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Associations of urinary phthalate metabolites and lipid peroxidation with sperm mitochondrial DNA copy number and deletions

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Abstract

Background: Phthalates, a chemical class of plasticizers, are ubiquitous environmental contaminants that have been associated with oxidative stress. Mitochondria DNA copy number (mtDNA_{cn}) and DNA deletions (mtDNA_{del}) are emerging biomarkers for cellular oxidative stress and environment exposures.

Objectives: To examine associations of urinary phthalate metabolite and isoprostane concentrations on sperm mtDNA_{cn} and mtDNA_{del} in male partners undergoing assisted reproductive technologies (ART).

Methods: Ninety-nine sperm samples were collected from male partners undergoing ART at Baystate Medical Center in Springfield, MA as part of the Sperm Environmental Epigenetics and Development Study (SEEDS). Seventeen urinary phthalate metabolite concentrations were analyzed by the Centers for Disease Control using tandem mass spectrometry. Urinary 15-F_{2t}-isoprostane concentrations, a biomarker of lipid peroxidation, were measured using a competitive enzyme-linked immunosorbent assay. A triplex qPCR method was used to determine the relative quantification of mtDNA_{cn} and mtDNA_{del}.

Results: Sperm mtDNA_{cn} and mtDNA_{del} were positively correlated (Spearman rho = 0.31; p = .002). Adjusting for age, BMI, current smoking, race, and measurement batch, urinary monocarboxy-isononyl phthalate (MCNP) concentrations were positively associated with mtDNA_{cn} (β = 1.63, 95% CI: 0.14, 3.11). Other urinary phthalate metabolite and isoprostane concentrations were not associated with sperm mtDNA_{cn} or mtDNA_{del}.

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Conclusions: Among this cohort of male ART participants, those with higher MCNP had higher mtDNAcn; other phthalate metabolites and isoprostane were not associated with mtDNAcn and mtDNA_{del}. Given our relatively small sample size, our results should be interpreted with caution. Future research is needed to replicate the findings in larger studies and among sperm samples obtained from the general population.

Keywords

Phthalates; Sperm; Mitochondrial DNA; Mitochondrial copy number; Mitochondrial deletions

1. Introduction

Phthalate diesters are a class of synthetic organic chemicals used in industrial and consumer products that include flexible vinyl plastics, medical equipment, food packaging, and personal care products (Johns et al., 2015; Zota et al., 2014). Phthalate exposure is widespread, such that urinary phthalate metabolites have been detected in the majority of individuals from representative samples within the U.S. general population (CDC, 2010). In humans, urinary phthalate metabolite concentrations have been associated with adverse male reproductive outcomes such as shorter anogenital distance (Suzuki et al., 2012; Swan et al., 2015), abnormal sex steroid hormone synthesis (Meeker et al., 2009), lower serum testosterone (Meeker and Ferguson, 2014), reduced sperm motility, concentration (Cai et al., 2015; Pant et al., 2014), and increased DNA damage (Hauser et al., 2007). Additionally, studies of phthalate exposure animal models reported reduced sex organ weight (Shono and Taguchi, 2014), abnormal Leydig cell aggregation (Mahood et al., 2007), repression of sex determination (Wang et al., 2016), altered sex ratio (Dobrzynska et al., 2011), and delayed sexual maturity (Lee et al., 2004).

Oxidative stress, characterized by the disruption of redox signaling and control (Jones, 2006), has been proposed a potential mediator in phthalate-associated adverse male reproductive outcome. Oxidative stress measures have been associated with diminished semen quality, including sperm motility, concentration, and morphology (Agarwal et al., 2014). Isoprostanes, formed by the presence of free radicals and commonly used markers of lipid peroxidation in vivo (Kadiiska et al., 2005; Roberts and Morrow, 2002), have been reported to be strongly associated with urinary phthalate metabolite concentrations among pregnant women (Ferguson et al., 2014, 2015; Holland et al., 2016) as well as in our previous study among couples seeking fertility treatment (Wu et al., 2017c). Similarly, a study of couples planning pregnancy reported that select urinary phthalate metabolite concentrations in both males and females were correlated with urinary 8-Oxo-2'-deoxyguanosine concentrations, a biomarker of oxidative stress via DNA oxidation (Guo et al., 2014). It is currently unknown if phthalate exposure directly induces lipid peroxidation, or rather, it involves other intermediate biological targets that induce an imbalance in redox signaling.

Measures of the sperm mitochondrial genome may provide useful markers of oxidative stress, male fertility and environmental exposures. The mitochondrial genome is highly susceptible to oxidative damage due to its proximity to reactive oxygen species produced

during oxidative phosphorylation and lack of protective defenses such as histones and DNA repair mechanisms (Phillips et al., 2014a; Sutovsky et al., 2003). Mitochondria contain their own double-stranded 16.6 kb, circular, maternally-inherited genome that encodes 37 genes, which are involved in a host of biological functions including, most notably, ATP production and other homeostatic and signaling processes (Chen et al., 2012; Phillips et al., 2014b). The number of mitochondria vary widely across cell types and each mitochondrion can harbor between 1 and 10 copies of its genome (Phillips et al., 2014b; Robin and Wong, 1988). Sperm mitochondrial copy number (mtDNA_{cn}), the ratio of the number of mitochondrial genome copies to nuclear DNA (nDNA) copies, and percent of large deletions in the mitochondrial genome (mtDNA_{del}) have both been associated with oxidative stress measures (Abasalt et al., 2013; Bonanno et al., 2016), and have also been associated with male infertility (Rajender et al., 2010). In addition, leukocyte mtDNA_{cn} and mtDNA_{del} have also been associated with markers of oxidative stress (Lee et al., 2000a; Liu et al., 2003a; Wang et al., 2011), further supporting the sensitivity of mtDNA_{cn} and mtDNA_{del} to oxidative stress and their potential use as biomarkers of this imbalance. One hypothesis suggests that increases in mtDNA_{cn} may be a compensatory mechanism by which oxidative damage or mtDNA_{del} induce the propagation of the mitochondrial genome (Lee et al., 2000b). Furthermore, one recent study reported that urinary polycyclic aromatic hydrocarbons (PAH) metabolite concentrations were inversely associated with sperm mtDNA_{cn} among young Chinese men (Ling et al., 2017), indicating the potential responsiveness of sperm mtDNA_{cn} to environmental exposures as has been shown with leukocyte mtDNA_{cn} and air pollution (Hou et al., 2010, 2013; Zhong et al., 2016).

To further characterize the relation of phthalates with measures of male reproductive health and the role of oxidative stress, we examined the associations of urinary phthalate metabolite and isoprostane concentrations with mtDNA_{cn} and mtDNA_{del} in sperm among couples seeking reproductive assistance.

2. Methods and materials

2.1. Study population

Couples were recruited as part of the Sperm Environmental Epigenetics and Development Study (SEEDS) from the Baystate Medical Center In Vitro Fertility (IVF) Program in Springfield, Massachusetts between 2014 and 2017. Inclusion criteria for male participants were 18–55 years of age without vasectomy, and for female participants were 40 years old. Furthermore, both males and females were required to use their own gametes to be eligible to participate in the study. Written consents from eligible males and females interested in participating were obtained by attending physicians. This study was approved by the institutional review boards at Baystate Medical Center and at the University of Massachusetts Amherst.

2.2. Covariate assessments

Relevant demographic (race, age, height, weight) and medical history data (diagnosis of infertility) were collected by clinic personnel during the course of an IVF cycle for both partners. In addition, each partner was asked to complete an intake questionnaire regarding

lifestyle factors (current and past cigarette and alcohol use) prior to sample collection. Current smoking status was determined using a Cotinine ELISA kit (Calbiotech Cat#: CO096D) on a binary basis of smoker or non-smoker.

2.3. Urinary biomarker measurements

2.3.1. Phthalates—Men who agreed to participate provided a spot urine sample in a sterile polypropylene collection cup on the same day of semen sample procurement. Urine samples were vortexed, divided into several aliquots, and stored at -80°C . Urine samples were shipped overnight on dry ice to the National Center for Environmental Health of the Centers for Disease Control and Prevention (CDC), where quantification of urinary 1,2-Cyclohexane dicarboxylic acid diisononyl ester (DINCH) and phthalate metabolites was conducted via enzymatic deconjugation of the metabolites, solid-phase extraction, separation and detection using high performance liquid chromatography isotope dilution tandem mass spectrometry as described previously (Kato et al., 2005; Silva et al., 2013). The following urinary phthalate and DINCH metabolites and were analyzed: Mono(2-ethylhexyl) phthalate (MEHP); Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP); Mono(2-ethyl-5-oxohexyl) phthalate (MEOHP); Mono(2-ethyl-5-carboxypentyl) phthalate (MECPP); Monocarboxyisooctyl phthalate (MCOP); Mono-isononyl phthalate (MNP); Monobenzyl phthalate (MBzP); Mono (3-carboxypropyl) phthalate (MCPP); Monocarboxy-isononyl phthalate (MCNP); Mono-n-butyl phthalate (MBP); Mono-3-hydroxybutyl phthalate (MHBP); Mono-isobutyl phthalate (MiBP); Mono-hydroxyisobutyl phthalate (MHiBP); Mono-ethyl phthalate (MEP); Mono-methyl phthalate (MMP); cyclohexane-1,2-dicarboxylic acid-monomarboxy isooctyl ester (MCOCH); and cyclohexane-1,2-dicarboxylic acid-mono(hydroxyisononyl) ester (MHINCH).

Limits of detection (LOD) varied between batches and ranged from 0.1 to 0.9 ng/mL across all metabolites. Values falling under the LOD for each metabolite on each plate were replaced by the $\text{LOD}/\sqrt{2}$. Analytical standards, quality control (QC) materials prepared from spiked pooled urine, and reagent blank samples were included in each batch along with study samples. The QC concentrations, averaged to obtain one measurement of high-concentration QC and one of low-concentration QC for each batch, were evaluated with standard statistical probability rules (Caudill et al., 2008). The coefficient of variations for the phthalate measurement of QC materials ranged from 6.7% to 11.7% (low concentration standard) and 5.0–9.3% (high concentration standard).

Specific gravity was measured in urine by the CDC or by Baystate Reproductive Clinic using a handheld refractometer for participants (Atago Co., Ltd., Toko, Japan). The formula for dilution normalization of phthalate measurement by specific gravity is $P_c = P[(\text{SGm}-1)/(\text{SG}-1)]$ where P_c is the SG-corrected metabolite concentration (ng/mL), P is the measured phthalate metabolite concentration, SGm is the median SG value of all samples, and SG is the specific gravity value for that individual urine sample. Values were log transformed for analyses.

2.3.2. Isoprostane—A competitive enzyme-linked immunoassay was used to measure urinary total 15-F_{2t} isoprostane with pretreatment with β -glucuronidase as per the

manufacturer's protocol (Cat#: EA84, Oxford Biomedical Research). (Wu et al., 2017c). To increase the sample size used in our previous publication between phthalates and isoprostane (N = 50), we analyzed an additional 49 samples. Each plate was read twice on a SpectraMax M2 microplate reader (Molecular Devices). Samples were run in duplicate and those with coefficient of variations (CV) > 15% were repeated. Two control urines were used across all samples in this study (N = 99) to monitor batch effects. All values were corrected for urinary dilution using specific gravity as previously described above.

2.4. Sperm mitochondrial biomarker measurements

Semen samples were collected in a sterile plastic specimen cup after a recommended 2–3 day abstinence period, per standard IVF protocol. Motile sperm cells were isolated using a two-step gradient fractionation and DNA was isolated using our previously published protocol (Wu et al., 2015).

To assess sperm mtDNA_{cn} and mtDNA_{del}, we utilized the triplex design previously reported (Phillips et al., 2014b) to amplify mitochondria loci located within the MinorArc and MajorArc, respectively. To assess nDNA, we utilized RNase P Copy Number Reference Assay (ThermoFisher, cat# 4403326). A total of 10 ng of total sperm DNA was amplified with ProAmp Master Mix (ThermoFisher, cat#A30865) with final concentrations of 250 μM for MinorArc and MajorArc probes and primers and 1× concentration for RNase P in a 10 μl PCR reaction on a StepOnePlus Real-Time PCR machine (ThermoFisher); the following conditions were used: 95°C for 10 min for activation, then 95°C for 15 s, 55°C for 15 s and 60°C for 60 s for 40 cycles. For each PCR reaction, mtDNA_{cn} was determined via the following formula: $2^{-(\text{mean CT})}$, where $\text{CT} = (\text{RNase P CT} - \text{MinorArc CT})$. Similarly, mtDNA_{del} was calculated via: $2^{-(\text{mean CT})}$, where $\text{CT} = (\text{MinorArc CT} - \text{MajorArc CT})$. All samples were run in triplicate.

A six-point standard curve was used to evaluate the linearity and amplification efficiency of the triplex reaction. All standard curves were $R^2 > 0.999$ and amplification efficiencies of 95.6%, 94.6% and 94.1% for MajorArc, MinorArc and RNase P, respectively. Intra-assay CVs for MtDNA_{cn} and MtDNA_{del} were 2.42% and 2.87%, and inter-assay CVs were 7.82% and 8.88%, respectively.

2.5. Statistical analyses

To examine the distributions of SG-corrected urinary phthalate metabolite and isoprostane concentrations, geometric means and selected percentiles were calculated. For bivariate analyses, we examined the correlation between the exposures, outcomes, and covariates using Spearman's correlation and Wilcoxon rank sum test, where appropriate. Generalized linear regression models (GLM) were used to examine the relationship of mtDNA_{cn} and mtDNA_{del} with urinary phthalate meta-bolite and isoprostane concentrations. Covariates considered for inclusion were: age, body mass index (BMI), current smoking status (yes/no), race (white/non-white), and measurement batches. Analyses were performed using R (R Core Team, Vienna, Austria). Covariates were chosen based on biological plausibility or those found to be associated with exposures or outcomes in bivariate analyses p-value < .1.

3. Results

Table 1 presents the demographic and fertility status for the current study population of men seeking fertility treatment. The average age and BMI was 36.1 ± 5.3 years old and 29.6 ± 6.0 , respectively. The population was predominantly white (76%) and not current smokers (92%). Approximately 36% of individuals are classified as overweight (BMI 25–29.9), while close to 38% of individuals could be classified as obese (BMI ≥ 30). Approximately 27% of the population was diagnosed with male factor infertility.

The distributions of SG-corrected urinary phthalate metabolite and isoprostane concentrations (ng/mL) as well as sperm mtDNAcn (ratio of mtDNA copies per nuclear DNA copies) and mtDNA~~del~~ (percent of mtDNA deletions per total mtDNA copies) for male partners are shown in Table 2. Twelve of the fifteen phthalate metabolites were measured above the LOD in more than 90% of samples (MEOHP, MEHHP, MECPP, MBzP, MBP, MHBP, MHBP, MMP, MCOP, MEP, MCPP, and MCNP). Urinary phthalate concentrations in the current study are in line with those reported previously by our group (Wu et al., 2017b, 2017c) and others (CDC, 2010; Dodge et al., 2015). Lower urinary concentrations were observed for the two metabolites of DINCH, a phthalate alternative, with 38% > LOD for MCOCH and 63.1% > LOD for MHINCH. The geometric mean (interquartile range (IQR)) for mtDNAcn and mtDNA~~del~~ were 1.99 (1.72) copies and 17.0% (14.7%). Additionally, mtDNA~~del~~ and mtDNAcn were found to be significantly correlated (Spearman rho=0.31; p-value = .002).

In bivariate analyses, BMI, current smoking status, and race were all associated with at least one urinary metabolite concentration, but none were associated with either MtDNAcn or MtDNA~~del~~. Therefore, the multivariable models contained only age and measurement batch. Table 3 presents results from multivariable regression models for associations between mtDNAcn and urinary phthalate metabolite or isoprostane concentrations for the study population. Adjusting for age, BMI, current smoking status, race, and measurement batches, MCNP was positively associated with mtDNAcn ($\beta = 1.323$, 95% CI: 0.10, 2.55). No other statistically significant associations were found between mtDNAcn and urinary phthalate metabolite or isoprostane concentrations. Additional adjustment by BMI, current smoking, and race did not substantively impact the results. Table 4 presents results from multivariable regression models for associations between mtDNA~~del~~ and urinary phthalate metabolite or isoprostane concentrations for the study population. No statistically significant associations were found between mtDNA~~del~~ and urinary phthalate metabolite or isoprostane concentrations.

4. Discussion

In this study of male partners in the SEEDS cohort (n = 99), we observed a positive association between urinary concentrations of MCNP and mtDNAcn. Consistent with others (Ferguson et al., 2014, 2015; Holland et al., 2016) and our previous study among a subset of male and female members of the SEEDS cohort (n = 50 couples), we observed urinary phthalate metabolite concentrations to be positively associated with biomarkers of oxidative stress among adults of reproductive age (Wu et al., 2017c). Therefore, we hypothesized that

sperm mtDNA_{cn} and mtDNA_{del} would be positively associated with urinary phthalate metabolite and isoprostane concentrations. However, overall, sperm mtDNA_{cn} and mtDNA_{del} were not associated with urinary concentrations of phthalate metabolites or isoprostane, a marker of lipid peroxidation in this study.

4.1. Environmental exposures

Emerging data indicate that mitochondrial biomarkers may be influenced by environmental exposures (Wallace, 2016). For example, PAHs in indoor dust were inversely associated with leukocyte mtDNA_{cn} (Pieters et al., 2013). In contrast, coke-oven workers with occupational PAHs exposure displayed elevated lymphocytes mtDNA_{cn} compared to controls (Pavanello et al., 2013). Similarly, ambient particulate matter (PM) (Hou et al., 2010), black carbon (Zhong et al., 2016) and occupational exposure to benzene (Carugno et al., 2012; Shen et al., 2008) were found to be positively associated with leukocyte mtDNA_{cn} while elemental carbon levels were reported to be inversely related to leukocyte mtDNA_{cn} (Hou et al., 2013). Finally, prenatal PM exposure was reported to be inversely associated with placental mtDNA_{cn}, while no associations were found between PM exposure and cord blood leukocyte mtDNA_{cn} (Janssen et al., 2012), suggesting cell- and tissue-specific differences between exposures and mitochondrial biomarkers likely exist.

To our knowledge only one study to date has examined associations between environmental exposures and sperm mtDNA_{cn} and mtDNA_{del}. In a cross-sectional study of young Chinese men, four urinary PAH metabolites were reported to be inversely associated with sperm mtDNA_{cn}; however, no associations were found between PAHs and mtDNA_{del} (Ling et al., 2017). In our study, we observed a positive association between urinary MCNP and sperm mtDNA_{cn}. The parent compound of MCNP, di-isodecyl phthalate (DIDP), is a high molecular weight phthalate used in food packaging and other consumer products as a replacement for DEHP; not surprisingly, diet is one of the major routes of human exposure to DIDP (Sakhi et al., 2014). Potential pathways by which MCNP may influence sperm mtDNA_{cn} are unknown. Fewer studies implicate MCNP with negative health outcomes than do other phthalate metabolites, however our results suggest further investigation is warranted. Among pregnant women, urinary MCNP was inversely associated with free testosterone (Sathyanarayana et al., 2017), suggesting an anti-androgenic effect of MCNP. In mice, oral DIDP exposure was recently shown to increase ROS and decrease glutathione levels (Shen et al., 2017), suggesting a DIDP-dependent increase in oxidative stress. However, we have previously reported that other phthalate metabolites, but not MCNP, were associated with urinary isoprostane in men (N = 50) from SEEDS (Wu et al., 2017c) and in current study with a larger samples size (data not shown). Thus, our data do not support a role for isoprostane as a potential mediator in the association observed between MCNP and mtDNA_{cn}, although we cannot rule out the possibility of the involvement of other oxidative stress pathways.

Our previous findings in SEEDS have shown that anti-androgenic phthalate metabolites were associated with sperm DNA methylation and diminished embryo quality (Wu et al., 2017a). In the current study we observed null findings between phthalates and mitochondrial bio-markers, which may be due in part to the tight regulation of sperm mitochondria during

spermatogenesis, a 74 day process in humans in which male germ cells undergo extensive reprogramming events to progress from diploid spermatogonia to haploid spermatozoa. In addition to the morphological and epigenetic changes that occur during spermatogenesis, key regulators of the mitochondrial genome are down-regulated resulting in a depletion of mitochondria and mtDNA in mature sperm that largely occurs during the transition from round to elongated spermatid (Rajender et al., 2010). Approximately 22–80 mitochondria are found in mature mammalian sperm – a tenth of its original number (Bahr and Engler, 1970; St John et al., 2000) and form a tight helix around the midpiece to provide the necessary energy for motility via flagellar propulsion (Rajender et al., 2010). The active depletion of sperm mitochondria underlies the maternal inheritance of mtDNA (Song et al., 2014) and has also been proposed as a mechanism that protects mature sperm against ROS-mediated damage to sperm membrane and DNA (Tremellen, 2008). Indeed, higher sperm mtDNA has been associated with male infertility and poor semen parameters (Gabriel et al., 2012; Kao et al., 2004; May-Panloup et al., 2003; Zhang et al., 2016). Thus, a precise balance of mitochondria is essential to meet the energetic demands of motile sperm while safeguarding against oxidative damage; our overall data suggest that phthalate exposure may not affect this balance. Alternatively, it should be noted that sperm used in our study were obtained after gradient centrifugation, a process that removes abnormal sperm and concentrates the motile fraction representing those sperm with high fertilization capacity. Therefore, we cannot rule out the possibility that phthalates may influence mitochondrial biomarkers in unprocessed semen from the general population. Additionally, our study was limited to a single spot urine sample on the day of semen collection; thus, we recognize that the short half-life of phthalates may pose a concern for accurate exposure assessment; however, several reports indicate that a single spot urine sample sufficiently represents phthalate exposure over several months (Hauser et al., 2004; Hoppin et al., 2004; Teitelbaum et al., 2008).

4.2. Oxidative stress

Previous studies have shown that indices of oxidative stress are positively associated with mtDNA in both sperm (Abasalt et al., 2013; Bonanno et al., 2016) and somatic cells (Lee et al., 2000a; Liu et al., 2003b; Wang et al., 2011). Our overall results found no association between urinary isoprostane and sperm mtDNA and mtDNA. The lack of associations observed between urinary isoprostane and sperm mitochondrial biomarkers in our study is unknown but may be reflective of our biomarker used to assess lipid peroxidation. F₂-isoprostane is considered to be a gold standard of systemic oxidative stress in epidemiologic studies (Yin et al., 2005); however, due to the relatively short half-life of isoprostane, a single spot urine sample represents oxidative stress levels within a 24-h period (Basu and Helmersson, 2005). Thus, urinary isoprostane concentrations in our study may not accurately reflect the state of oxidative stress during the critical window (e.g., transition from round to elongated spermatids) when mitochondria and mtDNA are depleted. Such temporal issues have been proposed to also explain the lack of association between plasma isoprostane and mtDNA in leukocytes (Lakkur et al., 2014). Lastly, we also have to acknowledge that isoprostane is an indicator of systemic marker of oxidative stress and may not reflect localized lipid peroxidation such as 4-hydroxynonenal and malondialdehyde, commonly found in sperm (Moazamian et al., 2015).

5. Conclusions

To our knowledge, this is the first study to investigate the relationship of sperm mtDNA_{cn} and mtDNA_{del} with urinary phthalate metabolite and lipid peroxidation concentrations. Our results from 99 men of couples seeking fertility treatment show that mtDNA_{cn} is associated with MCNP, mtDNA_{del} is not associated with any phthalate metabolites, and isoprostane is not associated with either mtDNA_{cn} or mtDNA_{del}. Given our relatively small sample size, our results should be interpreted with caution. Additional studies are necessary to examine these associations in larger studies of men recruited from fertility clinics as well as the general population.

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References

- Abasalt HC, Gholamali JS, Maryam GC, 2013 Lipid peroxidation and large-scale deletions of mitochondrial DNA in asthenoteratozoospermic patients. *Indian J. Biochem. Biophys* 50, 492–499. [PubMed: 24772973]
- Agarwal A, Virk G, Ong C, du Plessis SS, 2014 Effect of oxidative stress on male reproduction. *World J. Men's Health* 32, 1–17. [PubMed: 24872947]
- Bahr GF, Engler WF, 1970 Considerations of volume, mass, DNA, and arrangement of mitochondria in the midpiece of bull spermatozoa. *Exp. Cell Res* 60, 338–340. [PubMed: 5422964]
- Basu S, Helmersson J, 2005 Factors regulating isoprostane formation in vivo. *Antioxid. Redox Signal* 7, 221–235. [PubMed: 15650410]
- Bonanno O, Romeo G, Asero P, Pezzino FM, Castiglione R, Burrello N, et al., 2016 Sperm of patients with severe asthenozoospermia show biochemical, molecular and genomic alterations. *Reproduction (Camb. Engl.)* 152, 695–704.
- Cai H, Zheng W, Zheng P, Wang S, Tan H, He G, et al., 2015 Human urinary/seminal phthalates or their metabolite levels and semen quality: a meta-analysis. *Environ. Res* 142, 486–494. [PubMed: 26275958]
- Carugno M, Pesatori AC, Dioni L, Hoxha M, Bollati V, Albetti B, et al., 2012 Increased mitochondrial DNA copy number in occupations associated with low-dose benzene exposure. *Environ. Health Perspect* 120, 210–215. [PubMed: 22005026]
- Caudill SP, Schleicher RL, Pirkle JL, 2008 Multi-rule quality control for the age-related eye disease study. *Stat. Med* 27, 4094–4106. [PubMed: 18344178]
- CDC, 2010 Centers for Disease Control and Prevention Fourth National Report on Human Exposure to Environmental Chemicals. Centers for Disease Control and Prevention, Atlanta ga.
- Chen X, Wei S, Yang F, 2012 Mitochondria in the pathogenesis of diabetes: a proteomic view. *Protein Cell* 3, 648–660. [PubMed: 22729395]
- Dobrzynska MM, Tyrkiel EJ, Pachocki KA, 2011 Developmental toxicity in mice following paternal exposure to di-n-butyl-phthalate (dbp). *Biomed. Environ. Sci.: BES* 24, 569–578. [PubMed: 22108425]
- Dodge LE, Williams PL, Williams MA, Missmer SA, Souter I, Calafat AM, et al., 2015 Associations between paternal urinary phthalate metabolite concentrations and reproductive outcomes among couples seeking fertility treatment. *Reprod. Toxicol. (Elmsford NY)* 58, 184–193.
- Ferguson KK, McElrath TF, Ko YA, Mukherjee B, Meeker JD, 2014 Variability in urinary phthalate metabolite levels across pregnancy and sensitive windows of exposure for the risk of preterm birth. *Environ. Int* 70, 118–124. [PubMed: 24934852]

- Ferguson KK, McElrath TF, Mukherjee B, Loch-Caruso R, Meeker JD, 2015 Associations between maternal biomarkers of phthalate exposure and inflammation using repeated measurements across pregnancy. *PLoS One* 10, e0135601. [PubMed: 26317519]
- Gabriel MS, Chan SW, Alhathal N, Chen JZ, Zini A, 2012 Influence of micro-surgical varicocele on human sperm mitochondrial DNA copy number: a pilot study. *J. Assist. Reprod. Genet* 29, 759–764. [PubMed: 22562241]
- Guo Y, Weck J, Sundaram R, Goldstone AE, Louis GB, Kannan K, 2014 Urinary concentrations of phthalates in couples planning pregnancy and its association with 8-hydroxy-2'-deoxyguanosine, a biomarker of oxidative stress: longitudinal investigation of fertility and the environment study. *Environ. Sci. Technol* 48, 9804–9811. [PubMed: 25068827]
- Hauser R, Meeker JD, Park S, Silva MJ, Calafat AM, 2004 Temporal variability of urinary phthalate metabolite levels in men of reproductive age. *Environ. Health Perspect* 112, 1734–1740. [PubMed: 15579421]
- Hauser R, Meeker JD, Singh NP, Silva MJ, Ryan L, Duty S, et al., 2007 DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. *Human. Reprod. (Oxf. Engl.)* 22, 688–695.
- Holland N, Huen K, Tran V, Street K, Nguyen B, Bradman A, et al., 2016 Urinary phthalate metabolites and biomarkers of oxidative stress in a mexican-american cohort: variability in early and late pregnancy. *Toxics* 4.
- Hoppin JA, Ulmer R, London SJ, 2004 Phthalate exposure and pulmonary function. *Environ. Health Perspect* 112, 571–574. [PubMed: 15064163]
- Hou L, Zhu ZZ, Zhang X, Nordio F, Bonzini M, Schwartz J, et al., 2010 Airborne particulate matter and mitochondrial damage: a cross-sectional study. *Environ. Health.: a Glob. Access Sci. Source* 9, 48.
- Hou L, Zhang X, Dioni L, Barretta F, Dou C, Zheng Y, et al., 2013 Inhalable particulate matter and mitochondrial DNA copy number in highly exposed individuals in beijing, china: a repeated-measure study. *Part. Fibre Toxicol* 10, 17. [PubMed: 23628000]
- Janssen BG, Munters E, Pieters N, Smeets K, Cox B, Cuypers A, et al., 2012 Placental mitochondrial DNA content and particulate air pollution during in utero life. *Environ. Health Perspect* 120, 1346–1352. [PubMed: 22626541]
- Johns LE, Cooper GS, Galizia A, Meeker JD, 2015 Exposure assessment issues in epidemiology studies of phthalates. *Environ. Int* 85, 27–39. [PubMed: 26313703]
- Jones DP, 2006 Redefining oxidative stress. *Antioxid. Redox Signal* 8, 1865–1879. [PubMed: 16987039]
- Kadiiska MB, Gladen BC, Baird DD, Graham LB, Parker CE, Ames BN, et al., 2005 Biomarkers of oxidative stress study iii. Effects of the nonsteroidal anti-inflammatory agents indomethacin and meclofenamic acid on measurements of oxidative products of lipids in ccl4 poisoning. *Free Radic. Biol. Med* 38, 711–718. [PubMed: 15721981]
- Kao SH, Chao HT, Liu HW, Liao TL, Wei YH, 2004 Sperm mitochondrial DNA depletion in men with asthenospermia. *Fertil. Steril* 82, 66–73. [PubMed: 15236991]
- Kato K, Silva MJ, Needham LL, Calafat AM, 2005 Determination of total phthalates in urine by isotope-dilution liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci* 814, 355–360.
- Lakkur S, Bostick RM, Roblin D, Ndirangu M, Okosun I, Annor F, et al., 2014 Oxidative balance score and oxidative stress biomarkers in a study of whites, african americans, and african immigrants. *Biomark.: Biochem. Indic. Expo. Response Susceptibility Chem* 19, 471–480.
- Lee HC, Yin PH, Lu CY, Chi CW, Wei YH, 2000a Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. *Biochem. J* 348 (Pt 2), 425–432. [PubMed: 10816438]
- Lee HC, Yin PH, Lu CY, Chi CW, Wei YH, 2000b Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. *Biochem. J* 348 (Pt 2), 425–432. [PubMed: 10816438]

- Lee KY, Shibutani M, Takagi H, Kato N, Takigami S, Uneyama C, et al., 2004 Diverse developmental toxicity of di-n-butyl phthalate in both sexes of rat offspring after maternal exposure during the period from late gestation through lactation. *Toxicology* 203, 221–238. [PubMed: 15363597]
- Ling X, Zhang G, Sun L, Wang Z, Zou P, Gao J, et al., 2017 Polycyclic aromatic hydrocarbons exposure decreased sperm mitochondrial DNA copy number: a cross-sectional study (marhcs) in chongqing, china. *Environ. Pollut. (Barking Essex: 1987)* 220, 680–687.
- Liu CS, Tsai CS, Kuo CL, Chen HW, Lii CK, Ma YS, et al., 2003a Oxidative stress-related alteration of the copy number of mitochondrial DNA in human leukocytes. *Free Radic. Res* 37, 1307–1317. [PubMed: 14753755]
- Liu CS, Tsai CS, Kuo CL, Chen HW, Lii CK, Ma YS, et al., 2003b Oxidative stress-related alteration of the copy number of mitochondrial DNA in human leukocytes. *Free Radic. Res* 37, 1307–1317. [PubMed: 14753755]
- Mahood IK, Scott HM, Brown R, Hallmark N, Walker M, Sharpe RM, 2007 In utero exposure to di(n-butyl) phthalate and testicular dysgenesis: comparison of fetal and adult end points and their dose sensitivity. *Environ. Health Perspect* 115 (Suppl. 1), 55–61. [PubMed: 18174951]
- May-Panloup P, Chretien MF, Savagner F, Vasseur C, Jean M, Malthiery Y, et al., 2003 Increased sperm mitochondrial DNA content in male infertility. *Hum. Reprod. (Oxf. Engl.)* 18, 550–556.
- Meeker JD, Hu H, Cantonwine DE, Lamadrid-Figueroa H, Calafat AM, Ettinger AS, et al., 2009 Urinary phthalate metabolites in relation to preterm birth in mexico city. *Environ. Health Perspect* 117, 1587–1592. [PubMed: 20019910]
- Meeker JD, Ferguson KK, 2014 Urinary phthalate metabolites are associated with decreased serum testosterone in men, women, and children from nhanes 2011–2012. *J. Clin. Endocrinol. Metab* 99, 4346–4352. [PubMed: 25121464]
- Moazamian R, Polhemus A, Connaughton H, Fraser B, Whiting S, Gharagozloo P, et al., 2015 Oxidative stress and human spermatozoa: diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. *Mol. Hum. Reprod* 21, 502–515. [PubMed: 25837702]
- Pant N, Kumar G, Upadhyay AD, Patel DK, Gupta YK, Chaturvedi PK, 2014 Reproductive toxicity of lead, cadmium, and phthalate exposure in men. *Environ. Sci. Pollut. Res. Int* 21, 11066–11074. [PubMed: 24816463]
- Pavanello S, Dioni L, Hoxha M, Fedeli U, Mielzynska-Svach D, Baccarelli AA, 2013 Mitochondrial DNA copy number and exposure to polycyclic aromatic hydrocarbons. *Cancer Epidemiol. Biomarkers Prevent.: Publ. Am. Assoc. Cancer Res. Cospons. Am. Soc. Prevent. Oncol* 22, 1722–1729.
- Phillips NR, Sprouse ML, Roby RK, 2014a Simultaneous quantification of mitochondrial DNA copy number and deletion ratio: a multiplex real-time pcr assay. *Sci. Rep* 4, 3887. [PubMed: 24463429]
- Phillips NR, Sprouse ML, Roby RK, 2014b Simultaneous quantification of mitochondrial DNA copy number and deletion ratio: a multiplex real-time pcr assay. *Sci. Rep* 4, 3887. [PubMed: 24463429]
- Pieters N, Koppen G, Smeets K, Napierska D, Plusquin M, De Prins S, et al., 2013 Decreased mitochondrial DNA content in association with exposure to polycyclic aromatic hydrocarbons in house dust during wintertime: from a population enquiry to cell culture. *PLoS One* 8, e63208. [PubMed: 23658810]
- Rajender S, Rahul P, Mahdi AA, 2010 Mitochondria, spermatogenesis and male infertility. *Mitochondrion* 10, 419–428. [PubMed: 20595008]
- Roberts LJ, 2nd, Morrow JD, 2002 Products of the isoprostane pathway: unique bioactive compounds and markers of lipid peroxidation. *Cell. Mol. Life Sci.: CMLS* 59, 808–820. [PubMed: 12088281]
- Robin ED, Wong R, 1988 Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. *J. Cell. Physiol* 136, 507–513. [PubMed: 3170646]
- Sakhi AK, Lillegaard IT, Voorspoels S, Carlsen MH, Loken EB, Brantsaeter AL, et al., 2014 Concentrations of phthalates and bisphenol a in norwegian foods and beverages and estimated dietary exposure in adults. *Environ. Int* 73, 259–269. [PubMed: 25173060]
- Sathyanarayana S, Butts S, Wang C, Barrett E, Nguyen R, Schwartz SM, et al., 2017 Early prenatal phthalate exposure, sex steroid hormones, and birth outcomes. *J. Clin. Endocrinol. Metab* 102, 1870–1878. [PubMed: 28324030]

- Shen M, Zhang L, Bonner MR, Liu CS, Li G, Vermeulen R, et al., 2008 Association between mitochondrial DNA copy number, blood cell counts, and occupational benzene exposure. *Environ. Mol. Mutagen* 49, 453–457. [PubMed: 18481315]
- Shen S, Li J, You H, Wu Z, Wu Y, Zhao Y, et al., 2017 Oral exposure to diisodecyl phthalate aggravates allergic dermatitis by oxidative stress and enhancement of thymic stromal lymphopoietin. *Food Chem. Toxicol.: Int. J. Publ. Br. Ind. Biol. Res. Assoc* 99, 60–69.
- Shono T, Taguchi T, 2014 Short-time exposure to mono-n-butyl phthalate (mbp)-induced oxidative stress associated with DNA damage and the atrophy of the testis in pubertal rats. *Environ. Sci. Pollut. Res. Int* 21, 3187–3190. [PubMed: 24310901]
- Silva MJ, Jia T, Samandar E, Preau JL, Jr., Calafat AM, 2013 Environmental exposure to the plasticizer 1,2-cyclohexane dicarboxylic acid, diisononyl ester (dinch) in U.S. Adults (2000–2012). *Environ. Res* 126, 159–163. [PubMed: 23777640]
- Song WH, Ballard JW, Yi YJ, Sutovsky P, 2014 Regulation of mitochondrial genome inheritance by autophagy and ubiquitin-proteasome system: implications for health, fitness, and fertility. *BioMed. Res. Int* 2014, 981867. [PubMed: 25028670]
- St John JC, Sakkas D, Barratt CL, 2000 A role for mitochondrial DNA and sperm survival. *J. Androl* 21, 189–199. [PubMed: 10714811]
- Sutovsky P, Manandhar G, Wu A, Oko R, 2003 Interactions of sperm perinuclear theca with the oocyte: implications for oocyte activation, anti-polyspermy defense, and assisted reproduction. *Microsc. Res. Tech* 61, 362–378. [PubMed: 12811742]
- Suzuki Y, Yoshinaga J, Mizumoto Y, Serizawa S, Shiraishi H, 2012 Foetal exposure to phthalate esters and anogenital distance in male newborns. *Int. J. Androl* 35, 236–244. [PubMed: 21696396]
- Swan SH, Sathyanarayana S, Barrett ES, Janssen S, Liu F, Nguyen RH, et al., 2015 First trimester phthalate exposure and anogenital distance in newborns. *Hum. Reprod. (Oxf. Engl.)* 30, 963–972.
- Teitelbaum SL, Britton JA, Calafat AM, Ye X, Silva MJ, Reidy JA, et al., 2008 Temporal variability in urinary concentrations of phthalate metabolites, phytoestrogens and phenols among minority children in the united states. *Environ. Res* 106, 257–269. [PubMed: 17976571]
- Tremellen K, 2008 Oxidative stress and male infertility – a clinical perspective. *Hum. Reprod. Update* 14, 243–258. [PubMed: 18281241]
- Wallace DC, 2016 Genetics: mitochondrial DNA in evolution and disease. *Nature* 535, 498–500. [PubMed: 27383787]
- Wang YC, Lee WC, Liao SC, Lee LC, Su YJ, Lee CT, et al., 2011 Mitochondrial DNA copy number correlates with oxidative stress and predicts mortality in non-diabetic hemodialysis patients. *J. Nephrol* 24, 351–358. [PubMed: 20954133]
- Wang YX, Zeng Q, Sun Y, You L, Wang P, Li M, et al., 2016 Phthalate exposure in association with serum hormone levels, sperm DNA damage and spermatozoa apoptosis: a cross-sectional study in china. *Environ. Res* 150, 557–565. [PubMed: 26654563]
- Wu H, de Gannes MK, Luchetti G, Pilsner JR, 2015 Rapid method for the isolation of mammalian sperm DNA. *BioTechniques* 58, 293–300. [PubMed: 26054765]
- Wu H, Ashcraft L, Whitcomb BW, Rahil T, Tougias E, Sites CK, et al., 2017a Parental contributions to early embryo development: influences of urinary phthalate and phthalate alternatives among couples undergoing ivf treatment. *Hum. Reprod. (Oxf. Engl.)* 32, 65–75.
- Wu H, Estill MS, Shershebnv A, Suvorov A, Krawetz SA, Whitcomb BW, et al., 2017b Preconception urinary phthalate concentrations and sperm DNA methylation profiles among men undergoing ivf treatment: a cross-sectional study. *Hum. Reprod. (Oxf. Engl.)* 32, 2159–2169.
- Wu H, Olmsted A, Cantonwine DE, Shahsavari S, Rahil T, Sites C, et al., 2017c Urinary phthalate and phthalate alternative metabolites and isoprostane among couples undergoing fertility treatment. *Environ. Res* 153, 1–7. [PubMed: 27875712]
- Yin H, Porter NA, Morrow JD, 2005 Separation and identification of f2-isoprostane regioisomers and diastereomers by novel liquid chromatographic/mass spectrometric methods. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci* 827, 157–164.
- Zhang G, Wang Z, Ling X, Zou P, Yang H, Chen Q, et al., 2016 Mitochondrial biomarkers reflect semen quality: results from the marches study in chongqing, china. *PLoS One* 11, e0168823. [PubMed: 28006017]

- Zhong J, Cayir A, Trevisi L, Sanchez-Guerra M, Lin X, Peng C, et al., 2016 Traffic-related air pollution, blood pressure, and adaptive response of mitochondrial abundance. *Circulation* 133, 378–387. [PubMed: 26660284]
- Zota AR, Calafat AM, Woodruff TJ, 2014 Temporal trends in phthalate exposures: findings from the national health and nutrition examination survey, 2001–2010. *Environ. Health Perspect* 122, 235–241. [PubMed: 24425099]

Table 1

Demographic and clinical characteristics of SEEDS male partners (n = 99).

	N	%
Age (years)		
< 30	13	13.1
30 – 40	64	64.6
> 40	22	22.2
Race		
White	75	75.8
Other	9	9.1
Refused/Missing	15	15.1
BMI (kg/m ²)		
< 25	20	20.2
25 – 30	36	36.3
> 30	38	38.4
Missing	5	5.1
Smoking		
Not current	91	91.9
Current	8	8.1
Infertility diagnosis		
Male-factor	27	27.3
Not male-factor	72	72.7

* BMI, body mass index was missing for n = 5.

Table 2

Distribution of specific gravity-adjusted urinary phthalate metabolite and isoprostane concentrations (ng/mL) and sperm mtDNAcn and mtDNA_{del} levels (n = 99).

Parent Ester	Metabolite	GM (95% CI)	% > LOD	Percentile ^a			
<i>HMW Phthalates</i>				25%	50%	75%	95%
DEHP	MEHP	1.35 (1.15, 1.59)	71.7	0.74	1.25	2.11	4.91
	MEOHP	4.77 (4.08, 5.59)	100	3.11	4.44	6.61	20.9
	MEHHP	7.13 (6.08, 8.35)	100	4.43	6.67	9.50	28.7
	MECPP	11.27 (9.62, 13.22)	100	6.93	10.5	15.9	52.5
DiNP	MCOP	18.13 (14.51, 22.64)	100	7.61	15.0	38.0	143
	MNP	1.03 (0.87, 1.23)	54.4	0.58	0.86	1.56	5.42
BBzP	MBzP	4.62 (3.75, 5.70)	100	2.32	4.56	9.57	23.4
DOP	MCP	1.88 (1.58, 2.24)	100	1.02	1.72	3.01	7.30
DiDP	MCNP	2.89 (2.53, 3.30)	100	1.90	2.78	4.13	9.47
<i>LMW Phthalates</i>							
DBP	MBP	9.12 (7.71, 10.79)	98.1	6.13	9.43	14.0	29.0
	MHBP	0.75 (0.66, 0.86)	98.1	0.46	0.78	1.09	2.22
DiBP	MiBP	8.42 (7.11, 9.97)	71.8	4.67	8.51	12.4	39.0
	MHiBP	1.87 (1.57, 2.23)	90.3	1.05	1.86	2.87	9.47
DMP	MMP	2.29 (1.94, 2.71)	100	1.43	2.07	4.03	6.89
DEP	MEP	26.71 (21.13, 33.75)	93.2	10.6	22.1	50.4	193
<i>Phthalate Alternatives</i>							
DiNCH	MCOCH	0.65 (0.55, 0.78)	38.8	0.34	0.49	0.97	5.17
	MHINCH	0.84 (0.67, 1.04)	63.1	0.38	0.68	1.26	11.2
<i>Isoprostane</i>							
	15-F2t	4.17 (3.60, 4.84)	100	2.66	3.62	6.03	19.5
<i>mtDNA</i>							
	mtDNA _{cn} ^a	1.99 (1.66, 2.38)	100	1.33	1.78	3.05	8.93
	mtDNA _{del} ^b	17.0 (15.0, 19.0)	100	11.7	19.1	26.4	33.8

LOD, limit of detection; GM, geometric mean; HMW, high molecular weight; LMW, low molecular weight.

^aRatio of mitochondria DNA copies per nuclear DNA copies.

^bPercent of mitochondrial DNA deletions per total mitochondrial DNA copies.

Table 3

Adjusted effect estimates (95% CI) for urinary phthalate metabolite and isoprostane concentrations and mtDNAcn levels.

<i>Metabolite</i>	Model 1^a	Model 2^b
MEHP	0.37 (−0.66, 1.39)	0.29 (−1.04, 1.61)
MEOHP	−.002 (−1.07, 1.07)	−0.04 (−1.43, 1.35)
MEHHP	−0.13 (−1.19, 0.93)	−0.25 (−1.61, 1.13)
MECPP	−0.11 (−1.18, 0.97)	−0.22 (−1.55, 1.12)
MCOP	0.37 (−0.42, 1.17)	0.30 (−0.69, 1.30)
MNP	0.48 (−0.53, 1.49)	0.27 (−0.96, 1.50)
MBzP	0.44 (−0.37, 1.25)	0.49 (−0.54, 1.52)
MCPP	0.03 (−0.96, 1.01)	−0.31 (−1.53, 0.92)
MCNP	1.32 (0.10, 2.55) *	1.63 (0.14, 3.11) *
MBP	0.15 (−0.89, 1.18)	0.22 (−1.04, 1.48)
MHBP	0.12 (−1.14, 1.38)	−0.11 (−1.73, 1.51)
MiBP	−0.23 (−1.29, 0.82)	−0.22 (−1.46, 1.02)
MHiBP	−0.20 (−1.19, 0.80)	−0.44 (−1.64, 0.76)
MMP	0.32 (−0.61, 1.24)	0.53 (−0.72, 1.79)
MEP	0.23 (−0.50, 0.95)	0.43 (−0.54, 1.40)
MCOCH	0.90 (−1.23, 3.02)	0.83 (−1.76, 3.43)
MHINCH	0.19 (−1.65, 2.02)	0.05 (−2.19, 2.28)
Isoprostane	−0.09 (−0.21, 0.04)	−0.12 (−0.26, 0.03)

* Values are statistically significant; $p < .05$.

^a Adjusted for age and measurement batch.

^b Adjusted for age, BMI, current smoking, race, and measurement batch.

Table 4

Adjusted effect estimates (95% CI) for urinary phthalate metabolite and isoprostane concentrations and mtDNA_{del} levels.

<i>Metabolite</i>	Model 1^a	Model 2^b
MEHP	2.10 (−0.08, 4.28)	1.46 (−1.19, 1.61)
MEOHP	0.28 (−2.04, 2.59)	0.02 (−2.76, 1.35)
MEHHP	0.65 (−1.65, 2.94)	0.37 (−2.39, 1.13)
MECPP	0.52 (−1.79, 2.83)	0.35 (−2.33, 1.12)
MCOP	0.78 (−0.93, 2.49)	0.80 (−1.20, 1.30)
MNP	0.91 (−1.26, 3.09)	0.95 (−1.50, 1.50)
MBzP	0.72 (−1.03, 2.48)	0.99 (−1.07, 1.52)
MCPP	0.63 (−1.49, 2.75)	0.68 (−1.77, 0.92)
MCNP	2.11 (−0.57, 4.79)	2.49 (−0.53, 3.11)
MBP	1.56 (−0.65, 3.76)	1.47 (−1.04, 1.48)
MHBP	1.48 (−1.23, 4.19)	2.01 (−1.20, 1.51)
MiBP	1.52 (−0.74, 3.78)	1.26 (−1.22, 1.02)
MHiBP	0.35 (−1.79, 2.49)	0.69 (−1.72, 0.76)
MMP	0.13 (−3.23, 3.49)	−0.11 (−4.34, 1.79)
MEP	0.90 (−0.65, 2.46)	0.48 (−1.47, 1.40)
MCOCH	1.36 (−3.23, 5.95)	1.11 (−4.11, 3.43)
MHINCH	0.45 (−3.51, 4.41)	−0.78 (−5.26, 2.28)
Isoprostane	0.02 (−0.25, 0.30)	0.02 (−0.28, 0.316)

^a Adjusted for age and measurement batch.

^b Adjusted for age, BMI, current smoking, race, and measurement batch.