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An exploratory analysis of urinary organophosphate ester metabolites and oxidative stress among pregnant women in Puerto Rico

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Abstract

Background: Organophosphate esters (OPEs) are used as flame retardants and plasticizers. Oxidative stress, the imbalance of reactive oxygen species and antioxidants, measured prenatally has been associated with adverse birth outcomes including preeclampsia and preterm birth. We are the first study to investigate the relationship between OPEs and oxidative stress among pregnant women.

Methods: Pregnant women 18–40 yrs. were recruited in Northern Puerto Rico (n=47) between 2011 and 2015. OPE concentrations of: bis(2-chloroethyl) phosphate (BCETP), bis(1-chloro-2-propyl) phosphate (BCPP), bis(1,3-dichloro-2-propyl) phosphate (BDCPP), dibutyl phosphate (DNBP), and diphenyl phosphate (DHP) and biomarkers for oxidative stress, 8-hydroxy-2'-

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Conflicts of Interest

The authors declare no conflicts of interest.

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deoxyguanosine (8-OHdG) and 8-isoprostane were measured in urine up to three times during pregnancy. Associations between oxidative stress biomarkers and OPEs were assessed using linear mixed models adjusted for specific gravity, age, BMI, and income.

Results: Metabolites BCEtP, BDCPP, and DPHP were frequently detected (>97%). OPE metabolite concentrations remained stable over time (Intraclass correlation coefficients (ICCs): 0.51–0.60). Metabolites BCEtP, BCPP, and DPHP were associated with an increase in 8-isoprostane and OHdG. An interquartile range (IQR) increase in BDCPP was associated with a 21% increase in 8-isoprostane ($p<0.01$), while an IQR increase in DPHP and BCPP was associated with a 12% increase ($p=0.04$, $p=0.08$, respectively). IQR increases in BDCPP and DPHP were also associated with an 18 and 19% increase in OHdG, respectively ($p<0.01$).

Conclusion: OPE metabolites were frequently detected and our results suggest that exposure to OPEs is associated with higher levels of oxidative stress. Further investigation into these relationships and birth outcomes is warranted.

Keywords

Organophosphate Esters; Oxidative Stress; 8-isoprostane; Prenatal

1. Introduction

Organophosphate esters (OPEs) are frequently added to consumer and industrial products such as flame retardants (FRs), plasticizers, and surfactants (van der Veen and De Boer, 2012). In the early 2000s, evidence of bioaccumulation and adverse health effects associated with exposure to another class of flame retardant chemicals, polybrominated diphenyl ethers (PBDEs), led to PBDEs voluntary and mandatory phase-out of U.S and EU markets (Stubbings et al., 2018; USEPA, 2009). As the use of PBDEs diminished, OPEs became a popular FR alternative and by 2008 were classified as high production volume chemicals with production exceeding 1000 tons per year in the EU (Pantelaki and Voutsas, 2018; Papagni et al., 2015). For example, triphenyl phosphate (TPHP), tris(2-chloroethyl) phosphate (TCEP), and tris(1,3-dichloroisopropyl) phosphate (TDCIPP) are commonly used FRs and also frequently added to lacquers, paints, glues, and hydraulic fluids (He et al., 2018; Mendelsohn et al., 2016; Wei et al., 2015).

During manufacturing, OPEs are added or physically mixed with other compounds and are not covalently bound to the product(s). Weak bonds allow these semi-volatile compounds to escape into the environment via leaching, abrasion, or volatilization which has led to widespread exposure (Cequier et al., 2015; He et al., 2018; Stapleton et al., 2008). OPEs are rapidly metabolized in the body into dialkyl and diaryl phosphate esters, or hydroxylated OPEs (Hou et al., 2016; Reemtsma et al., 2011). These metabolites have been detected in nearly 100% of urine samples among men, women, and children (Cequier et al., 2015; Hoffman et al., 2017; Meeker et al., 2013b; Ospina et al., 2018; Phillips et al., 2018). Exposure to OPEs has been associated with endocrine disruption, adverse reproductive health effects, and birth outcomes (Carignan et al., 2017; Hoffman et al., 2018; Ingle et al., 2018; Meeker and Stapleton, 2009). TCEP and TDCIPP are also classified as carcinogens

(State of California Environmental Protection Agency Office of Environmental Health Hazard Assessment, 2017).

While the evidence of adverse health effects associated with OPE exposure expands, few studies have explored possible pathways by which these outcomes occur. Oxidative stress, the imbalance of reactive oxygen species (ROS) and antioxidants, has been a suggested mechanism linking chemical exposure and adverse pregnancy outcomes, such as preeclampsia, preterm birth, and intrauterine growth restriction (Ferguson et al., 2015; Mert et al., 2012; Pathak et al., 2010; van 't Erve et al., 2019). Animal and *in vitro* studies have shown exposure to TPHP and TCEP have induced oxidative stress in male mice and Leydig cells (Chen et al., 2015a, 2015b). Oxidative stress results in the damage of mitochondrial and nuclear DNA, as well as lipid peroxidation (Dev Banerjee et al., 2001). Two biomarkers frequently used in epidemiologic studies to quantify oxidative stress are 8-isoprostane, a secondary end product of lipid peroxidation, and 8-hydroxy-2'-deoxyguanosine (8-OHdG), a nucleoside released after damaged DNA is repaired (Il'yasova et al., 2012; Mizuno and Kataoka, 2015; Niki, 2014; Valavanidis et al., 2009).

To the best of our knowledge, only one prior human study has assessed the relationship between OPEs and oxidative stress and found positive significant correlations ($p < 0.01$) between OPE metabolites and 8-OHdG among 221 adults living in close proximity to an e-waste recycling site in southern China (Lu et al., 2017). In our present work we expand upon this prior study by including an additional biomarker for oxidative stress and a more robust statistical analysis. The aim of this exploratory analysis was to characterize the association of five urinary OPE metabolites: bis(2-chloroethyl) phosphate (BCEtP), bis(1-chloro-2-propyl) phosphate (BCPP), bis(1,3-dichloro-2-propyl) phosphate (BDCPP), dibutyl phosphate (DNBP), and diphenyl phosphate (DPHP), and two biomarkers for oxidative stress, 8-isoprostane and 8-OHdG among pregnant women in Puerto Rico.

2. Methods

Participants were a subset of pregnant women from the Puerto Rico Testsite for Exploring Contamination Threats (PROTECT), an ongoing longitudinal prospective cohort. Recruitment and inclusion criteria have previously been described in detail (Cantonwine et al., 2014; Meeker et al., 2013a). Briefly, pregnant women (18–40 years) were recruited from seven prenatal clinics in Northern Puerto Rico between 2011–2015. Women were excluded from participation if they reported use of oral contraceptives three months prior to pregnancy, conceived by way of *in vitro* fertilization, or had known medical or pregnancy complications. Research protocols were approved by the ethics and Research Committees of the University of Puerto Rico, participating clinics, the University of Michigan School of Public Health, and Northeastern University. The analysis of deidentified specimens at the Centers for Disease Control and Prevention (CDC) laboratory for OPEs was determined not to constitute engagement in human subjects research. The study was described in detail to all participants and informed consent was obtained from all participants.

Urine collection and analysis for OPEs has been described previously (Ingle et al., 2019). Briefly, women provided up to three spot urine samples throughout pregnancy (Visit 1:

n=48, visit 2: n=46, and visit 3: n=47). Specific gravity was measured at the University of Puerto Rico with a hand-held digital refractometer (Atago Co., Ltd., Tokyo, Japan). Samples were aliquoted and frozen at -80°C before shipment to the CDC's National Center for Environmental Health laboratory (Atlanta, Georgia) where five OPE metabolites: BCEtP, BCPP, BDCPP, DNBP, and DPHP were quantified. The analytical approach has been described elsewhere (Jayatilaka et al., 2017; Ospina et al., 2018). Metabolites were extracted by automated off-line solid phase extraction, isolated by reversed phase high-performance liquid chromatography, and quantified by isotope dilution-electrospray ionization tandem mass spectrometry. Accuracy and precision were assessed by using reagent blanks and quality control materials of high (15 ng/mL) and low (4 ng/mL) concentrations. Limits of detection (LOD) were 0.1 ng/mL for all metabolites. Approximately 70% of DNBP concentrations were below LOD and excluded from analysis. For all other metabolites, concentrations below LOD were imputed to the LOD divided by the square root of two (Hornung and Reed, 1990).

Analytical protocols for oxidative stress biomarkers 8-isoprostane and 8-OHdG have been described in detail previously (Ferguson et al., 2014; Watkins et al., 2015). Prior to quantification of 8-isoprostane, samples were hydrolyzed and affinity purified. Biomarkers 8-isoprostane and 8-OHdG were analyzed using enzyme immunoassay by Cayman Chemical (Ann Arbor, MI). LODs were 2.7 pg/mL for 8-isoprostane and 33 pg/mL for 8-OHdG. All concentrations were above LOD.

Descriptive statistics for women's demographic characteristics were calculated. Distributions of urinary OPE metabolites and biomarkers for oxidative stress were calculated using geometric means, 95% confidence intervals (CIs), and select percentiles. OPEs, 8-isoprostane, and 8-OHdG concentrations are presented as unadjusted and adjusted for specific gravity (SG) as: $C_{SG} = C * [(SG_M - 1) / (SG_i - 1)]$, where C_{SG} = SG-corrected urinary metabolite concentration, C = urinary metabolite concentration, SG_M = mean SG for the population, and SG_i = SG for an individual sample (Boeniger et al., 1993). Spearman correlation coefficients were used to assess the relationship between OPEs and oxidative stress biomarkers. Intraclass correlation coefficients (ICCs) and 95% CIs were calculated for OPEs, 8-isoprostane, and 8-OHdG (unadjusted and SG-adjusted) to assess variability of concentrations within women over time. Metabolites and biomarkers for oxidative stress presented as right-skewed and were transformed by the natural logarithm for further statistical testing.

Associations between OPEs and biomarkers for oxidative stress were evaluated using linear mixed models with random intercepts to account for correlation among individuals over time. Crude and adjusted models were constructed using one OPE metabolite (independent variable) and oxidative stress biomarker (dependent variable). Covariates for adjusted models were selected *a priori* and through bivariate testing (data not shown) (Hoffman et al., 2017; Ospina et al., 2018). Final covariates for adjusted models were SG, age, body mass index kg/m^2 (BMI), gestational age, and income. Missing covariates for continuous variables were imputed with their median values. To ease interpretability, our results are presented as the percent change in oxidative stress biomarker with an interquartile range (IQR) increase in OPE metabolite concentration. To assess any changes in association

between visits, models were associations were also evaluated stratified by visit. Analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC) and R version 3.3.5.

3. Results

Demographic characteristics of an overlapping subset of the PROTECT cohort have been previously reported (Ingle et al., 2019). Briefly, the cohort was comprised primarily of White Hispanic (58%), non-smokers (81%) in their late 20s (Median=27 years) who were slightly overweight prior to pregnancy (median BMI=26 kg/m²) (Supplemental Table 1). The majority of women (75%) reported an annual income below \$40,000, while over 70% reported at least some college education or technical school. Gestational age for the three visits ranged from the second to third trimester. However, for the majority of women, gestational age at each visit fell within the second trimester (Visit 1: Median=17 weeks, Visit 2: Median=24 weeks, and Visit 3: Median=26 weeks).

Distribution of unadjusted and SG-adjusted OPEs and biomarkers of oxidative stress are depicted in Table 1. BCEtP, BDCPP, and DPHP were detected in most samples (>97%) while BCPP was less frequently detected (30%>LOD). Concentrations for BCEtP, BDCPP, and DPHP were also higher compared to BCPP. The GM concentration for DPHP (GM=1.51 ng/mL) was 6 times higher than mean concentrations of BCPP (GM=0.26 ng/mL). BCEtP and BDCPP had similar mean concentrations (GM=1.12 ng/mL and GM=1.15 ng/mL, respectively). Geometric mean concentrations of OHdG were 124.65 ng/mL, while GM concentrations for 8-isoprostane were 266.08 pg/mL.

Correlations among OPE metabolites were weak, yet significant (0.16 r 0.35; p 0.001). ICCs for OPE metabolites and biomarkers of oxidative stress can be found in Table 2. All OPE metabolites remained moderately stable over time (0.51 ICC 0.60). However, when OPEs were adjusted for SG, ICCs for BCEtP became weaker (ICC=0.47). Concentrations of 8-isoprostane (ICC=0.33) and 8-OHdG (ICC=0.15) were less stable over time for participants.

All OPEs metabolites were associated with an increase in 8-isoprostane (Figure 1). An IQR increase in BDCPP was associated with a 21% increase in 8-isoprostane (95% CI: 20.43, 35.82, p =0.004). IQR increases in DPHP and BCPP were both associated with a 12% increase in 8-isoprostane (95% CI: 0.72, 25.79, p =0.04; 95% CI: -1.12, 28.0, p =0.08, respectively). All OPE metabolites except BCPP were also associated with an increase of 8-OHdG (Figure 2). IQR increases in BDCPP and DPHP were associated with 18 and 19% increases in 8-OHdG (95% CI: 6.78, 30.01; p =0.002; 95% CI: 8.98, 29.45; p =0.0001), respectively.

Associations in stratified models were highest for visits one and three (Supplemental Table 3). Associations for all OPE metabolites for both biomarkers were smallest during visit two (none statistically significant). An IQR increase in BDCPP was associated with a 6% increase in 8-isoprostane for visit one (95% CI: 1.8, 10.4, p =0.01). However, and IQR increase in DPHP was associated with an 18.7% increase in 8-isoprostane (95% CI: 6.5, 33.3, p =0.003) for visit three. For visit one, an IQR increase in BDCPP and DPHP was

associated with a 5.5 and 10.4% increase in 8-OHdG (95% CI: 2.8, 8.21, $p<0.001$ and 95% CI: 2.7, 19.7, $p=0.01$, respectively). However, the largest increase (20.8%) was observed for an IQR increase of DPHP for visit three (95% CI: 1.8, 31.0, $p<0.001$).

4. Discussion

OPE metabolites BCEtP, BDCPP, and DPHP were frequently detected among the pregnant women in our cohort. There were weak-to-moderate correlations among OPE metabolites, yet concentrations remained relatively stable over time. All OPE metabolites were associated with an increase in 8-isoprostane and three metabolites were associated with an increase in 8-OHdG. However, metabolites BDCPP and DPHP were associated with the largest increase in both biomarkers for oxidative stress.

We have previously compared the distribution and correlations of OPE metabolites from our cohort with other studies (Ingle et al., 2019). Briefly, concentrations of BCEtP in our sample were nearly 3-fold those of women from the National Health and Nutritional Examination Study (NHANES) in 2013–2014 (GM=0.38 ng/mL) (Ospina et al., 2018). Concentrations of DPHP were considerably higher than those from NHANES (0.92 ng/mL) and a small cohort of pregnant women ($n=23$) in Shanghai, China (GM=1.1 ng/mL), yet similar to a cohort of pregnant women ($n=349$) from North Carolina (NC) (GM= 1.42 ng/mL) (Feng et al., 2016; Hoffman et al., 2018; Ospina et al., 2018). In our study, BDCPP concentrations were higher than pregnant women in China (GM=1.2 ng/mL) and considerably lower than pregnant women in NC (GM=1.80 ng/mL) (Feng et al., 2016; Hoffman et al., 2018). We observed moderate temporal stability of OPE metabolites throughout three study visits, which is considerably higher than we have previously reported with only two visits (0.03 ICC 0.34) (Ingle et al., 2019). Similar stability of our current findings has been observed in a sample of pregnant women ($n=59$) in Rhode Island for BDCPP (ICC=0.60) and DPHP (ICC=0.43) (Romano et al., 2017).

Concentrations of 8-isoprostane remained relatively stable over the three study visits, however 8-OHdG was less stable over time. The reproducibility of our biomarkers for oxidative stress are slightly lower than a cohort of pregnant women in Boston for both 8-isoprostane (ICC=0.60) and 8-OHdG (ICC=0.32) (Ferguson et al., 2015). This is unexpected as our samples spanned a shorter window throughout pregnancy. However, 8-isoprostane concentrations were higher in our sample compared to the Boston cohort (GM=189 pg/mL), yet similar for 8-OHdG (GM=130 ng/mL). A study of pregnant Mexican-American women in California had substantially higher 8-isoprostane levels (GM=4.6 $\mu\text{g/g}$) compared to our women (8-OHdG not measured) (Holland et al., 2016). Differences in concentrations are possibly a result of our samples undergoing affinity purification prior to quantification. The variations in distributions may also be due to differences in demographic characteristics and sample size.

The association of OPEs and oxidative stress has only been studied in one prior cohort. Lu et al. observed weak-to-moderate correlations among urinary OPE metabolites: BCEtP ($r=0.50$), BDCPP ($r=0.48$), DNBP($r=0.21$), and DPHP ($r=0.44$) and 8-OHdG concentrations among 175 adults residing in close proximity to an e-waste dismantling area in southern

China (Lu et al., 2017). Our correlation findings were similar, as we observed significant correlations for BCEtP ($r=0.31$), and DPHP ($r=0.43$) (data not shown). We also observed significant correlations for metabolites BCPP, BDCPP, and DPHP with 8-isoprostane ($r=0.23$ and $r=0.34$, respectively).

All metabolites measured in this analysis were associated with an increase in at least one biomarker for oxidative stress. Stratified models revealed larger increases in oxidative stress biomarkers and OPE metabolites for visits one and three compared to visit two. Some animal and *in vitro* studies have also found associations with OPEs and oxidative stress. A study of male mice who ingested TPHP (one parent compound of DPHP) and TCEP (parent compound of BCEtP) had significant decreases in liver concentrations of glutathione (GSH), and increases of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), which are indicative of oxidative stress (Chen et al., 2015a). Similar increases were also observed among mouse Leydig cells for SOD, CAT, GPX, and GST when exposed to TPHP and BCEtP (Chen et al., 2015b). Taken together with our results, it is possible that OPE exposure induces oxidative stress which elicits an increase in antioxidant enzyme activities. However, it is also plausible that OPEs induce oxidative stress by way of gene transcription. Both mouse and Leydig cell studies also observed an increase in transcription of antioxidant genes (Sod1, Sod2, Gpx1, Gpx2, and Cat) when exposed to OPEs (Chen et al., 2015b, 2015a). A study of chicken embryos exposed to 2-ethylhexyl diphenyl phosphate (EHDPP), another parent compound of DPHP, found dysregulation of OGG1, a gene also associated with oxidative stress (Shen et al., 2019).

Our study is not without limitations. Our sample size was modest, yet we did have repeated measures for each participant which resulted in an increase of power. The majority of our samples were taken during the second and third trimester which may have missed some early critical windows of vulnerability. However, we did have corresponding measurements for both OPE and oxidative stress biomarker for each woman, therefore increasing the accuracy and precision of our findings. Some studies have also questioned the reliability of 8-OHdG as an adequate biomarker due to the variability in results from different laboratory methods as well as the timing and frequency of urine collection (Barregard et al., 2013). However, a study comparing many biomarkers for oxidative stress suggests 8-OHdG, when corrected for urine dilution, is a reliable biomarker when using spot urine samples as it had the highest reproducibility ($ICC=0.96$) compared to other frequently used biomarkers (Martinez-Moral and Kannan, 2019). It has also been argued that elevated 8-isoprostane concentrations are the result of both oxidative stress and inflammation and further parsing out between the two is preferred (van 't Erve et al., 2019).

Apart from the previous study solely assessing the relationship between OPE metabolites and 8-OHdG, the present study is the most robust to examine the potential relationship between OPE metabolites and oxidative stress. To the best of our knowledge, we are the first to examine the relationship with OPEs and 8-isoprostane. Our use of urine samples for OPEs and oxidative stress measures is also preferred over other biospecimens such as plasma (Klawitter et al., 2011; Morrow et al., 1990). The prospective design of this study allowed our capture of repeated measurements for both OPE metabolite and biomarkers for oxidative

stress which allowed participants to serve as their own reference over time, thus increasing the accuracy of our estimates. Finally, measuring biomarkers for both DNA damage (8-OHdG) and lipid peroxidation (8-isoprostane) provides insight on the various pathways in which OPEs may be associated with oxidative stress.

5. Conclusions

In the present work, we observed positive associations with all OPE metabolites and at least one biomarker for oxidative stress. Metabolites BDCPP and DPHP had the strongest association with both 8-isoprostane and 8-OHdG. It is possible that OPE metabolites elicit oxidative stress by affecting antioxidant enzyme activities or altering gene transcription. Although this is the most robust study to date, future investigations should focus on a more comprehensive quantification of oxidative stress and explore any possible adverse birth outcomes related to OPEs which may be mediated by oxidative stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Organophosphate ester metabolites were widely detected among pregnant women.
- Metabolite concentrations remained relatively stable over the study period.
- All metabolites were associated with an increase in 8-isoprostane.
- Most metabolites were associated with an increase in 8-hydroxydeoxyguanosine.

Figure 1.

Percent change (95% confidence interval) in urinary 8-isoprostane concentrations with an interquartile range increase in urinary OPE metabolite concentrations.

Regression coefficients (95% CIs) from linear mixed models were imputed to the percent change in 8-Isoprostane with an interquartile range increase in OPE metabolite concentrations. Models included unadjusted OPE metabolite concentrations and covariates: SG, age, BMI, gestational age, and income.

Figure 2.

Percent change (95% confidence interval) in urinary 8-OHdG concentrations with an interquartile range increase in urinary OPE metabolite concentrations.

Regression coefficients (95% CIs) from linear mixed models were imputed to the percent change in 8-OHdG with an interquartile range increase in OPE metabolite concentrations. Models included unadjusted OPE metabolite concentrations and covariates: SG, age, BMI, gestational age, and income.

Distribution of urinary organophosphate ester (OPE) metabolites and oxidative stress biomarkers among 48 pregnant women in Puerto Rico (n=141 samples)

Table 1.

	N < LOD ^a	% < LOD	GM	95% CI	Percentiles					
					5th	25th	50th	75th	95th	Max
Unadjusted OPEs										
BCEtP	0	0	1.12	(0.94, 1.33)	0.29	0.56	0.91	2.23	7.22	35.70
BCPP	29	20.57	0.26	(0.22, 0.30)	0.07	0.14	0.27	0.43	1.53	3.57
BDCPP	2	1.42	1.15	(0.98, 1.34)	0.29	0.65	1.20	1.94	4.67	16.50
DHPH	4	2.84	1.51	(1.20, 1.90)	0.15	0.77	1.30	3.23	22.00	90.20
SG-Adjusted OPEs										
BCEtP	0	0	1.15	(0.98, 1.36)	0.29	0.59	0.93	2.12	7.62	30.83
BCPP	29	20.57	0.26	(0.23, 0.31)	0.07	0.13	0.26	0.45	1.57	3.08
BDCPP	2	1.42	1.85	(1.02, 1.37)	0.32	0.61	1.19	2.22	4.82	14.93
DHPH	4	2.84	1.56	(1.26, 1.95)	0.25	0.73	1.39	3.09	20.33	85.69
SG ^b			1.02	(1.02, 1.02)	1.01	1.02	1.02	1.02	1.03	1.03
Oxidative Stress Biomarkers										
8-isoprostane	0	0	257.8	(230.9, 287.97)	68.97	173.3	292.3	388.1	649.1	984.2
8-OHdG	0	0	120.8	(109.6, 133.1)	48.16	79.58	121.1	184.4	298.5	797.4
SG-Adjusted Oxidative Stress Biomarkers										
8-isoprostane	0	0	266.1	(240.8, 294.0)	93.12	181.3	277.7	386.1	621.2	2024.8
8-OHdG	0	0	124.7	(114.8, 135.3)	61.50	90.75	121.3	160.1	285.4	841.7

^aLimits of detection (LOD) were 0.10 ng/mL for all OPE metabolites. LOD was 2.7 pg/mL for 8-isoprostane. LOD was 33 ng/mL for 8-OHdG. GM: Geometric mean; CI: Confidence interval, SG: specific gravity; OHdG: 8-Hydroxy-2'-deoxyguanosine;

^bn=3 missing

Table 2.

Intraclass correlation coefficients (ICCs) for OPE metabolites and biomarkers for oxidative stress among 48 pregnant women contributing up to three urine samples (n=141)

	Unadjusted		SG Adjusted	
	ICC	95% CI	ICC	95% CI
OPEs				
BCEtP	0.51	(0.35, 0.67)	0.47	(0.31, 0.63)
BCPP	0.59	(0.44, 0.72)	0.54	(0.38, 0.69)
BDCPP	0.43	(0.26, 0.61)	0.38	(0.21, 0.58)
DPHP	0.60	(0.45, 0.73)	0.36	(0.21, 0.55)
Oxidative Stress				
Biomarkers				
8-isoprostane	0.33	(0.18, 0.53)	0.13	(0.03, 0.42)
8-OHdG	0.15	(0.04, 0.43)	0.02	(0.00, 0.99)

SG: Specific gravity; CI: confidence interval; Visit 1: n=48 measurements, Visit 2: n=46 measurements, Visit 3: n=47 measurements