Brigham and Women's Hospital.[30,32]

Thyroid Hormone Measurements

A total of 1,443 plasma samples from 439 pregnant women were assayed for thyroid function parameters at the University of Michigan Clinical Ligand Assay Service Satellite (CLASS) Lab (Ann Arbor, MI). These samples were collected at up to four study visits. We measured TSH as well as total triiodothyronine (T3) and T4 using an automated

chemiluminescence immunoassay (Bayer ADVIA Centaur; Siemens Health Care Diagnostics, Inc.). FT4 was measured using direct equilibrium dialysis followed by radioimmunoassay (IVD Technologies).

The manufacturer only provided a non-pregnant normal range of 0.35-5.50 uIU/mL for TSH. In the absence of trimester-specific reference ranges, the American Thyroid Association (ATA) recommended in 2011 the following TSH reference ranges: first trimester, 0.1-2.5 μIU/mL; second trimester, 0.2-3.0 μIU/mL; and third trimester, 0.3-3.0 μIU/mL.[33] However, the ATA recently recommended an upper limit of ~ 4.0 μIU/mL when TSH pregnancy references are not available.[34] The laboratory pregnancy reference ranges for FT4 were: first trimester, 0.7-2.0 ng/dL; second trimester, 0.5-1.6 ng/dL; and third trimester, 0.5-1.6 ng/dL. In an exploratory analysis, we defined subclinical hypothyroidism as TSH exceeding the ATA recommended upper limit of 4 μIU/mL in any trimester in combination with a normal FT4 concentration within trimester-specific reference ranges.[34] The limits of detection (LOD) were 0.01 μIU/mL for TSH, 0.1 ng/mL for T3, 0.3 μg/dL for T4, and 0.1 ng/dL for FT4. The inter-assay coefficients of variation (CV) for all hormones ranged from 2.3% (for total T3) to 10.4% (for FT4) and the intra-assay CVs ranged from 1.2% (for total T3) to 12.3% (for FT4).[25]

Statistical Analyses

All analyses were performed using R version 3.3.2. Fetal growth indicators included in our study are secondary outcomes in the original nested case-control study of preterm birth. Thus, to correct for potential over-representation of preterm birth cases and to make the present study more representative of the base LIFECODES cohort, we applied to all analyses inverse probability weights representing the inverse sampling fraction for selection of cases (90.1%) and controls (33.9%) from the base population.[35,36]

To assess the bivariate associations between population characteristics and birth weight z-scores, we used linear regression models adjusted for gestational age at delivery. We examined the distributions of raw ultrasound measurements and thyroid hormone parameters by calculating selected percentiles at each study visit of sample collection as well as at delivery (for birth weight). The empirical distributions of total T3 and T4 approximated normality. The

distributions of TSH and FT4 were positively skewed and were natural log transformed (ln) for all statistical analyses. We calculated percentiles and tested the differences in mean thyroid hormone concentrations between women who underwent ultrasound scans at visit 3 and/or visit 4 vs. those who did not undergo scans at either visit using linear mixed models (LMMs) with subject-specific random intercepts.

In cross-sectional analysis, we explored the relationships between continuous thyroid hormone concentrations measured at each of the four study visits and birth weight z-scores using stratified (by study visit) multivariable linear regression models. We included maternal age, race/ethnicity, body mass index (BMI) at initial study visit, and fetal sex *a priori*. Additional covariates –such as health insurance provider, educational attainment, parity, and smoking and alcohol use in pregnancy– were retained in models if their inclusion resulted in ≥10% change in the main beta estimates using a forward step-wise selection procedure. Crude models were adjusted for gestational age at time of sample collection. Final models were adjusted for maternal age (continuous), race/ethnicity (White/Black/Other regardless of Hispanic origin), BMI at initial study visit (continuous), fetal sex (male/female), health insurance provider (private/public), and parity (no previous pregnancy/one previous pregnancy/more than one previous pregnancy). Given previously published data showing the potential for fetal sex to modify the associations between maternal thyroid function and fetal growth,[14] we investigated differences in these relationships by fetal sex by including an interaction term in each cross-sectional model.

Our second analysis explored the relationships between repeated measures of thyroid hormones and fetal growth z-scores using linear mixed models (LMMs) with one growth indicator regressed on one thyroid hormone per model. Given that ultrasound measurements at visit 2 tended to be more homogeneous with regard to estimated fetal weight and to remain consistent with previous analyses of fetal growth indicators within this cohort,[30] we presented results from repeated measures models using thyroid hormone and ultrasound measurements from visits 3-4. For models with fetal weight as the outcome, we created vectors for each subject with repeated measures of estimated fetal weight at visits 3 and 4 and birth weight at delivery. Since maternal blood samples were not collected at delivery, we imputed these concentrations by using the last observation carried forward method. That is, hormone concentrations from visit 4 were imputed for missing values at delivery. Final models included a subject-specific random

intercept and slope for gestational age – chosen based on Akaike Information Criterion (AIC) – and were adjusted for the same covariates as those in our cross-sectional analysis. All LMMs were repeated with the addition of an interaction term for fetal sex to investigate potential sex differences in these relationships. We also reported results from LMMs that included visit 2 through delivery for comparison.

Since thyroid hormones directly influence fetal tissue metabolism[4] and are regulated to remain within a narrow range,[37] we hypothesized that the degree of hormonal variability within individual pregnant women may influence fetal growth. To explore this hypothesis, we calculated the ratios of intra-individual variation to inter-individual variation (= within-person variance/between-person variance) for samples collected at visits 1 through 4. We regressed natural log-transformed variability ratios for each hormone on repeated measures of fetal growth z-scores using fully adjusted LMMs with the same random effects and covariates as those used in the main repeated measures analysis. Results from models utilizing repeated measurements from visits 2 through delivery as well as visits 3 for delivery were presented. All associations were considered statistically significant at the 5% level.

RESULTS

As previously reported, our study participants were predominately white (56%), of a healthy BMI (<25 kg/m^2; 53%), held private health insurance (80%), and many were college educated (38%) (**Table V.1**). In our bivariate analyses presented in **Table V.1**, we observed significantly lower birth weight z-scores among babies born to Black women compared to White women and to women who reported smoking in pregnancy versus those who reported no smoking. Significantly greater birth weight z-scores were detected among babies born to overweight (25-30 kg/m^2) and obese (>30 kg/m^2) women compared to women with a healthy BMI, to women with at least one previous pregnancy compared to women with no previous pregnancies, and in female versus male babies. We did not find a significant difference in birth weight between women with subclinical hypothyroidism in gestation (N=10) and those without (N=426). Due to the limited number of women with subclinical hypothyroidism, we did not pursue this investigation in subsequent analyses.

We reported distributions of raw fetal growth indicators (not z-scored) as well as thyroid hormone concentrations by study visit of sample collection in **Table V.2**. The weighted median (25^{th}-75^{th} percentile) gestational age at delivery was 38.9 weeks (37.9-40.0 weeks) and for birth weight, 3345 g (2945-3660 g). Thyroid function parameters were highly detected in this study population (percent detected for total T4 and T3 = 100%, TSH = 99.5%, and FT4 = 98%). The weighted median (25^{th}-75^{th} percentile) concentrations of the four parameters in the overall study population were: TSH, 1.26 µIU/mL (0.85-1.79 µIU/mL); FT4, 1.08 ng/dL (0.85-1.32 ng/dL); T4, 10.2 µg/dL (9.1-11.5 µg/dL); and T3, 1.56 ng/mL (1.31-1.89 ng/mL). More women had ultrasound scans at visit 2 (N = 389 for HC and AC; N=390 for FL; N = 324 for EFW) compared to visit 3 (N = 201 for all growth indicators) and visit 4 (N = 221 for HC; N = 223 for AC, FL, and EFW). All women had birth weight measurements (N=439). Thyroid hormone concentrations did not significantly differ between women without ultrasound scans at visits 3 and 4 (N = 146) compared to women with scans during at least one of these visits (N = 293) (**Table V.S1**). Thus, the potential for differential bias resulting from the inclusion of ultrasound metrics obtained after visit 2 (i.e., those obtained for clinical indications and not uniformly measured for all subjects) is limited.

Table V.3 presents results from our fully adjusted cross-sectional analysis in which we explored the associations between thyroid hormone parameters sampled at each study visit and birth weight z-scores. In measurements collected at visits 1-3, we observed significant inverse associations between FT4 and birth weight, with the greatest association detected at visit 1. At this visit, a unit increase in ln-transformed FT4 was associated with almost a half a z-score decrease in birth weight (β = -0.41; 95% confidence interval [CI] = -0.64, -0.18), or approximately 175 grams (g) based on the birth weight standard deviation at 40 weeks estimated for the reference population.[32] The inverse associations for total T4 were weaker than those observed for FT4, although a significant association observed at visit 3 (β = -0.08; 95% CI = -0.13, -0.02). In contrast to the inverse associations observed for free and total T4, total T3 was generally positively associated with birth weight z-scores. We detected a significant association for T3 at visit 3, at which point a unit increase in T3 was associated with a 0.35 z-score (95% CI = 0.01, 0.69) or approximately 149-gram increase in birth weight. No significant associations

were detected for TSH at any of the four study visits of sample collection. There were no significant interactions with fetal sex (data not shown).

In **Table V.4**, we reported our results from repeated measures analysis of thyroid function parameters and z-scored fetal growth indicators (HC, AC, and EFW) from visits 3-4 and delivery (birth weight). Similar to cross-sectional associations, FT4 was inversely associated with fetal growth indicators, with significant estimates found for EFW (β = -0.14; 95% CI = -0.26, -0.02), HC (β = -0.17; 95% CI = -0.31, -0.02), and AC (β = -0.16; 95% CI = -0.29, -0.02). We also observed significant inverse associations between T4 and HC (β = -0.05; 95% CI = -0.09, 0.00) and AC (β = -0.05; 95% CI = -0.10, 0.00). While repeated measures associations were generally inverse for TSH and positive for total T3, the beta estimates for these hormones were not significant. We also found no significant associations between thyroid function parameters and FL in our study population. Finally, we did not observe any significant interactions with fetal sex (data not shown). For comparison, we presented results from our repeated measures analysis utilizing samples and ultrasound measurements from visits 2 through 4 and delivery in **Table V.S2**. The only significant association in that analysis was observed between FT4 and HC (β = -0.12; 95% CI = -0.23, -0.01).

In our variability analysis, we found higher inter-individual variation compared to intra-individual variation for all hormones, with total T4 displaying the greatest variability overall (**Table V.S3**). Upon regressing the variability ratio (an index of an individual's hormone variation) on repeated measures of fetal growth z-scores, we observed that a unit increase in the variability ratio for total T4 was significantly associated with reduced EFW (β = -0.29; 95% CI = -0.58, -0.01), HC (β = -0.41; 95% CI = -0.74, -0.08), and FL (β = -0.43; 95% CI = -0.77, -0.08) in models utilizing repeated measurements from visits 3 through delivery (**Table V.S4**). Similar results were observed for models additionally including visit 2 ultrasound measurements (**Table V.S5**). We did not detect significant associations for any of the other thyroid hormones in this secondary analysis.

DISCUSSION

In this prospective study of pregnant women without overt thyroid disease, we observed consistent inverse associations between FT4 and fetal growth indicators. Specifically, FT4 was inversely associated with birth weight in cross-sectional models stratified by study visit as well as with estimated fetal weight in repeated measures models. Not surprisingly, given that head and abdominal circumference are the major constituents of estimated fetal weight, the models also found these metrics to be similarly inversely associated. . We observed weaker inverse associations for total T4, and a positive relationship between total T3 at median 26 weeks of gestation and birth weight. No significant associations were observed for TSH. Finally, we did not detect any different effects by fetal sex, although the lack of an interaction may be due to sample size limitations.

Studies investigating the effects of subclinical thyroid dysfunction in pregnancy on fetal growth have produced variable results. Subclinical hypothyroidism (elevated TSH and euthyroid FT4) in early pregnancy (< 20 weeks of gestation) has been associated with smaller head circumference and birth length in 1,107 mother-child pairs,[8] and in the third trimester has been linked to higher rates of intrauterine growth restriction (IUGR; estimated fetal weight < 10^{th} percentile for gestational age) and low birth weight (LBW; < 2500 g) in another large study.[9] High maternal TSH, defined by study-specific cutoffs (i.e., exceeding reference ranges or 90^{th} percentile), in the first trimester has also been associated with LBW[10] and small for gestational age (SGA; birth weight < 10^{th} percentile for gestational age) infants.[11] In contrast to these findings, a recent study found that subclinical hypothyroidism in early pregnancy was associated with a greater risk for large for gestational age (LGA; birth weight > 90^{th} percentile for gestational age) in male newborns.[14]

Results have also been conflicting for maternal hypothyroxinemia (normal TSH with low FT4). One study reported a higher risk for SGA,[8] while others have found larger birth weights among these babies compared to those born to euthyroid mothers.[12,13] Despite these results, studies conducted within other large birth cohorts have reported no associations between maternal subclinical hypothyroidism or hypothyroxinemia and similar growth metrics at delivery (e.g., birth length, head circumference, LBW, or SGA).[15-18] In the current study, we did not observe an association between subclinical hypothyroidism and birth weight, which may be in part due to limitations in sample size. Additionally, we did not evaluate hypothyroxinemia in our

analysis as only two women had FT4 levels below the trimester-specific reference ranges. Moreover, while estimates were generally inverse, we did not detect any associations between TSH in pregnancy and birth weight or repeated growth measurements. These dissimilar results may be due in part to differences in study sample size, laboratory assays utilized to measure free hormones (e.g., chemiluminescence immunoassay vs. radioimmunoassay), timing of sample collection in pregnancy, regional iodine status, or varying cutoff points used to define subclinical thyroid disease (e.g., different percentile and/or concentrations thresholds).

The more consistent findings from these studies, however, have been among generally euthyroid women in whom increases in FT4 have been associated with smaller birth weight.[14,19-21] In pregnant women without overt thyroid disease, Shields et al.[21] observed that maternal FT4 at 28 weeks of gestation was inversely associated with birth weight in a dose-dependent manner. Maternal FT4 within the normal range in early pregnancy (median ~13 weeks) was also inversely associated with birth weight in the Generation R cohort[20] and in a recent study among a community-based cohort of pregnant women living in Amsterdam[14]. Additionally, Haddow et al.[19] found that babies born to euthyroid pregnant women with second-trimester FT4 concentrations in the highest quintile had the lowest birth weight. Our results from the present study support these findings. In pregnant women without overt thyroid disease, we detected inverse associations between maternal FT4 and birth weight z-scores at three time points in pregnancy (median at median 10, 18, and 26 weeks of gestation).We also found consistent inverse associations between FT4 and estimated fetal growth as well as head and abdominal circumferences in repeated measures analysis.

Maternal thyroid hormones can indirectly and/or directly influence fetal growth and development. The placenta is a thyroid hormone-responsive organ, evident by its high binding capacity for T3 and concentration of deiodinase enzymes needed to regulate the transplacental passage of maternal thyroid hormones to the developing fetus. [38,39] Prior to the formation of the fetal thyroid gland in the second trimester, the fetus relies solely on the trans-placental supply of maternal thyroid hormones,[39] which are integral in the maintenance and function of the human placenta in early pregnancy.[3] *In vitro* studies have shown that maternal T3 may play a physiological role in placentation by regulating trophoblast proliferation and presumed invasion

and decidual remodeling.[38,40] Inadequate trophoblast invasion leads to restricted perfusion which would be a risk factor for IUGR among other gestational abnormalities.[38,41]

During the second half of gestation, thyroid hormones influence intrauterine growth by stimulating fetal metabolism (i.e., consumption of oxygen and glucose), affecting fetal bioavailability of growth-related hormones (e.g., growth hormones and prostaglandins) and growth factors (e.g., insulin-like growth factors), and by indirectly regulating fetal tissue accretion and differentiation near term.[4] The direct effects of thyroid hormones on skeletal growth and tissue differentiation have been shown in animal studies in which the lack of thyroid hormones (specifically, T3) in mice resulted in skeletal abnormalities[42] and in fetal sheep, thyroidectomy showed delayed bone maturation and altered bone strength and density.[4,43] While free T3 (FT3) is the biologically active thyroid hormone,[44] plasma sample volume constraints precluded its measurement in our study population. Furthermore, FT3 is generally not routinely assessed due to its low sensitivity and specificity for diagnosing hypothyroidism.[45,46] For total T3, we observed a positive association with birth weight in samples collected at 26 weeks. Currently, we are not aware of published data on the relationship between total T3 and fetal growth. However, our findings contrast with the null associations reported between unbound T3 and the risk for LBW and SGA neonates in a population-based cohort of pregnant women.[10]

The underlying physiology behind our observed inverse associations between free and total T4 and the fetal growth indicators is unclear. In our cross-sectional analyses, we observed that associations between FT4 and birth weight z-scores were strongest in the first trimester (in terms of both magnitude and significance) – a unit increase in ln-transformed FT4 measured at median 10 weeks of gestation was associated with an approximate 175-gram decrease in birth weight. While the majority of fetal weight gain occurs after approximately 24 weeks of gestation,[32,47] our results suggest that maternal FT4 concentrations in early pregnancy may have a lasting impact on fetal growth across gestation. These effects may be explained by altered placental function, which has an indirect impact on fetal growth by modifying nutritional transport and metabolism.[48] A crude marker of placental function is placental weight, which through surface area influences the capacity of nutrient transfer to the fetus [48] and correlates closely with birth weight.[49,50] In a cohort of 321 euthyroid pregnant women, Bassols et al. observed an inverse association between maternal FT4 and placental weight, suggesting a

potential role of maternal thyroid hormones in regulating placental growth and more indirectly, fetal growth.[51] Higher maternal FT4 in early pregnancy has also been found to influence placental function later in pregnancy.[3] Specifically, among pregnant women without thyroid disease, a higher maternal FT4 at median 13 weeks of gestation was associated with increased vascular resistance in the latter half of pregnancy, and thus, may be a potential risk factor for impaired placentation and/or vascularization.[3] Maternal FT4 may also influence fetal growth via the effects of deiodinase activity in tissues, including the placenta, on maternal metabolism. The FT3 to FT4 ratio is an index of thyroid hormone metabolism and the peripheral conversion of T4 to T3 by deiodinase enzymes.[52,53] Previous studies have suggested that higher FT4 and a lower FT3 to FT4 ratio in normal pregnancy are associated with reduced maternal BMI and metabolic parameters (e.g., hemoglobin A1c and triglycerides).[51] In turn, these metabolic markers, including maternal BMI, have been found to positively influence placental weight and fetal growth.[54] Thus, it is possible that within our cohort higher FT4, coupled with potentially lower deiodinase activity, may indirectly influence fetal growth via altered maternal metabolism.[19] Our findings for FT4 may be also explained by other factors determining thyroid hormone bioavailability *in utero* (e.g., transporter or receptor proteins) or additional thyroid hormone-dependent processes involved in fetal growth that are not captured by the biochemical markers of thyroid function available in our study. Further research is required to characterize the underlying mechanisms by which FT4 may influence fetal growth in additional cohorts of generally euthyroid pregnant women.

Finally, results from our variability analysis support the findings from a previous longitudinal investigation among 132 pregnant women showing markedly higher inter-individual variation compared to corresponding intra-individual variation for all hormones assayed.[22] Similar to our study, Boas et al.[22] observed the greatest overall variability (both within- and between) for total T4 and the largest variability ratio for FT4. Our repeated measures analysis revealed that a unit increase in within-subject variance in total T4 was associated with decreased head circumference, femur length, and estimated fetal weight z-scores. These results suggest that increased variability of total T4 within the normal range may have an impact on fetal bone maturation especially since similar associations were not observed for fetal abdominal circumference, which predominately captures liver size.[55] Together, our findings from this

secondary analysis have implications for trimester-specific reference intervals. That is, current reference ranges based on population data may not be sensitive markers of thyroid dysfunction at the individual-level since they markedly exceed the ranges of intra-individual variation.[22,56] Additionally, the smaller intra-individual variations of thyroid hormones relative to inter-individual variations suggest that a woman's thyroid hormone levels remain within a narrow range in pregnancy. While additional plasma measurements per woman may reduce exposure misclassification, our variability findings suggest that these effects may be marginal and that study funds may be better utilized by recruiting more study participants rather than collecting additional measurements per subject. Additional studies are required to determine the public health and clinical impact of these findings, and to explore whether accounting for the hormonal trajectories within each woman in reference intervals (i.e., via creating individual-level reference intervals) may help mitigate the potential adverse birth outcomes associated with subclinical thyroid dysfunction.

Our study was limited by sample size for the evaluation of subclinical thyroid disease (e.g., subclinical hypothyroidism and hypothyroxinemia) as well as by the fewer ultrasound measurements available at later time points in pregnancy. As previously reported, ultrasound scans at visits 3 (median 26 weeks) and 4 (median 35 weeks) were not routine and were requested if pregnancy complications were suspected. However, receiving an ultrasound scan later in pregnancy was not associated with thyroid hormone concentrations in our study population, thereby reducing the possibility of differential bias in our results. Additionally, we did not measure maternal anti-thyroid antibodies, the presence of which may increase the risk of adverse maternal and birth outcomes independent of thyroid function.[34] Strengths of our study include the availability of up to four repeated measures of thyroid hormone concentrations, which allowed us to investigate potential windows of susceptibility in pregnancy to thyroid hormone disruption. Repeated ultrasound measurements, standardized to those from a large population of pregnant women in Boston, allowed us to explore the influence of subclinical changes in thyroid function in gestation on intrauterine growth. Finally, our assay method of measuring FT4 using direct equilibrium dialysis followed by radioimmunoassay is preferred over traditional immunoassays as it is not influenced by serum binding proteins, which increase dramatically in pregnancy.[57,58]

CONCLUSIONS

Among pregnant women without clinical thyroid disease, we observed consistent inverse associations between FT4 and fetal growth indicators, including birth weight. Future animal and human health studies are needed to elucidate the biological mechanisms underlying the relationship between FT4 and fetal growth in generally euthyroid pregnancies.

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TABLES AND FIGURES

Table V.1. Associations between population characteristics (N=439 pregnant women) and birth weight z-scores

Population Characteristics		N (%)[b]	Association with birth weight z-score	
			β (95% CI)	p-value[c]
Age	18-24 years old	54 (13)	reference	
	25-29 years old	92 (21)	0.26 (-0.07, 0.60)	0.12
	30-34 years old	176 (40)	0.32 (0.02, 0.62)	0.04
	35+ years old	117 (27)	0.30 (-0.03, 0.62)	0.07
Race/Ethnicity	White	247 (56)	reference	
	Black	75 (17)	-0.38 (-0.64, -0.12)	<0.01
	Other	117 (27)	-0.05 (-0.27, 0.17)	0.67
Education Level[a]	High School	67 (15)	reference	
	Technical School	76 (17)	-0.13 (-0.47, 0.21)	0.45
	Junior College or Some College	127 (30)	0.21 (-0.09, 0.51)	0.18
	College Graduate	159 (38)	0.21 (-0.08, 0.50)	0.16
Health Insurance Provider[a]	Private	344 (80)	reference	
	Public	83 (20)	-0.24 (-0.48, 0.00)	0.05
BMI at Initial Visit[a]	<25 kg/m^2	223 (53)	reference	
	25-30 kg/m^2	113 (26)	0.31 (0.08, 0.54)	<0.01
	>30 kg/m^2	99 (21)	0.34 (0.10, 0.59)	<0.01
IVF	No	414 (95)	reference	
	Yes	25 (6)	-0.01 (-0.43, 0.41)	0.97
Fetal Sex	Male	198 (46)	reference	
	Female	241 (54)	0.27 (0.08, 0.46)	<0.01
Parity	No previous pregnancies	197 (45)	reference	
	One previous pregnancy	144 (34)	0.37 (0.16, 0.58)	<0.001
	More than one previous pregnancy	98 (21)	0.41 (0.16, 0.66)	<0.01
Tobacco Use[a]	No smoking in pregnancy	402 (93)	reference	
	Smoked in pregnancy	31 (7)	-0.42 (-0.8, -0.03)	0.03
Alcohol Use[a]	No alcohol use in pregnancy	412 (95)	reference	
	Alcohol use in pregnancy	18 (5)	0.13 (-0.32, 0.57)	0.58
Subclinical Hypothyroidism[a,d]	No	426 (98)	reference	
	Yes	10 (2)	0.01 (-0.67, 0.69)	0.98

Abbreviations: BMI, Body Mass Index

[a]Missing observations: N=10 for education level, N=12 for insurance provider, N=4 for BMI, N=9 for alcohol use, N= 6 for tobacco use, and N=3 for subclinical hypothyroidism.

[b]Proportions weighted by preterm birth case-control sampling probabilities to represent the general sampling population.

[c]p-value for the difference in mean birth weight z-score in each category compared to the reference using linear regression models adjusted for gestational age at delivery.

[d]Defined as TSH levels > 4 μIU/mL with FT4 within the normal trimester-specific reference range.

Table V.2. Weighted distributions of thyroid hormone and ultrasound measurements by study visit of sample collection.

	N	Selected Percentiles					
		25th	50th	75th	90th	95th	Max
Visit 2 (median 18 weeks)							
Ultrasound Measurements							
Head Circumference (mm)	389	141	148	159	166	173	202
Abdominal Circumference (mm)	389	119	127	137	148	154	177
Femur Length (mm)	390	25	27	29	31	32	40
Estimated Fetal Weight (g)	324	221	253	290	334	361	528
Thyroid Hormones[a]							
TSH (µIU/mL)	317	0.98	1.36	1.91	2.52	3.15	6.08
FT4 (ng/dL)	368	0.92	1.13	1.30	1.54	1.77	8.96
T4 (µg/dL)	357	9.50	10.6	11.8	12.7	13.6	16.4
T3 (ng/mL)	301	1.36	1.60	1.90	2.16	2.28	2.86
Visit 3 (median 26 weeks)							
Ultrasound Measurements							
Head Circumference (mm)	201	231	248	263	280	288	307
Abdominal Circumference (mm)	201	207	221	239	255	263	280
Femur Length (mm)	201	46	50	54	57	60	68
Estimated Fetal Weight (g)	201	810	978	1200	1442	1619	2032
Thyroid Hormones[a]							
TSH (µIU/mL)	306	0.90	1.29	1.70	2.31	2.86	8.73
FT4 (ng/dL)	349	0.81	1.00	1.18	1.44	1.97	12.5
T4 (µg/dL)	333	9.20	10.3	11.4	12.7	13.5	21.7
T3 (ng/mL)	286	1.36	1.62	1.90	2.15	2.37	2.79
Visit 4 (median 35 weeks)							
Ultrasound Measurements							
Head Circumference (mm)	221	308	317	326	333	340	366
Abdominal Circumference (mm)	223	302	317	333	349	361	393
Femur Length (mm)	223	65	68	71	74	75	77
Estimated Fetal Weight (g)	223	2323	2671	3034	3478	3643	4384
Thyroid Hormones[a]							
TSH (µIU/mL)	270	0.97	1.35	1.92	2.44	2.71	6.22
FT4 (ng/dL)	344	0.77	0.96	1.17	1.43	1.58	6.19
T4 (µg/dL)	337	8.90	10.0	11.4	12.7	13.6	20.2
T3 (ng/mL)	239	1.40	1.66	2.00	2.21	2.4	3.22
Birth (median 38 weeks)							
Birth Weight (g)	439	2946	3345	3660	3909	4139	4720

Analyses weighted by preterm birth case-control sampling probabilities.

[a]Number of samples per hormone varied due to limitations in sample volume.

Table V.3. Weighted multivariate cross-sectional associations between thyroid function parameters and birth weight z-scores by study visit of sample collection (N=439 pregnant women)

Hormones	Visit 1 (median 10 weeks)			Visit 2 (median 18 weeks)			Visit 3 (median 26 weeks)			Visit 4 (median 35 weeks)		
	N	**β (95% CI)**	**p-value**	**N**	**β (95% CI)**	**p-value**	**N**	**B (95% CI)**	**p-value**	**N**	**β (95% CI)**	**p-value**
TSH[a]	303	-0.02 (-0.13, 0.10)	0.79	308	0.02 (-0.17, 0.20)	0.86	297	-0.14 (-0.36, 0.09)	0.23	259	-0.16 (-0.38, 0.05)	0.13
FT4[a]	359	**-0.41 (-0.64, -0.18)**	**<0.001**	357	**-0.26 (-0.46, -0.05)**	**0.01**	339	**-0.24 (-0.44, -0.05)**	**0.02**	330	-0.10 (-0.27, 0.07)	0.25
T4	349	-0.01 (-0.06, 0.05)	0.79	346	-0.06 (-0.12, 0.00)	0.06	324	**-0.08 (-0.13, -0.02)**	**0.01**	323	-0.05 (-0.11, 0.00)	0.06
T3	290	0.25 (-0.09, 0.59)	0.15	292	0.25 (-0.09, 0.59)	0.16	277	**0.35 (0.01, 0.69)**	**0.04**	228	0.18 (-0.13, 0.50)	0.26

All models adjusted weighted by preterm birth case-control sampling probabilities and adjusted for gestational age at time of sample collection, maternal age, race/ethnicity, BMI at initial study visit, insurance provider, parity, and fetal sex.
[a]ln-transformed prior to analysis.

Table V.4. Weighted multivariate repeated measures associations between thyroid function parameters and fetal growth z-scores (N=439 pregnant women; measurements from visits 3 through delivery)

Hormones	Estimated Fetal Weight (V3-delivery)			Head Circumference (V3-V4)			Abdominal Circumference (V3-V4)			Femur Length (V3-V4)		
	N	β (95% CI)	p-value	N	β (95% CI)	p-value		β (95% CI)	p-value	N	β (95% CI)	p-value
TSH[a]	555	-0.12 (-0.30, 0.05)	0.17	294	-0.06 (-0.26, 0.14)	0.55	296	-0.17 (-0.38, 0.05)	0.13	296	-0.06 (-0.29, 0.17)	0.61
FT4[a]	693	**-0.14 (-0.26, -0.02)**	**0.02**	**361**	**-0.17 (-0.31, -0.02)**	**0.02**	**363**	**-0.16 (-0.29, -0.02)**	**0.03**	363	-0.10 (-0.26, 0.05)	0.18
T4	671	-0.04 (-0.08, 0.01)	0.09	**346**	**-0.05 (-0.09, 0.00)**	**0.048**	**348**	**-0.05 (-0.10, 0.00)**	**0.04**	348	-0.03 (-0.08, 0.02)	0.23
T3	493	0.21 (-0.05, 0.48)	0.11	263	0.15 (-0.13, 0.42)	0.29	265	0.16 (-0.15, 0.47)	0.31	265	0.06 (-0.27, 0.39)	0.71

All analyses weighted by preterm birth sampling probabilities. Linear mixed models include random subject specific random intercept and slope, and were adjusted for gestational age at time of sample collection/delivery, maternal age, race/ethnicity, body mass index (BMI) at initial study visit, insurance provider, parity, and fetal sex.

[a]ln-transformed prior to analysis.

SUPPLEMENTAL MATERIAL

Table V.S1. Differences in thyroid hormone distributions by availability of ultrasound scans at visit 3 and/or visit 4

Thyroid Hormone	Median (IQR)	
	No ultrasound scans at V3 and V4 (N = 146 women)	**Ultrasound scans at V3 and/or V4 (N = 293 women)**
TSH	1.25 (0.83, 1.84)	1.27 (0.87, 1.73)
FT4	1.09 (0.85, 1.32)	1.08 (0.85, 1.32)
T4	10.3 (9.30, 11.3)	10.1 (8.90, 11.6)
T3	1.55 (1.30, 1.88)	1.57 (1.31, 1.89)

* $p<0.05$ for a significant difference in thyroid hormone concentrations between the two groups based on linear mixed models with subject-specific random intercepts.

Table V.S2. Weighted multivariate repeated measures associations between thyroid function parameters and fetal growth z-scores (measurements from visits 2 through delivery)

Hormones	Estimated Fetal Weight (V2-delivery)			Head Circumference (V2-V4)			Abdominal Circumference (V2-V4)			Femur Length V2-V4		
	N	β (95% CI)	p-value	N	β (95% CI)	p-value	N	β (95% CI)	p-value	N	β (95% CI)	p-value
TSH[a]	834	0.01 (-0.12, 0.13)	0.89	573	0.00 (-0.13, 0.13)	0.95	575	0.01 (-0.13, 0.15)	0.92	576	0.03 (-0.10, 0.17)	0.63
FT4[a]	1017	-0.07 (-0.17, 0.02)	0.14	685	**-0.12 (-0.23, -0.01)**	**0.03**	687	-0.07 (-0.19, 0.05)	0.25	688	-0.05 (-0.16, 0.07)	0.41
T4	985	-0.02 (-0.05, 0.02)	0.35	660	-0.02 (-0.06, 0.02)	0.32	662	-0.01 (-0.06, 0.03)	0.47	663	-0.02 (-0.06, 0.02)	0.41
T3	757	0.11 (-0.09, 0.32)	0.28	527	0.15 (-0.06, 0.37)	0.16	529	0.21 (-0.02, 0.45)	0.08	530	-0.13 (-0.37, 0.10)	0.26

All analyses weighted by preterm birth sampling probabilities. Linear mixed models include random subject specific random intercept and slope, and were adjusted for gestational age at time of sample collection, maternal age, race/ethnicity, body mass index (BMI) at initial study visit, insurance provider, parity, and fetal sex.

[a]ln-transformed prior to analysis.

Table V.S3. Median values of indices of hormonal variability

Thyroid Hormone	Within-subject variance	Between-subject variance	Variability Ratio[a]
TSH	0.11	0.65	0.17
FT4	0.07	0.22	0.31
T4	0.69	3.03	0.23
T3	0.03	0.14	0.24

[a]Variability ratio = within-subject variance/between-subject variance

Table V.S4. Weighted multivariate repeated measures associations between variability ratio of thyroid function parameters and fetal growth z-scores (measurements from visits 3 through delivery)

Variability Ratio[c]	Estimated Fetal Weight (V3-delivery)			Head Circumference (V3-V4)			Abdominal Circumference (V3-V4)			Femur Length (V3-V4)		
	N[d]	**β (95% CI)**	**p-value**	**N[d]**	**β (95% CI)**	**p-value**	**N[d]**	**β (95% CI)**	**p-value**	**N[d]**	**β (95% CI)**	**p-value**
TSH[a]	717	0.03 (-0.31, 0.36)	0.88	348	-0.12 (-0.54, 0.31)	0.59	350	-0.06 (-0.54, 0.41)	0.80	350	0.21 (-0.26, 0.69)	0.38
FT4[b]	817	-0.01 (-0.06, 0.04)	0.78	403	-0.01 (-0.07, 0.05)	0.85	405	0.01 (-0.05, 0.08)	0.66	405	-0.02 (-0.08, 0.05)	0.60
T4[a]	795	**-0.29 (-0.58, -0.01)**	**0.04**	391	**-0.41 (-0.74, -0.08)**	**0.02**	393	-0.09 (-0.43, 0.26)	0.62	393	**-0.43 (-0.77, -0.08)**	**0.02**
T3[a]	672	0.31 (-0.02, 0.63)	0.07	323	0.25 (-0.12, 0.62)	0.19	325	0.22 (-0.2, 0.63)	0.30	325	0.41 (0.00, 0.82)	0.05

Linear mixed models include random subject specific random intercept and slope, and were adjusted for gestational age at time of sample collection/delivery, maternal age, race/ethnicity, body mass index (BMI) at initial study visit, insurance provider, parity, and fetal sex.

[a]ln(ratio + 1)

[b]ln(ratio)

[c]Variability ratio = within-woman variance/between-women variance

[d]N = total number of samples/measurements.

Table V.S5. Weighted multivariate repeated measures associations between variability ratio of thyroid function parameters and fetal growth z-scores (measurements from visits 2 through delivery)

Variability Ratio[c]	Estimated Fetal Weight (V2-delivery)			Head Circumference (V2-V4)			Abdominal Circumference (V2-V4)			Femur Length (V2-V4)		
	N[d]	β (95% CI)	p-value	N[d]	β (95% CI)	p-value	N[d]	β (95% CI)	p-value	N[d]	β (95% CI)	p-value
TSH[a]	1021	-0.05 (-0.34, 0.24)	0.74	652	-0.20 (-0.49, 0.10)	0.19	654	-0.21 (-0.55, 0.13)	0.22	655	0.09 (-0.24, 0.42)	0.58
FT4[b]	1160	-0.02 (-0.06, 0.03)	0.41	746	-0.03 (-0.08, 0.02)	0.22	748	0.00 (-0.06, 0.05)	0.88	749	-0.04 (-0.09, 0.01)	0.12
T4[a]	1129	**-0.25 (-0.50, 0.00)**	**0.05**	725	-0.23 (-0.49, 0.03)	0.08	727	-0.08 (-0.35, 0.20)	0.57	728	**-0.36 (-0.63, -0.09)**	**0.01**
T3[a]	958	0.15 (-0.13, 0.44)	0.3	609	0.00 (-0.28, 0.29)	0.99	611	0.09 (-0.24, 0.42)	0.61	612	-0.12 (-0.45, 0.20)	0.45

Linear mixed models include random subject specific random intercept and slope, and were adjusted for gestational age at time of sample collection/delivery, maternal age, race/ethnicity, body mass index (BMI) at initial study visit, insurance provider, parity, and fetal sex.

[a]ln(ratio + 1)

[b]ln(ratio)

[c]Variability ratio = within-woman variance/between-women variance

[d]N = total number of samples/measurements.

CHAPTER VI. Conclusions

SUMMARY OF FINDINGS

This dissertation presents findings from four studies aimed at investigating the relationships between environmental phthalate exposure, maternal thyroid function parameters, and adverse birth outcomes. Coupled with previous research on this topic, these results provide valuable insight into the possible role of subclinical thyroid dysfunction in mediating the relationships between environmental phthalate exposure and preterm birth and fetal growth. While additional epidemiological investigations are required, the present dissertation work has potential implications for medical and public health policies aimed at reducing the social and economic burdens of endocrine-related adverse birth outcomes.

Environmental Phthalate Exposure and Maternal Thyroid Hormones. The preliminary investigation of Aim 1 explored the relationships between urinary phthalate metabolites and maternal thyroid hormone concentrations in 106 pregnant women from Northern Puerto Rico. In the repeated measures analysis that included biomarker measurements collected at up to two time points in pregnancy, we observed that MCPP, a nonspecific metabolite of several long-chain phthalates, was inversely associated with FT3 (the principal bioactive thyroid hormone). We also found that study visit of sample collection significantly modified the relationships between certain urinary phthalate metabolites and maternal thyroid hormone concentrations. Specifically, we detected stronger inverse associations for both FT3 and FT4 at median 26 weeks of gestation compared to those observed at median 18 weeks, with significant negative estimates observed between FT3 and MCPP and MCOP as well as between FT4 and metabolites of DEHP. No associations were reported for TSH in any of our analyses.

Results from the follow-up study conducted among the larger, dissertation cohort of 439 pregnant women in Boston, MA support the previous investigation showing the potential for environmental phthalate exposure to disturb circulating levels of thyroid hormones in pregnant women. However, compared to the inverse associations observed in the Puerto Rico cohort, phthalate metabolites were largely positively associated with free and total thyroid T4 in the subsequent repeated analysis using biomarker measurements collected at up to 4 time points in pregnancy. While no relationships were observed for TSH in the earlier study, we found inverse associations between this hormone and DEHP metabolites as well as MiBP and MCPP. Due to sample volume constraints, we did not measure FT3 concentration in the Boston cohort. The discrepant results observed between these two analyses of Aim 1 may be due to differences in: population size, number of serial biological samples available as well as the timing of sample collection in pregnancy, phthalate exposure levels, laboratory methods used to measure free hormones (chemiluminescence immunoassay vs. radioimmunoassay), and/or population demographic characteristics.

While these repeated measures analyses conducted among two disparate cohorts of pregnant women provide suggestive evidence of the possible thyroid-altering effects of phthalates in gestation and represented some of largest studies on this topic to date, our analyses have potential limitations. For example, we did not assess the iodine status of our participants; notably, this trace element is essential for normal thyroid function.[1] However, we have no reason to believe that iodine status would influence phthalate exposure (and vice versa) and thus, iodine concentrations would not be a confounder in the phthalate-thyroid hormone relationship. Notably, in a study conducted among a representative sample of U.S. adult men and women, iodine excretion had a negligible impact on the significant relationships observed between phthalate metabolites and thyroid hormone levels.[2] Additionally, as previously mentioned in Chapter III, the evaluation of circulating thyroid hormones as a sole indicator of thyroid toxicity may have also posed an additional limitation to our analyses.[3] Measurements of peripheral thyroid hormones may not fully capture the phthalate-induced effects on thyroid homeostasis since blood levels of thyroid hormones do not always correspond to actions at the receptor, such as regulation of gene expression and the developmental processes in which they are involved.[3-5] However, given the limited amount of data on this subject and the infeasibility of collecting more

specific markers during pregnancy, levels of circulating thyroid hormone measurements may serve as the most appropriate biomarker of thyroid disruption in large-scale, epidemiological investigations among pregnant women. Finally, these two studies were conducted among pregnant women in Northern Puerto Rico as well as predominately white women in Boston, MA, which may have implications for the generalizability of results.

Overall, the findings for Aim 1 call for additional human health and animal studies to resolve the direction of the specific relationships between urinary phthalate metabolites and maternal thyroid hormone concentrations, to further elucidate periods of vulnerability in pregnancy to phthalate exposure, and to reveal the specific biological pathways of phthalate action on thyroid function in pregnant women. Furthermore, the implications of these findings on maternal and fetal health need to be determined.

Maternal Thyroid Hormones and Preterm Birth. The analysis of Aim 2 assessed the effects of subclinical alterations in thyroid function parameters on the risk of preterm birth in 439 pregnant women without clinical thyroid disease participating in a nested case-control study of preterm birth in Boston, MA. In our characterization of the hormonal patterns across gestation, we observed temporal differences in the trends of TSH and FT4 in early pregnancy between women who delivered preterm (cases) vs. at term (controls). Specifically, at approximately median 10 weeks of gestation we found higher concentrations of TSH and lower concentrations of FT4 in cases compared to controls. In particular, the hormonal trajectories in women delivering at term were consistent with what has been reported in the medical literature. As mentioned previously in Chapter IV, the observed variations in the hormonal patterns between the two groups may indicate a lack of thyroidal response to human chorionic gonadotropin (hCG; the pregnancy hormone) in women delivering preterm.[6] However, additional studies are required to confirm these findings and to identify the mechanisms involved.

Our stratified analysis by study visit of sample collection revealed potential windows of susceptibility for preterm birth, especially in early pregnancy. In samples collected at median 10 weeks of gestation, we observed that a unit decrease in FT4 was associated with a twofold increase in the odds of overall preterm birth. Additionally, we observed positive associations between T3 and the odds of overall preterm birth at median 10 and 26 weeks of gestation. We

did not detect any significant associations for TSH. One of the strengths of this study was our investigation of these relationships by subtype of preterm birth (spontaneous preterm birth vs. preterm birth due to aberrant placentation ["placental" preterm birth]). Here, we observed odds ratios that were greater in magnitude and more precise in models of spontaneous preterm alone, specifically for FT4 and total T3. While these findings may be due to differences in sample size between the two stratified analyses (N=49 for spontaneous vs. N=33 for placental preterm birth), our results for spontaneous preterm birth suggest that changes in these hormone concentrations during gestation may have particular consequences for spontaneous preterm labor and/or preterm premature rupture of the membranes (PPROM).

Results from our nested case-control study add to the body of literature, which to date lacks data on the effects of trimester-specific subclinical alterations in individual parameters of thyroid function, especially in late gestation, on the risk of preterm birth. However, potential limitations of this investigation should be noted. Due to volume constraints of the biological samples, we did not assess the thyroid autoimmunity of our study participants. The presence of anti-thyroid autoantibodies have been found to modify the relationships between circulating thyroid hormone concentrations and adverse birth outcomes.[7,8] Furthermore, our analysis of associations by subtype of preterm birth is limited by sample size. Additional studies in larger, prospective birth cohorts are required to more confidently determine the extent to which these associations differed by obstetric presentation of preterm birth, which would provide insight into the potential biological pathways involved.

While we observed consistently elevated odds of preterm birth for total T3 in our analyses, it is possible that these results may be due to unmeasured or residual confounding. That is, conceivably, these observed relationships may be due to the effects of other biological factors (e.g., deiodinase enzyme activity or thyroid transport proteins) that are physiologically relevant to both preterm birth as well as circulating levels of maternal T3. Published studies on this topic to date have predominately assessed TSH and free hormones (FT4 and FT3) as these are sensitive markers of thyroid dysfunction.[9] We are aware of no studies that have previously explored the effects of total hormones on the risk of preterm birth (or any adverse birth outcomes). While both unbound T4 and T3 initiate biological responses, FT3 is considered the main bioactive thyroid hormone as it binds to thyroid hormone receptors in responsive tissues.[10]

Given our findings from this study, further research in qualitatively disparate populations of pregnant women is necessary to tease out the relationships between total hormones, in particular T3, and preterm birth.

In conclusion, our results for Aim 2 support previous studies showing the potential for subclinical changes in thyroid hormone concentrations in pregnancy to influence the risk of preterm birth. Our stratified analyses suggest that these effects may vary by gestational age and clinical presentation of preterm birth. Additional human health and animal studies may consider taking these findings into account in their investigations of the mechanism(s) by which subclinical thyroid dysfunction influences the pathogenesis of preterm birth.

Maternal Thyroid Hormones and Fetal Growth. Aim 3 examined the extent to which thyroid function parameters, collected at up to four times points in pregnancy, were associated with birth weight and repeated measurements of fetal growth in 439 pregnant women without clinical thyroid disease. In this prospective study, we observed consistent inverse associations between FT4 and fetal growth indicators. Specifically, FT4 was inversely associated with birth weight in stratified analysis by study visit as well as with estimated fetal weight and head and abdominal circumferences in repeated measures models. We observed weaker inverse associations for total T4, and a positive relationship between total T3 at median 26 weeks of gestation and birth weight. No significant associations were observed for TSH. In contrast to what has been reported previously,[11] we did not detect any different effects by fetal sex, although the lack of an interaction may be due to sample size limitations. Finally, we did not find a significant difference in birth weight between women with subclinical hypothyroidism (TSH > 4 μIU/mL with normal FT4; N=10) vs. those without (N=426). Due to the small sample size, we did not pursue this investigation in additional analyses.

While the data on the influences of subclinical thyroid dysfunction on intrauterine and delivery indices of fetal growth are conflicting, the inverse associations we observed for FT4 are consistent with those previously reported in generally euthyroid women.[11-14] A novel finding from our analysis was the effects of FT4 in early pregnancy on fetal growth across gestation. As mentioned in Chapter V, the underlying physiology behind our observed inverse associations between free and total T4 and the fetal growth indicators is unclear and may involve altered

placental function, deiodinase activity, and/or maternal metabolism. However, it is also possible that these findings can be explained by other factors determining thyroid hormone bioavailability *in utero* (e.g., transporters or receptors), lack of thyroidal response to other pregnancy hormones such as hCG, and/or additional thyroid hormone-dependent processes involved in fetal growth. Undoubtedly, future research is required to identify the underlying mechanisms that drive the inverse relationships observed between FT4 and fetal growth in euthyroid pregnant women.

The analyses of Aim 3 included the largest number of repeated thyroid hormone and ultrasound measurements on this topic to date, and the standardization of fetal growth indices to a larger population of pregnant women in Boston, MA[15] allowed us to combine measurements to analyze these associations longitudinally. Despite the novelty of our research, this investigation was not without limitations. As previously noted for analyses of Aim 2, we did not assess thyroid autoimmunity in our study population. Notably, anti-thyroid autoantibodies may increase the risk of adverse maternal and birth outcomes independent of thyroid function.[16] Additionally, the inclusion of visits 3 (median 26 weeks) and 4 (median 35 weeks) ultrasound measurements, which were not routine and requested if gestational abnormalities were suspected, may impact the generalizability of these results. However, we did not observe differences in the mean concentrations of the four thyroid function parameters between women with scans at these visits versus those without, thereby limiting the potential for differential bias in our results.

Overall, this Aim 3 investigation was consistent with prior studies showing the potential for subclinical alterations in maternal thyroid function parameters to influence fetal growth. Furthermore, as noted in Chapter V, our findings showing smaller intra-individual variability of thyroid hormones compared to inter-individual variability across pregnancy have implications for study design (i.e., recruiting additional subjects vs. collecting more repeated measurements) as well as for trimester-specific reference intervals (i.e., population-level reference intervals may not accurately capture thyroid dysfunction at the individual-level). Since our results may be more generalizable to pregnant women who have medical indications that require ultrasounds later in pregnancy, additional investigations are required to confirm our results in larger, prospective birth cohorts.

Integration of dissertation findings. Together, the results from the three aims of this dissertation research suggest that subclinical changes in maternal thyroid function attributable environmental exposure to phthalates influence the risk of preterm birth and impact ultrasound and delivery indices of fetal growth. Specifically, we found that certain phthalate metabolites, including DEHP metabolites, were associated with decreased levels of FT4. In turn, we found that a unit decrease in FT4 was associated with nearly two times the odds of preterm birth in women without diagnosed thyroid disease. Thus, even slight changes in unbound T4 may have deleterious effects on birth outcomes. While additional studies are required to confirm these findings in qualitatively disparate populations of pregnant women, placental metabolism of maternal thyroid hormones may be underlying these observed relationships. The placenta regulates both the quantity and composition of thyroid hormones transported to the fetus via activating and deactivating deiodinase enzyme activity.[17] In thyroid deficiencies, however, the placenta lacks complete compensatory mechanisms necessary to optimize placental transfer of maternal thyroid hormones to the growing fetus.[17] It is also possible that these findings from Aims 1 and 2 may be due to other biological mechanisms involved in both the pathology of preterm birth as well as thyroid dysfunction, such as inflammation. Notably, in a separate analysis within this dissertation cohort of pregnant women, phthalate exposure was associated with increased cytokines, including IL-6, a marker of peripheral inflammation.[18] Additional research is necessary to elucidate the biological processes involved in relationships between phthalate-induced thyroid hormone disruption and preterm birth.

Interestingly, in both Aims 2 and 3 we observed consistent significant associations between total T3 (free plus unbound) and preterm birth as well as fetal growth indices. In Aim 2, total T3 was associated with more than three times the odds of preterm birth, and results from Aim 3 showed that total T3 measured at median 26 weeks of gestation was associated with an approximate 149-gram increase in birth weight. Since TSH and FT4 are the predominant markers of thyroid function measured in clinical settings, studies investigating the effects of total T3 on birth outcomes are lacking. However, it is possible that dysfunctional placental metabolism or issues in the upstream conversion of T4 to T3 by deiodinase enzymes may be contributing to the strong associations observed for total T3 in these studies.

Finally, in this research we performed a number of statistical comparisons, and there is the potential that some of the observed associations may be due to chance. We did not adjust for multiple comparisons (i.e., Type I error) using methods such as Bonferroni correction because our outcomes and exposures were correlated and these methods would have been too conservative. Furthermore, these studies were conducted to explore novel hypotheses utilizing repeated measures designs – representing some of the first of such studies in the field – and were conducted for discovery purposes. Correcting for multiple comparisons would have masked subtle associations and thus, would have precluded the exploration of these relationships in studies aimed to confirm and/or replicate our findings.

PUBLIC HEALTH IMPACT

Endocrine-disrupting chemicals contribute substantially to disease and dysfunction, and can have transgenerational effects.[17-19] In the United States, the annual economic burden attributable to diseases associated with endocrine-disrupting chemical exposure was estimated at over 2% of the gross domestic product (GDP) or $340 billion.[17] The cost associated with phthalate exposure was approximately $56 billion – second behind the $240 billion contributed by polybrominated diphenyl ether (PBDE) exposure.[17] This is likely a conservative estimate as the analysis for phthalates did not include the cost of hormone-related adverse pregnancy outcomes such as preterm birth and impaired fetal growth. In response to increasing scientific research and subsequent public concern about the toxicity of phthalates, various regulatory actions directed at restricting the use of certain phthalates in consumer products, particularly those concerning infants and children, have been recommended and/or enacted in the United States and abroad.[20,21] Despite the reformulation of products within the last decade, there are still significant gaps in the data regarding the human toxicity of phthalate alternatives.[22] Nonetheless, reduction of exposure to phthalates and increased production of alternative chemicals can decrease the staggering healthcare costs as well as alleviate the lifelong consequences of disease and dysfunction associated with these low-dose, ubiquitous exposures.

In 2012, the United Nations Environment Programme (UNEP) in conjunction with the World Health Organization (WHO) published a report on the state of the science of endocrine disrupting chemicals.[23] Partly in response to the documented increase in the worldwide incidence

of endocrine-related diseases and disorders, including adverse birth outcome such as preterm birth and low birth weight, the document highlighted the significant gaps in research necessary to identify possible environmental causes of disease.[23] Specifically, it was noted that at the time,"[t]here [wa]s very little epidemiological evidence to link [endocrine-disrupting chemical] exposure with adverse pregnancy outcomes…"(p. ix).[23] While additional epidemiological investigations on this topic have been published since the release of this report,[18] including two investigations conducted among the main dissertation cohort,[24,25] few human health studies have explored the potential mechanisms through which endocrine disruptors may influence pregnancy outcomes. Characterizing biological pathways of chemical action will aid the scientific and medical communities in identifying and developing interventions necessary to reduce the burden of adverse maternal and neonatal outcomes caused by ubiquitous environmental chemical exposure.

The current dissertation fills a crucial gap in the literature regarding the potential mechanisms by which phthalates may affect birth outcomes. Our results in two disparate cohorts of pregnant women showed that phthalates have subtle effects on the biochemical markers of thyroid function in gestation. Our follow-up studies in the main dissertation cohort revealed that even slight changes in these thyroid function parameters – akin to those attributable to phthalate exposure – may have deleterious effects on birth outcomes among generally euthyroid pregnant women.

Thyroid dysfunction is common in women of reproductive age, especially pregnant women.[9] Despite the potential adverse effects of overt and subclinical disease in pregnancy and studies showing the cost-effectiveness of universal screening,[9,26] testing all women for thyroid disease remains a controversial topic.[27] Our results from Aim 3 showing greater inter-individual variability than intra-individual variability in thyroid hormone concentrations across pregnancy coupled with our findings of positive associations between within-woman variability in total T4 and fetal growth, may have implications for the creation of trimester-specific reference intervals. That is, current reference intervals based on population data may not be sensitive markers of thyroid dysfunction at the individual-level.[28] Currently, the American Thyroid Association (ATA) recommends screening only for high-risk pregnant women with certain risk factors (e.g., a history of thyroid disease dysfunction, known thyroid antibody positivity, obesity, etc.).[16]

Opposing views of screening stem from uncertainties in the effectiveness of thyroxine treatment in pregnancy as well as the heterogeneous results from studies investigating the maternal and neonatal health effects of subclinical thyroid disease.[29] Potentially complicating these medical decisions based on the synthesis of epidemiological literature are the varied cutoff points (i.e., concentration vs. percentile) used to define subclinical disease, differences in the timing of sample collection, dissimilarities in the iodine status of study populations, as well as discrepancies in the laboratory assay methods. Utilizing the largest number of repeated hormone and ultrasound measurements to date and implementing analytically accurate free hormone assays, the results from this dissertation research necessitate future studies aimed at investigating the health and economic benefits of universal screening as well as subsequent treatment of subclinical thyroid disease in pregnant women.

OVERALL CONCLUSIONS

The increasing incidence in endocrine-related disorders coupled with the exponential rise in the global development and use of high production volume chemicals, many of which have known endocrine disrupting properties,[30] call for policies aimed at reducing exposure to these chemicals among susceptible populations such as pregnant women. The present dissertation provides additional data on the possible pathways through which one class of chemicals, in particular phthalates, may act to influence downstream birth outcomes. More specifically, these results suggest that environmental phthalate exposure in pregnancy may influence neonatal outcomes via thyroid hormone disruption. Additional longitudinal and multigenerational studies are required to identify the particular biological mechanisms involved in phthalate-induced thyroid hormone disruption (i.e., actions at the receptor or metabolic effects); quantify the extent to which thyroid disruption, in concert with other biological mechanisms such as inflammation, mediates these relationships; and to identify the appropriate screening and medical interventions necessary to reduce the burden of thyroid-related adverse pregnancy outcomes.

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