Bisphenol A Exposure, Adipogenic Mechanism and Effect on Childhood Adiposity

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Abstract

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Bisphenol A (BPA) is a common component in plastic consumer products and epoxy resin linings. Initially developed in the 1930s-40s as a synthetic hormone treatment, it is now widely considered an endocrine disrupting chemical (EDC). A growing body of epidemiological literature suggests that ubiquitous exposures to BPA may be contributing to the global epidemic of obesity, with children a particularly vulnerable population. Obesity in children, defined by a body mass index (BMI) greater than or equal to the 95th percentile for age and sex, is an epidemic of great concern in the United States. As with other chemicals, the prenatal and early life period are critical windows of exposure to BPA; however, the mechanism by which BPA may influence the development of body size in children remains unclear. Experimental studies have found that BPA influences adipogenesis in both murine and adult human preadipocyte cell lines and BPA is hypothesized to play a role in enhancing adipogenic regulation by nuclear receptors such as *peroxisome proliferator-activated receptor gamma* (*PPARγ*). While the timeline of the processes involved in adipogenesis in humans is not universally agreed upon, it is accepted that *PPARγ* is highly expressed in adipose tissue and considered to be the master regulator of adipogenesis. To answer the question of both timing and developmental origin of BPA effects on adipogensis, we employed both an epidemiological approach, and experimental methodologies using primordial cell lines, mesenchymal stem cells (MSCs). Our study characterizes early life exposures to BPA, explores the adipogenic mechanism of BPA in human MSCs via cellular morphometrics and *PPARγ* gene expression, and identifies associations between early life exposure to BPA and childhood obesity and adiposity.

For our epidemiological assessments, we studied a birth cohort of African American and Dominican mother and child dyads in New York City. BPA was measured in spot urine samples collected during pregnancy and at child ages 3, 5, and 7 years, from mothers and children (n=568 dyads) in the Columbia Center for Children's Environmental Health (CCCEH). We compared BPA concentrations

across paired samples. We explored relationships between BPA and the class of phthalate chemicals, another common plasticizer.

BPA was detected in nearly all urine samples from prenatal third trimester and childhood ages 3 years, 5 years and 7 years. Prenatal urinary BPA concentrations were significantly lower than postnatal urinary BPA concentrations ($p<0.001$). BPA and phthalate metabolites were correlated prenatally and at 3, 5, and 7 years (all p-values ≤ 0.02). BPA concentrations were correlated with phthalate metabolite concentrations prenatally, and at 3, 5 and 7 years(all p-values < 0.05). Geometric means of BPA were higher among African Americans than among Dominicans in prenatal ($p<0.01$), 5 year ($p<0.001$) and 7 year ($p=0.02$) samples. Postnatal BPA concentrations were significantly higher among children with mothers who had never marrried marital status and were significantly higher in summer than in all other seasons (all p-values < 0.05). These findings reveal widespread BPA exposure in an inner-city minority population.

Our *in vitro* experiment was a feasibility study which sought to determine whether exposure to BPA by human umbilical cord mesenchymal stem cells (HUMSC) induces morphological changes and *PPARγ* gene expression during adipogenesis. An anonymous sample of n=18 umbilical cords was collected at delivery from mothers registered at New York-Presbyterian Sloane Hospital for Women and New York-Presbyterian Allen Hospital in New York City. HUMSCs were harvested from umbilical cords using an adhesion technique. HUMSCs were then induced in culture to differentiate into adipocytes using: a standard differentiation induction mix medium, a negative vehicle control medium, a positive control medium and experimental control media. Differences in cell surface area and cell count in all cultures were assessed using ImageJ software (version 1.49n, 2014). Gene expression of *PPARγ* in all cultures was evaluated by RT-PCR. Cell morphometric results were based on 11,676 cells from 3 umbilical cord samples. *PPARγ1* and *PPARγ2* gene expression was assessed during differntiation phase and early terminal phase adipogenesis (0 to 72 hours). Cell morphometrics were assessed during middle to late terminal phase adipogenesis (days 14 and 21). No differences in cell count were observed for experimental conditions compared to standard induction medium. A significant decrease in surface area was seen in cells exposed to 100 μM concentration of BPA as compared to exposure to standard induction medium at day 14 ($t=-37.02$ $p=0.001$). Differences in cell surface area were not observed at

day 21. A twofold increased expression of *PPARγ1* was observed in cells exposed to 10 μM concentration of BPA by 72 hours of adipogenic induction which was higher than the increase in expression observed for cells exposed to the positive control induction medium containing 10 μM concentration of rosiglitazone. All induction media conditions had negligible effects on *PPARγ2* expression. As BPA increases expression of *PPARγ1* in HUMSCs during the transition into the early terminal differentiation phase of adipogenesis, HUMSCs may be an approximate target tissue for evaluating BPA effects in adipogenesis.

Finally, using a longitudinal research design, we analyzed the possible effect of prenatal and postnatal BPA exposures, measured in urine, on childhood anthropometric outcome measures. Participants in the CCCEH have been followed since the third trimester of pregnancy, providing us with anthropometric data on children from birth through the age of seven years. Available anthropometric outcome measures include body mass index z-scores (BMIZ) at 5 and 7 years, as well as fat mass index (FMI), percent body fat (%BF), and waist circumference (WC) at 7 years. Prenatal urinary BPA concentrations were positively associated with child age 7 FMI (beta=0.31 kg/m^2, p-value=0.04, [95%CI 0.01, 0.60]), %BF (beta=0.79, p-value=0.04, [95%CI 0.03, 1.55]), and WC (beta=1.29 cm, p-value=0.01, [95%CI 0.29, 2.30]). Child urinary BPA concentrations were not associated with childhood BMI or other anthropometric outcomes. As the prenatal exposures were associated with childhood measures of adiposity, prenatal BPA exposure may have an effect on adiposity as children age that cannot be determined by the use of BMI alone. Our results suggest BPA may contribute to the developmental origins of obesity and adiposity.

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Dedication

for my father

Dedicated to Alexander W. Hoepner, who attended every graduation but did not live to see these important days of my life: marriage, two births and completion of the DrPH. This one's for you, Dad.

Chapter 1: Introduction

There has been a growing concern over obesogens, or chemicals in the environment suspected of having endocrine disrupting properties related to obesity, possibly by induction of adipogenesis (Grun and Blumberg 2006). Bisphenol A (BPA) is considered an endocrine disrupting chemical (EDC) due to its similarity to estrogen's molecular structure and actions via nuclear receptors, both hormone and non-hormone. Preliminary data suggest that BPA is an obesogen, acting through induction of *peroxisome proliferator-activated receptor gamma* (*PPARγ*) expression with a resultant increase in adipogenesis (Masuno et al. 2002; Phrakonkham et al. 2008; Riu et al. 2011; Sargis et al. 2010). Beginning in the 1950s, BPA was used in the manufacture of polycarbonate plastics and epoxy resins. Present day estimates place annual production of BPA at approximately eight billion pounds worldwide with release to the environment exceeding one million pounds per year (EPA 2010; Vandenburg et al. 2010). Exposure is ubiquitous in the United States (US).

There has been an unprecedented rise in the global obesity rate since the 1970s. Obesity in the United States is an epidemic. In adults, obesity is classified as body mass index (BMI: weight(kg) divided by height(m) squared) greater than or equal to 30, and in children, it is defined as BMI at or above the 95th percentile of the sex-specific Centers for Disease Control and Prevention (CDC) BMI-for-age growth charts. Obesity in the US is an epidemic in both adults and children.

Childhood obesity prevalence in the US in the first decade of the 21st century has risen to 17% (Ogden et al. 2014; Skinner and Skelton 2014). Long-term risks of childhood obesity include type 2 diabetes and reduced adult life expectancy. Obesity in children can also lead to metabolic syndrome, arthritis, cancer, and cardiovascular disease. US National Health and Nutritional Examination Survey (NHANES) data has shown that obesity rates are higher among minority and low income children than among children as a whole (Anderson and Butcher 2006). Correspondingly, according to National Longitudinal Survey of Youth data, there was a rapid increase from 1986 to 1998 in overweight (i.e. BMI between 85th and 95th percentiles), particularly among African American and Hispanic children (Strauss and Pollack 2001). From 2003 to 2007, obesity prevalence among Hispanic children increased by 24.2% (Singh et al. 2010).

Given the growing concerns over childhood obesity coupled with preliminary evidence that BPA may act as an obesogen, we conducted an epidemiological study to evaluate whether prenatal exposure to BPA was associated with body size during early childhood in a longitudinal birth cohort of African American and Dominican mothers and children (n=568). Further, to better understand potential mechanisms, we also conducted *in vitro* studies to explore the effect of BPA on adipogenesis in mesenchymal stem cells (MSCs) that were derived from human umbilical cords. We hypothesize that:

***Hypothesis 1*:**

There will be evidence of widespread exposure to BPA in both mothers and children enrolled in the Columbia Center for Children's Environmental Health longitudinal birth cohort and BPA urinary concentrations will be associated with prenatal and childhood socio-demographic and seasonal predictors.

Specific Aim 1:

Analyze associations between urinary BPA concentrations and questionnaire data collected prenatally and at childhood ages 3 years, 5 years and 7 years.

***Hypothesis 2*:**

Exposure to BPA *in vitro* will induce adipogenesis via increased expression of *peroxisome proliferator-activated receptor gamma* (*PPARγ*) in adipocytes derived from human umbilical cord mesenchymal stem cells (HUMSCs).

Specific Aim 2a:

In a feasibility study, establish that BPA quantitatively enhances HUMSC conversion to adipocytes by exposing HUMSCs to BPA.

Specific Aim 2b:

In a feasibility study, establish that *PPARγ* expression increases in BPA-exposed HUMSCs.

Hypothesis 3:

BPA concentration in maternal urine collected during pregnancy will be significantly associated with increases in BMI Z-score, fat mass, percent body fat, and waist circumference in the children enrolled in CCCEH at ages 5 and 7 years.

Specific Aim 3:

Evaluate associations between maternal prenatal BPA urinary concentrations and anthropometric outcomes in the CCCEH cohort. Anthropometric outcomes include BMI-Z scores, fat mass, body fat percentage and waist circumference.

Section 1.1: Rationale for Hypotheses

Sources of exposure to BPA

BPA ($C_{15}H_{16}O_2$), a phenol with two hydroxyl groups, was first synthesized in Russia by Aleksander Dianin in 1891 (Michalowicz 2014). During the 1930s-40s, the estrogenic activity of BPA was confirmed and studied in rats for use as a synthetic estrogenic hormonal treatment in humans (Rubin 2011). The current application of BPA as a key component in polycarbonate plastics and epoxy resins was developed by chemists working independently at Bayer in Germany and General Electric in the US (Brunelle 2005). Through the process of esterification, the BPA monomer functions as a plastic hardener in polycarbonate plastics. The polymerization of BPA into a liquid epoxy resin of bisphenol A diglycidyl ether (BADGE) allows for a pliable material that can be formed into liners and coatings. BPA is brominated to form tetrabromobisphenol A (TBBPA) for use in flame retardant materials. BPA is also added to mixtures of other plasticizers. However, the specific formulae used in plastic processing is often proprietary such that the quantity of raw BPA is not information that is usually made available to the consumer.

Ubiquitous commercial applications of BPA in everyday consumer products include, but are not limited to, reusable plastic food and beverage containers, toys, dental sealants, eyeglass lenses, plastic stretch films, consumer electronics, digital media (CDs, DVDs), automobiles, medical equipment, food and beverage can linings and glass jar tops (Vandenberg et al. 2007; Vandenberg et al. 2010). BPA has been found in fresh and pre-packaged foods, including infant formula in Europe, Asia and the US (Kuo and Ding, 2004; Makris et al. 2013; Noonan at al. 2011; Rudel et al. 2011; Schecter et al. 2010; Vandenberg 2007; Wilson et al. 2007). Industrial uses of BPA include pipe material for water and sewage, as well as a stabilizer in vinyl chloride (Michalowicz 2014). Lastly, the use of BPA as an ink developer in thermal paper products such as cash register receipts has led to persistent BPA

contamination in paper materials such as newspaper, food cartons, toilet paper, paper towels and napkins made with recycled thermal paper (Ehrlich et al. 2014; Liao et al. 2012). It is estimated that nearly 30% of thermal paper products re-enter the consumer market through the manufacture of recycled paper (Liao et al. 2012). This pervasive use has resulted in detectable concentrations of BPA in 92.6% of 2003-2004 NHANES subjects via dermal contact, inhalation, and dietary intake, with dietary exposure of primary concern (Vandenberg et al. 2010; Calafat et al. 2008; Wilson et al. 2003; Zalko et al. 2003).

Hydrolysis of the ester bonds between BPA molecules in polycarbonate plastics may occur through normal use, in addition to exposure to friction, vibration, alkaline conditions or extreme temperatures. Newly fabricated polycarbonate is likely to have microscopic fractures in the surface of the material. Molecular damage increases the chance for BPA to leach into any materials which come in contact with polycarbonate surfaces. Additionally, food items themselves may cause BPA to leach from plastic materials. Acidic conditions may cause stress on epoxy resin and polycarbonate, while high-fat foods may attract the lipophilic BPA molecule.

Exposures to BPA are widespread with high detection rates in humans. Based on 2003-2004 NHANES results, children had higher urinary BPA concentrations than adolescents and adults (Calafat et al. 2008). Additionally, 96% of pregnant women in the 2003-2004 NHANES had detectable levels of urinary BPA concentration (Woodruff et al. 2011). BPA can cross the placenta and human exposure has been quantified in breast milk, sweat, serum, plasma, urine, semen, ovarian follicular fluid, umbilical cord blood, placental tissue, and amniotic fluid (Genuis et al. 2012; Ikezuki et al. 2002; Lathi et al. 2014; Schönfelder et al. 2002; Vandenberg 2007, 2010; Vandentorren et al. 2011; Veiga-Lopez et al. 2015).

Ingestion of food and beverages contaminated by BPA is considered the main source of exposure. Results from a study of US preschool children ages 23 to 64 months, suggest that diet contributes 95% of childhood exposure to BPA and that solid food is a significant contributor (Morgan et al. 2011). Associations of increased urinary BPA concentrations were found in pregnant women with higher frequencies of canned food consumption (Braun et al. 2011). Foods and beverages contaminated by packaging have been shown to be the main source of ingested BPA through laboratory experiments, product replacement and dietary intervention studies (Carwile et al. 2009; Carwile et al. 2011; Christensen et al. 2012; Cooper et al. 2011; Geens et al. 2012; Rudel et al. 2011). However, exposure to

BPA through personal care products, dust and air were cited as possible reasons why BPA concentrations were not lowered in a dietary intervention (Sathyanarayana et al. 2013). For example, young children are commonly observed to engage in frequent hand-to-mouth behaviors such that toys or other objects containing BPA and/or covered with dust containing BPA may be put into their mouths. Additionally, Braun et al. (2011) found higher urinary BPA concentrations in pregnant women with the occupation of cashier. Cashiers and retail workers may be at higher risk of cumulative BPA exposure due to handling of thermal receipt paper and currency papers, in addition to normal dietary consumption of BPA contaminated food. Further, food industry workers may be at higher risk of dermal transfer of BPA through handling of contaminated papers, as well as handling of food for customers. Eating food after handling thermal paper receipt and using hand sanitizer has been found to increase levels of serum and urinary BPA (Hormann et al. 2014). Individual dietary BPA exposure may have multiple routes. Thus, while dietary intake of exposed foods is of main concern, dermal contact with products containing BPA and inhalation or ingestion of BPA in dust are also known routes of exposure to BPA (Michalowicz 2014; Wilson et al. 2003; Zalko et al. 2011).

BPA metabolism

Dependent on route of exposure, BPA may circulate in the bloodstream prior to metabolism by the liver (dermal and inhaled routes) or may be delivered to the liver prior to circulation (oral route). Glucoronidation of BPA occurs via a hepatic microsomal enzyme, specifically the UDP-glucuronosyltransferase isoform UGT2B1 (Matsumoto et al. 2002). The main metabolite of the unconjugated parent BPA monomer is BPA glucoronide. Only the lipophilic free BPA is widely believed to be bioactive with endocrine disrupting effects. With a half-life of 6 hours and hydrophilic nature, glucuronidated BPA is not considered to bio-accumulate in the human body (Völkel et al. 2002). However, cumulative exposures to BPA over time and though different routes must be considered. Free BPA may not be completely eliminated from the human body and varying levels of body fat may account in part for the variability in urinary BPA concentrations. Sathyanarayana et al. (2013) hypothesized that the storage in and release of BPA from tissue with a high fat content may be one reason that BPA did not decline following a dietary intervention (Sathyanarayana et al. 2013; Stalhut et al. 2009).

Recent research using experimental pharmacokinetic evidence suggests that total daily human exposure to BPA may not be from diet alone and could be much higher than previously understood (Taylor et al. 2011). Physiologically based toxicokinetic (PBTK) models for BPA concentration have been developed for predicting BPA bioavailability and developing glucurodination ability in infants and children (Nachman et al. 2014). The PBTK models, which considered age-dependent parameters such as body weight and organ contributions including adipose tissue weight/organ blood flow, suggest that infants and young children have increased internal exposure to unconjugated BPA compared to adults (Edginton and Ritter 2009; Mielke and Gundert-Remy 2009). In addition to age-related differences, the biology of pregnancy must be taken into account when studying early life exposures.

Animal studies have shown there is a limited capacity for glucuronidation of BPA by the fetus and neonate as the full complement of UDP-glucuronosyltransferases do not emerge until after birth (Coughtrie et al. 1988; Matsumoto et al. 2002). Results from *ex vivo* human placental perfusion models show that maternal BPA glucoronide has limited capacity to cross the placenta (Balakrishnan et al. 2010; Corbel et al. 2014). This suggests fetal exposure to conjugated BPA may not be of concern for fetal exposure near term because the human placentae in the perfusion experiments were collected at delivery. Placental perfusion studies also found that during the rapid, bidirectional passive diffusion of free BPA across the placenta, it is either insufficiently glucoronidated during passage to the fetal compartment or it may be absorbed by the placenta (Balakrishnan et al. 2010; Corbel et al. 2014). Woodruff et al. (2011) reported a lower BPA geometric mean in pregnant women at varying trimesters compared to non-pregnant women (2.53 µg/L, n=86 vs. 2.89 µg/L, n=489, respectively) enrolled in NHANES which may be a result of accumulation in tissue such as fat or the placenta, or as suggested by the authors, in the fetus itself. Much remains unknown regarding human feto-placental metabolism of BPA during pregnancy.

Childhood obesity epidemic

Obesity in adults and children has risen to epidemic proportions in the US. By the 1990s it became apparent that, since the 1960s, there has been an 8 percent increase in the prevalence of obesity in adults (Flegal et al. 1998). Obesity in children became epidemic over the same time span.

An increase from 17% to 27% prevalence of obesity in children aged 6 to 11 years was found when triceps skinfold data from the National Health Examination Survey (NHES) cycle 2 (1963 to 1966) were compared to NHANES II (1976 to 1980) (Gortmaker et al. 1987). A decade later, the use of BMI data from NHANES III (1988 to 1991) indicated the prevalence of childhood obesity at or above the 95th percentile increased to 11% (Troiano et al. 1995). Using the National Longitudinal Survey of Youth (NSLY) from 1986 to 1998 the estimated yearly rate of increase for children aged 4 to 12 years with BMI at or above the 95th percentile ranged from 3.23 to 5.85 depending on race and ethnicity (Strauss and Pollack 2001). NHANES data from 1999 to 2012 revealed the prevalence of BMI at or above the 95th percentile was 11.1% and 17.5% in children ages 2 to 5 years and ages 6 to 11 years, respectively (Skinner and Skelton 2014). While Ogden et al. (2012) had similar obesity prevalence findings from NHANES 2009-2010 of 12.1% and 18.2% in children ages 2 to 5 years and ages 6 to 11 years and further calculated obesity prevalence at the highest BMI cut-off of 97th percentile to be 9.7% and 13.0%, respectively, they concluded that overall from 1999 to 2010 obesity prevalence had stabilized. Upon examination of NHANES 2011-2012 data in 2014, Ogden et al. determined that a significant decrease in obesity prevalence had occurred for children ages 2 to 5 years (8.4%) but remained at a plateau of 17.7% for ages 6 to 11 years. Regardless, obesity in nearly 1 of 10 pre-school children remains of concern. The importance of tracking and understanding obesity in early childhood years was underscored by Early Childhood Longitudinal Study results showing 12.4% of children entering kindergarten in 1998-1999 were already classified as obese (Cunningham et al. 2014). There is general agreement that causes of obesity are multifactorial. It is likely that genetics, early physical development, energy imbalance, socialization, food security, cultural differences in diet, learned food behaviors, built environment and environmental exposures may each have a role to play (Gillman et al. 2008; Janesick and Blumberg 2011). In addition to energy imbalance, there have been multiple changes in food production, packaging, availability and marketing to children as well as changes in socioeconomic and socio-cultural influences on family dynamics and eating behavior, all of which may affect obesity rates.

Adipogenesis

The laying down of and over-abundant accumulation of adipocytes peripherally in the body are the hallmarks of obesity. Mature adipocytes are characterized by a single large lipid compartment and exclusive production of the proteins adiponectin and leptin. Adipogenesis is the biological mechanism by which mesenchymal stem cells (MSCs) develop into preadipocytes and ultimately into adipocytes. First discovered in 1968 by Friedenstein, MSCs can be derived from a variety of sources: embryonic tissue, umbilical cord Wharton Jelly, placenta, adipose tissue, peripheral blood and bone marrow (Wang et al. 2012). MSCs will differentiate into multiple possible cell lineages both under normal *in vivo* and experimental *in vitro* conditions including: bone, cartilage, muscle, ligament, tendon, adipose, and stroma. The pluripotency of human MSCs makes them an ideal cellular model for studying the developmental influences on adipocyte formation.

In the human body, MSCs are stimulated to undergo the process of commitment to adipocyte differentiation by a metabolic state of excessive energy uptake and elevated glucose uptake over an extended period of time as well as, a variety of extracellular signaling factors (Tang and Lane 2012). Adipogenesis is described as a two-phase process: 1) in the *determination phase*, the MSC histochemically commits to pre-adipocyte conversion while still morphologically indistinguishable from the precursor cell and 2) in the *terminal differentiation phase*, the pre-adipocyte develops the complete characteristics of the mature adipocyte (Rosen and MacDougald 2006). The timeline for transition from determination phase to terminal differentiation is not well-defined nor agreed upon in the literature. However, experimental evidence points to days 0 to 5 post-induction as a possible determination phase, a possible transition during days 3 and 7, and days 7 to 21 as the principal timeframe for terminal differentiation which can be further defined as early, intermediate and late differentiation (Biemann et al. 2014; Dani 1999; Karahuseyinoglu et al. 2008; Morreno-Navarrete and Fernández-Real 2012; Morrison and Farmer 1999; Ntambi and Kim 2000).

While adipogenesis leads to an increased number of adipocytes; the same metabolic factors influencing adipogenesis also cause hyperplasia of existing mature adipocytes in order to store more fat while inverse metabolic factors can decrease mature adipocyte volume (Spalding et al. 2008; Tang and Lane 2012). Additionally, the number of adipocytes remains constant in both lean and obese individuals

regardless of weight loss, therefore Spalding et al. (2008) have concluded that lifetime adipocyte number is determined in childhood and adolescence. In adulthood, adipocyte number stays constant and weight gain is attributed to increases in adipocyte volume (Arner and Spalding 2010). This provides further evidence that early childhood adipogenesis processes are essential to understanding the childhood obesity epidemic. The use of primitive human umbilical cord MSCs may thus better inform the science of environmental influences on early childhood adipogenesis compared to MSCs derived from adult tissue.

Peroxisome proliferator-activated receptor gamma (PPARγ)

PPARγ, which binds target genes as a heterodimer with retinoid X receptor, is considered the "master" nuclear receptor integral to adipocyte differentiation (Grün and Blumberg 2007; Rosen and MacDougald 2006). *PPARy* is believed to be an early marker of adipogenesis with expression beginning at the end of the determination phase and leading into the terminal differentiation phase of adipogenesis (Gregoire et al. 1998). Ligand activation of *PPARy* is thought to be required to induce adipogenesis but not to maintain *PPARy*-dependent gene expression in mature adipocytes (Rosen and MacDougald 2006). *PPARγ* is a permissive group class II nuclear receptor with the largest ligand binding pocket (LBP) among nuclear receptors at 1400Å which may allow for less discriminate binding to structurally diverse ligands with low affinity (Zaveri and Murphy 2007). However, it is accepted in the literature that a high affinity endogenous ligand has not been isolated for *PPARy* and recent experimental results suggest that ligand binding may not be necessary for *PPARy* to induce adipogenesis (Morrison and Farmer 1999; Walkey and Spiegelman 2008).

Two isoforms of *PPARγ* are known. *PPARγ1* is continuously expressed in many tissues while *PPARγ2* is expressed exclusively in adipose tissue. The two isoforms express nearly identical polypeptides except for 30 additional amino acids on the N terminus of the *PPARγ2* polypeptide (Farmer 2006). Both *PPARγ1* and *PPARγ2* have approximately identical binding affinities, however *PPARγ2* has higher adipogenic activity which is likely attributable to the additional amino acids in the N terminus (Mueller et al. 2002). In the absence of *PPARγ2*, *in vitro* and *in vivo* studies suggest that *PPARγ1* can compensate and similarly promote adipogenesis (Mueller et al. 2002; Zhang et al. 2004).

The transition from the determination phase to the terminal differentiation phase of adipogenesis may be explained by changes in expression levels of both *PPARγ1* and *PPARγ2* (Farmer 2006). The two *PPARγ* isoforms work in an adipogenic feedback loop with transcription factors *CAAT/enhancer binding proteins* (*C/EBP*) *α* and *β*. *PPARγ1* is induced during the first 16 to 48 hours of the determination phase and is believed to act on *C/EBPα* which induces *PPARγ2* into a cross-regulation feedback loop while *C/EBPβ* expression simultaneously promotes *PPARγ2* (Farmer 2006; Morreno-Navarrete and Fernández-Real 2012; Morrison and Farmer 1999; Tang and Lane 2012). *PPARγ2* is activated as terminal differentiation begins, from 24 to 72 hours after induction of adipogenesis (Morrison and Farmer 1999; Tang and Lane 2012). Given *PPARγ* has a promiscuous binding affinity and multiple opportunities for influence on the adipocyte life cycle suggests that there is potential for an influence on adipogenesis by environmental agents.

Experimental Models for Effect of BPA on Adipogenesis

BPA has been shown to accelerate adipocyte conversion in murine *3T3-L1* preadipocytes (Masuno et al. 2002). The mechanism by which BPA could affect adipogenesis is inconclusive based on cell experiments but induction of *PPARγ* is a suspected pathway (Chamorro-Garcia et al. 2012; Phrakonkham et al. 2008; Riu et al. 2011; Sargis et al. 2010; Somm et al. 2009). Evidence from animal experiments has confirmed that prenatal exposure to BPA leads to increased body weight, adiposity, and metabolic syndrome effects (Angle et al. 2013; Miyawaki et al. 2007; Rubin et al. 2001; Somm et al. 2009; van Esterik et al. 2014; Wei et al. 2011). Epigenetic transgenerational inheritance of obesity was observed in F3 generation rats after F1 generation female ancestors were exposed to a plastic derived mixture of compounds including BPA (Manikkam et al. 2013). Additionally, it has been shown that prenatal exposure to BPA increased the expression of *PPARγ* in the mouse liver (García-Arevalo et al. 2014).

Prior research in non-human preadipocyte cell lines has used known agonist pharmaceutical rosiglitazone as a positive control when testing the effect of BPA on *PPARγ* expression during adipogenesis (Pereira-Fernandes et al. 2013; Taxvig et al. 2012; Wright et al. 2000). Rosiglitazone has a greater effect on *PPARγ* expression than BPA at the same concentrations (Pereira-Fernandes et al.

2013; Taxvig et al. 2012). Additionally, when used together in ligand binding assays, BPA does not compete with rosiglitazone for binding to *PPARγ* (Wright et al. 2000). However, experiments in the murine preadipocytes (*3T3-L1*) have determined that BPA has an agonistic effect on *PPARγ* (Phrakonkham et al. 2008; Riu et al. 2011; Sargis et al. 2010). In contrast, induction experiments in multipotent murine mesenchymal stem cells of the C3H10T1/2 lineage have shown, dependent on stage of adipogenesis, both a reduction in and lack of differentiation effect by BPA (Biemann et al. 2012, Biemann et al. 2014). However, when BPA was included in an induction mixture Biemann et al. (2014) observed increased *PPARγ2* expression. Much still needs to be determined regarding whether and how BPA induces *PPARγ* expression and if this is a potential mechanism whereby BPA exposures induce adipogenesis. Additionally, the use of murine preadipocytes by the majority of studies, i.e. cells already determined to progress to adipocytes, does not necessarily enhance the science of BPA exposure inducing multipotent mesenchymal stem cells in humans.

While there are a growing number of published experiments using human adipose lineage cells (adipose stromal cells, preadipocytes, adipocytes), to date there is only one published article which has investigated the effect of BPA on adipogenesis in MSCs derived from adult bone marrow cells (Boucher et al. 2014a; Boucher et al. 2014b; Chamorro-García et al. 2012; Ohlstein et al. 2014; Wang et al. 2013). The aforementioned researchers examining the effect of BPA on adipogenesis in human cells have each used different cell lines, different means of harvesting cells and different age categories of donors (i.e. adult, child). Hence, it is not possible to synthesize the results into a conclusive representation. The studies which used adipose lineage cells all found a positive effect of BPA on adipogenesis through different mechanisms (Boucher et al. 2014a; Boucher et al. 2014b; Ohlstein et al. 2014; Wang et al. 2013). In contrast, Chamorro-García et al. (2012) found that while BPA did not promote adipogenesis in adult human MSCs, it had an adipogenic effect in *3T3-L1* cells. Although Wang et al. (2013) experimented with adipocytes freshly harvested from children undergoing abdominal surgery, the literature lacks any research on BPA adipogenic effects on MSCs obtained during early childhood development. Using human umbilical cord MSCs could be a start towards a more comprehensive understanding of how early life BPA exposures may influence adipogenesis in humans.

Epidemiologic Evidence for Effect of BPA on Childhood Obesity

While it is likely that obesity in humans is a multi-factorial outcome, it is hypothesized that endocrine disruptors may be playing a role. As early as 1987, an environmental cause for obesity was proposed (Gortmaker et al. 1987). Anderson and Butcher (2006) hypothesized that an environmental change increasing the risk of obesity must have begun sometime in the 1980s and continued through the 1990s. Using these lines of thinking along with theories for developmental origins of disease, evidence has shown that brief exposure early in life to environmental EDCs, especially those with estrogenic effects, causes an increase in bodyweight as mice age (Newbold et al. 2007). Furthermore, the "environmental obesogen hypothesis" acknowledges the role prenatal exposure to BPA plays in adipogenesis and suggests that it may be linked to a persistent enhancement of adipocyte differentiation (Grün and Blumberg 2007). Due to their increased metabolic rate and reduced body size relative to food consumption, infants and children are most vulnerable when considering adverse effects of EDCs found in diet such as BPA.

NHANES researchers began measuring adult and child BPA urinary concentrations in 2003. However, there is little information on prenatal and early childhood BPA exposure among minority populations in the US. NHANES reports on BPA concentrations among different race/ethnic groups but analyses are limited to adults and children ages 6 years and older (Calafat et al. 2008). The Children's Total (Aggregate) Exposure to Persistent Pesticides and Other Persistent Organic Pollutants (CTEPP) study of preschool children in North Carolina and Ohio has published on BPA exposure, but data was not analyzed based on race, ethnicity or gender (Wilson et al. 2003; Wilson, personal communication, 2011) . A multiethnic longitudinal study of girls aged 6 years and older in the US by Wolff and Teitelbaum had detectable concentrations of BPA in 95% of subjects (Teitelbaum et al. 2008; Wolff et al. 2010). However, none of the three aforementioned children's studies included obesity as an outcome. NHANES data has shown that African American and Latino children have the highest rates of obesity in the US with Latino children having had the highest percent increase in obesity rates from 2003 to 2007 according to data from the National Survey of Children's Health (Shaibi et al. 2010; Skinner and Skelton 2014). According to the 2010 US Census, African Americans and Latinos are the dominant minority populations in NYC (25.5% and 28.6%, respectively) (US Census Bureau, 2012). Based on the existing research,

there is need for epidemiological investigation of whether BPA exposure during sensitive periods of development, such as *in utero* and early childhood, may lead to obesity. The research also needs to address the BPA exposure associations in populations of minority children who are at highest risk for obesity.

The scientific evidence linking postnatal BPA exposure to childhood obesity is growing (Eng et al. 2013; Harley et al. 2013, Li et al. 2013; Trasande et al. 2012; Wang et al. 2012; Wells et al. 2013). For example, recent epidemiologic population studies using NHANES 2003-2008 data suggest associations between childhood BPA exposure and childhood obesity in the US (Bhandari et al. 2013; Eng et al. 2013, Trasande et al. 2012, Wells et al. 2013). However, data on prenatal BPA exposure associations with childhood obesity in humans not only remains scarce but a variety of anthropometric outcomes have been used (Braun et al. 2014; Eng et al. 2013; Harley et al. 2013; Philippat et al. 2014; Valvi et al. 2013). In the only two US studies investigating BPA exposure and obesity, the anthropometric outcomes were: BMI trajectories and waist circumference (Braun et al. 2014) and BMI, waist circumference, fat mass and body fat percent (Harley et al. 2013). Both studies analyzed relationships with prenatal urinary BPA concentrations and childhood urinary BPA concentrations: at ages 1 and 2 years (Braun et al. 2014) and at ages 5 and 9 years (Harley et al. 2013). However, only Harley et al. (2013) found associations with BPA. Contrary to expectations, Harley et al. (2013) reported that prenatal concentrations were inversely related to 9 year BMI z-score and body fat percent for girls only. Among the non-US studies of prenatal BPA exposure effects on childhood body size outcomes, subjects were drawn from populations in Europe. Philippat et al. (2014) found no effects of prenatal urinary BPA concentrations on height, weight, and abdominal circumference measurements from prenatal ultrasounds as well as six measurements between birth and 3 years of age in a sample of boys in France. In Spain, Valvi et al. (2013) found a positive association between prenatal urinary BPA concentrations and measurements of waist circumference and BMI z-score at 4 years, but not with the waist circumference and BMI z-score at 14 months.

The use of BMI as a comparative anthropometric outcome in both prenatal and postnatal exposure studies is further complicated by varying standards. The US researchers used the 2000 CDC references for calculating BMI z-score and BMI percentile (Braun et al. 2014; Harley et al. 2013). In

Spain, BMI z-scores were based on World Health Organization references. Within the same country, different standards have been used. Two Chinese cross-sectional studies of childhood obesity found positive associations between urinary BPA concentrations and BMI in school aged children: 8 to 15 years old (Wang et al. 2012) and 4 to 12 years old (Li et al. 2013). However, Wang et al. (2012) determined normal, overweight and obese using BMI defintions from the Working Group on Obesity in China and Li et al. (2013) used BMI percentile from a published reference for Shanghai children. Thus, the already limited results on the relationship of BPA with obesity have marked discrepancies based on location, timing of biomarker collection and method of determining childhood body size.

There are relatively few studies on the relationship between prenatal BPA exposure and childhood obesity in minorities. Further, studies in minority populations of the association between prenatal BPA exposure and child body size are inconclusive. In the Cincinnati-based Health Outcomes and Measures of the Environment (HOME) study, a prospective cohort composed primarily of white (67%) and African American (27%) children (n=297), prenatal urinary BPA concentrations were not associated with BMI at ages 2-5 years (Braun et al. 2014). As described above, body size outcomes at age 9 years were inversely related to prenatal BPA concentrations in girls, yet positively associated with childhood age 9 years BPA concentrations in both sexes in the CHAMACOS birth cohort of primarily (98%) Latina mothers and children (Harley et al. 2013). To date, no other study has published results for the analysis of BPA effects on childhood body size, in particular distinct measures of adiposity, in a birth cohort composed entirely of more than one minority group.

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Chapter 2: Urinary Concentrations of Bisphenol A in an Urban Minority Birth Cohort in New York City, Prenatal Through Age 7 Years

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Abstract

Background: Despite growing concern over potential health effects associated with exposures to the endocrine disruptor, bisphenol A (BPA), insufficient information is available on determinants of BPA concentrations among minority populations in the US.

Objectives: To describe concentrations and predictors of BPA in an inner-city longitudinal birth cohort.

Methods: We analyzed spot urines for total BPA collected during pregnancy and child ages 3, 5, and 7 years from African Americans and Dominicans (n=568) enrolled in the Columbia Center for Children's Environmental Health birth cohort and residing in Northern Manhattan and the South Bronx. Adjusting for specific gravity, generalized estimating equations were used to compare BPA concentrations across paired samples and linear regression analyses were used to determine relationships between BPA, season of sample collection, socio-demographic variables and urinary concentrations of phthalate metabolites.

Results: BPA was detected in $\geq$ 94% of samples. Prenatal concentrations were significantly lower than postnatal concentrations. Geometric means were higher among African Americans compared to Dominicans in prenatal ($p=0.008$), 5 year ($p<0.001$) and 7 year ($p=0.017$) samples. Geometric means at 5 and 7 years were higher ($p=0.021$, $p=0.041$ respectively) for children of mothers never married compared to mothers ever married at enrollment. BPA concentrations were correlated with phthalate metabolite concentrations at prenatal, 3, 5 and 7 years (p-values <0.05). Postnatal BPA concentrations were higher in samples collected during the summer.

Conclusions: This study shows widespread BPA exposure in an inner-city minority population. BPA concentration variations were associated with socio-demographic characteristics and other xenobiotics.

Keywords: Bisphenol A; Urine; Child; Prenatal; Minority

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1. Introduction

Bisphenol A (BPA), a xenoestrogenic monomer, was originally investigated for efficacy as a synthetic hormone treatment during the 1930s-40s. Today BPA is used commercially as a key component in manufacturing polycarbonate plastics and epoxy resins. BPA has applications in everyday consumer products such as baby bottles, toys, dental sealants, eyeglass lenses, reusable water bottles, plastic stretch films, consumer electronics, digital media (CDs, DVDs), automobiles, medical equipment, food and beverage can linings and glass jar tops (Vandenberg 2007, 2010). Approximately 4 million tons of BPA are produced annually (Vandenberg 2010). Global market prices and demand for polycarbonate are on the rise and expected to grow at an average annual rate of 5.8 percent to 4.9 million tons by 2015 (CMAI 2010).

This widespread use has resulted in significant exposures (Calafat et al. 2008). Known routes of exposure include dermal and inhalation, but dietary intake is of primary concern (Wilson et al. 2003; Zalko et al. 2011). BPA has been found in fresh and pre-packaged foods, including infant formula in Europe, Asia and the United States (US) (Kuo and Ding, 2004; Noonan at al. 2011; Rudel et al. 2011; Schecter et al. 2010; Vandenberg 2007; Wilson et al. 2007). Results from a recent study of US preschool children ages 23 to 64 months, suggest that diet contributes 95% of childhood exposure to BPA and that solid food is a significant contributor (Morgan et al. 2011). Human maternal exposure has been quantified in breast milk, serum, plasma, urine, ovarian follicular fluid, amniotic fluid and placental tissue (Ikezuki et al. 2002; Schönfelder et al. 2002; Vandenberg 2007, 2010, Vandentorren et al. 2011). Using data from the 2003-2004 NHANES which includes Mexican American and non-Hispanic Black women, Woodruff et al. found detectable concentrations of BPA in 96% of pregnant women (Woodruff et al. 2011). The discovery of BPA in human pregnancy fluids and tissues led researchers to determine that conjugated BPA can cross the placenta (Ikezuki et al. 2002; Schönfelder et al. 2002). However, there is also evidence that the use of catheters during delivery may introduce BPA into the mother's body (Vandentorren et al. 2011). The biologic half-life of BPA has long been accepted to be approximately 4 hours. To date, there is no conclusive evidence regarding clearance rate of BPA from the developing human fetus or child.

Experimental and preliminary epidemiological studies suggest associations between BPA exposure and numerous adverse health effects, including cardiovascular disease, breast cancer,

metabolic disorders, male sexual function, polycystic ovary syndrome, recurrent miscarriages, female adult obesity, endometrial hyperplasia and thyroid effects (Alonso-Magdalena et al. 2010; Crofton 2008; Hiroi et al. 2004; Lang et al. 2008; Li et al. 2010; Melzer et al. 2010; Matsumoto et al. 2005; Sugiura-Ogasawara et al. 2005; Takeuchi et al. 2004; Vandenberg 2007). A positive association has been shown between BPA exposure and higher estrogenic gene expression in male adults (Melzer et al. 2011).

There is little prior information on prenatal and early childhood exposure to BPA among minority populations in the US. Much of the current prenatal data comes from Asia and Europe (Casas et al. 2011; Ikezuki et al. 2002; Padmanabhan et al. 2008; Schönfelder et al. 2002; Yamada et al. 2002; Ye et al. 2008, 2009). Both the US National Health and Nutritional Examination Survey (NHANES) and the Canadian Health Measures Survey report on BPA concentrations among different ethnic groups but are limited to analyses of BPA in adults and in children ages 6 years and older (Calafat et al. 2008; Health Canada, 2010). Another study limited to girls aged 6 years and older of multiple ethnicities in the US by Wolff and Teitelbaum had detectable concentrations of BPA in 95% of subjects (Teitelbaum et al. 2008; Wolff et al. 2010). In the US, the Children's Total (Aggregate) Exposure to Persistent Pesticides and Other Persistent Organic Pollutants (CTEPP) study of preschool children in North Carolina and Ohio has published on BPA exposure, but did not analyze data based on race, ethnicity or gender (Wilson, personal communication, 2011).

Additionally, little is known about inter-correlations between BPA concentrations and other chemical exposures that might affect childhood development. While Woodruff et al. performed analyses on groups of chemicals in the same chemical classes from NHANES samples, they did not run examine the relationships between phenols and phthalates (Woodruff et al. 2011). There is growing concern regarding effects from mixtures of chemical exposures in the literature (Casals-Casas and Desvergne 2011; Kavlock et al. 1996; Landrigan et al 2003).

In this paper we report on BPA concentrations in a minority, low income birth cohort of African American and Dominican mothers and children living in New York City. We also identify associations between BPA concentrations and season of sample collection, socio-demographic characteristics and phthalate metabolites, a family of chemicals shown to be weakly associated with BPA in prior studies

(Braun et al. 2011a) and which should be considered for confounding effects when analyzing BPA in health outcomes research.

2. Material and methods

2.1. Study design and population

Participants (*n*=568) were selected from the Mothers and Newborns longitudinal cohort study of the Columbia Center for Children's Environmental Health (CCCEH) based in Northern Manhattan and the South Bronx, New York (Perera et al. 2003; Whyatt et al. 2003). The mother was selected for our analysis if she had a spot urine sample analyzed for BPA prenatally and her child was selected if he or she had at least one sample analyzed at age 3, 5 or 7 years. For the first five years of the CCCEH study, enrolled participants were contacted every 3 months after delivery for questionnaire follow-up and every 6 months thereafter, with biological samples collected from the children at age 3, 5, and 7 years. Biological samples were collected from the children regardless of any prior missed collection intervals. At the time of our analysis, the 7 year old follow-up was not yet complete.

Study protocols for the CCCEH cohort are described elsewhere (Perera et al. 2003; Whyatt et al. 2003). Briefly, the study enrolled pregnant women 18–35 years old who self-identified as either African American or Dominican and had resided in Northern Manhattan or the South Bronx for at least 1 year before pregnancy. Women were excluded at enrollment if they reported that they smoked cigarettes or used other tobacco products during pregnancy, used illicit drugs, had diabetes, hypertension or known HIV, or had their first prenatal visit after the 20th week of gestation. A 45 minute baseline questionnaire was administered to the mother by a trained bilingual interviewer during the third trimester of pregnancy and at each follow-up interval (child age 3, 5 and 7 years). Medical records of the mother and infant at delivery were abstracted by the research staff to ascertain birth outcomes.

2.2. Urinary biomarker collection

In the mothers, urine was collected during the third trimester of pregnancy between 1999 and 2006 (mean gestational age: 34.7 weeks; SD: 3.4). In the children, urine was collected concurrent with the follow-up questionnaires between 2001 and 2010. The samples were all spot urine samples collected at varying times of day. The date, but not the exact time of collection, was consistently available. The total (free plus conjugated) BPA urinary concentrations (ng/ml) were measured at the National Center for

Environmental Health, Centers for Disease Control and Prevention (CDC). Urine samples were analyzed using online solid-phase extraction coupled to high-performance liquid chromatography, and detected with isotope-dilution tandem mass spectrometry with peak focusing as previously described (Calafat et al. 2008). The assay limit of detection (LOD) was 0.4 ng/ml. For results below LOD, the value of LOD/2 was substituted where applicable, consistent with prior analyses (Whyatt et al. 2003; Whyatt et al. 2009). Specific gravity, as a measure of urinary dilution, was quantified at room temperature at Columbia University with a handheld refractometer (PAL 10-S, Atago, Bellevue, WA).

The following phthalate metabolites were also measured in the spot urine samples at CDC as previously described (Kato et al. 2005; Silva et al. 2007): mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), mono-benzyl phthalate (MBzP), mono-3-carboxypropyl phthalate (MCPP), mono-*n*-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP) and mono-ethyl phthalate (MEP). The LODs ranged from 0.2 to 1.2 ng/ml for all phthalate metabolites. The value of LOD/2 was assigned to the few phthalate metabolite concentrations below LOD. We multiplied the reported MEP and MBzP concentrations by 0.66 and 0.72, respectively, to correct for the inadequate purity of the analytic standards used (Calafat, personal communication, 2012). Due to a higher rate of non-detectable results, MEHP was dropped from analysis in favor of the other three metabolites of di-2-ethylhexyl-phthalate (DEHP): MECPP, MEHHP and MEOHP.

Study procedures, questionnaires and collection of biological samples were explained to each subject at enrollment and a signed consent, approved by the IRB of Columbia University Medical Center and the CDC, was obtained.

2.3. Statistical analysis

BPA concentrations were natural log-transformed and z-scored urinary specific gravity measures were included in regression models as a covariate. Specific gravity values have a very narrow range and cluster tightly around 1.0, which can cause instability in estimating model constants. Specific gravity values were z-score transformed, which does not alter the beta coefficients for the other variables in the model, but does stabilize the estimate of the model constant. Means of the log-transformed BPA data were calculated and exponentiated to calculate geometric mean BPA concentrations. Natural log-

transformed BPA concentrations at each age interval of collection were compared by generalized estimating equations to account for repeated measures within individuals at different ages. The BPA concentration at child age 3 years was used as the referent group due to the consistently higher geometric mean we observed at this age.

To determine Pearson correlations between BPA samples at each collection (ie. prenatal vs. each child sample; child vs. each later child sample) we used the formula: $BPA_{\text{Specific gravity adjusted/log-transformed}} = LN(BPA_{raw} * [(\text{mean for age specific gravity}-1)/(\text{individual specific gravity}-1)])$ (Hauser et al. 2004). For example, the prenatal BPA was first corrected with prenatal specific gravity in the algorithm and then natural-log transformed.

Socio-demographic predictors of BPA concentrations at each time point were assessed using two separate regression analyses. The first was a series of linear regression models including one socio-demographic predictor variables as well as standardized specific gravity as the correction covariate. The second was a series of linear regression GLM models including season of collection as a categorical model with summer as the reference group. Socio-demographic variables we included were: race/ethnicity classified as African American or Dominican, child's sex, income classified as above or below $20,000 per year, marriage status classified as never or ever married at enrollment, maternal education classified as no high school diploma or high school diploma or higher education level, and breastfeeding status classified as ever or never within 3 months of the child's birth. Season of urine collection was coded using June to August (summer) as the reference group with three month units composing Fall (September to November), Winter (December to February) and Spring (March to May) (Wolff, 2010). Partial correlation coefficients from linear regression models for the association between log-transformed BPA and log-transformed phthalate metabolites, adjusted for specific gravity (as a covariate), are reported.

We considered results with $p < 0.05$ to be statistically significant. All of our analyses were performed using PASW Statistics version 18.0 (SPSS Inc., Chicago, IL, USA)

3. Results

3.1. Study population characteristics

Of the 568 participants, we measured BPA concentration in n=375 prenatal samples, n=408 age 3 year samples, n=401 age 5 year samples, and n=318 age 7 year samples. Demographic characteristics during the prenatal period are presented in Table 1. There were no significant differences in demographic characteristics for those children with all 3 childhood urine samples analyzed compared to those with fewer than 3 childhood samples.

BPA was detected in 94% of prenatal samples, 98% of 3 year and 5 year samples, and 96% of 7 year samples. BPA concentrations (ng/ml) are presented in Table 2. The geometric mean BPA remained stable in prenatal samples collected between 1999 and 2006 and a temporal trend was not observed (Figure 1, Supplemental Table S1). Similarly, for each of the follow-up samples collected from 2001 to 2009 (age 3), 2003 to 2010 (age 5) and 2005 to 2010 (age 7) there were no temporal trends in geometric mean BPA concentrations (Supplemental Table S2).

Maternal prenatal BPA concentrations were not correlated with any of the child concentrations (all p-values $\geq$ 0.40). Among children, only the age 3 and 7 year concentrations were weakly correlated (r=0.164, p-value=0.012) while the 3 and 5 year concentrations and 5 and 7 year concentrations did not correlate (p=0.823, p=0.079 respectively). Maternal prenatal BPA concentrations were significantly lower than those of their children at all ages (see Table 2). Child BPA concentrations at age 3 years were significantly higher than repeat samples at ages 5 and 7 years. However, BPA concentrations did not significantly differ at ages 5 and 7 years. When complementary analyses were done in subjects with BPA concentrations at all three postnatal ages (n=227) to account for the differing sample sizes at ages 3, 5 and 7 years the correlation and GEE results were similar.

3.2. Socio-demographic predictors of BPA

Table 3 shows BPA concentration by demographic characteristics and season. Adjusting for specific gravity, BPA geometric means concentrations were higher among African American as compared to Dominicans in prenatal (p=0.008), 5 year (p<0.001) and 7 year (p=0.017) samples but not in 3 year samples (p=0.89) (Table 3). Geometric means at 5 and 7 years were higher (p=0.021, p=0.041 respectively) for children of mothers never married at cohort enrollment compared to mothers ever married at cohort enrollment. Comparisons of BPA concentrations by child's sex, maternal education at

enrollment, and household income at enrollment did not reveal significant differences at any assessment interval.

3.3. Season of collection predictors of BPA

After adjusting for specific gravity, calendar season of urine collection predicted log-transformed BPA concentration. Geometric means for urine collections during the summer at ages 3, 5 and 7 years were consistently higher than those collected in all other seasons (p-values range: < 0.001 to 0.034) (Table 3). When the non-summer seasons were collapsed into a single category, regression analyses showed that BPA concentrations in samples collected in summer months remained significantly higher than concentrations in samples collected in non-summer months: 3 years: β: 0.4 ng/ml, CI: (0.65, 0.25), p<0.001; 5 years: β: 0.30 ng/ml, CI: (0.50, 0.11), p=0.002; 7 years: β: 0.50 ng/ml, CI: (0.72, 0.29), p<0.001.

3.4. Phthalate correlations

Table 4 presents partial correlation coefficients for natural log-transformed BPA compared to natural log-transformed urinary metabolites of phthalates after adjustment for specific gravity. Prenatal BPA concentrations were weakly but significantly correlated with phthalate metabolite concentrations after adjusting for specific gravity. Similar to the prenatal findings, childhood BPA concentrations were weakly but significantly correlated with all urinary phthalate metabolite concentrations after adjusting for specific gravity at ages 3, 5 and 7 years. The highest correlations were with the DEHP metabolites at age 3 years.

4. Discussion

Results show BPA to be a pervasive contaminant with 94% or higher detection rates in this study population of inner-city mothers and children. We found that geometric means for maternal prenatal BPA concentrations were significantly lower than paired children's postnatal concentrations. BPA in pregnant women may be temporarily diverted from the excretory process due to transfer across the placenta. Human placenta does not act as a barrier to BPA (Schönfelder et al. 2002). Studies of radiolabeled-BPA in pregnant CD1 mice determined that after 24 hours, only 6% of BPA was excreted in urine while a disaccharide conjugate of BPA accounted for 60%, 20% and 10% of radioactivity in placenta, amniotic fluid and fetuses, respectively (Zalko, 2003). In humans, placenta has only a synctiotrophoblast

monolayer separating the maternal and fetal blood vessels as opposed to the mouse placenta which has three layers. Early in pregnancy, amniotic fluid is comparable to maternal or fetal plasma and may be composed of secretions from the umbilical cord, placental membrane and developing epithelium (Beall et al, 2007). Towards the end of pregnancy, an estimated 10 ml/day of amniotic fluid cross into the maternal circulation via the transmembranous pathway (Beall et al. 2007; Underwood et al. 2005). While the literature has yet to describe it in humans, there remains the possibility that pregnant women may have less BPA in their urine due to transfer across the placenta. Alternatively, lower total BPA urinary concentration in the mothers than in their children may be related to the fact that diet is the main pathway of exposure to BPA (Morgan et al. 2011) and children eat more relative to body weight than adults (US EPA, 2011). Such a trend has been observed for other non-persistent chemicals to which diet is the main source of exposure (CDC 2012). In contrast, some animal pharmacokinetic evidence suggests that total daily human exposure to BPA cannot be from diet alone and is much higher than previously understood (Taylor et al. 2011).

Our unadjusted geometric means are similar to data from two other studies comparing prenatal and childhood BPA concentrations. A European Spanish cohort of mothers and their 4 year old children, reported lower urinary concentrations of BPA in spot urine samples collected prenatally from the mothers as compared to their children [2.2 ng/mL, n=120 vs. 4.2 ng/mL, n=30, $p<0.05$, respective medians] (Casas et al. 2011). In a Cincinnati, Ohio study of 240 mother-child dyad spot urine samples, the mean prenatal BPA concentration was 2.0 µg/L and the childhood mean of ages 1, 2, and 3 years was 4.1 µg/L, while the median age 3 year BPA concentration was 2.6 µg/L (Braun et al. 2011b).

Our overall prenatal BPA geometric mean of 1.8 ng/ml is in accordance with findings from other investigators. In a US study looking at repeated measures of BPA in 249 women both prenatally and postnatally, Braun et al. (2011b) found median BPA concentrations of 1.8 µg/L (16 weeks), 1.7 µg/L (26 weeks) and 1.2 µg/L (at birth). Using NHANES data, Woodruff et al. (2011), reported a lower geometric mean in pregnant women compared to non-pregnant women (2.53 µg/L, n=86 vs. 2.89 µg/L, n=489, respectively). After adjusting for creatinine and socio-demographics (age, race/ethnicity, education, smoking, parity, BMI, albumin, duration of fasting prior to sample collection) the BPA concentration

disparity increased between pregnant women (1.63 µg/L, n=72) and non-pregnant women (2.83 µg/L, n=371) (Woodruff et al. 2011).

Due to the metabolic characteristics of pregnant women and young children, we controlled for urinary specific gravity as opposed to creatinine in our regression and correlation analyses and this could result in some differences across studies. Renal clearance of creatinine is directly proportional to and dependent on glomerular filtration, such that changes in renal clearance that commonly occur during pregnancy could also cause changes in creatinine concentrations (Jatlow et al. 2003; Mahalingaiah et al. 2008). Creatinine, a product of muscle metabolism, relies on the liver and the kidney which are still maturing in children. Childhood levels of creatinine tend to be lower with more variability over time compared to levels in non-elderly adults. Creatinine comparisons in the NHANES III (1988-1994) study describe a mean of 102.1 mg/dL in children ages 6 to 11 years, whereas adult means were consistently higher by decade of age from 20 to 49 years (Barr et al. 2005). Little is known about BPA renal clearance during human pregnancy. Specific gravity is a unit-less ratio of the density of urine to the density of water, dependent on fluid intake, renal perfusion and renal function and thus should be less influenced by muscle mass and muscle retention.

At age 3 years, the children in our study had significantly higher BPA concentrations than they did at follow-up ages 5 and 7 years, a difference that may be related to changes in behavior as children age. While young children tend to have greater oral contact with their world than older children and adults, diet should still be considered the primary route of BPA exposure in young children (Reed et al. 1999). It is known that children between the ages of 3 and 6 years eat more relative to body weight than adults (US EPA, 2011). Additionally, children under the age of 6 years tend to have less varied diets (Goldman, 1995). Diet in pre-school children may be more dependent on the feeding behaviors of their mothers whereas school-aged children are able to make food choices outside the presence of their mothers. Adult pregnant female eating behaviors are also likely to be different than those of children.

In our study population, African Americans had consistently significantly higher BPA concentrations than Dominican Americans, except among 3 year olds where BPA was elevated for African Americans but not significantly. Additionally we saw higher concentrations among older children of never married mothers compared to older children of ever married mothers. This suggests that BPA

concentrations may vary by socioeconomic status. However, we did not see any differences based on household income nor maternal education. In contrast, other studies have found BPA concentration to be negatively associated with income and maternal education (Calafat et al. 2008; Braun et al. 2011b). Little is known about how dietary differences across race/ethnicity and maternal characteristics may affect BPA exposures in pregnancy or childhood. Our results have implications for guiding sociodemographic considerations in future health effects studies of BPA exposures.

We found postnatal BPA concentrations to be higher during the summer months than during the rest of the year. This could be due to different dietary patterns among children during the summer relative to other seasons. It could also be related to diurnal variation in renal clearance of BPA by season. This finding is consistent with previously reported BPA urinary geometric means in a population of prepubertal girls from three sites in the US, including New York City (Wolff et al. 2010).

Urinary concentrations of BPA and phthalate metabolites were correlated. These findings are similar to Braun et al. (2011a) in the US but are in contrast to those of Casas et al. (2011) in Spain. Given the ubiquitous presence of both BPA and phthalates in the environment, it is not surprising that they would both be widely detectable in our study population. However, the observation of correlations between BPA concentrations and these phthalate metabolites highlights the complex issue of analyzing the health effects of chemical mixtures in people.

We saw a consistent pattern of BPA exposure for race/ethnicity, marital status and season of collection despite no correlation between BPA concentrations in repeat samples. High variability and poor interclass correlations for BPA concentrations in spot urine samples have been reported by others, however spot urine samples may adequately reflect BPA exposure at the group level (Braun et al. 2011a; Teitelbaum et al. 2008; Ye et al. 2011). If, as expected, the errors in measures of BPA concentrations are non-differential, when BPA concentrations are analyzed as a dependent variable in the regression models, the standard errors for the regression coefficients are expected to be increased, resulting in lower statistical power (Cook and Campbell, 1979). Our significant findings indicate it is likely the differences are greater than we can detect due to the poor reliability of the biomarker. Regardless, our use of single spot urines for BPA analysis provides clear evidence that the children in our study

population are exposed to BPA between ages 3 and 7 years. Cumulative exposure effects on health and development beginning prenatally and over a lifetime are a concern which should be investigated further.

Limitations of this study include the use of single spot urines and the lack of recording time of day for urine collection. The data on time of urine collection are not available for the prenatal samples or the age 3 samples, and are only available for a minority of the samples collected at ages 5 and 7. Diurnal variations in urinary BPA concentrations have been reported and it is possible collection time may confound our results. We also did not previously collect any dietary data but we have begun administering food frequency assessments among cohort children at older ages and these may give insight on dietary behaviors in future analyses.

5. Conclusion

We present evidence that BPA exposure is widespread among this cohort of inner-city mothers and children. Variations in BPA concentrations were associated with socio-demographic characteristics and exposures to other xenobiotics. The data presented here suggest specific areas for analytical attention due to confounding and covariate measurement issues. These analyses also demonstrate the critical need for studies to consider the effects of exposures to chemical mixtures and potentially complex interactions among exposures and socio-demographic characteristics. New statistical methods to analyze the effects of chemical mixtures are urgently needed. Further analyses will be undertaken in the cohort to determine the effects of early life exposure to BPA on health outcomes.

Disclosure Statement

The authors have no actual or potential financial or nonfinancial conflicts of interest to disclose.

Role of the funding source

Study sponsors had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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Figure 1. Temporality of Prenatal BPA Urinary Concentration. Unadjusted prenatal BPA concentration with LOESS Curve through a point by point scatterplot to describe the lack of variation by collection year.

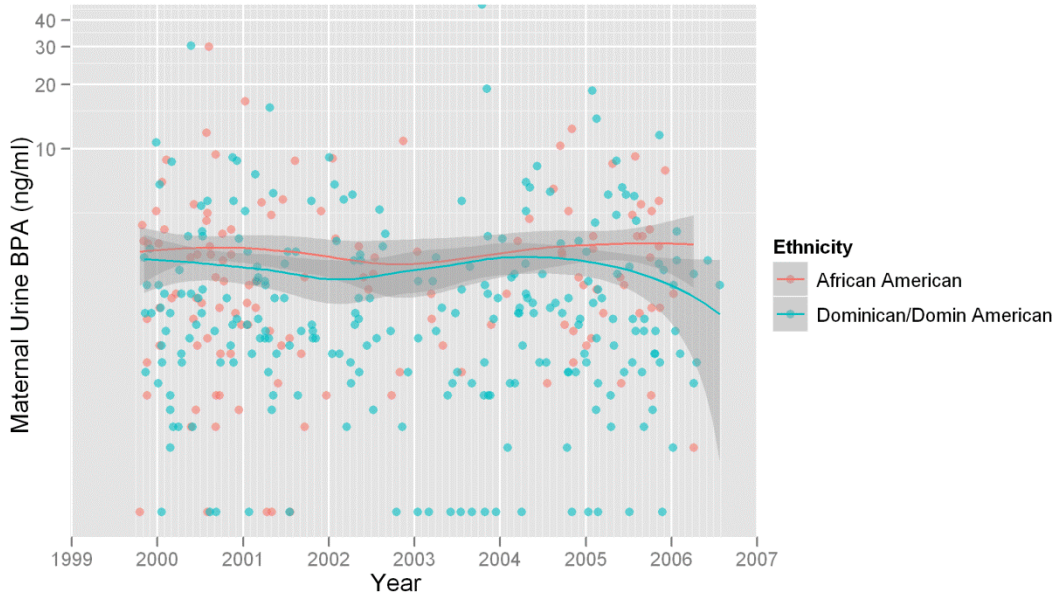

Table 1. Subject demographics

Characteristic [***Categorical: n(%)***]	**All subjects (n=568)**	**Prenatal (n=375)**	**3 Year (n=408)**	**5 Year (n=401)**	**7 Year (n=318)**
Race/Ethnicity					
Dominican	351 (61.8)	244 (65.1)	246 (60.3)	246 (61.3)	176 (55.3)
African American	217 (38.2)	131 (34.9)	162 (39.7)	155 (38.7)	142 (44.7)
Gender of Child					
Female	305 (53.7)	202 (53.9)	219 (53.7)	217 (54.1)	168 (52.8)
Male	263 (46.3)	173 (46.1)	189 (46.3)	184 (45.9)	150 (47.2)
Maternal Education[a]					
< HS	197 (34.7)	134 (35.7)	147 (36.0)	135 (33.7)	107 (33.6)
Marital Status[a]					
Never Married	374 (65.8)	252 (67.2)	271 (66.4)	265 (66.1)	214 67.3)
Household Income[a]					
< $20K	385 (67.8)	240 (64.0)	280 (68.6)	276 (68.8)	223 (70.1)
Breastfed					
Ever by 3 Months	360 (63.4)	--	251 (61.5)	243 (60.6)	185 (58.2)
[***Continuous: median(IQR)***]					
Median Age At Urine Collection (years)	--	24.7 (8)	3.0 (0.2)	5.0 (0.1)	7.0 (0.1)
Median Gestational Age At Urine Collection[a] **(weeks)**	--	39 (1)	--	--	--

[a] Missing from all subjects: Maternal Education n=9, Marital Status n=2, Household Income n=39, Breastfed n=100, Gestational Age n=4

Table 2. Unadjusted BPA concentrations (ng/ml) in spot urine samples collected from the mother during the 3rd trimester of pregnancy and from the child at ages 3-7 years

	#>LOD (%)	GM (95%CI)	25%	50%	75%	95%	p-value[a]
Prenatal	351/375 (94%)	1.8 (1.7, 2.0)	1.0	1.8	3.5	9.0	<0.001
Child age 3	398/408 (98%)	3.7 (3.3, 4.2)	1.8	3.8	7.4	29.7	- -
Child age 5	392/401 (98%)	3.2 (2.9, 3.6)	1.7	3.1	6.4	18.3	0.048
Child age 7	306/318 (96%)	2.9 (2.6, 3.3)	1.4	2.7	6.0	19.1	0.001

[a] P-values for difference in the geometric mean between age groups calculated by GEE analysis of log-transformed BPA using age 3 as the referent group.

Table 4. Correlations of ln-transformed urinary BPA and phthalate metabolites adjusted for specific gravity

Table 3. Geometric mean urinary BPA concentrations by demographic characteristics and season, within age group

Prenatal Characteristic [GM[95% CI]]	Prenatal	3 Year	5 Year	7 Year
Race/Ethnicity				
Dominican[a]	1.6 (1.4, 1.9)	3.4 (2.9, 4.0)	2.8 (2.4, 3.1)	2.3 (2.0, 2.8)
African American	2.3 (1.9, 2.7)*	4.1 (3.4, 4.8)	3.9 (3.4, 4.6)**	3.6 (3.0, 4.4)*
Gender of Child				
Female[a]	1.8 (1.5, 2.1)	3.5 (3.0, 4.2)	3.2 (2.8, 3.7)	2.7 (2.3, 3.3)
Male	1.9 (1.6, 2.2)	3.8 (3.2, 4.5)	3.1 (2.7, 3.6)	3.0 (2.5, 3.6)
Maternal Education				
< HS[a]	1.9 (1.6, 2.2)	3.5 (3.0, 4.2)	3.3 (2.8, 3.9)	2.9 (2.3, 3.6)
HS +	1.8 (1.6, 2.1)	3.7 (3.2, 4.3)	3.1 (2.7, 3.5)	2.8 (2.4, 3.3)
Marital Status				
Ever Married[a]	1.7 (1.4, 1.9)	4.1 (3.3, 5.0)	2.5 (2.1, 3.0)	2.3 (1.8, 2.8)
Never Married	1.9 (1.7, 2.2)	3.4 (3.0, 4.0)	3.5 (3.2, 4.0)*	3.2 (2.7, 3.7)*
Household Income				
< $20K[a]	1.8 (1.5, 2.0)	3.6 (3.2, 4.2)	3.3 (2.9, 3.7)	2.9 (2.5, 3.3)
>$20K	2.1 (1.7, 2.5)	3.9 (3.2, 4.9)	3.0 (2.4, 3.7)	2.8 (2.2, 3.7)
Season of Collection				
Summer[a]	2.1 (1.7, 2.5)	5.1 (4.3, 6.0)	4.0 (3.4, 4.6)	4.1 (3.4, 4.9)
Fall	1.7 (1.4, 2.0)	3.4 (2.9, 4.0)**	2.9 (2.5, 3.3)*	3.0 (2.5, 3.6)*
Winter	1.8 (1.5, 2.1)	3.1 (2.6, 3.7)**	2.9 (2.5, 3.4)*	2.3 (1.9, 2.8)**
Spring	1.8 (1.6, 2.2)	3.2 (2.8, 3.7)**	3.0 (2.7, 3.4)*	2.2 (1.9, 2.7)**

[a] Reference group; *p<0.05, **p≤0.001; p-values for differences in geometric mean calculated using GLM analysis of log-transformed BPA and adjusted for specific gravity.

Biomarkers [r (p-value)]	**Prenatal (n=362)**	**3 Year (n=346)**	**5 Year (n=337)**	**7 Year (n=314)**
DEHP metabolites				
MEHHP	0.208 (<0.001)	0.304 (<0.001)	0.287 (<0.001)	0.182 (0.001)
MECPP	0.188 (<0.001)	0.344 (<0.001)	0.294 (<0.001)	0.157 (0.005)
MEOHP	0.239 (<0.001)	0.308 (<0.001)	0.295 (<0.001)	0.189 (0.001)
Non-DEHP metabolites				
MBZP	0.191 (<0.001)	0.135 (0.012)	0.154 (0.005)	0.131 (0.020)
MCPP	0.224 (<0.001)	0.288 (<0.001)	0.255 (<0.001)	0.153 (0.007)
MnBP	0.267 (<0.001)	0.267 (<0.001)	0.300 (<0.001)	0.261 (<0.001)
MIBP	0.220 (<0.001)	0.234 (<0.001)	0.269 (<0.001)	0.272 (<0.001)
MEP	0.169 (0.001)	0.159 (0.003)	0.144 (0.008)	0.262 (<0.001)

Urinary concentrations of bisphenol A in an urban minority birth cohort in New York City, prenatal through age 7 years

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Supplementary Material

Table S1. Prenatal BPA Geometric Means Over Time. The geometric means by collection year of urinary BPA collected from pregnant women enrolled in the Columbia Center for Children's Environmental Health (CCCEH) birth cohort.

Collection Year	N	Geometric Mean	Lower 95% CI	Upper 95% CI
1999	15	2.04	1.25	3.34
2000	88	1.93	1.55	2.39
2001	59	1.71	1.32	2.20
2002	39	2.00	1.50	2.66
2003	38	1.26	0.85	1.85
2004	53	1.97	1.55	2.50
2005	70	2.06	1.62	2.62
2006	13	1.51	1.00	2.28

Table S2. Childhood BPA Geometric Means Over Time. The geometric means by collection year of urinary BPA collected from children enrolled in the CCCEH birth cohort at ages 3, 5 and 7 years.

Age	Collection Year	N	Geometric Mean	Lower 95% CI	Upper 95% CI
3 years					
	2001	30	4.61	3.19	6.65
	2002	78	3.52	2.60	4.77
	2003	63	4.08	3.18	5.24
	2004	63	4.60	3.49	6.06
	2005	46	4.35	3.15	5.99
	2006	31	2.96	2.02	4.34
	2007	37	2.86	1.85	4.44
	2008	44	2.82	2.00	3.98
	2009	16	3.57	2.59	4.92
5 years					
	2003	28	4.30	3.00	6.17
	2004	102	3.61	2.93	4.45
	2005	78	3.27	2.57	4.16
	2006	66	2.71	2.09	3.52
	2007	46	3.67	2.82	4.77
	2008	41	2.70	2.10	3.48
	2009	40	2.54	1.86	3.47
7 years					
	2005	33	3.50	2.28	5.37
	2006	92	2.93	2.31	3.73
	2007	83	2.72	2.18	3.41
	2008	70	2.66	2.02	3.51
	2009	40	3.17	2.17	4.63

Chapter 3: Bisphenol A, Adipogenesis and *Peroxisome Proliferator-Activatated Receptor Gamma* Expression in Human Umbilical Cord Mesenchymal Stem Cells: A Feasibility Study

Abstract

Background: Exposure to the endocrine disruptor bisphenol A (BPA) has been associated with adiposity in epidemiological and experimental studies, but the underlying mechanism is unclear.

Objectives: This is the first known feasibility study to evaluate whether exposing freshly sourced human umbilical cord mesenchymal stem cells (HUMSCs) to BPA during standard adipogenic induction promotes adipogenesis.

Methods: Eighteen umbilical cords were sucessfully collected anonymously from mothers at delivery and propogated. Mesenchymal stem cells were harvested using an adhesion technique. HUMSCs were induced in culture to differentiate into adipocytes and exposed to BPA. Morphometric differences in all cultures were assessed using ImageJ software (version 1.49n, 2014). Gene expression of peroxisome *proliferator-activated receptor gamma* (*PPARγ*) in cultures was evaluated by RT-PCR. Separate promoters for *PPARγ1* and *PPARγ2* were used to explore time dependence of BPA-induced adipogenesis in HUMSCs. *PPARγ1* and *PPARγ2* gene expression was assessed during early terminal phase adipogenesis (0 to 72 hours). Cell morphometrics were assessed during middle to late terminal phase adipogenesis (days 14 and 21).

Results: Cell morphology results are based on 11,676 cells from 3 umbilical cord samples. No differences in cell count were observed for experimental conditions compared to standard induction medium at day 14 or day 21. A significant decrease in surface area was seen in cells exposed to 100 μM concentration of BPA as compared to exposure to standard induction medium at day 14 (t=-37.02 p=0.001). Differences in cell surface area were not observed at day 21. A twofold increased expression of *PPARγ1* was observed in cells exposed to 10 μM concentration of BPA by 72 hours of adipogenic induction which was higher than the increase in expression observed for cells exposed to the positive control induction medium containing 10 μM of rosiglitazone. All induction media conditions had negligible effects on *PPARγ2* expression.

Conclusions: BPA increases expression of *PPARγ1* gene expression HUMSCs during the transition into the early terminal differentiation phase of adipogenesis. This feasibility study suggests human umbilical cords may be a relevant target tissue for evaluating BPA effects in adipogenesis.

Introduction

BPA is a key component in polycarbonate plastics and epoxy resins with common use in consumer plastic products resulting in significant human exposure (Calafat et al. 2008; Vandenberg et al. 2007, 2010). Exposure pathways include oral, dermal and inhalation, with dietary intake the primary route (Wilson et al. 2003; Zalko et al. 2011). Prenatal exposure may occur when conjugated BPA crosses the placenta *in utero* or by use of catheters during delivery (Ikezuki et al. 2002; Schönfelder et al. 2002; Vandentorren et al. 2011). Previous studies have shown that bisphenol A (BPA), a suspected obesogen, accelerates adipocyte conversion in murine *3T3-L1* preadipocytes (Masuno et al. 2002). Others reported that in *3T3-L1* cells BPA had an agonistic effect on the master adipogenic nuclear receptor, *peroxisome proliferator-activated receptor gamma* (*PPARγ*) (Chamorro-Garcia et al. 2012; Phrakonkham et al. 2008; Riu et al. 2011; Sargis et al. 2010).

Adipogenesis is the biological mechanism in which mesenchymal stem cells (MSCs) develop into preadipocytes and ultimately into adipocytes. Adipogenesis is comprised of two-phases with transition from the determination phase to terminal differentiation. In the determination phase, the MSCs histochemically commit to adipogenesis. The terminal differentiation phase produces mature adipocytes. However, the timeline for transition is neither well-defined nor agreed upon in the literature. The determination phase occurs during days 0 to 5 post-induction, while a transition occurs at some point during days 3 and 7, and days 7 to 21 are widely considered the principal timeframe for terminal differentiation which can be further delineated into early, intermediate and late differentiation (Biemann et al. 2014; Dani 1999; Karahuseyinoglu et al. 2008; Morreno-Navarrete and Fernández-Real 2012; Ntambi and Kim 2000).

The transition from the determination phase to the terminal differentiation phase of adipogenesis appears to be explained by changes in expression levels of the two isoforms of *PPARγ*: *PPARγ1* and *PPARγ2* (Farmer 2006). *PPARγ1* is induced during the first 16 to 48 hours of the determination phase and is believed to induce *PPARγ2* in a cross-regulation feedback loop (Farmer 2006, Morreno-Navarrete and Fernández-Real 2012, Morrison and Farmer 1999, Tang and Lane 2012). *PPARγ2* is activated as terminal differentiation begins, from 24 to 72 hours after induction of adipogenesis (Morrison and Farmer 1999; Tang and Lane 2012). The *PPARγ* class of nuclear receptors has a large ligand binding pocket

(LBP) likely allowing for promiscuous binding affinities and multiple opportunities for influence on the adipocyte life cycle by multiple potential ligands. This could explain how adipogenesis can be influenced by environmental agents.

While there is growing literature investigating the effect of BPA on adipogenesis using human adipose cell lines (adipose stromal cells, preadipocytes, adipocytes), to date there is only one published article using primary MSCs which are from adult bone marrow (Boucher et al. 2014a; Boucher et al. 2014b; Chamorro-García et al. 2012; Ohlstein et al. 2014; Wang et al. 2013). The literature lacks any research on BPA adipogenic effects on MSCs obtained during early childhood development. Techniques for preserving mammalian umbilical cords and isolating human umbilical cord MSCs (HUMSCs) proved difficult until 2011 when De Bruyn et al. published successful results for isolating HUMSCs based solely on the plastic adhesion properties of MSCs (DeBruyn et al. 2011). As HUMSCs can differentiate into adipocytes under the correct conditions, the facile ability to successfully isolate HUMSCs is highly desirable as a starting point for a mechanistic study of the actions of BPA on adipocytes.

HUMSC morphological and histochemical changes during conventional *in vitro* adipogenesis have been extensively described by Karahuseyinoglu et al.(2008). However, to date, the literature lacks any research on BPA adipogenic effects on MSCs derived from the human umbilical cord. Here we describe results from a novel *in vitro* feasibility study of BPA induction in adipogenic HUMSC cultures, where BPA exposure was found to affect adipocyte propagation, adipocyte morphology, and *PPARγ* expression.

Materials and Methods

Human subjects

Umbilical cords (n=18) were collected from Feb 2013 to June 2013 from mothers registered at New York-Presbyterian Sloane Hospital for Women (n=5) and New York-Presbyterian Allen Hospital (n=13) in New York City. The samples were acquired as part of the Columbia Center for Children's Environmental Health study of RNA extraction procedures and methylation levels in anonymous cord blood samples. All collection protocols were reviewed and approved by the Institutional Review Board of Columbia-Presbyterian Medical Center and all umbilical cords were collected anonymously at delivery. First, umbilical cord blood was collected from the umbilical vein. Following the method of deBruyn et al.

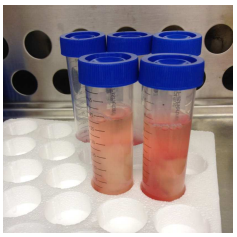
(2011), umbilical cords were then cut into one to three segments of 5 to 10 cm each and individually stored at room temperature in sterile 50 ml conical tubes (BD Falcon) containing 10x phosphate-buffered saline (PBS) (Lonza) supplemented with antibiotic/antimycotic (AB/AM) solution (HyClone). Umbilical cords were processed in the lab within 12 hours of collection (see Figure 1).

Cell culture

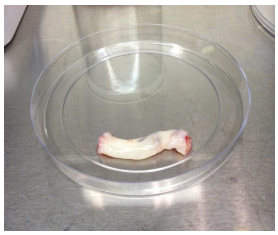
HUMSCs were harvested from umbilical cords using an adhesion technique as previously described (deBruyn et al. 2011). Umbilical cords were longitudinally sectioned and scored under sterile conditions to expose the inner Wharton's Jelly. Clotted blood was removed from the blood vessels. Cord sections were plated individually in 10 cm^2 Petri plates (Fisher) containing Dulbeccco's modified Eagle medium with 1 g/L glucose without L-glutamine (DMEM) (Lonza), 15% fetal bovine serum (FBS) (Lonza, Fisher). 2 nM L-glutamine (HyClone) and 0.5% AB/AM and incubated at 37°C with 5% humidified CO_2. On Day 5, cord sections were discarded, cell growth and plate adherence was confirmed by microscopy and the medium was renewed by 50% in order to remove debris but retain cellular growth factors. On Day 13, the medium was completely renewed. HUMSCs were then fed weekly with 50% renewed medium until they reached subconfluence (typically 30-40 days). At subconfluence, cells were detached using TrypLE™ Select solution (Gibco) and expanded 1:2 by plating in 75 cm^2 flasks (BD Falcon). HUMSCs were passaged until P2 and then prepared for cryopreservation in liquid nitrogen.

We confirmed growth of multipotent mesenchymal stem cells per the criteria of the International Society for Cellular Therapy: (1) plastic-adherent when maintained in standard culture conditions; (2) expression of *CD105*, *CD73* and *CD90*, and lacking expression of *CD45*, *CD34*, *CD14* or *CD11b*, *CD79a* or *CD19* and *HLA-DR* surface molecules (FlowCellect Human Mesenchymal Stem Cell Characterization Kit, EMD Millipore); (3) *in vitro* differentiation to osteoblasts, adipocytes, and chondroblasts (StemPro Chondrogenesis Differentiation Kit, Gibco; StemMACS AdipDiff Media, Miltenyi Biotec; adipogenesis media recipe, Hoepner; StemPro Osteogenesis Differentiation Kit, Gibco) (Dominici et al. 2006). Differentiation of HUMSCs was verified via microscope inspection after staining with Alizarin Red (American MasterTech) for osteoblasts, Oil Red O (American MasterTech) for adipocytes, and Alcian Blue pH 2.5 (American MasterTech) for chondroblasts (see Figure 2).

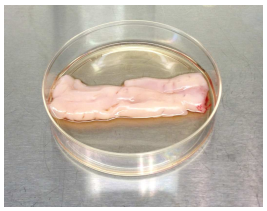
Figure 1. Harvesting of mesenchymal stem cells



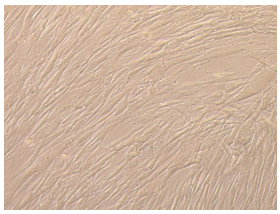
Post-Collection
Samples



Cord Section
Pre-Processing

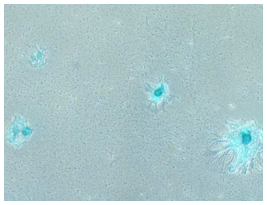


First Media Change
Day 5

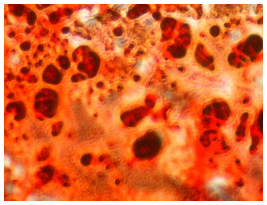


Human Umbilical Cord
Mesenchymal Stem Cells (10x)

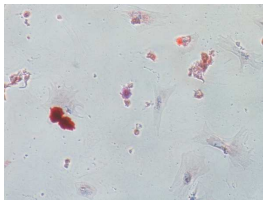
Figure 2. Multipotent differentiation



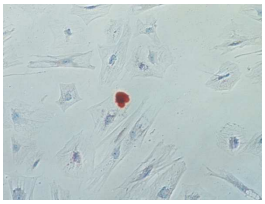
Chondrocytes
Alcian Blue pH2.5 Stain (10x)



Osteocytes
Alizarin Red Stain (10x)



Adipocytes
Commercial Induction Media
Oil Red O Stain (10x)



Adipocytes
Recipe Induction Media
Oil Red O Stain (10x)

Cryopreservation

When a minimum count of 1.05 x10^6 HUMSCs was achieved in each flask as determined by hematocytometer, cells were stored in 1.8 ml cryovials (Nunc) containing 1 ml of cryoprotective agent (CPA) and 1.05 x10^6 cells. CPA consisted of 10% dimethyl sulfoxide (DMSO) (Fisher), 15% FBS, 40% 0.25M Sucrose (Fisher), and 35% DMEM. Cryovials were placed in a Mr. Frosty (Nalgene) and put in a -80°C freezer to chill 1°C/min over a minimum of 24 hours. Cells were stored long term in liquid nitrogen.

Propogation of HUMSCs

HUMSCs were removed from liquid nitrogen, thawed and plated in 75 cm^2 flasks with DMEM, 15% FBS. 2 nM L-glutamine, and 0.5% AB/AM and incubated at 37°C with 5% humidified CO_2 (Seshareddy et al. 2008). Cell viability was determined with Trypan Blue stain (Sigma Aldrich). HUMSCs were cultured from a minimum of P6 through a maximum of P8 in preparation for adipogenesis studies.

Adipogenic differentiation and experimental conditions

HUMSCs were expanded to a minimum of 21.0 x10^6 cells at which point they were seeded in fresh growth culture medium on 3 x 15mm^2 Petri dishes (BD Falcon). The cultures were allowed to rest in the incubator for 2 to 3 days before the experimental conditions were applied.

On day 1, one culture was selected for baseline and HUMSCs were trypsinized and total RNA isolated (RNeasy Mini Kit, Qiagen). The remaining 20 cultures were divided into four sets and each set was treated with one of five experimental media: induction medium alone ("IDX"), negative control induction medium containing 0.1% DMSO ("DMSO"), positive control induction medium containing 10 µM rosiglitazone (Cayman) ("ROSI"), experimental induction medium containing 10 µM BPA (Crescent) ("BPA10"), and experimental induction medium containing 100 µM BPA ("BPA100"). Induction medium was prepared using DMEM with 9% FBS, 0.1% 3-isobutyl-1-methylxanthine (IBMX) (Sigma Aldrich) dissolved in DMSO, 0.1% dexamethasone (Fisher brand) dissolved in DMSO, 0.05% human insulin (Sigma Aldrich). A time course was carried out. Sets were incubated for 1, 3, 48 or 72 hous at which point cells were trypsinized and RNA extracted.

Quantification and morphology

HUMSCs were expanded to allow for seeding of 6-well plates (BD Falcon) with a minimum of 4x10^5 cells per well under experimental conditions in triplicate in preparation for staining after

adiogenesis. Four different experimental media were used: IDX, IDX+ROSI, BPA10, BPA100. Seeding and differentiation of the HUMSCs was done concurrent with plating for experimental conditions for RNA extraction. Each experimental condition and timepoint was performed and stained in triplicate. HUMSCs were fed with experimental media every 3 to 4 days for two intervals within late termination phase of adipogenesis: 14 days and 21 days. Late termination was chosen to allow for optimal staining of lipid granules and vacuoles by Oil Red O.

At the end of each interval, wells were washed with PBS and cells were fixed with 10% formalin (Fisher) for 30 minutes and stained with Oil Red O. Modified Mayer's Hematoxylin (American MasterTech) was used as counterstain. Plates were then assessed under a light microscope and photographed. Five areas of each well were reviewed consistently: each quadrant and the center of the field. All cells in each image were counted. Three cells were selected from each image, measured in triplicate and averaged. Images were analyzed for quantification and morphology using ImageJ version 1.49n (National Institutes of Health, Bethesda, MD, USA).

Quantitative RT-PCR

Total RNA was extracted from baseline and experimental cultures at 0, 1, 3, 48 and 72 hours using RNeasy Mini Kit (Qiagen). RNA was quantified using a NanoDrop® 2000 UV-Vis Spectrophotometer (NanoDrop, Wilmington, DE, USA). All RNA concentrations were adjusted to 263.2 ng/ul. Prior to complementary DNA (cDNA) synthesis, 4 ug of RNA were treated with DNase I (Invitrogen) to eliminate genomic DNA contamination. Reverse transcription of the RNA was performed using SuperScript® First Strand Synthesis System for RT-PCR (Invitrogen) and remaining RNA was degraded using RNase H (Invitrogen). Sybr® Select Master Mix (Applied Biosystems) was added to primers specific to *PPARγ1* and *PPARγ2* for amplification and gene expression using a 7900HT Fast Real-Time PCR System. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as the reference gene (Jozefcuk and Adjaye 2011). Primer sequences for the adipogenic genes of interest were: *PPARγ1* Forward 5'-AAAGAAGCCAACATAAACC-3', *PPARγ1* and *PPARγ2* Reverse 5'-GGATCTCTCCGTAATGGAAG-3', *PPARγ2* Forward 5'-ATTCTCCTATTGACCCAGAAAGCG-3'.

Laboratory protocols

All laboratory protocols are available in Appendix A.

Statistical analysis

Pearson's correlations and paired samples t-tests were conducted to compare cells across timepoints and experimental conditions. For all analyses, we considered results with $p<0.05$ to be statistically significant. Analyses were performed using IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA).

Results

Umbilical cords were primarily collected from morning deliveries (92%). The majority of samples were considered "complete" with three umbilical cord sections collected (n=18, 49%). In three instances, the umbilical cords were of sufficient length to provide only one section (8%). The average umbilical cord section length was 8 cm.

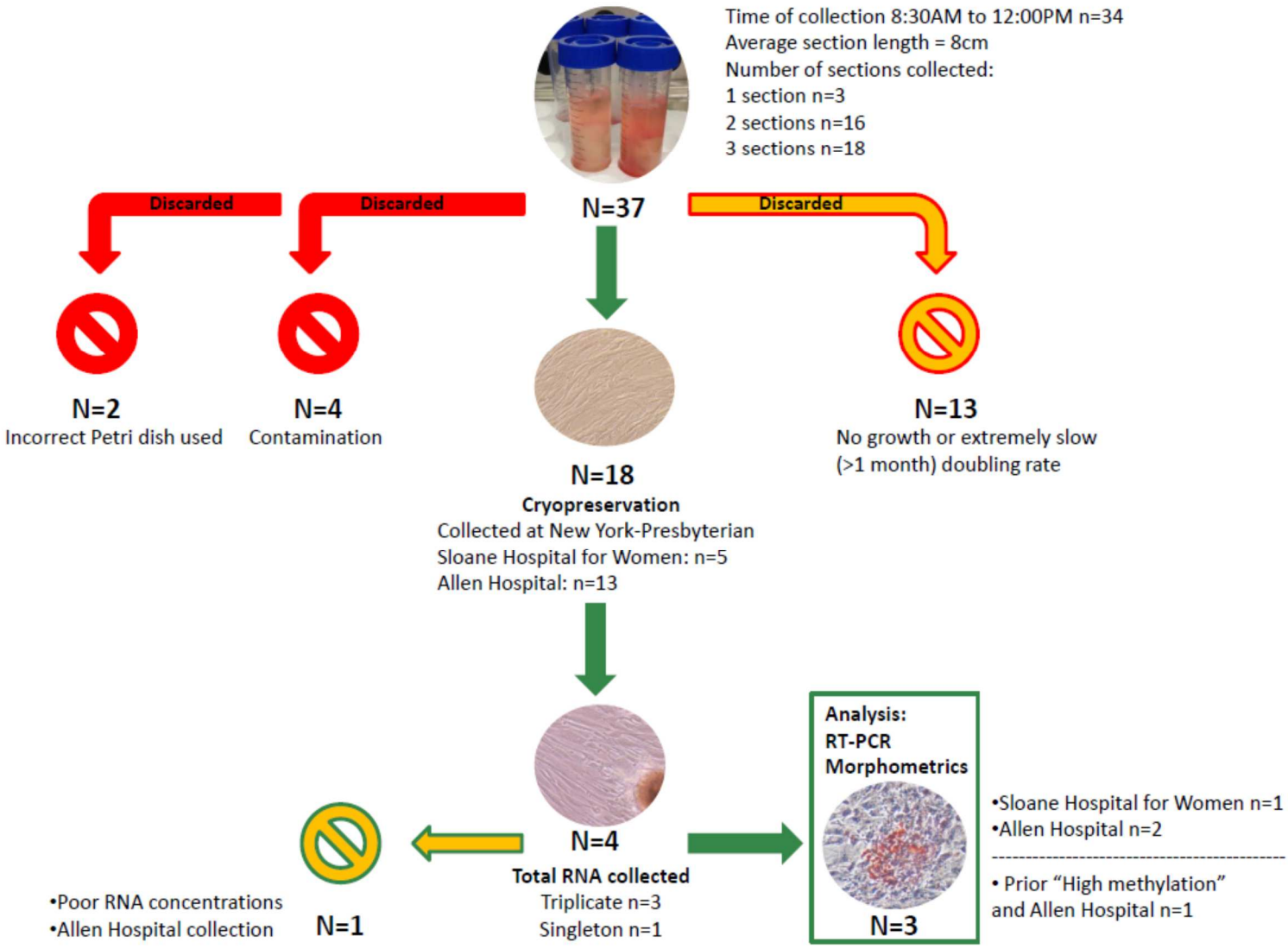
Of the 37 umbilical cords collected and cultured, 13 samples were not considered viable due to insufficient MSC doubling for cryopreservation. The first two samples were discarded due to laboratory supply error and an additional four samples were discarded due to contamination. The remaining 18 samples were cryopreserved in liquid nitrogen. Of the 18 cryopreserved samples, four samples were selected for the BPA experiments (see Figure 3). A concentration of 10 μM BPA was used to simulate a typical human exposure and the tenfold higher concentration 100 μM BPA was chosen to test for *in vitro* cytotoxic effects at a concentration albeit still considered within the range of expected human exposure based on the literature (Chamorro-Garcia et al. 2012; Teeguarden and Hanson-Drury 2013).

Cell characteristics

1. Cell count

A total of 11,676 cells from three umbilical cord samples were counted, with 5851 (50%) cells counted from samples stained at day 14 and 5825 (50%) cells counted from samples stained at day 21 (see Supplemental Table S1, Supplemental Figures S1a and S1b). Mean cell counts for each sample are displayed in Supplemental Figures S5a and S5b. Total cell counts for each sample are displayed in Supplemental Figures S6a and S6b. No differences in cell count were seen for experimental conditions compared to standard induction medium IDX at day 14 or day 21. A significant decrease in cell count was observed between day 14 and day 21 for ROSI ($t=4.85$ $p=0.04$; $t=4.450$ 0.05) and B100 ($t=9.07$ $p=0.01$) indicating possible issues with cell viability under the experimental conditions.

Figure 3. HUMSC Flow Chart



2. Cell surface area

Cellular surface area was measured in 216 cells with 81 cells measured for each experimental condition per timepoint (See Supplemental Table S1, Supplemental Figures S1a and S1b). A significant increase in cell surface area was seen under exposure to B100 as compared to exposure to standard induction medium IDX at day 14 (t=-37.02, 95% C.I. (-1357.73, -1075.02), p=0.001). No differences in cell surface area were seen for experimental conditions compared to IDX at day 21. Additionally, in cells exposed to B10, overall mean cell count at day 14 and overall mean cell surface area at day 21 were inversely correlated (r=-0.998 p=0.04), again indicating possible issues with cell viability under the experimental conditions.

RNA expression of PPARγ

Synthesis of cDNA and RT-PCR analysis of *PPAR γ1* and *PPARγ2* expression was conducted in triplicate on one sample. The sample was chosen prior to cell morphology analysis. Expression of *PPARγ1* increased under all experimental conditions at 48 and 72 hrs, with a twofold increase seen for cells exposed to B10 by 72 hrs (see Supplemental Figure S4a). The increase in expression observed for cells exposed to B10 was higher than that observed for cells exposed to the positive control of ROSI. *PPARγ2* expression negligibly increased only for cells exposed to ROSI (see Supplemental Figure S4b).

Discussion

Trying to understand the underlying causes of the obesity epidemic has led some investigators to consider the possibility that exposure to environmental endocrine disruptors, such as BPA, may play a role in the process. In our study, we tested the hypothesis that BPA exposure would act to promote adipogenesis in primary mesenchymal cells by altering *PPARγ* expression. We also measured morphological changes in these cells in response to BPA exposure.

No differences in cell counts were observed for experimental conditions compared to standard induction medium at day 14 or day 21. However, cell count depletion at day 21 was seen across all experimental conditions suggesting that the adipogenic medium may not have been able to sustain adipocytes as they entered maturation in the late terminal differentiation phase. The finding that cells exposed to B10 had an inverse correlation between overall mean cell count at day 14 and overall mean cell surface area at day 21 suggests that cell signaling and communication may have been influenced

either by exposure or by the death of neighboring cells. While a significant decrease in cell surface area was observed for B10 compared to IDX at day 14, differences in cell surface area were not observed at day 21.

Consistent with cell morphology results for B10 exposure, *PPARγ1* expression was highest for this exposure at 48 and 72 hrs, with a two-fold effect seen by 72 hrs. The literature notes that *PPARγ1* is continuously expressed in a variety of tissues and specifically plays a role in both the determination phase and terminal phase of adipogenesis (Morrison and Farmer 1999; Wang et al. 2014). In addition, while it is believed that *PPARγ2* is activated at the commencement of terminal differentiation, *in vivo* studies suggest that in the absence of *PPARγ2*, *PPARγ1* can compensate (Mueller et al. 2002; Zhang et al. 2004). Thus, while *PPARγ2* was not expressed in appreciable amounts by 72 hrs, the results for *PPARγ1* expression are in accordance with current literature.

This study has successfully replicated the ability to culture mesenchymal stem cells from freshly harvested human umbilical cords. A main strength of this study is that it provides proof of concept that a multi-stage experiment on the effect of BPA exposure on adipogenesis in mesenchymal cells is feasible. In studying adipogenesis, there are essentially no non-invasive approaches to acquiring primary target tissue. Epigenetic studies are also limited to easily acquired tissue, such as blood. However, blood and other surrogate tissues raise questions of relevance. In fact, a previous study by us demonstrated that methylation patterns in the promoter regions of *PPARγ* and a second gene involved in adipogenesis, *delta-like protein 1* (*DLK1)*, differed greatly between white blood cells and mesenchymal cells isolated from umbilical cords (Martinez et al. in preparation). Thus, an additional strength of this study is the collection of umbilical cord tissue, an otherwise discarded tissue, which enabled the study of the progenitor to the target adipose tissue. Finally, the samples were harvested anonymously which allowed for the rapid collection of 37 fresh samples within four months.

Conversely, anonymous collection was also a limitation, as the analysis could not take into account maternal demographics, method of delivery, gestational age, parity, maternal anthropometrics or maternal medical history. The two different hospital locations from which samples were collected also have different characteristics. The maternity patients at Sloane Hospital for Women tend to have high risk pregnancies, whereas maternity patients at Allen Hospital may have more heterogenous pregnancy

risks. Additionally, the collection rate at Sloane Hospital for Women was slower than that at Allen Hospital, perhaps due to the greater concern of staff with attending to the medical needs of high risk deliveries. Therefore, there may have been collection bias with the umbilical cord samples.

Additonal limitations are the natural variability encountered in fresh samples used *in vitro* and development of lab techniques, at times *ad hoc*, to account for the variations between samples. The quantity, length, thickness, coiled nature and bloodiness of umbilical cord sections varied between samples. Also, mesenchymal stem cell cultures did not reach subconfluence in a uniform amount of time. Each sample had a different rate of doubling meaning there were inconsistent doubling rates leading to P2 and cryopreservation. Thus, the time it took for each sample to reach P2 may be dependent on a number of factors. While the circumstances involved in each sample collection were recorded, without the analysis of a larger sample size it is not possible to determine which factors may be most important to control for during collection and culturing.

Laboratory supplies complicated technique. As this was a study of BPA exposure and BPA is a component of many plastics, it was important to be sure the laboratory materials were made of plastic that is not known or believed to contain BPA. Other than the few exceptions when glass materials could be used (i.e. beakers, graduated cylinders, flasks), plastic supplies were made of polypropylene, polyethylene or polystyrene. An unintended laboratory supply problem was the lack of consistent availability of FBS. As noted in the Cell Culture section of the Methods, two brands of FBS were used to make the medium used in culture. The Lonza brand of FBS was initially available but after approximately one year of working with the cultures, Lonza discontinued their FBS. The Fisher brand of FBS was selected to replace the Lonza brand of FBS. It is unknown whether the change in FBS brand may have had an effect on the doubling rate, metabolism or adipogenesis of the HUMSCs.

A number of techniques necessitated troubleshooting as this is the first known study to investigate BPA exposures during HUMSC adipogenesis. Although the time to finalize umbilical cord collection was rapid (4 months), the HUMSCs had to be frozen in liquid nitrogen until staining techniques and RNA extraction protocols were tested. It is unknown whether long term cryopreservation of HUMSCs may affect their ability to thrive in culture. However, it was noted anecdotally that higher RNA concentrations were detected via spectrophotometry prior to liquid nitrogen freezing compared to post-

thaw. This necessitated the unanticipated inclusion of further concentration of RNA via a SpeedVac® Concentrator (Savant Instruments, Farmingdale, NY, USA) and testing of that protocol using serial dilutions. If RNA was concentrated with the SpeedVac, the RNA concentrations were measured again using an Epoch Spectrophotometer (BioTek) which lended efficiency by allowing the measurement of up to 18 samples simultaneously. However, the use of the Epoch Spectrophotometer also meant using 1.5 to 2.0 ul of sample rather than the 1ul of sample needed for the single sample measurement in the NanoDrop®.

A number of RT-PCR conditions were tested for appropriateness. Primers had to be evaluated prior to the final experiment. Informed by the literature (Chamorro-Garcia et al. 2012; Leyvraz et al. 2010) and prior experiments, 3 different sets of *PPARγ1* and *PPARγ2* forward and reverse primer pairs were built using the UCSC Genome Bioinformatics database and tested for robustness of expression results. While the cell morphometrics were determined by a single unblinded researcher, per the original research plan. Since the results were not confirmed by a second blinded investigator, there may be unintentional researcher bias based on known experimental condition. Lastly, it is evident that with the small number of samples, results cannot be considered conclusive. However, the results do give an early insight into the potential effects of BPA exposure on adipogenesis in a relevant target tissue.

Were this feasibility study of the effect of BPA exposure on adipogensis in HUMSCs continued as a larger scale experiment with an increased sample size, possibly as part of an epidemiological study, there are a number of ideal conditions one should take under advisement.

- Purchase all laboratory supplies in advance and in bulk. This will avoid the possibility of delays due to shipping and handling, out of stock materials, or discontinuation of materials.
- If it is not possible to use only glass laboratory supplies, insure that all laboratory supplies are made of a plastic known or believed not to contain BPA, such as polypropylene, polyethylene or polystyrene.
- Compare the in-house laboratory "recipe" medium to commercially available complete medium under experimental conditions, i.e. use the two types of media in parallel experiments for staining purposes and total RNA extraction.

- Do not conduct an anonymous umbilical cord collection. It would be more desirable and strengthen the results to be able to analyze the data baed on variations due to factors which the researcher can design the study to control for and which cannot be controlled for including but not limited to: gestational age, race/ethnicity, maternal age, maternal medical conditions, gravidity and parity, season of delivery, time of day of collection, maternal BPA exposure (lifetime vs during pregnancy).
- Standardize the amount of elapsed time between umbilical cord collection and laboratory processing of the cord sections. While the method stated by deBruyn et al. (2011) had an elapsed time of 24 hours and the current feasibility study did not exceed an elapsed time of 12 hours, it may be more prudent to standardize the protocol with an even shorter elapsed time.
- The staining protocol in Appendix A was written for triplicate samples on a 6 well plate. It may be more efficient to use an alternate plate such as a 12 well plate.
- Do not conduct the experiment post-cryopreservation of the subconfluent HUMSCs. Rather, make every effort to run the BPA exposure experiments on HUMSCs that have never been cryopreserved. Alternatively, it would be advisable to run parallel experiments comparind never cryopreserved HUMSCs to cryopreserved HUMSCs to determine if there are any differences due to cryopreservation medium and freeze/thaw techniques.

Conclusion

The results of this feasibility study are the first to demonstrate an alternative successful method of investigating effects of BPA exposure on adipogenesis in target tissue. We found that exposure of HUMSCs to a 10 µM concentration of BPA during adipogenesis induced *PPARγ1* gene expression during the transition into the early terminal differentiation phase of adipogenesis. Having overcome challenges to implementing experimental strategies, this study serves as a solid foundation for future experiments. For this reason, detailed study protocols are provided in Appendix 1. Further studies should be conducted to elucidate the effects of a wider range of BPA concentrations over a larger number of adipogenesis timepoints. Future investigations will advance our understanding of how BPA may be contributing to the obesity pandemic.

Acknowledgements

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Supplemental Material

Bisphenol A, Adipogenesis and *Peroxisome Proliferator-Activatated Receptor Gamma* Expression in Human Umbilical Cord Mesenchymal Stem Cells: A Feasibility Study

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Table S1. Cell characteristics

Cell Count Mean (SD)	**IDX**	**ROSI**	**B10**	**B100**
Day 14	205 (±134)	199 (±126)	312 (±183)	192 (±91)
Day 21	144 (±146)	107 (±99)	120 (±89)	116 (±102)
Cell Surface Area (μm^2) Mean (SD)	**IDX**	**ROSI**	**B10**	**B100**
Day 14	9.04×10^3 ($\pm 4.93 \times 10^3$)	8.56×10^3 ($\pm 4.42 \times 10^3$)	10.41×10^3 ($\pm 7.43 \times 10^3$)	10.26×10^3 ($\pm 4.80 \times 10^3$)
Day 21	15.32×10^3 ($\pm 12.93 \times 10^3$)	13.43×10^3 ($\pm 11.29 \times 10^3$)	17.43×10^3 ($\pm 12.20 \times 10^3$)	14.41×10^3 ($\pm 10.48 \times 10^3$)

Figure S1a. Adipogenesis at day 14 (10x)

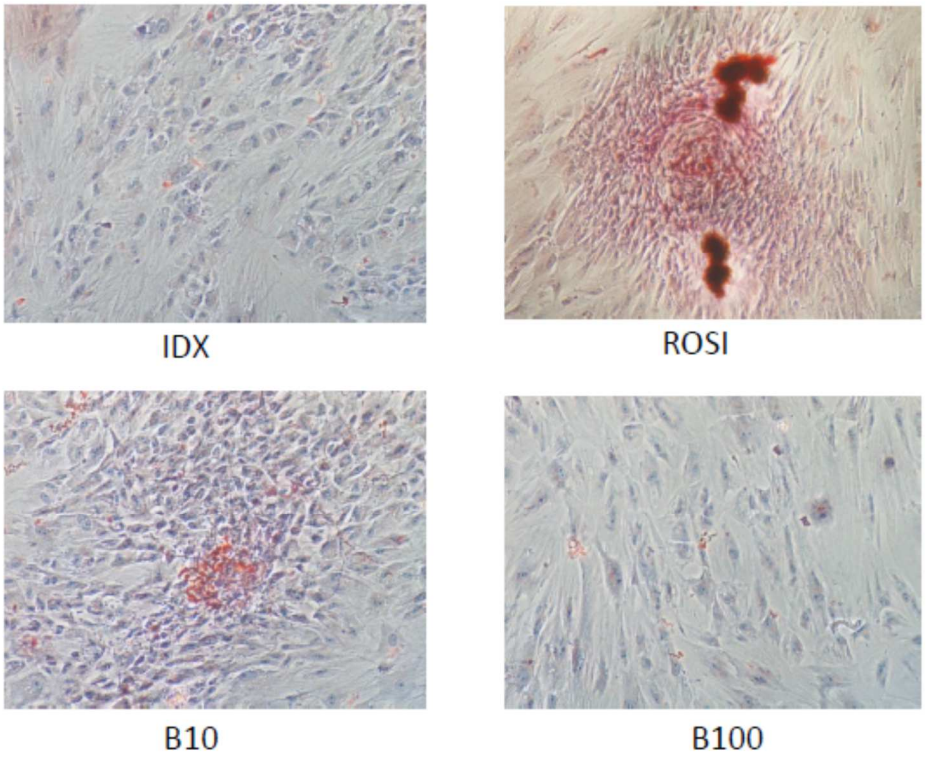


Figure S1b. Adipogenesis at day 21 (10x)

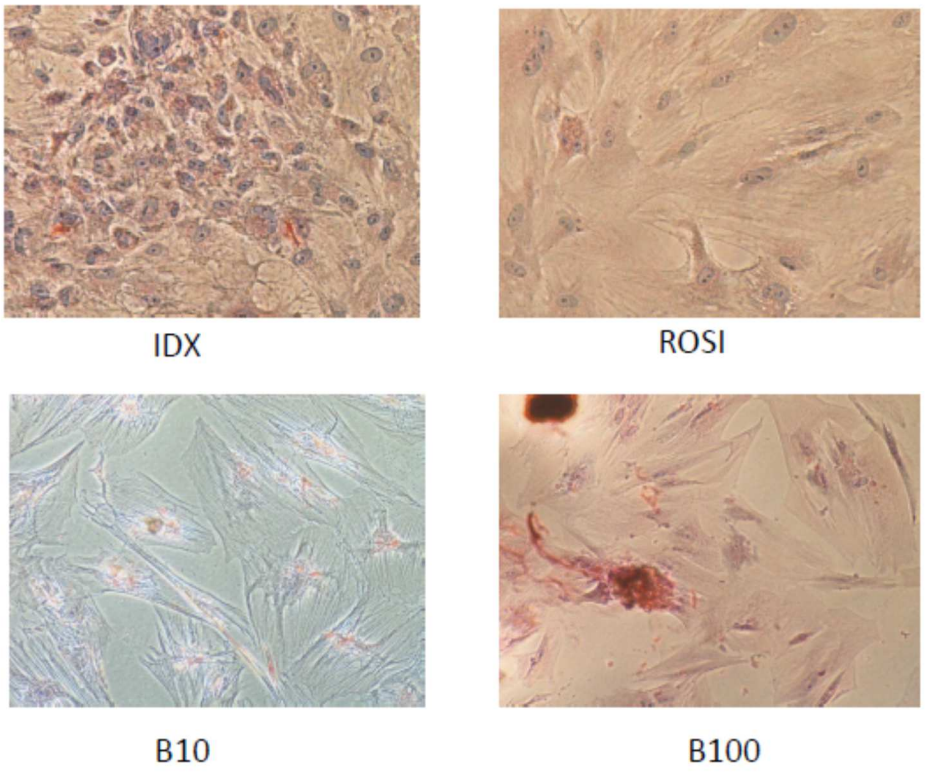
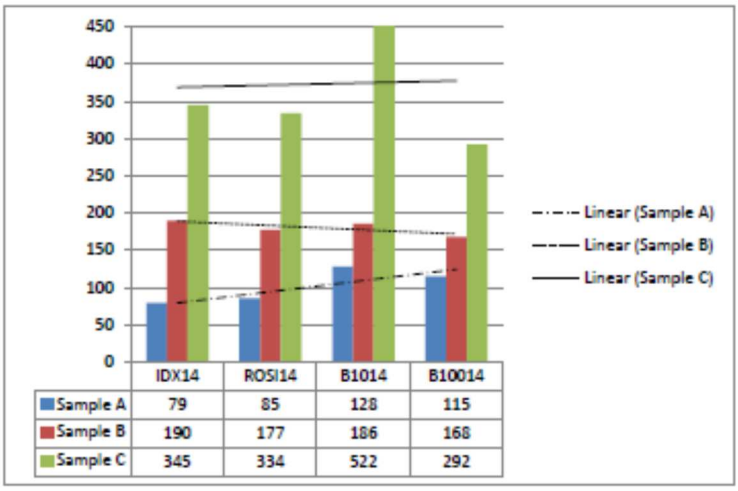
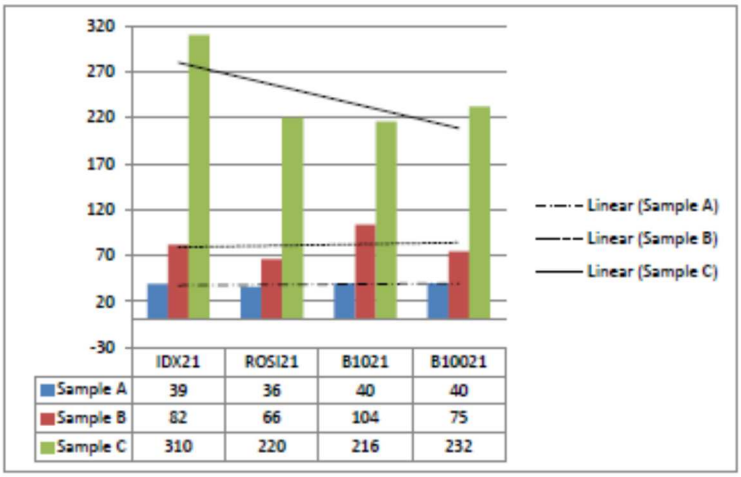


Figure S2a. Mean cell counts at day 14 for each sample*



	IDX14	ROSI14	B1014	B10014
Sample A	79	85	128	115
Sample B	190	177	186	168
Sample C	345	334	522	292

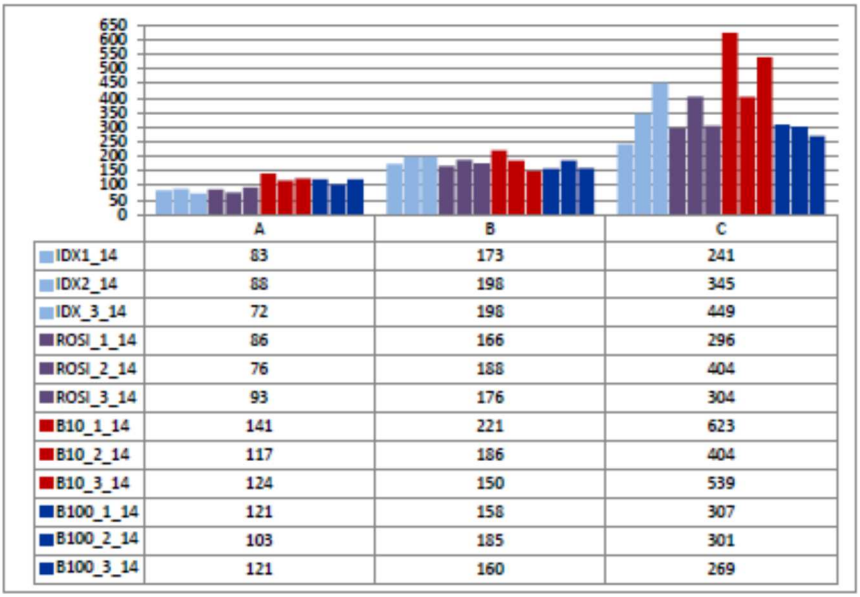
Figure S2b. Mean cell counts at day 21 for each sample*



	IDX21	ROSI21	B1021	B10021
Sample A	39	36	40	40
Sample B	82	66	104	75
Sample C	310	220	216	232

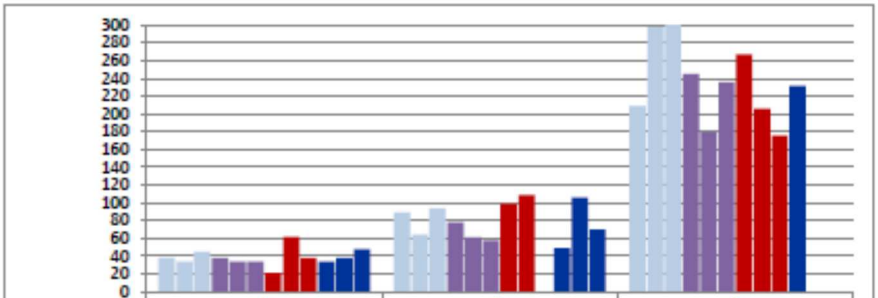
* Sample Characteristics:
Sample A: morning delivery at Sloane Hospital for Wome, 2 umbilical cord sections of 8 cm each, 7h45m between collection and plating, 1.5 months to P2 cryopreservation, classified as "good growth"
Sample B: morning delivery at Allen Hospital, 2 umbilical cord sections of 7 cm each, umbilical cord sections were twisted with appearance of possible fat tissue on 1st section and 2nd section was difficult to incise and score, 4h between collection and plating, 2.5 months to P2 cryopreservation, cultured during technical difficulties with incubator, classified as "good growth"
Sample C: morning delivery at Allen Hospital, 3 umbilical cord sections of 7 cm each, possible fat tissue on section 2/3, 6h between collection and plating, 2.5 months to P2 cryopreservation, cultured during technical difficulties with incubator, classified as "good growth" and "high methylation"
Samples B and C were collected on the same day, 2 hours apart

Figure S3a. Total cell counts at day 14 for each triplicate*



	A	B	C
IDX1_14	83	173	241
IDX2_14	88	198	345
IDX_3_14	72	198	449
ROSI_1_14	86	166	296
ROSI_2_14	76	188	404
ROSI_3_14	93	176	304
B10_1_14	141	221	623
B10_2_14	117	186	404
B10_3_14	124	150	539
B100_1_14	121	158	307
B100_2_14	103	185	301
B100_3_14	121	160	269

Figure S3b. Total cell counts at day 21 for each triplicate*



	A	B	C
IDX_1_21	38	89	209
IDX_2_21	34	64	298
IDX_3_21	45	94	305
ROSI_1_21	38	78	245
ROSI_2_21	34	61	179
ROSI_3_21	34	58	236
B10_1_21	21	99	267
B10_2_21	62	109	206
B10_3_21	38		176
B100_1_21	34	49	232
B100_2_21	38	106	
B100_3_21	48	70	

* Sample Characteristics:
Sample A: morning delivery at Sloane Hospital for Wome, 2 umbilical cord sections of 8 cm each, 7h45m between collection and plating, 1.5 months to P2 cryopreservation, classified as "good growth"
Sample B: morning delivery at Allen Hospital, 2 umbilical cord sections of 7 cm each, umbilical cord sections were twisted with appearance of possible fat tissue on 1st section and 2nd section was difficult to incise and score, 4h between collection and plating, 2.5 months to P2 cryopreservation, cultured during technical difficulties with incubator, classified as "good growth"
Sample C: morning delivery at Allen Hospital, 3 umbilical cord sections of 7 cm each, possible fat tissue on section 2/3, 6h between collection and plating, 2.5 months to P2 cryopreservation, cultured during technical difficulties with incubator, classified as "good growth" and "high methylation"
Samples B and C were collected on the same day, 2 hours apart

Figure S4a. *PPARγ1* expression*

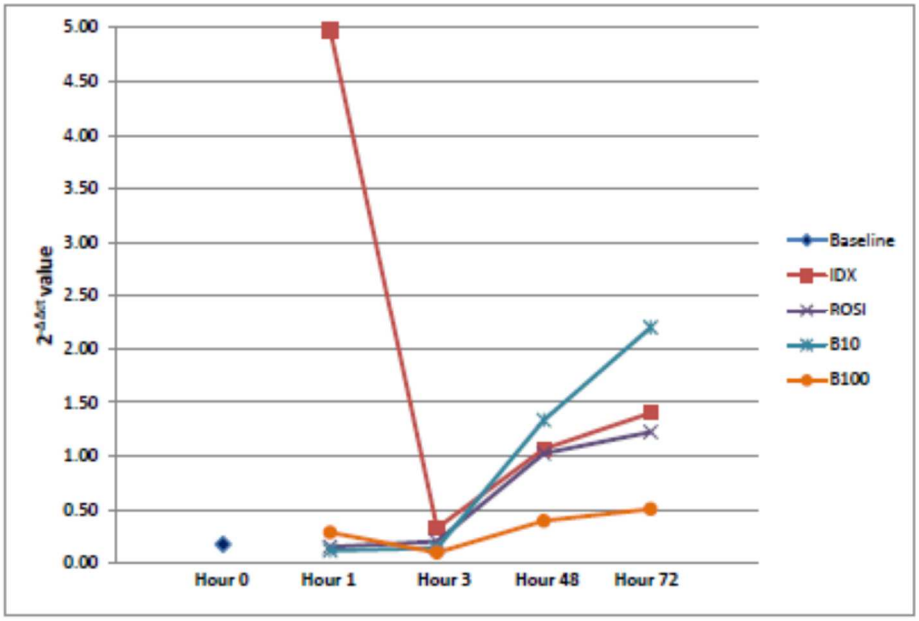
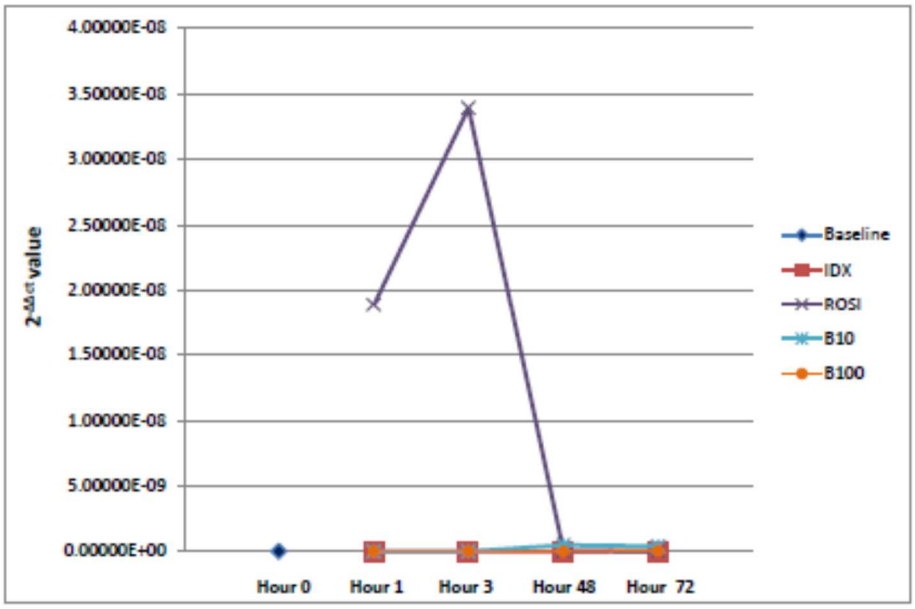


Figure S4b. *PPARγ2* expression*



* Sample Characteristics:
Sample C: morning delivery at Allen Hospital, 3 umbilical cord sections of 7 cm each, possible fat tissue on section 2/3, 6h between collection and plating, 2.5 months to P2 cryopreservation, cultured during technical difficulties with incubator, classified as "good growth" and "high methylation"

Chapter 4: Bisphenol A and Adiposity in an Inner-City Birth Cohort

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Abstract

Background: It has been suggested that early life exposure to the endocrine disruptor bisphenol A (BPA) may contribute to development of obesity. Prospective evidence in humans on this topic is limited.

Objectives: We prospectively examined prenatal and early childhood BPA exposures in relation to childhood measures of adiposity in the Columbia Center for Children's Environmental Health (CCCEH) New York City birth cohort.

Methods: BPA concentrations were measured in prenatal (n=375) and child ages 3 (n=408) and 5 years (n=518) spot urine samples. Childhood anthropometric outcomes included body mass index z-scores (BMIZ) at 5 and 7 years, and fat mass index (FMI), percent body fat (%BF), and waist circumference (WC) at 7 years. Multiple linear regression was used to evaluate associations.

Results: Prenatal urinary BPA concentrations were positively associated with child age 7 FMI (beta=0.31 kg/m^2, p-value=0.04, [95%CI 0.01, 0.60]), %BF (beta=0.79, p-value=0.04, [95%CI 0.03, 1.55]), and WC (beta=1.29 cm, p-value=0.01, [95%CI 0.29, 2.30]), but not with birth weight, age 5 year BMIZ, age 7 year BMIZ, or change in BMIZ between ages 5 and 7 years (all p-values > 0.1). Child urinary BPA concentrations were not associated with childhood anthropometric outcomes (all p-values > 0.05).

Conclusions: Analyses of the CCCEH longitudinal birth cohort found associations between prenatal urinary BPA concentrations and FMI, %BF and WC. Our results suggest that prenatal BPA exposure may contribute to developmental origins of adiposity. These findings are consistent with a number of prior studies and raise concern about the pervasiveness of this endocrine-disrupting chemical.

Introduction

Obesity in children, defined by a body mass index (BMI) greater than or equal to the 95th percentile for age and sex, is an epidemic of great concern in the United States. According to the Robert Wood Johnson Foundation, childhood obesity rates (ages 2-19 years) have more than tripled between the years 1980 and 2010 (RWJF 2012). Long-term risks of childhood obesity include metabolic syndrome, type 2 diabetes, cardiovascular disease and reduced adult life expectancy, with children of minority status at greater risk for becoming obese (Strauss and Pollack, 2001; Singh et al. 2010; Shaibi et al. 2007). The 2007-2008 overall prevalence of obesity was 20.3% and prevalence of overweight was 17.6% for New York City (NYC) public school children, with African American and Hispanic children having higher odds of being obese than white children (O.R.=1.11 and O.R.=1.48, respectively) (Rundle et al. 2012). While overeating and inactivity play a role, they do not fully explain the obesity epidemic (Gillman et al. 2008; Janesick and Blumberg 2011). It has been hypothesized that early life exposure to endocrine disruptors, such as bisphenol A (BPA), may be contributing to the obesity epidemic (Grün and Blumberg 2006). Endocrine disruptors may also lead to altered metabolic regulation early in life (Chevalier and Fénichel 2014).

BPA is a known endocrine disrupting chemical and a key component in polycarbonate plastics and epoxy resins which are commonly used in consumer plastic products resulting in significant human exposure (Calafat et al. 2008; Vandenberg et al. 2007, 2010). Exposure pathways include oral, dermal and inhalation, with dietary intake the primary route (Wilson et al. 2003; Zalko et al. 2011). Prenatal exposure may occur when conjugated BPA crosses the placenta *in utero* or by use of catheters during delivery (Ikezuki et al. 2002; Schönfelder et al. 2002; Vandentorren et al. 2011).

Cross-sectional epidemiological studies suggest that BPA exposure is associated with obesity in children (Bhandari et al. 2013; Eng et al. 2013; Li et al. 2013; Trasande et al. 2012; Wang et al. 2012; Wells et al. 2013). However, because diet is an exposure route, reverse causality cannot be ruled out as an explanation for these cross-sectional findings as obese children may have greater exposure to BPA due to higher dietary intakes. Existing longitudinal data on effects of prenatal BPA exposure on postnatal adiposity are limited due to dissimilarities in outcome measures (Braun et al. 2014; Chou et al. 2011;

Harley et al. 2013; Snijder et al. 2013; Lee et al. 2013; Philippat et al. 2014; Tang et al. 2013; Valvi et al. 2013).

We hypothesized that prenatal and early childhood BPA exposure would be positively associated with childhood adiposity at ages 5 and 7 years in a NYC inner-city birth cohort.

Methods

Study design and population

Study subjects are participants in the prospective birth cohort of the Columbia Center for Children's Environmental Health (CCCEH) in Northern Manhattan and the South Bronx, New York. Mother-child pairs were selected if a maternal prenatal urine sample was analyzed for BPA (n=375). We additionally included children if BPA concentration had been measured in urine samples collected at child age 3 (n=408) and/or 5 years (n=518). The CCCEH study design is described elsewhere (Perera et al. 2003; Whyatt et al. 2003). Briefly, we enrolled 727 women ages 18–35 years old during their third trimester of pregnancy. Women were included if they self-identified as either African American or Dominican and had resided in Northern Manhattan or the South Bronx for at least 1 year before pregnancy. Exclusion criteria included mother's report of: cigarette smoking or use of other tobacco products during pregnancy, illicit drug use, diabetes, hypertension, known HIV, or a first prenatal visit after the 20th week of gestation. Medical records of the mother and infant at delivery were abstracted by the research staff to ascertain prenatal medical history and birth outcomes. For the first five years of the CCCEH study, we contacted enrolled participants every 3 months after delivery for questionnaire follow-up and every 6 months thereafter, with biological samples collected biennially from the children beginning at age 3 years. We have documented loss to follow-up status and reasons for missed intervals.

Data collection was completed through age 7 years in August 2013 and laboratory measurement of urinary BPA has been completed for all samples. Study procedures, questionnaires and collection of biological samples were explained to each subject at enrollment and a signed consent, approved by the IRB of Columbia University Medical Center and the Centers for Disease Control and Prevention (CDC), was obtained.

Urinary biomarker collection

For mothers, urine was collected during the third trimester of pregnancy between 1999 and 2006 (mean gestational age: 34 weeks; SD: 3) and concurrent with administration of the baseline questionnaire by trained bilingual interviewers. In children, urine was collected concurrent with follow-up questionnaires between 2001 and 2010. All samples were spot urine samples collected at varying times of day. The date, but not the exact time of collection, was available.

The total (free plus conjugated) BPA urinary concentrations (ng/mL) were measured as previously described (Calafat et al. 2008). The limit of detection (LOD) was 0.4 ng/mL. Specific gravity, as a measure of urinary dilution, was quantified at room temperature at Columbia University with a handheld refractometer (PAL 10-S, Atago, Bellevue, WA).

We also measured the following metabolites of di-2-ethylhexyl phthalate (DEHP) in the urine samples at CDC as previously described as they were previously shown to be correlated with BPA: mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), and mono-2-ethyl-5-oxohexyl phthalate (MEOHP) (Braun et al. 2011; Hoepner et al. 2013; Kato et al. 2005; Silva et al. 2007). The LODs ranged from 0.2 to 1.2 ng/mL. BPA was below LOD in urine prenatally (6%) and at childhood ages 3 years (2%) and 5 years (2%). For the few BPA and DEHP metabolotes results below LOD, the value of LOD/2 was substituted, consistent with prior analyses (Whyatt et al. 2003; Whyatt et al. 2009).

Anthropometric measurements

Weight prior to pregnancy was self-reported by the mother during the third trimester. Birth weight was collected from the hospital medical record. We collected body size measurements and/or body composition data for children aged 5 years (n=498) and 7 years (n=511). A Detecto Cardinal 750 digital scale/stadiometer (Cardinal Scale Manufacturing Company, Webb City, MO) was used to collect standing height measurements at ages 5 and 7 years until January 2010. After January 2010, we assessed standing height with the Holtain-Harpenden Wall Mounted Stadiometer Counter 602VR (Holtain Limited, Crymych, UK). Body composition values, including fat mass, were collected with the Tanita Digital Body Mass Indicator Scale BC-148 (Tanita Corporation of America, Arlington Heights, IL) at age 7 years. Children wore light clothing and no shoes. Body mass index z-scores (BMIZ) for study children were

quantified based on reference data from the CDC growth charts using the SAS programs provided by the CDC (C.D.C., 2011). Fat mass index (FMI) was calculated using the algorithm [fat mass(kg)/height(m)^2] (VanItallie et al. 1990). Waist circumference (WC) was measured at a level midway between the lower rib margin and iliac crest using a non-stretchable measurement tape.

Statistical analysis

The values for BPA and the molar sum of DEHP metabolite concentrations (∑DEHP) were natural log-transformed for analysis to correct for their non-normal distribution. We standardized the specific gravity values by z-score transformation in order to stabilize the estimate of the model constant. The standardized specific gravity values were included in regression models as a covariate except in the case of analyses using tertiles of BPA concentrations as the predictor variables. As using percentiles is a method of ordering by rank, we arithmetically adjusted BPA concentrations at each urine collection interval to calculate tertiles by first accounting for urinary dilution and then natural log-transforming using the formula: Specific gravity adjusted/log-transformed BPA=LN(BPA $_{raw}$ * [(mean specific gravity for population at collection interval-1)/(individual specific gravity-1)]) (Hauser et al. 2004). In other words, the prenatal BPA concentration was first specific gravity adjusted through the above formula and the result was natural-log transformed.

For childhood BPA exposure, we calculated the mean of BPA concentrations at ages 3 and 5 years. In the absence of two childhood urinary BPA concentrations, the single existing concentration was used (n=51).

Linear regression analyses were conducted to determine whether maternal prenatal urinary BPA concentrations predicted birth weight, BMIZ at ages 5 and 7 years and the change in BMIZ (ΔBMIZ) from age 5 to 7 years, as well as FMI, percent body fat (%BF) and WC at age 7 years. Additionally, linear regression was used to analyze whether child urinary BPA concentrations from age 3 years or the mean of ages 3 and 5 years predict BMIZ at ages 5 and 7 years, ΔBMIZ from age 5 to 7 years, and FMI, %BF and WC at age 7 years.

The decision to evaluate maternal prenatal urinary BPA concentrations separately from child was informed by our prior analyses in the CCCEH birth cohort confirming prenatal BPA concentrations and childhood BPA concentrations were not correlated and prenatal BPA concentrations were significantly

lower than those of their children (Hoepner et al. 2013). However, we separately assessed as potential confounders the prenatal BPA concentrations in childhood predictor regression models and the childhood BPA concentrations in the prenatal predictor regression models and found no confounding. In addition, we did not find an association between prenatal BPA concentrations with maternal weight gain during pregnancy therefore we did not consider it to be a confounder variable in our analysis.

Variables were included in the model if they were significantly ($p<0.05$) associated with the outcome or caused a greater than 10% change in the effect size of the predictor variable. The following variables were controlled for in the analyses of childhood anthropometric measures at ages 5 and 7 years: specific gravity, ln ∑DEHP, ethnicity, dichotomous pre-pregnancy obesity status, child sex, birth weight, and gestational age. Additionally, we controlled for height in the analyses of %BF and WC at age 7 years. To assess the representativeness of the subset analyzed, T-tests and chi-square tests were performed to compare subjects with and without prenatal and postnatal BPA concentrations and with and without anthropometric outcomes at 5 years and 7 years of age.

We stratified analyses by child sex in order to explore differences in associations by sex that had been found in other studies (Harley et al. 2013). Based on our prior analyses showing significantly higher BPA concentrations (prenatal, 5 year and 7 year) in African Americans versus Dominicans, we also stratified our models by race/ethnicity (Hoepner et al. 2013). Possible interaction between BPA concentration and child sex were assessed for all BMIZ and 7 year body composition outcomes.

We conducted sensitivity analyses using baseline data and logistic regression models to estimate inverse probability weighting (IPW) for successful follow-up to assess potential bias of effect estimates due to loss to follow-up and missing anthropometric data (Curtis et al. 2007; Hernan et al. 2004; Robins et al. 2000; Rundle et al. 2012; Widen et al. 2015). To estimate the weights, we included all variables in each of our final models, mother's satisfaction with living conditions, mother's years of school completed at time of pregnancy, as well as geographic information system variables using 2000 US Census block group data aggregated to 1 km radial neighborhood buffers around the home: linguistic isolation and neighborhood socioeconomic status (Rundle et al. 2012).

For all analyses, we considered results with $p<0.05$ to be statistically significant. Analyses were performed using IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA) and Stata version 13.0 (StataCorp, College Station, TX, USA).

Results

Demographic and clinical characteristics of the 369 participants with prenatal BPA concentrations and available birth outcomes data are presented in Table 1. Table 2 shows the characteristics of the study participants grouped by BPA concentration interval and age at which anthropometric measures were collected. There was no significant difference between subjects with and without prenatal and postnatal BPA concentrations and those with and without anthropometric outcomes at 5 years and 7 years of age.

Prenatal BPA concentration vs. child anthropometry

Prenatal BPA concentrations were not associated with birth weight (see Supplemental Material, Table S1). Results from analyses of fully adjusted ln-transformed prenatal BPA concentrations and child anthropometric outcomes are shown in Table 3. Overall, prenatal BPA concentrations were not significantly associated with BMIZ at ages 5 years or 7 years or ΔBMIZ from 5 to 7 years. Prenatal BPA concentration was positively associated with FMI at age 7 years (beta=0.31 kg/m^2, p-value=0.04, [95%CI 0.01, 0.60]). Prenatal BPA concentration was also positively associated with %BF at age 7 years (beta=0.79, p-value=0.04) and with WC at age 7 years (beta=1.29 cm, p-value=0.01).

To determine whether the FMI results were due to the fat mass or the height component of the index, we substituted the fat mass itself as the dependent variable in the model. Prenatal BPA concentrations were positively associated with fat mass regardless of the addition of height as a covariate (adjusting for height: beta=0.55 kg, p-value=0.02; without adjustment for height: beta=0.57 kg, p-value=0.03). Additionally, we analyzed the relationship between prenatal BPA concentrations and height, but observed no association.

Prenatal tertile analysis

Linear regression analysis using tertiles of prenatal BPA concentrations as predictor variables, with the first tertile as the reference, suggested positive linear associations with FMI, percent body weight and WC (see Table 3).

Prenatal differences by child sex and race/ethnicity

After stratifying by sex, among girls there was a positive association between prenatal BPA concentrations and 7 year FMI and WC (see Supplemental Material, Table S2). Among boys, there was no association between prenatal BPA concentrations and anthropometric outcomes. The interaction between sex and BPA concentrations was significant as seen in Supplemental Material, Table S3. Stratification by race/ethnicity, showed a positive association for Dominicans between prenatal BPA concentrations and 7 year FMI, %BF and WC (see Supplemental Material, Table S2). Among African Americans, there was no association between prenatal BPA concentrations and anthropometric outcomes. The interaction between race/ethnicity and BPA concentrations was significant as seen in Supplemental Material, Table S4.

Postnatal BPA concentration vs. child anthropometry

Child BPA concentrations had a borderline negative association with ΔBMIZ from 5 to 7 years (beta=-0.10, p-value=0.06, [95%CI -0.02, 0.005]). Child BPA concentrations were not associated with FMI, %BF or WC at age 7 years (see Supplemental Material, Table S5).

Postnatal tertile analysis

Linear regression analysis using tertiles of child BPA concentrations as predictor variables, with the first tertile as the reference, was consistent with the borderline negative association with ΔBMIZ from 5 to 7 years, showing that higher exposure may be associated with lower ΔBMIZ (see Supplemental Material, Table S5).

Postnatal differences by child sex and race/ethnicity

When we conducted analyses stratified by sex and ethnicity we found a negative association between child urinary BPA concentrations and ΔBMIZ from 5 to 7 years for girls and no associations for boys (see Supplemental Material, Table S6). We observed a borderline negative association between child urinary BPA concentrations and ΔBMIZ from 5 to 7 years for African Americans and no associations for Dominicans (see Supplemental Material, Table S6). Stratification by race/ethnicity revealed a positive association of child BPA concentrations with BMIZ at 5 years in African Americans (see Supplemental Material, Table S6). However, interaction terms for postnatal BPA concentrations and sex or race/ethnicity were not significant.

IPW Analysis

Additionally, weighting the data by the inverse probability of follow-up and complete anthropometric and biomarker data collection by 7 years did not appreciably change the size of the effect estimates (data not shown).

Discussion

Among boys and girls, we observed a positive association between prenatal urinary BPA concentration and childhood FMI, %BF and WC at age 7 years. This result suggests that prenatal BPA exposure is associated with overall body fat and central adiposity, accounting for height. It is notable that we used the CDC growth chart data for our BMIZ statistical comparison. While the CDC revised the original 1977 growth chart by adding more nationally representative children in 2000, the majority of the underlying reference group is still comprised of data from children as of 1975 (Kuczmarski et al. 2000). Thus, the standard curve of the CDC growth chart has moved to the right and more than 5% of childen are above the 95th percentile. However, contrary to our hypotheses, we found that maternal urinary BPA concentrations were not associated with birth weight, childhood BMIZ at ages 5 and 7 years, and ΔBMIZ from age 5 to 7 years. The differences in results across anthropometric outcomes may reflect differences in the construct validity of body composition versus BMI as measures of child adiposity. BMIZ alone may not be the best measure of adiposity in prepubertal children (Mueller et al. 2013). The few pediatric studies in the US that have evaluated BMI versus FMI have determined they are not equivalent measures for determining excess adiposity in children (Weber et al. 2013). This is particularly important when considering sex and race/ethnicity as the body composition components of fat mass and lean body mass differ between sexes and across "population ancestry" groups (Weber et al. 2013). There is limited literature on birth cohort studies of childhood obesity outcomes related to BPA exposure and only one study includes longitudinal analyses of prenatal BPA exposure effects on school aged boys and girls (Braun et al. 2014; Harley et al. 2013; Philippat et al. 2014; Valvi et al. 2013). Minority populations are also underrepresented in BPA exposure research.

In our analysis, we found sex-specific and race/ethnicity-specific associations with prenatal BPA exposures and age 7 years anthropometric outcomes measures. In the Cincinnati-based Health Outcomes and Measures of the Environment (HOME) study, a prospective cohort composed primarily of

white (67%) and African American (27%) children (n=297), prenatal urinary BPA concentrations were not associated with BMI at ages 2-5 years (Braun et al. 2014). In the aforementioned HOME study an increase in childhood BMIZ slope per prenatal BPA tertile was observed but sex-specific associations were weak and based on a small sample size (Braun et al. 2014). However, accelerated growth may be due to a variety of factors including fat mass and fat free mass that can only be determined with body composition techniques.

Conversely, the "INfancia y Medio Ambiente" (INMA) population-based birth cohort study in Spain found a positive association between prenatal urinary BPA concentration and body size outcomes BMI and WC at age 4 years and no sex differences (Valvi et al. 2013). Researchers in France did not identify an association of prenatal urinary BPA with weight or WC at age 3 years in a birth cohort subset of boys only (Philippat et al. 2014). In the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) birth cohort consisting primarily (98%) of Mexican American mothers and children, researchers found prenatal urinary BPA concentrations to be inversely related to 9 year BMIZ and %BF for girls only; concurrent urinary BPA concentrations were positively related to 9 year BMI, fat mass and WC for both sexes (Harley et al. 2013). Similar to our results, early childhood (5 year) urinary BPA concentrations in the CHAMACOS cohort were not related to late childhood (9 year) anthropometric outcomes. In addition, *in vitro* and *in vivo* experimental studies have shown positive associations between BPA exposure and adipogenesis (Masuno et al. 2002, 2005; Riu et al. 2011; Rubin et al. 2001; Somm et al. 2009; García-Arevalo et al. 2014). Taken together with the existing literature, our results are an important addition to the growing literature on the potential role of BPA in the developmental origin of overweight, obese and adipose deposition.

The longitudinal cohort design of this study is a major strength as prior cross-sectional studies (Bhandari et al. 2013; Li et al. 2013; Trasande et al. 2012; Wang et al. 2012) may be biased due to associations between body size and higher food intake, leading to higher exposures to BPA from food. As such, a strength of our study is that we have been able to collect both repeated urinary BPA concentrations and directly assess anthropometrics outcomes in childhood, with the addition of body composition measures via bioimpedance at 7 years of age. The analyses found consistent results across the outcomes of FMI, %BF, and WC, although these are quite inter-correlated. The lack of findings with

BMI z-score and the known issues in using this measure to judge adiposity in children argue for the routine inclusion of body composition and WC measures in childhood obesity research.

Another strength of our study is our ability to control for socioeconomic and additional environmental factors, including urinary phthalate concentrations measured concurrently with BPA. Additionally, our study design provides the opportunity for analysis of adiposity in under-studied inner-city minority populations. For example, there is little prior information on prenatal and early childhood exposure to BPA and effects on body size outcomes among minority populations in the US, even though from 2003 to 2007, obesity prevalence among Hispanic children increased by 24.2% (Singh et al. 2010). Furthermore, although the National Health and Nutrition Examination Survey reports on BPA concentrations among different ethnic groups, it is limited to adults and children ages 6 years and older (Calafat et al. 2008). To date, no other study has evaluated the potential BPA effects on childhood body size in a birth cohort composed entirely of more than one minority group. According to the 2010 US Census, African Americans and Hispanics are the dominant minority populations in NYC (25.5% and 28.6%, respectively) (US Census Bureau, 2012). In our NYC-based birth cohort, composed of African Americans and Dominicans mother-child dyads currently being followed through adolescence, we found race/ethnicity-specific associations between prenatal BPA exposures and age 7 years anthropometric outcomes measures.

The use of spot urine samples is a potential limitation of the research presented here; prior literature finds a low interclass correlation (ICC) for BPA between serial urine samples from minority populations of pregnant women (ICC=0.27) (Meeker et al. 2013) and children (ICC=0.22) (Teitelbaum et al. 2008). If, as expected, the exposure misclassification because of variability of urinary BPA concentrations is non-differential in regards to the outcome, bias towards the null is expected. Thus, due to the poor reliability of the biomarkers, the findings for FMI, %BF and WC are likely to be underestimates of the true effects.

While our study was limited by the lack of available dietary data during pregnancy and childhood, accurate dietary data reporting is extremely difficult to acquire from young children given age-related development of language skills and recall ability. Dietary measures would also require quantification of BPA in food items, which was outside the scope of this study. Another possible limitation is that body

composition at age 7 years was assessed with bioelectrical impedance analysis which has been validated in some populations, but has not been compared to gold-standard reference methods in a population similar to ours (Haroun et al. 2009). There are also sex differences in pubertal development that may be associated with body composition (Ahmed et al. 1999). Earlier puberty in girls may lead to an estrogen-mediated increase in body fat (Kaplowitz 2008). Plasma concentrations of the hormone leptin, which regulates body fat and may be influenced by androgens, increases in girls and decreases in boys as pubertal development continues paralleling sex-specific changes in fat (Blum et al. 1997; Kaplowitz 2008). Hormonal and precocious puberty data was not available for this cohort. Therefore, puberty cannot be ruled out as a possible confounder of the sex-specific association observed between prenatal BPA concentrations and 7 year FMI and WC. As in any longitudinal study, we had missing anthropometric and biomarker data from the children in our cohort such that our sample size did not remain consistent across analyses. However, IPW analysis suggests that loss to follow-up did not bias our results.

Conclusions

Our results contribute to the growing literature studying prenatal and early life exposure to BPA, a known endocrine disruptor, and childhood obesity. In our longitudinal birth cohort, we found positive associations between prenatal urinary BPA concentrations and adiposity measures at age 7 years: FMI, %BF, and WC. These associations were sex-specific and race/ethnicity-specific in our cohort. Urinary BPA concentrations were positively associated with adiposity measures at age 7 years among girls (prenatal and postnatal BPA) and among Dominicans (prenatal BPA). Our findings suggest that prenatal BPA exposure may have an effect on adiposity as children age, an effect which cannot be observed by BMI-based measures alone. As the CCCEH cohort population ages into adolescence and emerging adulthood, follow-up studies would provide useful information to better understand the factors that may relate to obesity in this group of African American and Hispanic mothers and children.

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Table 1. Characteristics of mothers and children with available birth weight and/or prenatal weight gain, and prenatal urinary BPA measures (N=369)

Categorical variables: N (%)	
Sex of child	
Female	201 (54.5)
Male	168 (45.5)
Race/Ethnicity	
African-American	131 (35.5)
Dominican	238 (64.5)
Foreign Born [a]	
US Born	162 (44)
Foreign Born	206 (56)
Maternal Pre-pregnancy Obesity (BMI >30 kg/m^2)[a]	
Yes	75 (20.3)
No	285 (77.2)
Parity	
Nulliparous	168 (45)
Multiparous	201 (54)
Continuous variables mean (SD)	
Birth weight in g	3365 (475)
Maternal Pre-pregnancy BMI[a] in kg/m^2	25.80 (6.00)
Prenatal BPA in ng/mL	3.03 (4.16)

[a] # subjects missing data on each variable: Foreign Born n=1, Pre-pregnancy Obese n=9, Pre-pregnancy BMI n=9.

Table 2. Characteristics of subjects with urinary BPA measures and childhood anthropometric outcomes

	Prenatal BPA		Age 3 Years BPA	Age 3 and/or 5 Years BPA
	5 Year Anthropometry (N=300)	7 Year Anthropometry (N=308)	5 Year Anthropometry (N=317)	7 Year Anthropometry (N=325)
Categorical variables: N (%)				
Sex of Child				
Female	165 (55)	164 (53)	164 (52)	173 (53)
Male	135 (45)	144 (47)	153 (48)	152 (47)
Race/Ethnicity				
African-American	113 (38)	113 (37)	131 (41)	136 (42)
Dominican	187 (62)	195 (63)	186 (59)	189 (58)
Maternal Pre-pregnancy Obesity (BMI $\geq$ 30 kg/m^2)				
Yes	64 (21)	65 (21)	71 (22)	71 (22)
No	236 (79)	243 (79)	246 (78)	254 (78)
Child Overweight/Obese Status				
Overweight (BMI 85th to 95th %ile)	49 (16)	64 (21)	52 (16)	58 (18)
Obesity (BMI $\geq$ 95th %ile)	54 (18)	72 (23)	67 (21)	86 (26)
No	197 (66)	172 (56)	198 (63)	181 (56)
Continuous variables				
Mean Birth Weight (gms) (SD)	3356 (480)	3371 (487)	3381 (492)	3385 (499)
Mean BMI Z-score (SD)	0.42 (1.42)	0.79 (1.15)	0.58 (1.37)	0.86 (1.15)
Mean BMI (SD)	16.52 (2.73)	17.93 (3.43)	16.78 (2.82)	18.19 (3.62)
Median BMI Percentile (inter-quartile range)	69.47 (32.36, 92.13)	81.12 (53.38, 94.27)	74.88 (37.40, 93.11)	81.17 (54.24, 95.72)
Mean Fat Mass (kg) (SD)	--	7.19 (3.92)	--	7.47 (4.09)
Mean FMI [a] (SD)	--	4.51 (2.18)	--	4.67 (2.25)
Mean Percent Body Fat [a] (SD)	--	24.13 (6.06)	--	24.53 (6.14)
Mean Waist Circumference [a] (cm) (SD)	--	23.50 (8.27)	--	23.50 (8.43)
Mean Prenatal BPA (ng/mL) (SD)	3.12 (4.42)	3.06 (4.35)	--	--
Mean Child BPA (ng/mL) (SD)	--	--	8.12 (12.70)	3 year: 8.05 (12.53) 5 year: 5.35 (6.51) 3 and/or 5 year: 6.72 (7.43)

[a] # subjects with prenatal BPA concentration and missing data on each variable at age 7 years: fat mass index n=8, fat mass n=8, percent body fat n=8, waist circumference n=11; # subjects with mean childhood BPA concentration and missing data on each variable at age 7 years: fat mass index n=22, fat mass n=22, percent body fat n=22, waist circumference n=28.

Table 3. Associations between prenatal urinary BPA concentrations and child anthropometric outcomes

BPA measures	BMI Z-score at age 5 Beta Coefficient (95% CI)	BMI Z-score at age 7 Beta Coefficient (95% CI)	BMI Z-score change ages 5-7 Beta Coefficient (95% CI)	FMI at age 7 Beta Coefficient (95% CI)	Percent Body Fat at age 7 Beta Coefficient (95% CI)	Waist Circumference (cm) at age 7 Beta Coefficient (95% CI)
Continuous ln-transformed BPA concentrations[1]						
Prenatal BPA	(N=300) 0.04 (-0.16, 0.24)	(N=308) 0.11 (-0.04, 0.26)	(N=279) 0.06 (-0.06, 0.18)	(N=300) 0.31* (0.01, 0.60) p=0.04	(N=300) 0.79* (0.03, 1.55) p=0.04	(N=297) 1.29* (0.29, 2.30) p=0.01
Tertiles of specific gravity-adjusted ln-transformed prenatal BPA concentrations (in ng/mL)[2]						
<0.33	Reference	Reference	Reference	Reference	Reference	Reference
0.33-0.98	-0.29 (-0.70, 0.12)	-0.10 (-0.42, 0.21)	0.16 (-0.09, 0.40)	0.004 (-0.61, 0.40)	0.13 (-1.45, 1.71)	0.89 (-1.21, 3.00)
>0.98	-0.09 (-0.51, 0.32)	0.10 (-0.22, 0.42)	0.16 (-0.09, 0.41)	0.47 (-0.14, 1.09)	0.73 (-0.86, 2.32)	1.93 (-0.20, 4.06)

[1] All analyses controlled for: maternal variables: pre-pregnancy obesity, race/ethnicity, ∑DEHP, urinary specific gravity; child variables: sex, birth weight, gestational age. Body fat % and waist circumference analysis also controlled for height.

[2] All analyses controlled for: maternal variables: pre-pregnancy obesity, race/ethnicity, ∑DEHP; child variables: sex, birth weight, gestational age. Body fat % and waist circumference analysis also controlled for height.

*p < 0.05 #p<0.1

Supplemental Material

Bisphenol A and Adiposity in an Inner-City Birth Cohort

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Table S1. Associations between prenatal urinary BPA concentrations and birth outcomes

BPA measures	Birthweight (g) Beta Coefficient (95% CI)
Continuous ln-transformed BPA concentrations	
Prenatal BPA	(N=333) -47.15 (-104.19, 9.90)
Tertiles of specific gravity-adjusted ln-transformed prenatal BPA concentrations (in ng/mL)	
<0.33	Reference
0.33-0.98	-86.40 (-206.28, 33.47)
>0.98	-77.75 (-197.24, 41.74)

The following variables were controlled for in the analysis of continuous ln-transformed BPA concentrations and birth weight: standardized specific gravity, ∑DEHP, ethnicity dichotomous pre-pregnancy obesity status, baby sex, maternal foreign born, gestational age, maternal pregnancy weight gain. The following variables were controlled for in the analysis of tertiles of BPA concentrations and birth weight: ∑DEHP, ethnicity dichotomous pre-pregnancy obesity status, baby sex, maternal foreign born, gestational age, maternal pregnancy weight gain.

After full adjustment in the linear regression model, ln-transformed prenatal BPA concentrations were not associated with birth weight. Linear regression analysis using tertiles of BPA as predictor variables support the lack of association between prenatal urinary BPA concentration and birthweight.

Table S2. Associations[1] between ln-transformed prenatal urinary BPA concentrations and child anthropometric outcomes stratified by sex and race/ethnicity

BPA measures (ng/mL)	BMI Z-score at age 5 Beta Coefficient (95% CI)	BMI Z-score at age 7 Beta Coefficient (95% CI)	BMI Z-score change ages 5-7 Beta Coefficient (95% CI)	FMI at age 7 Beta Coefficient (95% CI)	Percent Body Fat at age 7 Beta Coefficient (95% CI)	Waist Circumference (cm) at age 7 Beta Coefficient (95% CI)
Girls						
Prenatal BPA	(N=165) 0.02 (-0.26, 0.30)	(N=164) 0.12 (-0.10, 0.33)	(N=153) 0.04 (-0.14, 0.20)	(N=161) 0.48* (0.5, 0.91) p=0.03	(N=161) 0.74 (-0.32, 1.81)	(N=160) 1.45* (0.5, 2.85) p=0.04
Boys						
Prenatal BPA	(N=135) 0.09 (-0.20, 0.37)	(N=144) 0.10 (-0.12, 0.32)	(N=126) 0.03 (-0.14, 0.21)	(N=139) 0.06 (-0.35, 0.47)	(N=139) 0.73 (-0.40, 1.87)	(N=137) 1.07 (-0.47, 2.61)
Dominican						
Prenatal BPA	(N=187) 0.01 (-0.24, 0.27)	(N=195) 0.18# (-0.01, 0.38) p=0.07	(N=174) 0.13 (-0.03, 0.28)	(N=193) 0.39* (0.01, 0.76) p=0.04	(N=193) 1.18* (0.25, 2.11) p=0.01	(N=189) 1.98* (0.64, 3.32) p<0.01
African American						
Prenatal BPA	(N=113) 0.05 (-0.27, 0.38)	(N=113) -0.02 (-0.29, 0.24)	(N=105) -0.06 (-0.25, 0.12)	(N=107) 0.12 (-0.41, 0.64)	(N=107) -0.11 (-1.52, 1.31)	(N=108) -0.06 (-1.63, 1.52)

[1] All analyses controlled for: maternal variables: pre-pregnancy obesity, race/ethnicity, ∑DEHP, urinary specific gravity; child variables: sex, birth weight, gestational age. Body fat % and waist circumference analysis also controlled for height.
*p < 0.05 #p<0.1

Table S3. Comparison of stratification and interaction by child sex for associations[1] between ln-transformed prenatal urinary BPA concentrations and child anthropometric outcomes at age 7 years

Age 7 Years Beta Coefficient (95% CI)	Full Sample	Girls	Boys	p-value (BPA) x (Sex)
BMI Z-score	0.11 (-0.04, 0.26)	0.12 (-0.10, 0.33)	0.10 (-0.12, 0.32)	0.41
FMI	0.31* (0.01, 0.60) p=0.04	0.48* (0.5, 0.91) p=0.03	0.06 (-0.35, 0.47)	**0.04**
Percent Body Fat	0.79* (0.03, 1.55) p=0.04	0.74 (-0.32, 1.81)	0.73 (-0.40, 1.87)	0.51
Waist Circumference (cm)	1.29* (0.29, 2.30) p=0.01	1.45* (0.5, 2.85) p=0.04	1.07 (-0.47, 2.61)	0.32

[1] All analyses controlled for: maternal variables: pre-pregnancy obesity, race/ethnicity, ∑DEHP, urinary specific gravity; child variables: sex, birth weight, gestational age. Body fat % and waist circumference analysis also controlled for height.
*p < 0.05 #p<0.1

Table S4. Comparison of stratification and interaction by race/ethnicity for associations[1] between ln-transformed prenatal urinary BPA concentrations and child anthropometric outcomes at age 7 years

Age 7 Years Beta Coefficient (95% CI)	Full Sample	Dominican	African American	p-value (BPA) x (Race/Ethnicity)
BMI Z-score	0.11 (-0.04, 0.26)	0.18# (-0.01, 0.38) p=0.07	-0.02 (-0.29, 0.24)	0.06
FMI	0.31* (0.01, 0.60) p=0.04	0.39* (0.01, 0.76) p=0.04	0.12 (-0.41, 0.64)	0.13
Percent Body Fat	0.79* (0.03, 1.55) p=0.04	1.18* (0.25, 2.11) p=0.01	-0.11 (-1.52, 1.31)	**0.04**
Waist Circumference (cm)	1.29* (0.29, 2.30) p=0.01	1.98* (0.64, 3.32) p<0.01	-0.06 (-1.63, 1.52)	**0.01**

[1] All analyses controlled for: maternal variables: pre-pregnancy obesity, race/ethnicity, ∑DEHP, urinary specific gravity; child variables: sex, birth weight, gestational age. Body fat % and waist circumference analysis also controlled for height.
*p < 0.05 #p<0.1

Table S5. Associations between child urinary BPA concentrations and child anthropometric outcomes

BPA measures	BMI Z-score at age 5 Beta Coefficient (95% CI)	BMI Z-score at age 7 Beta Coefficient (95% CI)	BMI Z-score change ages 5-7 Beta Coefficient (95% CI)	FMI at age 7 Beta Coefficient (95% CI)	Percent Body Fat at age 7 Beta Coefficient (95% CI)	Waist Circumference (cm) at age 7 Beta Coefficient (95% CI)
Continuous ln-transformed BPA concentrations[1]						
3 year BPA	(N=317) 0.04 (-0.12, 0.20)					
Tertiles of specific gravity-adjusted ln-transformed 3 year BPA concentrations (in ng/mL)[2]						
<0.98	Reference					
0.98-1.73	-0.12 (-0.49, 0.53)					
>1.73	0.13 (-0.25, 0.51)					
Continuous ln-transformed BPA concentrations[1]						
Mean BPA (3-5 years)		(N=325) -0.01 (-0.15, 0.14)	(N=298) -0.10# (-0.20, 0.005) p=0.06	(N=303) 0.05 (-0.24, 0.34)	(N=303) 0.16 (-0.59, 0.91)	(N=297) -0.03 (-1.01, 0.96)
Tertiles of specific gravity-adjusted ln-transformed mean BPA concentrations (in ng/mL)[2]						
<1.05		Reference	Reference	Reference	Reference	Reference
1.05-1.78		0.21 (-0.11, 0.52)	-0.02 (-0.24, 0.20)	0.30 (-0.34, 0.93)	0.13 (-1.45, 1.71)	0.89 (-1.21, 3.00)
>1.78		0.01 (-0.31, 0.33)	-0.18 (-0.41, 0.05)	0.10 (-0.55, 0.76)	0.73 (-0.86, 2.32)	1.93 (-0.20, 4.06)

[1] All analyses controlled for: maternal variables: pre-pregnancy obesity, race/ethnicity, ∑DEHP, urinary specific gravity; child variables: sex, birth weight, gestational age. Body fat % and waist circumference analysis also controlled for height.

[2] All analyses controlled for: maternal variables: pre-pregnancy obesity, race/ethnicity, ∑DEHP; child variables: sex, birth weight, gestational age. Body fat % and waist circumference analysis also controlled for height.

*p < 0.05 #p<0.1

Table S6. Associations[1] between ln-transformed child urinary BPA concentrations and child anthropometric outcomes stratified by sex and race/ethnicity

BPA measures	BMI Z-score at age 5 Beta Coefficient (95% CI)	BMI Z-score at age 7 Beta Coefficient (95% CI)	BMI Z-score change ages 5-7 Beta Coefficient (95% CI)	FMI at age 7 Beta Coefficient (95% CI)	Percent Body Fat at age 7 Beta Coefficient (95% CI)	Waist Circumference (cm) at age 7 Beta Coefficient (95% CI)
Girls						
3 year BPA	(N=164) 0.05 (-0.18, 0.28)					
Mean BPA (3-5 years)		(N=173) -0.13 (-0.33, 0.07)	(N=161) -0.18* (-0.32, -0.04) p=0.01	(N=163) -0.15 (-0.52, 0.23)	(N=163) -0.13 (-1.09, 0.84)	(N=160) -0.15 (-1.50, 1.20)
Boys						
3 year BPA	(N=153) 0.01 (-0.22, 0.25)					
Mean BPA (3-5 years)		(N=152) 0.12 (-0.10, 0.34)	(N=137) -0.02 (-0.18, 0.14)	(N=140) 0.23 (-0.24, 0.69)	(N=140) 0.48 (-0.76, 1.71)	(N=137) 0.06 (-1.46, 1.57)
Dominican						
3 year BPA	(N=186) - 0.10 (-0.31, 0.11)					
Mean BPA (3-5 years)		(N=189) -0.10 (-0.29, 0.09)	(N=170) -0.07 (-0.20, 0.06)	(N=179) 0.004 (-0.37, 0.38)	(N=179) 0.02 (-0.93, 0.97)	(N=176) -0.12 (-1.45, 1.21)
African American						
3 year BPA	(N=131) 0.32* (0.06, 0.58) p=0.02					
Mean BPA (3-5 years)		(N=136) 0.18 (-0.06, 0.41)	(N=128) -0.16 (-0.33, 0.01) p=0.07#	(N=124) 0.15 (-0.33, 0.64)	(N=124) 0.50 (-0.79, 1.79)	(N=121) 0.31 (-1.16, 1.79)

[1] All analyses adjusted for: maternal variables: pre-pregnancy obesity, race/ethnicity, sum of DEHP metabolites, urinary specific gravity; child variables: sex, birth weight, gestational age. Body fat % and waist circumference analysis also adjusted for height. *p < 0.05 #p<0.1

Chapter 5: Conclusions and Future Directions

Conclusions

The epidemiological and experimental studies described in Chapters 2 through 4 were devised to further the field of BPA exposure science by integrating the environmental obesogen hypothesis with the developmental origins of health and disease hypothesis specifically relating to obesity (Gillman et al. 2008, Grün and Blumberg 2007). We followed the conceptual pathway of BPA from a study of human exposures in a longitudinal birth cohort (Chapter 2) to an *in vitro* feasibility investigation into mechanistic actions on a primary cell line during adipogenesis (Chapter 3) and culminated with a health outcomes analysis of childhood adiposity in the same birth cohort (Chapter 4). The mechanistic experiments described in Chapter 3 have laid a solid foundation for future research both in the laboratory and in the field. Our epidemiologic results add to the knowledge regarding disparities in health outcomes from BPA and other environmental toxins.

In Chapter 2, we provided evidence that BPA exposure is widespread among a longitudinal birth cohort of inner-city minority mothers and children. Variations in BPA concentrations in urine were associated with the socio-demographic characteristics of race/ethnicity and marital status, as well as the season of collection, and concurrent exposure to phthalate plasticizers. However, it is not ethical to collect target adipose tissue in pregnant mothers and their children to analyze effects of BPA exposure on adipogenesis. To compensate for this, the experimental study described in Chapter 3 was implemented.

Our study provides the first demonstratration of the effects of BPA exposure on adipogenesis in freshly collected target tissue from humans. In comparison to standard adipogenic induction medium, contrary to our hypotheses, a significant decrease in surface area was seen in cells exposed to BPA at day 14 of the terminal phase of adipogenesis However, exposing HUMSCs to BPA during the early terminal phase of adipogenesis induced *PPARγ1* gene expression through hour 72. It is known that *PPARy* is a permissive class of nuclear receptors for which a high affinity endogenous ligand has not been isolated (Walkey and Spiegelman 2008). Additionally, lifetime adipocyte number is determined in childhood and adolescence (Spalding et al. 2008) with weight gain attributed to increases in adipocyte volume (Arner and Spalding 2010). Our study of HUMSCs provides preliminary evidence for a mechanism of BPA effect on adiposity suggesting BPA may act on *PPARy* receptors, thereby inducing

morphological changes in cells undergoing adipogenesis. In particular, our third aim was to evaluate the associations between prenatal BPA exposure and childhood anthropometric outcomes of BMI-Z scores, fat mass, body fat percentage, and waist circumference.

Our analyses in Chapter 4 were conducted on data from the same longitudinal birth cohort used for the analyses presented in in Chapter 2. This is the first study to find positive associations between prenatal urinary BPA concentrations and adiposity measures of fat mass, body fat percentage and waist circumference at age 7 years. These associations were sex-specific to girls (prenatal and postnatal BPA) and race/ethnicity-specific to Dominicans (prenatal BPA). Our findings suggest that prenatal BPA exposure may have an effect on adiposity as children age, an effect which cannot be determined by the use of BMI alone.

However, our results are not consistent with results from longitudinal analyses of other US birth cohorts. The only other two US studies investigating BPA exposure and obesity vary in their anthropometric outcomes: BMI trajectories and waist circumference (Braun et al. 2014) and BMI, waist circumference, fat mass and body fat percent (Harley et al. 2013). Prenatal urinary BPA concentrations were not associated with anthropometric outcomes at ages 2-5 years in the Cincinnati-based Health Outcomes and Measures of the Environment (HOME) study, a prospective cohort composed primarily of white and African American subjects (Braun et al. 2014). In contrast, researchers at the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) found prenatal urinary BPA concentrations to be inversely related to 9 year BMI z-scare and body fat percent only for girls (Harley et al. 2013). Our study is the first to find positive associations between prenatal BPA exposure and child adiposity outcomes in an exclusively urban minority population.

Additionally, our experimental results lend support to our epidemiological findings. Confirmed ubiquitous prenatal exposure to BPA within a longitudinal birth cohort was shown to have an effect on increased childhood adiposity with a possible mechanism via *PPARy1* gene expression during adipogenesis. We continued to see the effect on adiposity in the birth cohort after controlling for factors that were associated with higher BPA concentrations, as well as controlling for factors that are generally associated with obesity outcomes. This suggests that prenatal in-utero environmental conditions have a deleterious effect on childhood adiposity, over and above the effect of socio-demographic factors. This

dissertation has successfully synthesized the hypotheses of environmental obesogens with the developmental origins of health and disease in terms of early life effects of BPA on childhood adiposity.

Our epidemiological studies (Chapters 2 and 4) were characterized by a number of strengths: first, access to a birth cohort which allowed for longitudinal comparisons of repeated biomarker samples and anthropometric outcomes; second, the availability of extensive data that allowed us to control for socioeconomic and additional environmental factors, including urinary phthalate concentrations measured concurrently with BPA.

These strengths created a rich data resource that allowed us to identify a seasonal increase in childhood BPA exposures during the summer. Water consumption from polycarbonate bottles during the summer has been positively associated with urinary BPA concentrations in females (Makris et al. 2013), a finding which is also relevant to our research on BPA exposures in pregnant women and adiposity effects in their children. As biopsying adipose tissue from pregnant women and children is clearly unethical, we were able to address the issue of potential mechanism through the culturing of mesenchymal stem cells from human umbilical cord tissue, thereby enabling an investigation into the effect of BPA on adipocytes as the progenitor to target adipose tissue.

Limitations existed for both our epidemiological and experimental studies due to the unavailability of data for potentially informative variables. For example, our epidemiological studies were limited by use of single spot urines rather than repeated samples. Prior literature finds a low interclass correlation (ICC) for BPA between serial urine samples from minority populations of pregnant women (ICC=0.27) (Meeker et al. 2013) and children (ICC=0.22) (Teitelbaum et al. 2008). If, as expected, the exposure misclassification because of variability of urinary BPA concentrations is non-differential in regards to the outcome, our results may be biased towards the null. Thus, due to the poor reliability of the biomarkers, the positive associations of BPA with the age 7 years adiposity measures are likely to be underestimates of the true effects. It is also possible that the urinary BPA concentrations had diurnal variations which we could not control for without data on collection time for the majority of urine samples. We also did not collect dietary data during pregnancy and childhood through age 7 years, and we are missing anthropometric and biomarker data from some children in the birth cohort. As hormonal and precocious puberty data was not available for this cohort sex differences in leptin and sex hormones during pubertal

development cannot be ruled out as possible confounders of the sex-specific association observed between prenatal BPA concentrations and 7 year FMI and WC. In the experimental study, the use of anonymous samples meant that demographic and medical record data from the donors were not available for analysis.

Finally, while minority populations are at greater risk of obesity and we have shown positive associations between BPA exposure and childhood adiposity in a minority population living in northern Manhattan and the Bronx, these results may not be generalizable to other populations of children. This is because bioelectrical impedance analysis of the type used in this study has not yet been compared to gold-standard reference methods in a population similar to ours (Haroun et al. 2009). However, at a minimum, we believe our results can be appropriately generalized to individuals belonging to African American and Dominican populations residing in New York City and in similar urban environments.

Future directions

Given that obesity is a multi-factorial disease, it is not expected that BPA is the only EDC contributing to the epidemic. Rather we expect that our research, notably the observed correlations of BPA with phthalates, demonstrates the critical need for future studies to consider the effects of exposures to chemical mixtures and potentially complex interactions among exposures, socio-demographic characteristics and obesity. As the birth cohort population ages into adolescence and emerging adulthood, follow-up studies are critical to evaluate whether the associations between prenatal BPA and child age 7 years fat mass and waist circumference persist over time. Based on findings in animal literature, the associations may become more pronounced as the children age into adolescence and adulthood and may have transgenerational effects (Angle et al. 2013; Manikkam et al. 2013).

As ours was the first study to show an association between prenatal exposure to BPA and the childhood adiposity outcomes of fat mass and waist circumference, the specific focus of future endeavors should be on abdominal visceral adipose tissue mass. Additionally, the child ages we studied were pre-pubertal and endogenous hormonal effects on adiposity during and after puberty cannot be discounted, particularly when considering the effects of an estrogen mimicking compound such as BPA. Further, upon entering puberty, sex differences may become more pronounced. And finally, the race/ethnic differences we observed may eventually be part of a synergy between puberty, BPA exposures and

known race/ethnicity variations in location of fat deposition (Staiano and Katzmarzyk 2012) in this birth cohort. Due to the accumulation of fat tissue around key internal organs, increased abdominal fat mass deposition is related to increased cardiometabolic health risks. Therefore, it is essential to better understand the relationship between BPA and abdominal fat mass as children mature into adolescence and adulthood.

To best elucidate the importance of adipose tissue storage location in the body, it is recommended that future research employ the use of magnetic resonance imaging (MRI) technology rather than continued use of bioelectrical impedance techniques. MRI is widely considered the gold standard methodology to measure fat tissue and is particularly useful for determining distribution across different body depots. As was seen in our study, BMI Z-score was not associated with BPA concentrations. However, the use of MRI would likely confirm the observed associations of BPA with fat mass and waist circumference, thereby further advancing the science towards a wider acknowledgement that fat compartment deposition should be given greater attention when assessing the effects on obesity of BPA and other environmental exposures.

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Appendix A: Laboratory Protocols

UMBILICAL CORD COLLECTION

1. Preparing Cord and Collecting Samples
After the placenta has been delivered and examined by the attending physician, sections of umbilical cord should be obtained as follows:

- Lay the umbilical cord on a flat surface.
- Using the rubber forceps and scissors from the Disposable Suture Removal kit to grasp and lift the umbilical cord, cut the cord into 3 sections. Using the ruler, confirm that each section is 5 to 10 cm in length.

2. Documenting and Capping Samples

- Place the vials into a plastic bag labeled UMBILICAL CORD
- Fill out the Umbilical Cord Tissue Collection Information Sheet

5. Delivering Samples to the Laboratory

- Deliver samples to the laboratory, making the effort not to expose them to extreme heat or cold during delivery.
- Make a call to the laboratory staff and let them know when the blood samples were delivered.
- Make a call to Lori and let her know when the cord samples were delivered.

PRE-ADIPOCYTE LAB STUDY
COLUMBIA CENTER FOR CHILDREN'S ENVIRONMENTAL HEALTH

UMBILICAL CORD SAMPLES

Subject ID _____ Tech Initials_____

Umbilical cord collected? 0 No 1 Yes

If yes:

a) date umbilical cord sections collected __ __ / __ __ / __ __ __ __

time umbilical cord sections collected __ __ : __ __

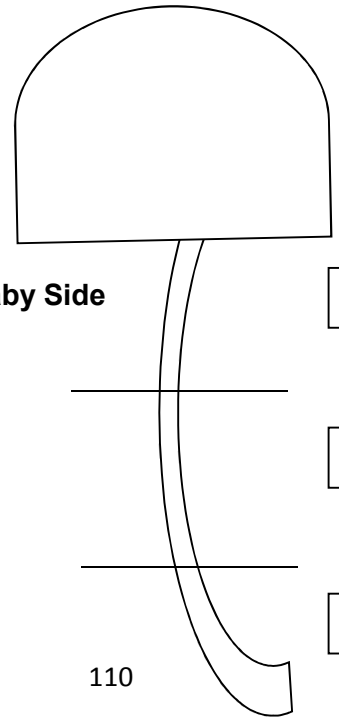
b) total number of cord sections bagged: ____

If no **or** if fewer than 3 sections bagged:

Reason umbilical cord not collected:

1 Failure to notify about delivery
2 Lost sample
3 No personnel
4 Delivery too fast
5 Difficulty with cord or placenta
6 Cord not long enough
7 Other _____

Placenta Maternal Side



Placenta Baby Side

1/3 Section Length: _____ cm

2/3 Section Length: _____ cm

3/3 Section Length: _____ cm

Umbilical Cord

Anonymous Umbilical Cord – Adipocyte Differentiation
Lab Protocol

Stock Solutions:

NOTE – if prepping collection solution and culture medium in same day, can use just one 10ml aliquot of AB/AM for entire process

1) Collection Solution for 20 tubes:
    a. Thaw 10 ml aliquot of AB/AM from -20 freezer
    b. Prepare PBS spout – all stored in culture room
    c. Materials – 1 x 10ml pipette, 1 x 25ml pipette, 1000 ml beaker, 20 x 50ml vials, pipette handle
    d. Ingredients:
        - 700 ml PBS
        - 7 ml AB/AM
    e. Pour 700 ml of PBS into beaker
    f. Pipette 7 ml of AB/AM into beaker
    g. Pipette 35 ml of solution into each 50ml vial

    [*For 10 tubes: 350 PBS, 3.5 AB/AM*]
2) Culture Medium:
    a. Thaw 10ml aliquot of AB/AM from -20 freezer
    b. Thaw 2 x 50ml aliquot of FBS from -20 freezer
    c. Thaw 10ml aliquot of L-Glutamine from -20 freezer
    d. Take container of DMEM from cold room and warm in water bath
    e. Materials – 1000ml beaker and aliquot containers or 1000ml capped jar
    f. Ingredients:
        - 500 ml DMEM
        - 89.82 ml FBS
        - 5.99 ml L-Glutamine
        - 2.99 ml AB/AM
3) 0.25M Sucrose:
    a. Autoclave 500 ml H_2O
    b. Weigh 42.79g of sucrose
    c. Mix sucrose with autoclaved water until completely dissolved
4) Cryopreservation Agent (CPA) Medium:
    a. Thaw 50ml aliquot of FBS from -20 freezer
    b. DMSO and 0.25M Sucrose on shelf above JD's bench
    c. Take container of fresh medium from cold room and warm in water bath
    d. Materials – 150ml aliquot containers
    e. Ingredients:
        - 28.33 ml DMSO
        - 41.66 ml FBS
        - 113.32 ml 0.25M Sucrose
        - 100 ml Fresh Media

Collection Prep:

1) In Ziploc Bag:
    - 4 x sterile 50 ml tube prepped with 35 ml PBS supplemented with 350 µL penicillin/streptomycin (3 tubes for collection, 1 tube for top-off)
    - 1 x Ruler
    - Umbilical Cord Log Sheet
2) Place Ziploc Bag in 4ºC cold room on shelf labeled "Boris/Lori/GAF"
    - Can remain for a maximum of 1 week

Summary of Protocol Post-Collection:
Day 1 = plated
Day 6 = change media
Weekly = change media until confluence
At subconfluence (Day 12-28 or longer) = trypsinize and give room for growth in T75s or T25s

Post-Collection:

(1) **Day 1** - Plate samples within 24 hours of collection

Materials: 1-3 x umbilical cord samples
1-3 x 10cm^2 Petri dishes for plating
1 x 10cm^2 dish (for cell culture) for sectioning – per sample
1x sterile scalpel
1x sterile forceps
Dulbecco's with 1.0g/L glucose (DMEM), 15% fetal bovine serum (FBS), 2nM L-glutamine, 0.5% antibiotic-antimycotic (AB-AM) solution (stock solution in 1000ml capped bottle in 4ºC cold room on shelf labeled "Boris/Lori/GAF")
2 beakers – 1 to stand sample tubes, 1 to discard excess PBS/AB/AM if needed
1 x 10ml Pipette + handle

a. Longitudinally section and score umbilical cord segments , cleaning clotted blood from the 3 vessels (2 umbilical arteries and one umbilical vein) to expose Wharton's Jelly
b. Plate dishes with each segment and 7ml of DMEM, FBS, L-Glut, AB-AM solution
c. Incubate with 5% CO2 for **5 days**

(2) **Day 6/Media Renewal 1 (MR1) -** Discard cord segments and renew medium 50:50

Materials: Plated cord samples
1 x sterile forceps
Dulbecco's with 1.0g/L glucose (DMEM), 15% fetal bovine serum (FBS), 2nM L-glutamine, 0.5% antibiotic-antimycotic (AB-AM) solution (stock solution in 1000ml capped bottle in 4ºC cold room on shelf labeled "Boris/Lori/GAF")
Large Petri dish to discard cord samples
T75 flask to discard spent media
2 x 10ml Pipette + handle

a. Pull up media to determine amount remaining
b. Remove the difference up to 5ml of spent media from each Petri dish to allow for a 50% renewal (ie. if there's 8ml remaining, remove 3ml in order for 5ml old medium to stay in culture)
c. Add a complementary amount of media up to 5ml to each culture dish
d. Incubate with 5% CO2

(3) **Day 13/MR2 -** Completely renew medium 100%

Materials: Plated cultures
Dulbecco's with 1.0g/L glucose (DMEM), 15% fetal bovine serum (FBS), 2nM L-glutamine, 0.5% antibiotic-antimycotic (AB-AM) solution (stock solution in 1000ml capped bottle in 4ºC cold room on shelf labeled "Boris/Lori/GAF")
T75 flask to discard spent media
2 x 10ml Pipette + handle

a. Pull up media to determine amount remaining, note this and discard ALL old media
b. Wash culture dish to displace and remove non-adherent cells, floating debris, blood cells, etc
c. Add 10ml fresh media to each culture dish
d. Incubate with 5% CO2

(4) **Weekly /MR3 to MR6** - Renew medium 50:50 until culture reaches subconfluence (80-90%)

Materials: Plated cultures

Dulbecco's with 1.0g/L glucose (DMEM), 15% fetal bovine serum (FBS), 2nM L-glutamine, 0.5% antibiotic-antimycotic (AB-AM) solution (stock solution in 1000ml capped bottle in 4ºC cold room on shelf labeled "Boris/Lori/GAF")
T75 flask to discard spent media
2 x 10ml Pipette + handle

a. Pull up media to determine amount remaining
b. Remove the difference up to 5ml of spent media from each Petri dish to allow for a 50% renewal (ie. if there's 8ml remaining, remove 3ml in order for 5ml old medium to stay in culture)
c. Add a complementary amount of media up to 5ml to each culture dish
d. Incubate with 5% CO2

(5) At subconfluence (post MR3 /~28 days) – Transfer to T75 or T25

T75 transfer from culture plate

a) Reserve medium from culture in 15 ml conical tube
b) 1 ml trypsin on plate – swirl, tap bottom, incubate for 3 minutes
c) Stop the trypsinization reaction: Add 10 ml FRESH medium while rinsing plate
d) Move all 11 ml from plate into 15 ml conical tube
e) Centrifuge at 6 RPM for 5 minutes (use existing 11ml blank next to hot water bath)
f) Remove supernatant (10 ml pipette then 1 ml pipette)
g) CAREFULLY resuspend pellet in original reserved medium – slowly resuspend using a 5 ml pipette
h) ***Either during 3 mins of incubation or 5 mins of centrifugation, put 10 ml of FRESH medium in T75 flask (or complementary amount needed to make 20 ml when added to the amount of reserve medium)

T25 transfer from culture plate

a) Reserve medium from culture in 15 ml conical tube
b) 1 ml trypsin on plate – swirl, tap bottom, incubate for 3 minutes
c) Stop the trypsinization reaction: Add 10 ml FRESH medium while rinsing plate
d) Move all 11 ml from plate into 15 ml conical tube
e) Centrifuge at 6 RPM for 5 minutes (use existing 11ml blank next to hot water bath)
f) Remove supernatant (10 ml pipette then 1 ml pipette)
g) CAREFULLY resuspend pellet in original reserved medium using up to 4ml of reserve only and slowly resuspend using a 5 ml pipette
h) ***Either during 3 mins of incubation or 5 mins of centrifugation, put 4 ml of FRESH medium in T25 flask (or complementary amount needed to make 8 ml when added to the amount of reserve medium)

Hemacytometer

- Use red 20µl or grey 10µl pipette – turn with white knob to get to 10 (black knob to lock amount)
- Use tips in red box for 20µl (color-coded to match the pipette handle) or tips that are for .1 to 10µl in non-color coded box
- Pull up 10µl of culture and place under coverslip on hemacytometer, watching that culture covers the clear glass area

Freezing protocol - ***for T75s***

a) Discard medium from culture
b) 2 ml trypsin in T75 – swirl, tap bottom, incubate for 3 minutes
c) Stop the trypsinization reaction: Add 20 ml FRESH medium while rinsing
d) Move all 22 ml from T75 by splitting 11 ml into 2 x 15 ml conical tube
e) Select one tube to count cells using hemacytometer – this determines amount of CPA and number of cryovials
f) Centrifuge tubes at 6 RPM for 5 minutes
g) While centrifuging, prepare CPA for resuspension of pellets and prepare Nunc cryovials
h) Remove supernatant (10 ml pipette then 1 ml pipette)
i) CAREFULLY resuspend pellet in appropriate amount of CPA medium – slowly resuspend using a 5 ml pipette
j) Dispense 1 ml of CPA-resuspension into each cryovial
k) Place cryovials in Mr. Frosty for slow freeze procedure
l) Put Mr. Frosty in -80C freezer for 24 hours minimum
m) After 24 hours move cryovials into liquid nitrogen tank

Freezing equations:

$$\frac{\text{Cell count for all 4 hemacytometer quads } / 4}{.0001 \text{ ml}} \times 22 \text{ ml} = \_\_\_\_\_ \text{ cells}$$

$$\frac{\text{\# cells from above}}{X} = \frac{1.05 \times 10^6 \text{ cells}}{1 \text{ ml}} \quad \text{OR} \quad \frac{\text{\# cells from above}}{1.05 \times 10^6 \text{ cells}} = \text{amount of CPA in ml [ie.1 cryovial per ml]}$$

Thaw protocol (from Seshareddy et al., 2008)

a) Remove cryovial from liquid nitrogen
b) Plunge cryovial in water bath with gentle swirling
c) Before the last ice crystal melts, wipe cryovial with ethanol and move to cabinet
d) Contents of cryovial are added dropwise to 10 ml of fresh media in a culture plate, using a 1 ml pipette and gentle swirling
e) Rinse the cryovial once with 1 ml of fresh media
f) Incubate with 5% CO2

Cell Viability Post-Thaw

a) Add 0.5 ml of Trypan Blue to an Eppendorf Tube
b) Add 0.5 ml of thawed cells in fresh media to Eppendorf Tube
c) Mix well, allow to sit for 10 minutes
d) Pipette mixture vigorously and place 10µl on hemacytometer
e) Count cells in 4 quadrants: Dead cells will take up the trypsin blue, viable cells will not

Anonymous Umbilical Cord – Adipocyte Differentiation
Experimental Protocol
(developed with assistance by Rosie Martinez)

Stock Solutions:
IBMX: Add 100 mg of IBMX to 0.90 mL DMSO
Dex: Add 10 mg of Dex to 25.47 mL DMSO
Rosi: Add 10 mg of Rosi to 2.52 mL DMSO
BPA (10 μM): Add 10 mg of BPA to 4.36 mL DMSO
BPA (10M): Add 200 mg to 87 μL DMSO

Recipes Using Stock Solutions:
Induction Media Alone (IDX- 100 mL)
90 mL DMEM
9 mL FBS
100 μL IBMX Stock
100 μL Dex Stock
50 μL Insulin
Fill Rest with 750 μL DMEM

Induction Media + DMSO (DMSO – 100 mL)
90 mL DMEM
9 mL FBS
100 μL IBMX Stock
100 μL Dex Stock
50 μL Insulin
100 μL DMSO
Fill Rest with 650 μL DMEM

Induction Media + Rosi (Rosi – 100 mL)
90 mL DMEM
9 mL FBS
100 μL IBMX Stock
100 μL Dex Stock
50 μL Insulin
100 μL Rosi Stock
Fill Rest with 650 μL DMEM

Induction Media + BPA (10 μM – 100 mL)
90 mL DMEM
9 mL FBS
100 μL IBMX Stock
100 μL Dex Stock
50 μL Insulin
100 μL BPA Stock
Fill Rest with 650 μL DMEM

Induction Media + BPA (100 μM – 100 mL)
90 mL DMEM
9 mL FBS
100 μL IBMX Stock
100 μL Dex Stock
50 μL Insulin
10 μL of 10 M BPA Stock
Fill Rest with 740 μL DMEM

Laboratory Procedure:

Note:
Prep Day=1-3 days prior to experiment to allow for MSC settling post trypsinization, attachment to plates, and at least one doubling. In general best practice is prep day=Friday and Day 1=Monday.
All labeling for RNA extraction should also be done on prep day, leaving everything set-up for early start on day 1.
Labeling convention - Type-Sample#-Timepoint (ie. R-99-1)
Type: M=media, I=Induction only, D=DMSO, R=Rosi, B10=BPA 10 µl, B100=BPA 100µl

(1) **Prep Day** - Count cells, trypsinize and plate in small petri dishes for experiment.
Materials: For each cell line:
21 x 15mm^2 Petri dishes
TrypLE
Dulbecco's with 1.0g/L glucose (DMEM), 15% fetal bovine serum (FBS), 2nM L-glutamine, 0.5% antibiotic-antimycotic (AB-AM) solution (stock solution in 1000ml capped bottle in 4ºC cold room on shelf labeled "Boris/Lori/GAF")
mL Pipette handle
1 x 2ml Pipette
3 x 25ml Pipette
1 x 10ml Pipette
1 x 20µl Pipetter
Hemacytometer
Counter
Light Microscope
2 x 15ml conical tubes

a. Trypsinize cells with 2ml TrypLE for 5 minutes in 37C, 5% CO2
b. Stop the trypsinization reaction: Add 20ml FRESH medium while rinsing flask
c. Split the 22ml into each 15ml tube
d. Remove 10µl for counting cells, determine how many cells are available for seeding (1million per plate) and dilution factor

$$\frac{\text{Cell count for all 4 hemacytometer quads} / 4}{.0001 \text{ ml}} \times 22 \text{ ml} = \_\_\_\_\_ \text{ cells}$$

1 million cells:5ml medium

e. Centrifuge conical tubes at 6 RPM for 5 minutes
f. Remove supernatant
g. Resuspend pellets in fresh medium according to dilution amount needed – slowly resuspend using a 5 ml pipette
h. Seed plates with 5ml per plate
i. Incubate in 37C, 5% CO2 for 2-3 days

(2) **Day 1**- Discard old media and feed with experimental media; count cells in baseline plate, RNEasy baseline cells
Materials: Plated MSCs
Experimental media (induction, DMSO, ROSI, BPA10µl, BPA100µl)
On ICE: Dulbecco's with 1.0g/L glucose (DMEM), 15% fetal bovine serum (FBS), 2nM L-glutamine, 0.5% antibiotic-antimycotic (AB-AM) solution (stock solution in 1000ml capped bottle in 4ºC cold room on shelf labeled "Boris/Lori/GAF")
mL Pipette handle
1 x 1ml Pipette
1 x 2ml Pipette

1 x 5ml Pipette
1 x 10ml Pipette
5 x 25ml Pipette
1 x 20µl Pipetter
Eppendorf tube
Hemacytometer
Counter
Light Microscope
1 x 15ml conical tube

a. Baseline Plate - Discard media, trypsinize cells with 2ml TrypLE for 5 minutes in 37C, 5% CO2
b. Stop the trypsinization reaction: Add 5ml COLD medium while rinsing plate, put media/cells in 15ml conical tube and place on ice for 10 minutes
c. Remove 1ml of media/cells from baseline conical tube and place in Eppendorf tube
d. Count cells from baseline plate to estimate doubling rate of cell line
e. Continue baseline tube using RNeasy Protocol
f. Remainder of plates, remove all media
g. Feed each plate with experimental media according to labels
h. Incubate all plates

(3) **Each Experimental Timepoint -** Discard media; RNEasy cells

Materials: Plated MSCs
On ICE:
Dulbecco's with 1.0g/L glucose (DMEM), 15% fetal bovine serum (FBS), 2nM L-glutamine, 0.5% antibiotic-antimycotic (AB-AM) solution (stock solution in 1000ml capped bottle in 4ºC cold room on shelf labeled "Boris/Lori/GAF")
mL Pipette handle
5 x 5ml Pipette
6 x 10ml Pipette
1 x 25ml Pipette
5 x 15ml conical tube

a. For each plate: Discard media, trypsinize cells with 2ml TrypLE for 5 minutes in 37C, 5% CO2
b. Stop the trypsinization reaction: Add 5ml COLD medium while rinsing plate, put media/cells in 15ml conical tube and place on ice for 10 minutes
c. Continue tubes using RNeasy Protocol

RNeasy Protocol: (Make sure everything is thoroughly labeled)

(developed with assistance by Rosie Martinez)

PREP:

I. RNAse bench and equipment (1000µL and 100 µL pipettes)

II. Prep ice tubs and discard (weighing) boat

III. Label all collection tubes as RNA+sample-initials-date on cap and side

IV. Label columns, collection tubes and Epp tubes to know which is which - pre-cool in -8 freezer

1) Harvest cells using normal trypsinization protocol
2) Spin 15 ml tube cold at 1000RPM for 5 minutes.
3) Elute off the supernatant, ensuring all of it is removed.
4) Disrupt pellet by adding 600-µL RLT Buffer and pipette to mix.
5) Pipette lysate onto QIAshredder column (Purple) and centrifuge for 2 minutes at full speed.
6) Add 600-µL of 70% ethanol to lysate in collection tube and mix by pipetting. **DO NOT CENTRIFUGE**.
7) Transfer 600-µL sample onto the RNeasy spin column (Pink) and centrifuge for 15 seconds at 10,000 RPM. Discard the flow through. Keep column.
8) **REPEAT** step 7 (can only do 600 µL at a time)
9) Add 700-µL RW1 Buffer to spin column and centrifuge for 15 seconds at 10,000 RPM and discard flow through.
10) Add 500-µL RPE Buffer to spin column and centrifuge for 15 seconds at 10,000 RPM and discard flow through.
11) Add 500-µL RPE Buffer to spin column and centrifuge for 2 minutes at 10,000 RPM and discard flow through.
12) Put column back in collection tube and centrifuge for 1 minute at full speed to dry to column.
13) Place RNeasy spin column in a new Eppendorf tube and add - directly into the center of the column so can know it can go through - 50-µL RNase-free water and centrifuge for 1 minute at 10,000 RPM.
14) Place **same water** over spin column again (directly in the center) and centrifuge for 1 minute at 10,000 RPM.
15) Place RNA samples on ice.

Nanodrop:

1) NO GLOVES on mouse or keyboard
2) PC - Desktop Icon - Admin - Health
3) Remove KimWipe from Nano, wipe with new KimWipe and distilled water
4) Software - click nucleic acid - do NOT load last workbook
5) Verifications - ok - make sure KimWipe gone
6) Click -> My Data -> Rosie/Lori folder (brings up old stuff - usually RNA first)
7) Drop down on right -> RNA, type sample #
8) Nanodrop the RNA samples using nanodrop on the nucelic acid selection.
9) "Blank" Nanodrop = pipette 1µL of RNase free H_2O, close, press "Blank" on screen
10) 1st sample -> 1µL sample, press "Measure", wipe clean
11) **REPEAT** steps 9 & 10 for all samples
12) When finished, wash Nanodrop with KimWipe and Distilled H_2O, fold KimWipe, place in middle of Nanodrop
13) Record the [RNA], 280 and 260 in Excel file
14) Place RNA samples in -80°C freezer.

cDNA Synthesis

STEP 1: DNase Prep (~1 hr)

when isolating RNA, there is always a chance of DA contamination, so you have to DNase

18µl -> 50% gel - 50% cDNA ***only ever have to run a gel x1

In DNase Prep Excel table - enter names of samples and ng/µl

1) Prepare gel for running cDNA
2) Turn on thermocycler (switch in back)
3) Turn on SpeedVac
4) RNase zap good Rainin pipetters - use red & green tips
5) UV PCR and/or Hydro water from JD's old PCR room
6) clean bench - EtOH, RNase zap spray
7) autoclaved PCR tubes & caps - clean razor blade

label PCR tubes with sample/row# from Excel sheet

8) autoclaved PCR rack - bottom drawer by sink - use red racks - stack 2 on top of each other
9) Ice bucket:
    - RNA samples - allow to thaw in ice, flick to mix, spin in tabletop centrifuge for a few seconds
    - RQ1 DNase Buffer
    - RQ1 DNase Stop Solution
    - Water DEPC treated
    - Silver tube tray for "RNA"
10) 1 vial DNase - keep cold in fridge
12) Fill PCR tubes according to DNase prep table amounts
    - **Start with H2O so can re-use tip for all tubes with H2O**
    - DNase Buffer (same tip)
    - RNA (new tips)
    - DNase (new tips) (last - *want to keep cold as long as possible*)
13) Cap tubes and put them in thermocycler
14) Thermocycler:

    Browse New Methods
        - highlight 37 - 30 minute run (37 C)
        - enter volume=18
        - start run now
15) Add 2µl DNase Stop to tubes while **IN** Thermocycler after run ends
16) Run Thermocycler on 65 - 10 4 rest (65 C, 10 mins, rest at 4C indefinitely) - remember to make volume in TC=20µl
17) Final volume = 18µl RNA solution + 2µl DNase Stop = 20µl

    10µl for cDNA synth and 10µl for gel
18) Take out volume for gel run - put in PCR tubes

**If have to store RNA before cDNA synthesis – can put in -80C but best to finish through to cDNA synthesis

STEP 2: cDNA Synthesis (~2 hr)

in cDNA synthesis Excel table - put the # of samples needed in top
usually add 1 more than required - i.e. if running 12 samples, use 13 in table

cDNA - make 2 master mixes (***do while thermocycler is running***)
reagents are in -20C freezer - allow to thaw
tubes can sit on ice as long as necessary before adding 2nd master mix
1) get more ice
2) add H2O cold
3) follow directions on cDNA synthesis table
"cDNA synthesis" program on Thermocycler
4) can freeze cDNA product in -20C until use it again

Running Gel

Small gel - up to 32 runs
Large gel - up to 100 runs
Can re-use gels if don't use all the slots - start on bottom of gel, not top if plan to save gel

Pipettes on gel bench all have orange tape - they **do not leave** the gel bench - concern that PCR product could contaminate other things in the lab
Rinse trays etc before using – get rid of lint/dust, etc

Make 1.5% Gel
Small gel: 1.50g agarose powder; 100ml TBE; 5µl EtBr
Large gel: 3.75g agarose powder; 250ml TBE; 12.5µl EtBr

1) **Always** add agarose powder **first** (to a flask at least 2x volume of gel need)
powder on table next to scale
spatula in gel container
2) Use 1.0 TBE (but really 0.5 TBE) on the left (green) - use graduated cylinder on the gel table
after adding liquid check for clumps on bottom (if clumps swirl before microwaving)
4 minutes in microwave, and watch flask - when gel fully dissolved it will be clear
Use heat protection for hands!
bubbles, then swirl - do not want "foamy"
let flask cool slightly
3) Add Ethidium bromide (EtBr) (on gel bench - use dedicated pipetter) and swirl
4) Pour into tray - make sure red bumpers are in place and balance bubble in center of circles
pour in cold room for it to harden faster
if there are bubbles, use a pipette tip to move bubbles to bottom of tray
5) Rinse empty agarose flask with H2O in sink before leaving it in sink so agarose does not harden in the flask
6) While waiting for gel to harden
Aliquot out loading dye
Loading dye should be 1:6
in general: **2.5 µl loading dye + 12.5µl sample**
Set pipetter to 13µl so no bubbles in tip
(*if 11 µl of samples each -> 8mL RNA + 3 mL loading dye -set pipetter to 10*)
(*if 10µl RNA solution, use 2µl loading dye, set pipetter to 11µl*)
8) When gel hardens
Pull out combs
Pull out tray
6) Fill black tray with TBE buffer
~600 mL for small gel
~1300 mL for large gel
Want enough buffer to completely cover gel
7) Add 7µl Gene Ruler aka "ladder" to first column
load - hover over well
pipetter to 1st stop, then slow to 2nd stop until bubble

8) Add 13µl each of samples + loading dye each
samples - same tips ok for samples - rinse in buffer
Remember - because of multichannel spacing when counting where samples go- have to skip spots on gel
9) Add negative controls - new tips
10) Add H2O - new tips
11) Setting on milliamp
Black (+) Red (-)
sample (-) charge - will run from negative to positive end
know gel is running if see bubbles from wire by the red
run 40 minutes (more time will just increase spread of bands)
12) Turn off, unplug everything, take to Santella lab for UV photo
13) Rinse trays in sink

PCR photo

In Santella lab - pw=Santella
Slide gel off in machine - follow directions on machine
Turn machine on in back: 1) turn UV switch on 2) Filter wheel #2
In computer: Fluorchem software
"acquire"
expose preview (change seconds - exposure time)
acquire image
Flash drive
file save as .tif
EtOH spray and wipe machine when remove gel (follow directions on machine)

*****JD's Room**
UV water - beige box - UV for 15 minutes
use clean gloves to remove items - PCR tubes, caps, H2O
when finished UV again

Primers

Make 100 µMol stock

(nM)10 = X = # µl ---> 100 µM stock

1:10 dilution used from stock - ie. 10µl stock + 90µl H2O (make 5 tubes from each primer)

All primer stocks previously made in 2013:

Summer 2013:

FWD Leyvraz PAPRG1

REV Leyvraz PPARG1/2

December 2013:

FWD Freyer PPARG2= 31.3nM(10)=314µl

FWD 'NEW' GAPDH= 36.9nM(10)=369µl

REV 'NEW' GAPDH= 39.89nM(10)=398µl

primers come as powder - use the tubes come in as stock tube

1) spin powder
2) add H2O to powder
3) Vortex Genie
   - push tubes all the way down in holes
   - high vortex level
   - spin for a little while
4) While vortexing - add 90µl H2O in each tube, use same pipette tip
5) After make dilutions, put all stock in -20C and put all dilutions except 1 of each in -20C
6) Dilutions that are kept out should go on ice

Step 3: **qPCR**

Fill out "qPCR plate setup" Excel file on computer and print out
ABI 7900= Santella lab qPCR machine on Google calendar - reserve as "Freyer Lab"

Prep Master Mixes
Follow directions on Master Mix Excel sheet
- change the **bold #** on top right box of Master Mix sheet - this is the # of samples

Amount of primer - JD suggests using 0.5 µl (standard middle of the road)
* Rosie used 0.25 µl -> [0.2 µM] in PCR -- low end of [primer] for PCR run
* Garcia used 0.625 µl

1) UV H2O in JD's room
2) Eppendorf tubes on ice
3) Addition steps (keep all on ice):
   i. H20
   ii. Sybr Green (keep covered with Al foil)
   iii. Primers - vortex before adding because they are cold and unmixed
   iv. mix up and down while in stop 1
4) Keep MMs on ice until ready to use

Remember slow pipetting to empty stop 1 then slow to empty stop 2
For multichannel - remember that multichannel pipette will skip wells on PCR plate
1) centrifuge cDNA in PCR tubes across from PCR machine
   - balance plates using metal holders
   - close door, select 1000rpm
   - start, wait for speed to hit 1000rpm
   - stop centrifuge
   - remove plates
2) Fill PCR plate with primer
2) Multichannel from PCR tubes directly to PCR plate
   straight down, hit bottom and slow release to stop 1, then stop 2
   noRT, H2O, positive control, etc -- add each component **ON** PCR plate
   mix up and down to stop 1 on pipetter
3) Spin PCR tray - centrifuge in Santella side room
   - use black tray - cover with plastic now?
   - run for 1 minute, then stop
   - when stops push lid open from bottom
   - remove black tray
4) Bring PCR plate and plastic cover to Santella lab
   - carefully place plastic on plate
   - want to see no bubbles, flat, correctly positioned
   - remove end tabs 1) paper 2) plastic
   - smooth out with brown paddle smoother
4) PCR computer - Click on program - SDS 2.4
   New run - Enter sample names on left
   Choose primer/SYBR pair on right (ex - GAPDH/SYBR, PPARG/SYBR - - > 1, 2)
   Next
   Instruments tab
      Connect to Instrument
      Open (machine)
      Insert PCR tray - align corner A1 with A1 on machine holder
      Start run - asks you to save filename/location
   Retrieve plate when run ends - can leave overnight if nobody has machine reserved for next morning

Speedvac

Turn refrigeration on about 1 hour in advance – takes about 20 minutes to reach -54C – want temperature at about -20C

Low Drying Rate (concentrator on=spin)
Turn top switch to "closed" (arrow points to left)
Turn on vacuum pump
Put samples in – remember to balance them
Concentrator on
Turn top switch to "open" (arrow points down)
When finished spinning (should take maybe 20 minutes or so*)

1) concentrator off + 2) top switch closed
1) turn pump off + 2) top switch open

Turn off refrigerator = middle of button – should be flat

Sample measurements:
Put RNA on ice
Measure volume with a pipette - flick, pipette up and down sample on side of tube
Quickly and briefly vortex AND THEN centrifuge (15 sec, 10,000 RPM)
Put RNA back on ice
Take to NanoDrop - can do multi up to 16 at a time in Santella lab
Remember to take 2ul pipette, tips and RNA free H2O for blanking

***20 minutes we got 50 ul to 15 ul
***30 minutes to dry

*[RNA]<65: aim for 20-25 minutes to get to 8-10ul
*[RNA]>65: aim for 20 minutes to get to 10-12ul

Santella Epoch Spectrophotometer Machine - Gen5 Softeare:
Kimwipe plate (only - no liquid) - before blank/after blank/after each sample set run

Blanks/samples - use 2ul (min 1.5 ul)

H2O blank - use 2-4 top wells, run blank; click 'ok' on temp
When green checkmark - passed; click 'approve'

Click RNA
PC will auto change to sample=blue
if use <16 wells for sample de-select wells not used
after finish running sample, click to exit on right side of screen

Save Excel files/rename samples while still at Santella lab
Save to USB

Dnase Prep

MM/DD/YYYY

Sample	Name	ng/µl	ul (4 ug)	10x Dnase buffer	Dnase	H2O	Final
1				1.8	1		18
2				1.8	1		18
3				1.8	1		18
4				1.8	1		18
5				1.8	1		18
6				1.8	1		18
7				1.8	1		18
8				1.8	1		18
9				1.8	1		18
10				1.8	1		18
11				1.8	1		18
12				1.8	1		18
13				1.8	1		18
14				1.8	1		18
15				1.8	1		18
16				1.8	1		18
17				1.8	1		18
18				1.8	1		18
19				1.8	1		18
20				1.8	1		18
21				1.8	1		18
22				1.8	1		18
23				1.8	1		18
24				1.8	1		18
25				1.8	1		18

37 C - 30 min
Add 2 ul Dnase stop
65 C - 10 min

10 ul for cDNA synth and 10 ul for gel

SpeedVac Worksheet
MM/DD/YYYY

Date	Name	Conc (ng/µl)	Starting Vol (ul)	RNA (ng)	Target Volume for 263.2 ng/µl	Measured Vol	Time	Delta Vol	H2O to Add	Measured Conc (ng/µl)

Speedvac concentration recommended for
<263.2 ng/µl
≥ 65 ng/µl

cDNA Synthesis Prep Table

MM/DD/YYYYY

	1 rxn (ul)	**N**
10 mM dNTP mix	1.25	
Oligo dT	1.25	
Dnased RNA (2 ug)	10.00	
Total Vol	12.50	

65°C for 5:00
Ice for >1:00

	1 rxn (ul)	**N**
10x RT buffer	2.50	
25 mM MgCl2	5.00	
0.1 M DTT	2.50	
RNaseOUT	1.25	
Total Vol	11.25	

Add 11.25 ul of second mix
42°C for 2:00
Aliquot 1.25 ul RT into fresh PCR tubes
Multichannel 20 ul of mix into RT containing tubes
Leave rest for no RT
42°C for 50:00
70°C for 15:00
Chill on ice
Briefly centrifuge
Add 1.25 ul of Rnase H to RT and no RT
37°C for 20:00

qPCR Master Mix Worksheet

MM/DD/YYYY

99	1 rxn (ul)	N
Sybr green	6.25	
H2O	4.25	
PPARG 1 Leyvraz F	0.50	
PPARG 1 Leyvraz R	0.50	
Template	1.00	
Total Vol	12.50	

99	1 rxn (ul)	N
Sybr green	6.25	
H2O	4.25	
PPARG 2 Freyer F	0.50	
PPARG 2 Leyvraz R	0.50	
Template	1.00	
Total Vol	12.50	

99	1 rxn (ul)	N
Sybr green	6.25	
H2O	4.25	
GAPDH F	0.50	
GAPDH R	0.50	
Template	1.00	
Total Vol	12.50	

IF doing single runs
Make all master mixes
Add 1 ul of cDNA to each PCR plate well (can multichannel)
Add H2O/control cDNA to plate well
Add 11.5 ul PPARG1 MM to cDNA
Add 11.5 ul PPARG2 MM to cDNA
Add 11.5 ul GAPDH MM to cDNA and H2O and control wells

IF doing duplicates/triplicates on same plate
Make all master mixes
Aliquot XX ul of each MM into 12 PCR tubes
Add X.X ul of cDNA to each aliquot (ie. If 36ul MM, 3.6 ul cDNA)
Add 12 ul aliquots to plate
Add 10.8 ul of GAPDH MM to no RT and H2O
Add 1.2 ul no RT cDNA/H2O

qPCR Plate Set-up (excerpted from 24 columns)

	1	2	3	4	5	6	7	8	9
A	sid		sid		sid		sid		sid
B	sid		sid		sid		sid		sid
C	sid		sid		sid		sid		sid
D	sid		sid		sid		sid		sid
E	sid rt		sid rt		sid rt		sid rt		sid rt
F	sid noRT		sid noRT		sid noRT		sid noRT		sid noRT
G	H2O								
H									
I									
J									
K									
L									
M									
N									
O									
P									

PPARG 1
PPARG 2
GAPDH

Thermocycler	
50°C	2 min
95°C	10 min
(x40)	
95°C	15 sec
60°C	1 min

Anonymous Umbilical Cord – Adipocyte Differentiation
Staining Protocol

Oil Red O

I. Culture Room

- Pipette off adipgenesis media and discard
- Rinse 1x with PBS

II. Fume Hood

1. Fix with 10% formalin for 30 minutes
2. While fixing, aliquot 25ml of Oil Red O to 50ml conical tube and heat in a water bath to 60°C
3. Remove 10% formalin and rinse 2x with DI H2O
4. Add propylene glycol for 2 minutes
5. Remove propylene glycol
6. Stain with Oil Red O for 6 minutes
7. Tip plate to remove Oil Red O
8. Add 85% propylene glycol (in DI H2O) for 1 minute
9. Remove 85% propylene glycol
10. Tip plate to rinse **gently** with DI H2O 2x. Do not rinse directly on cells.
11. Gently pipette from the edge of the well to stain with Modified Mayer's Hematoxylin for 1 minute
12. Remove Modified Mayer's Hematoxylin
13. Rinse with DI H2O until no more stain rinses off
14. Take photos quickly afterwards or there may be drying artifacts like bubbles appearing under the microscope. Can add 2-3ml of PBS to each plate and image them to insure integrity of lipid droplets.
15. Remove PBS and let plate air dry

Alcian Blue pH2.5

I. Culture Room

- Pipette off chondrogenesis media and discard
- Rinse 1x with PBS

II. Fume Hood

1. Fix with 10% formalin for 30 minutes
2. Remove 10% formalin and rinse 1x with PBS
3. Rinse 1x with DI H2O
4. Add 3% acetic acid for 3 minutes
5. Remove 3% acetic acid
6. Stain with Alcian Blue for 30 minutes
7. Remove Alcian Blue
8. Rinse 1x with DI H2O
9. Add Nuclear Fast Red for 3 minutes
10. Remove Nuclear Fast Red
11. Rinse 1x with DI H2O
12. Dehydrate through 3 changes of EtOH
13. Take photos

Alizarin Red

I. Culture Room

- Pipette off osteogenesis media and discard
- Rinse 1x with PBS

II. Fume Hood

1. Fix with 10% formalin for 30 minutes
2. Remove 10% formalin and rinse 1x with PBS
3. Rinse 2x with DI H2O
4. Stain with Alizarin Red for 1 to 5 minutes (check under microscope)
5. Remove Alizarin Red
6. Dehydrate with acetone for 15 seconds
7. Remove acetone
8. Add 1:1 xylene/acetone solution for 15 seconds
9. Remove 1:1 xylene/acetone solution
10. Clear slide/well through 3 changes of xylene
11. Take photos