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Associations between urinary phenol and paraben concentrations and markers of oxidative stress and inflammation among pregnant women in Puerto Rico

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Abstract

Phenols and parabens are used in a multitude of consumer products resulting in ubiquitous human exposure. Animal and *in vitro* studies suggest that exposure to these compounds may be related to a number of adverse health outcomes, as well as potential mediators such as oxidative stress and inflammation. We examined urinary phenol (bisphenol A (BPA), triclosan (TCS), benzophenone-3 (BP-3), 2,4-dichlorophenol (24-DCP), 2,5-dichlorophenol (25-DCP)) and paraben (butyl paraben (B-PB), methyl paraben (M-PB), propyl paraben (P-PB)) concentrations measured three times during pregnancy in relation to markers of oxidative stress and inflammation among participants in the Puerto Rico Testsite for Exploring Contamination Threats (PROTECT) project. Serum markers of inflammation (c-reactive protein (CRP), IL-1 β , IL-6, IL-10, and tumor necrosis factor- α (TNF- α)) were measured twice during pregnancy (n=105 subjects, 187 measurements) and urinary markers of oxidative stress (8-hydroxydeoxyguanosine (OHdG) and isoprostane) were measured three times during pregnancy (n=54 subjects, 146 measurements). We used linear mixed models to assess relationships between natural log-transformed exposure and outcome biomarkers while accounting for within individual correlation across study visits. After adjustment for urinary specific gravity, study visit, maternal pre-pregnancy BMI, and maternal education, an interquartile range (IQR) increase in urinary BPA was associated with 21% higher OHdG (p=0.001) and 29% higher isoprostane (p=0.0002), indicating increased oxidative stress. The adjusted increase in isoprostane per IQR increase in marker of exposure was 17% for BP-3, 27% for B-PB, and 20% for P-PB (all p<0.05). An IQR increase in triclosan (TCS) was associated with 31% higher serum concentrations of IL-6 (p=0.007), a pro-inflammatory cytokine. In

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contrast, IQR increases in BP-3 and B-PB were significantly associated with 16% and 18% lower CRP, a measure of systemic inflammation. Our findings suggest that exposure to BPA, select parabens, and TCS during pregnancy may be related to oxidative stress and inflammation, potential mechanisms by which exposure to these compounds may influence birth outcomes and other adverse health effects, but additional research is needed.

Keywords

bisphenol A; inflammation; oxidative stress; parabens; phenols

1. Introduction

The rate of preterm birth in Puerto Rico is 17%, one of the highest rates in the U.S. (March of Dimes, 2014) and the world (Blencowe et al., 2012). The island of Puerto Rico also has a high concentration of hazardous waste sites (US EPA, 2014; Padilla et al., 2011), raising the question of whether exposure to environmental chemicals plays a role in the high rates of preterm birth, as well as other prevalent health outcomes among this population. The Puerto Rico Testsite for Exploring Contamination Threats (PROTECT) program is an ongoing prospective birth cohort in Northern Puerto Rico designed to investigate relationships between chemical exposures during pregnancy, preterm birth, and other adverse pregnancy outcomes, as well as potential toxicological mechanisms for these relationships (Meeker et al., 2013). Potential mechanisms of interest include oxidative stress and inflammation, as these have been associated with both environmental contaminants and adverse birth outcomes (Al-Gubory et al., 2010; Bastek et al., 2011; Ferguson et al., 2014c). Previous research within the PROTECT project suggests that pregnant women in Puerto Rico may have higher urinary concentrations of the phenols triclosan (TCS), benzophenone-3 (BP-3), and 2,5-dichlorophenol (25-DCP), and similar urinary concentrations of bisphenol-A (BPA), 2,4-dichlorophenol (24-DCP), and parabens, compared to women of reproductive age from the US general population (Meeker et al., 2013). In the US, recent reports from the National Health and Nutrition Examination Survey (NHANES) suggest that exposure to a number of phenols and parabens is widespread, with detectable urinary concentrations present in the majority of participants (CDC, 2014).

BPA is a weakly estrogenic, high-volume chemical used to make polycarbonate plastics and epoxy resins that are present in a multitude of consumer products (CDC, 2014). In animal studies, BPA has been shown to affect a number of reproductive endpoints, but human studies have been limited (Cantonwine et al., 2013). BPA has also been associated with a number of markers of oxidative stress in both humans (Yang et al., 2009), and animals (Aboul Ezz et al., 2013; Hassan et al., 2012; Song et al., 2014).

TCS is a broad-spectrum antimicrobial used in a large number of personal care products and consumer goods, including soaps, toothpaste, mouthwash, deodorants, textiles, toys, and kitchenware (Dann and Hontela, 2011). Associations between TCS exposure and reproductive outcomes in humans have not been studied, although TCS is thought to have anti-inflammatory properties (Barros et al., 2010; Elwood et al., 2007; Modeer et al., 1996;

Mustafa et al., 2000; Wallet et al., 2013). Given that inflammation is a potential factor in preterm birth (Bastek et al., 2011), we might expect a possible protective relationship between urinary TCS concentrations and this outcome.

BP-3 is a UV-filter commonly used in sunscreens, cosmetics, and plastics (CDC, 2014). Animal studies suggest that BP-3 is weakly estrogenic and antiandrogenic (Krause et al., 2012), but health effects related to BP-3 exposure in humans have not been studied. In *in vitro* studies, BP-3 has been associated with markers of oxidative stress (Gao et al., 2013; Kato et al., 2006), but findings from an animal study suggested that BP-3 may have anti-inflammatory properties (Couteau et al., 2012).

24-DCP is a minor metabolite of 2,4-dichlorophenoxyacetic acid (2,4-D), a herbicide widely used in the US and elsewhere, while 25-DCP is a metabolite of 1,4-dichlorobenzene, a compound used in mothballs and room and toilet deodorizers (CDC, 2014). In human studies, maternal urinary 24-DCP and 25-DCP concentrations during pregnancy have been associated with decreased birth weight in boys (Philippat et al., 2012; Wolff et al., 2008) but not in girls, and one study observed no association between maternal urinary concentrations and gestational age (Wolff et al., 2008). However, 24-DCP has been associated with markers of oxidative stress (Bukowska, 2003).

Parabens are a class of chemicals widely used as antimicrobial preservatives in cosmetics and other personal care products, food, and pharmaceuticals (Calafat et al., 2010). Animal and *in vitro* studies have demonstrated that parabens are estrogenic (Boberg et al., 2010; Karpuzoglu et al., 2013), but studies on reproductive or pregnancy outcomes are limited. In humans, higher urinary concentrations of various parabens have been associated with increases in markers of oxidative stress (Kang et al., 2013).

Few human studies have explored measures of phenol and paraben exposure in relation to oxidative stress and inflammation despite the potential for these processes to be important mediators of numerous health effects, including atherosclerosis, cardiovascular disease, cancer, and pregnancy outcomes such as intrauterine growth restriction and preterm birth. The objective of the current study was to examine these associations by utilizing repeated measures of phenols, parabens, and markers of oxidative stress in urine samples collected up to three times per participant throughout pregnancy, and markers of inflammation in plasma samples collected up to two times per participant throughout pregnancy among women participating in the PROTECT project.

2. Methods

2.1 Study participants

Participants in the present study are pregnant women who have enrolled in the PROTECT project, an ongoing prospective birth cohort in Northern Puerto Rico. Recruitment practices and inclusion criteria have been previously described (Meeker et al., 2013). Briefly, pregnant women 18 to 40 years of age were recruited at less than 20 weeks gestation from 7 prenatal clinics and hospitals from 2010 to 2012. We excluded women who used oral contraceptives within three months prior to getting pregnant, used *in vitro* fertilization to get

pregnant, or had known obstetric or medical health conditions (e.g., heart conditions or diabetes). Women provided spot urine samples at three separate study visits (16-20, 20-24, and 24-28 weeks gestation). We collected blood samples from participants at visits 1 and 3, and administered questionnaires at all three visits. We followed participants until delivery and recorded detailed information on birth outcomes. This analysis comprises the first 141 women recruited into the study for which urinary phenol and paraben measurements and urinary specific gravity (SG) were available from at least one study visit, as participation was ongoing for some women. Markers of either oxidative stress or inflammation were available on 106 of these women. The ethics and research committees of the University of Michigan School of Public Health, University of Puerto Rico, Northeastern University, and participating hospitals and clinics approved all study protocols, and all subjects provided informed consent prior to participation.

2.2 Urinary Phenols and Parabens

Spot urine samples were collected in polypropylene containers, divided into aliquots, and frozen at -80°C until aliquots were shipped overnight on dry ice to the CDC for phenol and paraben analysis or to the University of Michigan for measurement of oxidative stress biomarkers. At the CDC, urine samples ($n=375$) were analyzed for five phenols (BPA, TCS, BP-3, 24-DCP, and 25-DCP) and three parabens (butyl paraben (B-PB), methyl paraben (M-PB), and propyl paraben (P-PB)) by online solid phase extraction-high-performance liquid chromatography-isotope dilution tandem mass spectrometry (Ye et al., 2006; Ye et al., 2005). The inter-assay coefficients of variation for urinary phenols and parabens range from 5 to 10%. Concentrations below the limit of detection (LOD) were assigned a value of $\text{LOD} / 2$ (Hornung and Reed, 1990). Women were not asked the time since their last void, although urinary specific gravity was measured at the University of Puerto Rico Medical Sciences Campus using a digital handheld refractometer (Atago Co., Ltd., Tokyo, Japan) as an indicator of urine dilution.

2.3 Oxidative Stress Biomarkers

A subset of 146 urine samples collected from 54 participants at visits 1 ($n=52$), 2 ($n=50$), and 3 ($n=44$) were analyzed for 8-hydroxydeoxyguanosine (OHdG), a marker of DNA oxidation, and 8-isoprostane (isoprostane), a marker of lipid peroxidation and global oxidative stress, by Cayman Chemical (Ann Arbor, MI). Urine samples were hydrolyzed and affinity purified prior to determination of isoprostane concentrations, and both OHdG and isoprostane were measured using enzyme immunoassay. The inter-assay coefficients of variation range from 4.5 to 24.3%. OHdG or isoprostane concentrations below the LOD were replaced by the $\text{LOD} / 2$ (Hornung and Reed, 1990).

2.4 Inflammation Biomarkers

At the time of the present analysis, a total of 187 blood samples from visits 1 ($n=103$) and 3 ($n=84$) were available from a subset of 105 participants for measurement of inflammation biomarkers. These samples were processed for the collection of plasma, which was divided into aliquots, frozen at -80°C , and shipped overnight on dry ice to the University of Michigan. C-reactive protein (CRP) and the cytokines interleukin (IL)-1 β , IL-6, IL-10, and tumor necrosis factor (TNF)- α were measured by the University of Michigan Cancer Center

Immunological Monitoring Core (Ann Arbor, MI). CRP was measured using a DuoSet enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN), and cytokines were measured using a Milliplex MAP High Sensitivity Human Cytokine Magnetic Bead Panel (EMD Millipore Corp., St. Charles, MO) with a Luminex L200 instrument (Luminex, Austin, TX). Cytokines were analyzed in duplicate and an arithmetic average of the two measurements was created for data analysis. The inter-assay coefficients of variation for the CRP and cytokine assays range from 5 to 19%. CRP and cytokine levels below the LOD were replaced by the LOD/ 2 (Hornung and Reed, 1990).

2.5 Statistical analysis

For descriptive analyses, phenol, paraben, and markers of oxidative stress concentrations were standardized to urinary specific gravity using the following formula: $P_s = P[(1.020 - 1)/(SG_i - 1)]$ where P_s is the SG-standardized concentration, P is the measured concentration, 1.020 is the median urinary specific gravity in this population, and SG_i is the individual sample urinary specific gravity. We calculated geometric means (GM) and standard deviations (GSD), as well as selected percentiles to describe distributions of SG-standardized urinary phenol and paraben concentrations. Distributions of oxidative stress and inflammation biomarkers, as well as correlations between these biomarkers, have been previously described (Ferguson et al., 2014a).

We used linear mixed models (LMM) to assess potential covariates, including maternal pre-pregnancy BMI, maternal education (categorical variables), and maternal age at the first study visit (continuous variable), in relation to repeated outcome measurements. We also used LMMs to examine associations between natural log-transformed urinary phenol and paraben concentrations and natural log-transformed markers of oxidative stress and inflammation. Each model had one marker of exposure (e.g. BPA) as a predictor of one outcome (e.g. OHdG) with a random intercept for subject ID to account for correlation among individuals across study visits. Baseline models included the study visit at which the sample was collected and urinary specific gravity as time-dependent covariates, while fully adjusted models included study visit, urinary specific gravity, as well as the time-invariant covariates maternal pre-pregnancy BMI, and education. Linear relationships between markers of exposure and outcome were evaluated by examining scatterplots of residual vs. predicted values from each model. Results are presented as the percent difference in oxidative stress or inflammation biomarker concentration (95% confidence interval) in non-transformed units per interquartile range (IQR) increase in natural log-transformed phenol or paraben concentration, calculated using the following formula:

$$\% \text{ difference per IQR increase} = \left(\left(75^{th} \text{ percentile} / 25^{th} \text{ percentile} \right)^{\beta} - 1 \right) \times 100.$$

We recently reported positive associations between urinary phthalate metabolites and markers of oxidative stress in the PROTECT cohort (Ferguson et al., 2014a). Accordingly, we examined Spearman correlations between urinary phthalate metabolites, phenols, and parabens within this study population. In sensitivity analyses we added individual phthalate metabolites that were correlated with urinary phenol or paraben levels, as well as

independent predictors of markers of oxidative stress, to our models to adjust for potential confounding due to co-exposure to these compounds. In addition, we investigated windows of susceptibility for phenol and paraben exposure in relation to inflammation and oxidative stress by including an interaction term for urinary phenol or paraben concentrations and study visit in LMM. All analyses were performed using SAS version 9.3 (Cary, NC).

3. Results

Demographic characteristics on similar subsets of this study population have been presented elsewhere (Cantonwine et al., 2014). Briefly, participants were on average 27.1 years of age, and the majority attended or graduated from college (82%), and had a BMI ≥ 25 kg/m² (57%) (Supplementary Material, Table S1). Urinary phenol and paraben distributions among currently enrolled PROTECT participants (n=141) were very similar to distributions among participants who also had measures of oxidative stress and/or inflammation (n=106)(Table 1). Urinary 24-DCP and 25-DCP were strongly correlated (Spearman $r = 0.79$, $p < 0.001$), while most other phenols were weakly to moderately correlated ($r = -0.02$ to 0.38) (Supplementary Material, Table S2). Correlations between urinary paraben concentrations ranged from 0.36 (MPB and B-PB) to 0.77 (M-PB and P-PB)(Supplementary Material, Table S2). With the exception of BPA, the majority of urinary phenols and parabens were not correlated or weakly correlated with urinary phthalate metabolite concentrations (Supplementary Material, Table S3). Urinary BPA concentrations were weakly or moderately correlated with all phthalate metabolites, with Spearman correlations ranging from 0.17 with both MCNP and MCOP to 0.36 with MiBP. Distributions of and correlations between markers for oxidative stress and inflammation in this study population have been presented elsewhere (Ferguson et al., 2014a). Briefly, the oxidative stress markers isoprostane and OHdG were detected in all urine samples analyzed and were moderately correlated with one another ($r = 0.43$, $p < 0.01$). The inflammation markers CRP and TNF- α were detected in all serum samples analyzed, while IL-1 β , IL-6, and IL-10 were detected in 64%, 97%, and 99% of serum samples, respectively. The majority of inflammation markers were very weakly to moderately correlated with one another ($r = -0.01$ to 0.40). Individual markers of inflammation were strongly correlated across study visits (visits 1 and 3), while markers of oxidative stress were weakly correlated across study visits (visits 1, 2, and 3) (Supplementary Material, Table S4).

3.1 Associations between Covariates and Markers of Oxidative Stress and Inflammation

Markers of oxidative stress were not significantly associated with demographic covariates (Table 2). Higher maternal pre-pregnancy BMI was significantly associated with higher concentrations of the pro-inflammatory markers IL-6 and CRP, while higher education was significantly associated with lower concentrations of IL-6 (Table 3).

3.2 Phenols and Parabens in Relation to Markers of Oxidative Stress

BPA was significantly associated with both markers of oxidative stress, with an IQR increase in BPA associated with 21% higher urinary OHdG ($p = 0.001$) and 29% higher urinary isoprostane ($p = 0.0002$) after adjustment for urinary SG, visit, maternal BMI, and education (Table 4). IQR increases in BP-3 and the parabens B-PB and P-PB were

significantly associated with 17%, 27% and 20% higher isoprostane ($p=0.05$, $p=0.01$, $p=0.02$), respectively, after adjustment for covariates. TCS, 24-DCP, 25-DCP, and M-PB were not associated with markers of oxidative stress (Table 4).

3.3 Phenols and Parabens in Relation to Markers of Inflammation

An IQR increase in TCS was associated with 32% higher IL-6 ($p=0.007$) after adjustment for covariates (Table 5). We observed a suggestive positive association between BPA and IL-6 in our crude analysis, but this association was somewhat attenuated in the fully adjusted model (Table 5). IQR increases in BP-3 and the parabens B-PB and P-PB were associated with 16%, 18%, and 14% lower CRP ($p=0.02$, $p=0.03$, $p=0.06$), respectively. BPA, 24-DCP, 25-DCP, and M-PB were not associated with markers of inflammation (Table 5).

3.4 Sensitivity Analyses

When we additionally controlled for individual urinary phthalate metabolites in models predicting markers of oxidative stress, associations between urinary phenol and paraben concentrations and OHdG did not change. In fact, when we included both BPA and individual di-2-ethylhexyl phthalate (DEHP) metabolites (mono-2-ethylhexyl phthalate, mono-2-ethyl-5-hydroxyhexyl phthalate, mono-2-ethyl-5-oxohexyl phthalate, or mono-2-ethyl-5-carboxypentyl phthalate) in models together, associations between the individual DEHP metabolites and OHdG were no longer significant. However, relationships between urinary BPA, BP-3, and M-PB with isoprostane were attenuated when select phthalate metabolite concentrations, particularly mono-n-butyl phthalate and mono-isobutyl phthalate, were included as covariates (data not shown).

We found a significant interaction between study visit and urinary concentrations of 25-DCP in relation to effects on urinary OHdG concentrations ($p<0.0001$). When we stratified this analysis by study visit, an IQR increase in urinary 25-DCP was marginally associated with 14% lower OHdG at visit 1 ($p=0.11$), associated with 35% lower OHdG at visit 2 ($p=0.004$), and 32% higher OHdG at visit 3 ($p=0.01$). There were no interactions between study visit and other urinary phenol or paraben concentrations in relation to OHdG. In addition, there were no interactions between study visit and any phenol or paraben in relation to isoprostane or markers of inflammation (data not shown).

4. Discussion

In the current study we evaluated relationships between urinary phenol and paraben concentrations and markers of oxidative stress and inflammation during pregnancy. We found that urinary BPA and paraben concentrations were associated with higher levels of oxidative stress biomarkers, while TCS was associated with higher concentrations of IL-6, a pro-inflammatory cytokine (Murphy et al., 2008). In contrast, BP-3 and two parabens were associated with lower concentrations of CRP, an acute phase protein formed in response to inflammation (Marnell et al., 2005). Our findings suggest that oxidative stress, and possibly inflammation, may be mechanisms by which select phenols and parabens may act.

4.1 Comparisons with other studies

In comparison to female adult 2011-2012 NHANES participants, women in the present study had higher median urinary concentrations of all measured markers of exposure, including approximately 2 times higher BPA, 24-DCP, and M-BP, 3-4 times higher P-PB and TCS, and 6 times higher 25-DCP concentrations (CDC, 2014). Women in the present study also had higher levels of isoprostane and CRP, lower levels of IL-10, but similar levels of other markers of inflammation and OHdG compared to pregnant women participating in a study of preterm birth in Boston (Ferguson et al., 2014a; Ferguson et al., 2014b).

Our strongest observations were between urinary BPA concentrations and markers of oxidative stress. A study among Korean adults reported similar associations between urinary BPA and increased OHdG and malondialdehyde (MDA), a marker of global oxidative stress (Yang et al., 2009). However, these relationships were only observed among post-menopausal women, not among premenopausal women or men. The authors hypothesized that lower estrogen levels in post-menopausal women would result in increased availability of estrogen receptors (ER) for binding BPA and subsequent adverse effects, including cellular responses that may trigger oxidative stress and inflammation (Yang et al., 2009). Our observed associations between BPA and oxidative stress among pregnant women, who presumably have higher estrogen concentrations compared to men and both pre and post-menopausal women, suggest that BPA may also act via non-ER-binding mechanisms. In a study of male rats, authors reported associations between prenatal BPA exposure and increased MDA and decreased superoxide dismutase in adulthood, indicating that BPA exposure during sensitive periods of fetal development may be related to increased oxidative stress later in life (Song et al., 2014). Although similar associations need to be explored in human studies, the findings from this animal study suggest that our observed relationship between urinary BPA concentrations and oxidative stress during pregnancy could have a long-term impact on children's health.

Our observed associations between urinary paraben concentrations and increased isoprostane are similar to previously reported associations between urinary parabens and increased MDA among both pregnant women and their newborns (Kang et al., 2013). This previous study also reported a weak association between ethyl paraben (E-PB) and OHdG (Kang et al., 2013) among pregnant women; we similarly observed a weak association between B-PB and OHdG.

We did not observe an association between BP-3 and markers of oxidative stress, although previous animal studies observed exposure-related decreases in glutathione and increases in catalase, both indicators of oxidative stress (Gao et al., 2013; Kato et al., 2006). We also did not observe an association between urinary 24-DCP concentrations and markers of oxidative stress, while an *in vitro* study reported a relationship between 24-DCP exposure and reduced glutathione levels (Bukowska, 2003). However, differences between these studies could be a result of the higher doses typically used in animal studies or considering different markers of oxidative stress. For example, we measured OHdG, a marker of DNA oxidation, and isoprostane, a marker of lipid peroxidation and global oxidative stress, while the discussed

animal studies measured glutathione, an antioxidant that is often decreased under conditions of oxidative stress (Dalle-Donne et al., 2006).

We observed an association between urinary TCS concentrations and higher levels of IL-6, a pro-inflammatory cytokine (Murphy et al., 2008) that has been linked to preterm birth and a number of other adverse birth outcomes (Prins et al., 2012). In contrast, previous *in vitro* studies suggest that TCS might have anti-inflammatory properties (Elwood et al., 2007; Modeer et al., 1996; Mustafa et al., 2000; Wallet et al., 2013), and one *in vitro* study specifically observed decreased IL-6 with TCS exposure in human peripheral whole blood (Barros et al., 2010). We also observed a positive, suggestive association between urinary BPA and plasma IL-6 concentrations, consistent with previous animal and *in vitro* studies in which BPA exposure has been associated with increased IL-6 in mouse hepatic tissue (Moon et al., 2012), and human adipose tissue (Ben-Jonathan et al., 2009; Valentino et al., 2013). However, inflammation is mediated by the release of a number of cytokines from surrounding cells and tissues, a complex process that may not be fully captured in *in vitro* studies. This limitation may complicate making comparisons across study types, and may possibly explain discrepancies between results from our *in vivo* human study and the described *in vitro* studies.

In the present study, urinary BP-3 and parabens were associated with lower plasma CRP concentrations, suggesting exposure may be related to decreased inflammation. These findings are consistent with those from a study in mice that reported inhibition of edema, an anti-inflammatory effect, associated with topical BP-3 application (Couteau et al., 2012). To our knowledge, no studies have examined associations between parabens and inflammation in general, or CRP specifically.

Exposure to various phenols and parabens has been associated with adverse reproductive outcomes in both animal and human studies, but the mechanisms by which these effects occur is unknown. In the present study we found associations between urinary BPA, and possibly paraben concentrations and increased oxidative stress, a potential mediator between exposure and reproductive outcomes. Increased oxidative stress during pregnancy has been associated with preeclampsia and intrauterine growth restriction in several human studies, both of which can result in a multitude of pregnancy complications such as preterm birth and health effects for the child later in life (Al-Gubory et al., 2010). We also observed an association between urinary TCS concentrations, which were relatively high in this population compared to similar female NHANES participants, and increased IL-6, a pro-inflammatory cytokine that has been linked to a number of birth outcomes such as preeclampsia and preterm birth (Prins et al., 2012). IL-6 may be an important mediator between TCS exposure and adverse birth outcomes by initiating an inflammatory cascade that may ultimately lead to spontaneous preterm birth (Challis et al., 2009). However, further research is needed to examine associations between TCS and reproductive outcomes, as well as clarify the inflammatory and anti-inflammatory properties of TCS *in vivo*.

Our observations also suggest that the relationship between urinary 25-DCP and OHdG concentrations, a measure of DNA oxidation, appeared to change over the course of pregnancy. At 20-24 weeks gestation, urinary 25-DCP concentrations were associated with

lower urinary OHdG indicating lower oxidative stress, while at 24-28 weeks gestation, 25-DCP was associated with higher urinary OHdG indicating higher levels of oxidative stress. One explanation for this difference could be chance, as the sample sizes for each study visit are small (visit 1, n=52; visit 2, n=50; visit 3, n=44). Otherwise, it is unclear why 25-DCP may be differentially related to DNA oxidation over the course of pregnancy.

4.2 Strengths and Limitations

Our present analysis was limited by a somewhat small sample size, as it comprises the first group of participants in the ongoing PROTECT project. However, we did measure markers of exposure and outcome at multiple points during pregnancy, increasing the accuracy of our exposure assessment and our power to detect significant relationships. Regardless, urinary phenol and paraben concentrations can vary considerably over pregnancy (Meeker et al., 2013), thus a greater number of urinary measurements could have further improved our exposure assessment. Levels of oxidative stress and inflammation biomarkers may also vary over pregnancy, and we measured markers of inflammation at only two time points. Increasing the number of outcome measurements during pregnancy would have also further improved our ability to detect associations. In addition, given the exploratory nature of these analyses, we made a large number of comparisons, increasing the possibility of chance significant findings. Because urinary phenol and paraben concentrations among women in the current study were generally much higher than concentrations found in women in the US, findings from this study may not be generalizable to populations of pregnant women with lower exposure.

Our analysis also had a number of strengths, most notably repeated measurements of urinary phenol, paraben, oxidative stress, and inflammation biomarkers across gestation. This study design allowed us to examine changes within individuals over time, as well as examine potential sensitive windows of exposure during pregnancy. We also measured multiple markers of both inflammation and oxidative stress, increasing our ability to identify different mechanisms by which phenols and parabens may be related to these processes and the subsequent health effects. *4.3 Conclusion*

Our findings suggest that exposure to BPA, select parabens, and TCS during pregnancy may be related to oxidative stress and inflammation, potential mechanisms by which exposure to these compounds may influence birth outcomes and adverse health effects. More research is necessary to confirm these associations, and to explore relationships between phenols, parabens, and adverse reproductive and birth outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

24-DCP	2,4-dichlorophenol
25-DCP	2,5-dichlorophenol
BP-3	benzophenone-3
BPA	bisphenol A
B-PB	butyl paraben
CPR	c-reactive protein
DEHP	di-2-ethylhexyl phthalate
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
GM	geometric mean
GSD	geometric standard deviation
IL	interleukin
IQR	interquartile range
LMM	linear mixed models
LOD	limit of detection
MDA	malondialdehyde
M-PB	methyl paraben
NHANES	National Health and Nutrition Examination Survey (NHANES)
OHdG	8-hydroxydeoxyguanosine
P-PB	propyl paraben
PROTECT	Puerto Rico Testsite for Exploring Contamination Threats
TCS	triclosan
TNF-α	tumor necrosis factor- α

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Highlights

- We measured exposure, oxidative stress, and inflammation biomarkers across pregnancy.
- An IQR increase in BPA was associated with 21% higher OHdG, 29% higher isoprostane.
- IQR increases in B-PB and P-PB were associated with 27% and 20% higher isoprostane.
- An IQR increase in TCS was associated with 31% higher IL-6, a pro-inflammatory cytokine.
- BP-3 and B-PB were associated with lower CRP, a measure of systemic inflammation.

Table 1

Distributions of SG-corrected urinary phenol and paraben concentrations among PROTECT participants who also have measures of oxidative stress or inflammation (n=106 subjects, 238 samples).

	<u>LOD</u>	<u>%>LOD</u>	<u>GM</u>	<u>GSD</u>	<u>5th</u>	<u>25th</u>	<u>50th</u>	<u>75th</u>	<u>95th</u>
bisphenol A (ng/mL)	0.4	98.7	2.86	2.29	0.86	1.68	2.67	4.48	13.7
triclosan (ng/mL)	2.3	93.3	27.3	7.78	1.53	4.90	26.5	148	913
benzophenone-3 (ng/mL)	0.4	100	58.0	7.57	4.00	13.3	34.5	209	2573
2,4-dichlorophenol (ng/mL)	0.2	98.7	1.70	3.21	0.35	0.71	1.45	3.60	17.3
2,5-dichlorophenol (ng/mL)	0.2	100	29.6	5.00	3.11	8.67	23.1	94.8	570
butylparaben (ng/mL)	0.2	75.6	1.03	7.84	<0.2	<0.2	0.60	6.27	37.6
methylparaben (ng/mL)	1.0	100	149	4.33	8.57	63.2	152	396	1320
propylparaben (ng/mL)	0.2	100	33.3	6.21	1.04	9.60	45.4	122	446

Table 2

Associations^a between covariates and urinary oxidative stress biomarker concentrations adjusting for urinary specific gravity and accounting for within individual correlation across study visits.

Oxidative Stress Biomarkers	OHdG		Isoprostane	
	% difference (95% CI)	p-value	% difference (95% CI)	p-value
BMI (kg/m2)	25	0.80		0.83
	>25 to 30	0.55	-11 (-44, 41)	0.61
	>30	0.51	-5 (-42, 56)	0.84
Maternal Education	ref		ref	
	college	0.58		0.97
HS/equivalent completed	-5 (-43, 59)	0.84	7 (-48, 123)	0.73
	-21 (-58, 46)	0.44	12 (-55, 176)	0.63
Maternal Age at V1 (year)	ref		ref	
	0 (-2, 2)	0.98	1 (-2, 4)	0.62

^a Results from linear mixed models are expressed as the percent difference in oxidative stress biomarker concentrations compared to the reference group for categorical variables, and per unit increase for continuous variables.

Table 3

Associations^a between covariates and serum inflammation biomarker concentrations accounting for within individual correlation across study visits.

Inflammation Biomarkers								
	IL-1β		IL-6		IL-10		p-value	
	% difference (95% CI)	p-value	% difference (95% CI)	p-value	% difference (95% CI)	p-value		
BMI (kg/m2)								
-25	-34 (-60, 9)	0.16	-54 (-73, -20)	0.02	-19 (-52, 36)	0.47		
>25 to -30	-42 (-66, 0)	0.11	-43 (-68, 2)	0.01	-1 (-43, 72)	0.42		
>30	ref	0.06	ref	0.07	ref	0.97		
Maternal Education								
college	13 (-32, 86)	0.44	-57 (-75, -27)	0.01	9 (-34, 81)	0.6		
HS/equivalent completed	63 (-24, 251)	0.64	-63 (-83, -16)	0.002	48 (-32, 220)	0.71		
less than HS	ref	0.21	ref	0.02	ref	0.33		
Maternal Age at V1 (years)	0 (-3, 3)	0.96	-2 (-5, 2)	0.28	1 (-2, 5)	0.51		
TNF-α								
CRP								
	% difference (95% CI)	p-value	% difference (95% CI)	p-value				
BMI (kg/m2)								
-25	1 (-34, 55)	0.89	-57 (-71, -35)	0.0004				
>25 to -30	-6 (-41, 49)	0.96	-45 (-64, -14)	0.0001				
>30	ref	0.79	ref	0.01				
Maternal Education								
college	-37 (-58, -5)	0.1	-9 (-40, 39)	0.08				
HS/equivalent completed	-32 (-64, 29)	0.03	-49 (-73, -4)	0.67				
less than HS	ref	0.24	ref	0.04				
Maternal Age at V1 (years)	-2 (-5, 0)	0.11	-1 (-4, 1)	0.34				

^aResults from linear mixed models are expressed as the percent difference in inflammation biomarker concentrations compared to the reference group for categorical variables, and per unit increase for continuous variables.

Table 4

Percent difference^a in urinary oxidative stress biomarker concentrations with IQR increase in Intraformed urinary phenol or paraben

	Baseline^b (n=54 subjects, 146 samples)			Adjusted^c (n=52 subjects, 140 samples)		
	% difference	95% CI	p-value	% difference	95% CI	p-value
OHdG						
BPA	23.4	11.1, 37.0	0.0001	20.8	8.21, 34.8	0.001
TCS	-1.92	-14.8, 12.8	0.78	-2.20	-15.6, 13.3	0.76
BP-3	4.74	-6.01, 16.7	0.40	5.28	-6.48, 18.5	0.39
24-DCP	-0.14	-10.5, 11.4	0.98	-0.57	-11.1, 11.2	0.92
25-DCP	-7.76	-18.6, 4.57	0.20	-7.70	-18.8, 4.93	0.22
B-PB	15.5	-0.18, 33.6	0.05	13.9	-1.80, 32.1	0.08
M-PB	6.41	-4.17, 18.1	0.24	5.77	-5.29, 18.1	0.32
P-PB	1.62	-9.98, 14.7	0.79	1.57	-10.7, 15.6	0.81
Isoprostane						
	% difference	95% CI	p-value	% difference	95% CI	p-value
BPA	30.2	15.0, 47.3	<.0001	29.2	13.5, 47.2	0.0002
TCS	0.87	-15.5, 20.3	0.92	0.52	-16.5, 21.1	0.96
BP-3	15.1	-0.08, 32.7	0.05	17.0	0.15, 36.6	0.05
24-DCP	12.4	-1.54, 28.2	0.08	11.4	-2.85, 27.7	0.12
25-DCP	4.02	-11.3, 22.0	0.63	2.49	-13.3, 21.1	0.77
B-PB	27.2	6.04, 52.6	0.01	27.3	5.46, 53.8	0.01
M-PB	10.5	-2.88, 25.6	0.13	12.6	-1.68, 29.1	0.09
P-PB	17.5	1.77, 35.7	0.03	20.4	3.39, 40.3	0.02

^aResults are from linear mixed models with random intercepts for subject ID to account for within individual correlation across study visits.

^bAdjusted for urinary SG and study visit;

^cAdjusted for urinary SG, study visit, maternal pre-pregnancy BMI, and maternal education.

Table 5

Percent difference^a in serum inflammation biomarker concentrations with IQR increase in Intransformed urinary phenol or paraben

	Baseline^b (n=105 subjects, 187 samples)			Adjusted^b (n=101 subjects, 181 samples)		
	% difference	95% CI	p-value	% difference	95% CI	p-value
IL-1β						
BPA	6.78	-5.94, 21.2	0.31	4.65	-7.91, 18.9	0.48
TCS	14.5	-4.59, 37.3	0.14	14.4	-4.51, 37.1	0.14
BP-3	-0.41	-14.8, 16.4	0.96	-0.75	-15.1, 16.1	0.92
24-DCP	1.51	-12.0, 17.2	0.84	2.18	-11.3, 17.7	0.76
25-DCP	-4.59	-18.1, 11.2	0.54	-3.40	-17.0, 12.5	0.65
B-PB	10.1	-8.57, 32.7	0.31	10.5	-8.11, 32.8	0.29
M-PB	-2.10	-15.8, 13.8	0.78	-3.63	-17.1, 12.1	0.63
P-PB	-0.21	-15.1, 17.3	0.98	-1.76	-16.5, 15.6	0.83
IL-6						
	% difference	95% CI	p-value	% difference	95% CI	p-value
BPA	15.0	-0.40, 32.9	0.06	12.5	-2.50, 29.7	0.11
TCS	29.9	6.09, 59.0	0.01	31.5	8.05, 59.9	0.007
BP-3	-7.10	-22.2, 10.9	0.41	-4.81	-19.9, 13.1	0.57
24-DCP	9.86	-6.55, 29.2	0.25	12.1	-4.07, 31.0	0.15
25-DCP	5.96	-11.0, 26.1	0.51	9.12	-7.76, 29.1	0.30
B-PB	13.1	-8.50, 39.9	0.25	15.8	-5.47, 41.7	0.15
M-PB	3.03	-13.2, 22.3	0.73	4.90	-11.2, 23.9	0.57
P-PB	-0.11	-16.9, 20.0	0.99	3.70	-13.4, 24.2	0.69
IL-10						
	% difference	95% CI	p-value	% difference	95% CI	p-value
BPA	-0.09	-12.3, 13.8	0.99	-1.20	-13.4, 12.7	0.85
TCS	11.2	-7.71, 34.0	0.26	13.6	-5.61, 36.8	0.17
BP-3	-2.03	-16.5, 14.9	0.80	-3.88	-18.1, 12.8	0.62
24-DCP	3.46	-10.6, 19.8	0.65	5.72	-8.57, 22.3	0.45
25-DCP	0.87	-13.8, 18.0	0.91	3.31	-11.6, 20.8	0.68
B-PB	6.24	-12.2, 28.6	0.53	5.28	-12.9, 27.2	0.59
M-PB	7.55	-7.81, 25.5	0.35	6.66	-8.62, 24.5	0.41
P-PB	1.12	-14.3, 19.3	0.89	-0.09	-15.4, 18.0	0.99
TNF-α						
	% difference	95% CI	p-value	% difference	95% CI	p-value
BPA	5.18	-1.21, 12.0	0.11	4.85	-1.70, 11.8	0.15
TCS	-1.71	-10.8, 8.29	0.72	-0.93	-10.2, 9.29	0.85
BP-3	-3.68	-11.5, 4.79	0.38	-2.13	-10.3, 6.77	0.62
24-DCP	0.79	-6.40, 8.55	0.83	1.06	-6.26, 8.95	0.78
25-DCP	0.82	-6.95, 9.24	0.84	1.27	-6.67, 9.89	0.76

	Baseline^b (n=105 subjects, 187 samples)			Adjusted^b (n=101 subjects, 181 samples)		
B-PB	6.33	-3.88, 17.6	0.23	5.69	-4.67, 17.2	0.29
M-PB	1.53	-6.37, 10.1	0.71	2.00	-6.18, 10.9	0.64
P-PB	-1.16	-9.32, 7.73	0.79	-0.83	-9.29, 8.42	0.85
CRP						
	% difference	95% CI	p-value	% difference	95% CI	p-value
BPA	6.83	-5.82, 21.2	0.30	5.10	-7.47, 19.4	0.44
TCS	11.9	-5.94, 33.2	0.20	8.93	-8.50, 29.7	0.33
BP-3	-13.1	-24.8, 0.29	0.05	-16.3	-27.5, -3.42	0.02
24-DCP	2.04	-11.1, 17.1	0.77	2.37	-10.6, 17.3	0.73
25-DCP	0.06	-13.5, 15.8	0.99	0.99	-12.6, 16.7	0.89
B-PB	-19.4	-32.0, -4.36	0.01	-17.5	-30.3, -2.27	0.03
M-PB	-6.90	-19.1, 7.19	0.32	-6.75	-19.0, 7.38	0.33
P-PB	-14.0	-26.0, -0.09	0.05	-13.6	-25.8, 0.50	0.06

^aResults are from linear mixed models with random intercepts for subject ID to account for within individual correlation across study visits.

^bAdjusted for urinary SG and study visit;

^cAdjusted for urinary SG, study visit, maternal pre-pregnancy BMI, and maternal education.