Mediation analysis for the relationship between urinary phthalate metabolites and type 2 diabetes via oxidative stress in a population in Jeddah, Saudi Arabia

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ABSTRACT

Human exposure to phthalates is ubiquitous and has received considerable attention due to their association with adverse health outcomes, including type 2 diabetes mellitus (T2DM). Nevertheless, earlier studies that link phthalate exposure to T2DM yielded ambiguous results. Furthermore, studies that associate phthalate exposure with oxidative stress and then with T2DM are scant. In this diabetic case-control study, urine samples collected from 101 individuals aged 28–68 years from Jeddah, Saudi Arabia, were analyzed to determine 20 phthalate metabolites (PhMs) and seven oxidative stress biomarkers (OSBs). Unconditional logistic regression was used to estimate odds ratios for the association between diabetes and urinary PhMs and OSBs in participants, stratified by age, gender, nationality, smoking status, occupation, and urinary creatinine. Twelve PhMs and five OSBs were found at detection rates above 50%, with geometric mean concentrations of 0.61–100 and 0.35–10.7 ng/mL (1.04–171 and 0.61–18.6 μg/g creatinine), respectively. Almost all exposures were significantly higher in diabetic cases than in controls. The 12 PhMs were positively associated with higher urinary concentrations of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8-iso-prostaglandin $F_{2\alpha}$ (8-PGF$_{2\alpha}$). Individuals in the 3rd and/or 4th quartile(s) for urinary concentrations of PhMs and OSBs showed 3.7- and 7.3-fold increase, respectively, in the odds of having diabetes compared with those in the 1st quartile. The rank order of association of PhMs/OSBs with diabetes followed the order of: mEP ≈ mBP > mEHP > mCPP > mECPP ≈ mEOHP ≈ mEHHP ≈ mIBP ≈ mMP > mCMHP ≈ mBzP and 8-OHdG > 8-PGF$_{2\alpha}$ ≈ 15-PGF$_{2\alpha}$. The relationship between phthalate exposure and risk of developing T2DM was mediated in part by phthalate-induced oxidative stress, especially 8-OHdG. Our study suggests that human exposure to phthalates is associated with increased oxidative stress which mediates the development of T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM), which accounts for over 90% of all diabetes, has become a major global public health concern (DeFronzo et al., 2015). The World Health Organization (WHO, 2016) estimated that the global prevalence of diabetes is expected to rise from 422 million cases in 2014 to 592 million cases by 2035. Although genetic predisposition and lifestyle choices are common risk factors, the escalation of T2DM concurrent with increased exposure to endocrine disrupting chemicals (EDCs) since the 1980s led to a novel postulation of the environmental etiology of T2DM (Li et al., 2018; Stojanoska et al., 2017; Sun et al., 2014). Phthalates, a group of environmental EDCs, are extensively used as plasticizers or solvents in personal care products (PCPs), food packaging, medical devices, and building

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materials (Guo et al., 2014; James-Todd et al., 2012). Due to their high production volume and migration from products, phthalate exposure is nearly ubiquitous in the U.S., European and Asian populations (Guo et al., 2011; James-Todd et al., 2018).

Phthalates have the ability to alter both adiposity and insulin resistance (James-Todd et al., 2016; Kim et al., 2013; Stahlhut et al., 2007; Yaghjyan et al., 2015). The association of phthalate metabolites (PhMs) with T2DM has been limited mostly to cross-sectional studies, but the results are somewhat divergent (Table S1). Some evidence suggests an association between the risk of developing T2DM and elevated urinary concentrations of mono-ethyl phthalate (mEP), mono-n-butyl phthalate (mBP), mono-isobutyl phthalate (mIBP), mono-benzyl phthalate (mBzP), and metabolites of di-2-ethylhexyl phthalate (ΣDEHP) (Chen et al., 2017; Dales et al., 2018; James-Todd et al., 2012, 2018; Svensson et al., 2011). Inverse associations with T2DM, however, also were reported for mBzP (Svensson et al., 2011), ΣDEHP (Dirinck et al., 2015; James-Todd et al., 2016), and mIBP (James-Todd et al., 2018). Two studies reported a lack of association between PhMs and the risk of developing gestational diabetes (Robledo et al., 2015; Shapiro et al., 2015). Several recent reviews highlighted the need for more detailed studies of the link between T2DM and phthalate exposure (Goodman et al., 2014; Sun et al., 2017; Thayer et al., 2012).

In vitro studies have shown that exposure to phthalates can induce reactive oxygen species and several biomarkers of oxidative stress via activation of peroxisome proliferator-activated receptors (PPARs) (Cho et al., 2015; Zhang et al., 2017). PPARs are regulators of lipid and glucose homeostasis, and insulin sensitivity (Sun et al., 2014; Zhang et al., 2017). Positive associations were shown between urinary PhM concentrations and oxidative stress biomarkers (OSBs) in several epidemiologic studies (Table S2), whereas inverse associations also were found between PhMs and OSBs (Ferguson et al., 2011; Holland et al., 2016). Malondialdehyde (MDA), 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8-iso-prostaglandin $F_2\alpha$ (8-PGF$_{2\alpha}$) were the most commonly measured OSBs in earlier studies (Table S2). Although oxidative stress was suggested as an outcome of phthalate exposure, and urinary PhM concentration was linked to T2DM, few studies have collectively assessed phthalate exposure, oxidative stress (especially seven different OSB markers), and T2DM in population-based studies.

In this study, we analyzed urine from a population of individuals of 28–68 years of age from Jeddah, Saudi Arabia, to probe the association of T2DM with concentrations of 20 PhMs and seven OSBs (Table S3). Specifically, urinary concentrations of 20 PhMs and seven OSBs were compared between diabetic cases and non-diabetic controls, and a logistic regression analysis was used to quantify odds ratios (ORs) for diagnosing diabetes after adjusting for confounders. Mediation analysis was performed to understand how oxidative stress influenced the relationship between phthalates and T2DM. We hypothesized that high urinary PhMs concentrations are linked to oxidative stress and increased odds of T2DM diagnosis.

2. Materials and methods

2.1. Study population

This is a community-based clinical case-control study involving the analysis of urinary PhMs ($n = 101$, with 54 T2DM cases and 47 controls) and OSBs ($n = 92$, with 52 T2DM cases and 40 controls). The participants' ages ranged from 28 to 68 years, and they were all residents of Jeddah, Saudi Arabia, during the period May 2015–May 2016. A survey questionnaire was used to collect information regarding diabetic status, age, gender, nationality, smoking status, and occupation. Diabetic cases were recruited during their routine checkup visit at diabetic clinics and were identified through a positive diagnosis by a physician from the analysis of fasting plasma glucose levels and then confirmed based on the medications taken to manage the condition. The controls were randomly selected from a non-diabetic population

and represented the target population in terms of sociodemographic distribution. The control group was recruited through voluntary participation, at an approximate 1:1 ratio and confirmed free of T2DM through the responses to questions on diabetic symptoms and/or lack of insulin or other hypoglycemic medications taken within the past three years. The controls were recruited from residents of Jeddah, Saudi Arabia. Any study participant with infectious disease or cardiovascular illness within the past three years was excluded from the study. Based on anecdotal information, body mass index (BMI) of participants did not vary considerably. Spot urine samples were collected in polypropylene (PP) conical tubes from cases and controls during 2015–2016, after the administration of the questionnaire. Institutional Review Board approval of the King Abdulaziz University was obtained before sample collection. All urine samples were stored in a freezer at −20 °C until chemical analysis.

2.2. Analytical methods of urinary PhMs and OSBs

The method for the analysis of urinary PhMs ($n = 101$) entailed enzymatic deconjugation, solid-phase extraction and HPLC-MS/MS detection. Twenty PhMs were measured, which comprised, phthalic acid (PA), low (LMW: mMP, mEP, mBP, mIBP, mIPrP, mPeP) and high (HMW: mEHP, mECPP, mEHHP, mEOHP, mCMHP, mCPP, mCHP, mBzP, mHxP, mHpP, mOP, mINP, mIDP) molecular weight phthalates (Table S3). The method for the analysis of seven biomarkers ($n = 92$) of oxidative damage to lipids (MDA, 8-PGF$_{2\alpha}$, 15-PGF$_{2\alpha}$, 8,15-PGF$_{2\alpha}$, 11-PGF$_{2\alpha}$), proteins (diY; o,o'-dityrosine), and DNA (8-OHdG) has been described elsewhere (Martinez and Kannan, 2018). Details of sample preparation and analysis are shown in the Supplementary Information. Table S3 shows details of the analytical standards and isotope labeled internal standards used in this study. Details of instrumental analyses and compound specific mass spectrometric parameters are shown in Table S4.

2.3. Quality assurance and quality control

An internal standard method was used to quantify the target analytes. The standard calibration curve was prepared with concentrations that ranged from 0.01 to 200 ng/mL. All standard calibration curves had the regression coefficients (r) of over 0.99. Samples that exceeded concentrations of the calibration range were diluted for reanalysis. The method limits of quantification (MLOQs) were estimated based on three factors: the lowest concentrations of the calibration standard with a signal-to-noise ratio of ≥10, volume of urine aliquoted for extraction, and the sample concentration factor. The MLOQs in urine ranged from 0.02 to 2.0 ng/mL for PhMs, and from 0.16 to 0.40 ng/mL for OSBs (Table S5). For every 20 samples, a procedural blank (HPLC grade water in place of urine) was analyzed to determine contamination that may arise from laboratory materials. Trace concentrations of PhMs (0.23–1.59 ng/mL) and MDA (0.45 ng/mL) were detected in procedural blanks (Table S5). The mean PA concentration in procedural blanks was as high as 21.8 ng/mL, potentially originating from enzymes and reagents used in the analysis. However, PA concentrations in samples were significantly higher than those found in the blanks. The concentrations of target compounds found in procedural blanks were deducted from measured concentrations in urine samples. Six pre-extraction matrix spikes were passed through the entire analytical procedure for every 20 samples by fortifying known concentrations of target analytes (10 ng/mL) in urine. Additionally, the Standard Reference Materials (SRMs) 3672 and 3673 (organic contaminants in smokers and non-smokers' urine, respectively), supplied by the National Institute of Standards and Technology, were analyzed for nine PhMs, for which certified values were available. A midpoint calibration standard was injected after every 20 samples as a check for instrumental drift in response factors (i.e., sensitivity) over time. A pure solvent (methanol) was injected after every 10 samples to monitor for sample-

Table 1
Urinary concentrations (ng/mL) of 20 phthalate metabolites (PhMs) and 7 oxidative stress biomarkers (OSBs) in a diabetic and non-diabetic population in Jeddah, Saudi Arabia.

Analyte (ng/mL)	Cases					Controls					p
	< MLOQ (%)[a]	Min	Median	Max	GM[b]	< MLOQ (%)	Min	Median	Max	GM	
mEHP	0.0	2.26	5.46	176	10.2	10.6	< 0.05	2.14	12.0	1.54	**< 0.01**
mEHHP	1.9	< 0.02	27.8	277	32.9	0.0	0.71	10.1	28.7	10.0	**< 0.01**
mEOHP	1.9	< 0.05	16.6	182	20.4	0.0	0.38	5.56	16.5	5.61	**< 0.01**
mECPP	0.0	0.31	37.9	415	41.5	0.0	0.83	10.9	30.4	11.3	**< 0.01**
mCMHP	0.0	0.47	15.3	42.4	12.7	0.0	0.48	5.53	10.4	5.01	**< 0.01**
Σ_5DEHP		**3.54**	**101**	**1060**	**127**		**2.43**	**34.5**	**90.8**	**34.1**	**< 0.01**
mEP	1.9	< 0.50	426	1250	310	0.0	4.29	32.6	45.9	27.3	**< 0.01**
mBP	0.0	0.48	60.2	316	67.0	0.0	2.89	27.4	42.2	22.5	**< 0.01**
mIBP	1.9	< 0.20	30.8	117	30.9	0.0	1.40	15.3	34.1	14.1	**< 0.01**
mCPP	1.9	< 0.05	2.52	10.7	2.26	0.0	0.09	0.70	1.53	0.67	**< 0.01**
mMP	1.9	< 2.00	11.5	28.8	11.2	14.9	< 2.00	5.52	10.4	4.65	**< 0.01**
PA	0.0	12.3	84.9	734	71.7	27.7	< 0.10	1.58	273	1.14	**< 0.01**
mBzP	22.2	< 0.20	1.61	16.1	1.30	48.9	< 0.20	0.20	4.49	0.25	**< 0.01**
mPeP	63.0	< 0.50	0.35	1.29	0.47	44.7	< 0.50	0.69	1.89	0.64	**< 0.01**
mIDP	98.2	< 0.02	0.01	1.44	0.02	97.9	< 0.02	0.01	2.84	0.02	0.91
mOP	100	–	–	–	–	97.9	< 0.10	0.07	0.20	0.07	0.28
mHxP	100	–	–	–	–	97.9	< 0.10	0.07	0.12	0.07	0.28
mIPrP	100	–	–	–	–	100	–	–	–	–	–
mCHP	100	–	–	–	–	100	–	–	–	–	–
mHpP	100	–	–	–	–	100	–	–	–	–	–
mINP	90.7	0.14	0.14	1.46	0.16	100	–	–	–	–	**< 0.05**
Σ_{20}PhMs[c]		**14.0**	**815**	**3200**	**745**		**14.8**	**125**	**388**	**115**	**< 0.01**
diY	0.0	0.52	1.92	3.77	1.73	2.5	< 0.40	2.17	5.86	2.01	0.11
8-OHdG	0.0	0.67	4.94	52.2	4.94	2.5	< 0.40	1.50	3.68	1.37	**< 0.01**
MDA	0.0	1.57	10.4	100	12.0	0.0	0.76	10.4	75.8	9.16	0.15
8,15-PGF$_{2\alpha}$	53.9	< 0.16	0.11	6.59	0.33	60.0	< 0.16	0.11	3.40	0.30	0.77
8-PGF$_{2\alpha}$	19.2	< 0.12	1.40	4.86	0.83	65.0	< 0.12	0.08	4.19	0.20	**< 0.01**
11-PGF$_{2\alpha}$	46.2	< 0.20	0.42	4.10	0.46	82.5	< 0.20	0.14	1.32	0.19	**< 0.01**
15-PGF$_{2\alpha}$	30.8	< 0.16	0.49	2.82	0.46	45.0	< 0.16	0.20	1.02	0.25	**< 0.01**
Σ_7OSBs[d]		**5.13**	**30.5**	**120**	**26.2**		**3.75**	**16.4**	**81.8**	**15.4**	**< 0.01**

$p < 0.05$ and sum concentrations are presented in bold font.
[a] MLOQ, method limit of quantification.
[b] GM, geometric mean.
[c] For urinary PhMs, $n = 101$, 54 cases and 47 controls.
[d] For urinary OSBs, $n = 92$, 52 cases and 40 controls.

to-sample carryover of target analytes.

2.4. Method performance

Relative recoveries of target chemicals in spiked urine samples and SRMs were calculated as the ratio of signal/response found for the target analyte and the corresponding internal standard (Table S5). The relative recoveries of PhMs spiked in urine ranged from 70% to 120%, and those of PhMs in SRMs were from 73% to 130% (Table S5). The relative recoveries of OSBs spiked in urine ranged from 98% to 114%. The relative standard deviation (RSD) of repeated analysis of samples was ≤15% for both PhMs and OSBs (Table S5).

2.5. Data analysis

Urinary concentrations of PhMs and OSBs with values below the MLOQ were replaced with MLOQ divided by square root of two, and log-transformed ($\chi + 1$) for further analysis. Creatinine concentration was used to adjust for urine dilution. If the data followed a normal distribution, a Student's *t*-test was used to examine differences in urinary concentrations of chemicals between cases and controls; otherwise, a Mann-Whitney *U* test was used. Based on logarithmic concentrations of target analytes, urinary PhMs and OSBs were categorized into four quartiles. Binary or multivariate unconditional logistic regression analysis was applied to estimate crude and adjusted ORs and 95% confidence intervals (CIs) by comparing the 2nd, 3rd, and 4th quartile concentrations of each analyte to that of the 1st quartile concentrations (the lowest quartile as the reference) between cases and controls. In regression models, (creatinine) unadjusted PhM concentrations were used, and models were adjusted for participants' age, gender, nationality, smoking status, occupation, and creatinine as covariates. Previous studies indicated that creatinine-adjusted PhM concentrations can introduce bias in regression models (Barr et al., 2005). The use of creatinine as a covariate for adjustment, however, can curtail noise in the intra-individual variability of spot urine determinants (O'Brien et al., 2016). Potential dose-response relationship was examined in tests for trend across quartiles by using the median log ($\chi + 1$) concentrations of analytes in each quartile as a continuous variable in the regression models. All analyses, including the Pearson's correlation, were conducted by SPSS 19.0 (SPSS Inc., Chicago, IL). A *p* value of < 0.05 denoted significance. Mediation analysis was performed using bootstrapping, and model 4 was run in Process (V3.2) with covariates in SPSS. Sensitivity analyses were performed to evaluate the robustness of these findings by excluding outliers (values that fell outside two standard deviations).

3. Results and discussion

3.1. Demographic factors

Demographic characteristics of the participants are detailed elsewhere (Li et al., 2018). Briefly, participants were 42.4 years of age, on average, at enrollment; 52.5% females; 38.6% active smokers; and 76.2% employed; and nationalities included Saudi Arabian (20.8%), Indian (40.6%) and Pakistani (25.7%). Age and smoking status were strongly associated with the diagnosis of diabetes. Other factors such as

Table 2

Pearson correlation coefficient (r) of urinary concentrations of 12 phthalate metabolites and 5 oxidative stress biomarkers (ng/mL, log-transformed) in a diabetic and non-diabetic population in Jeddah, Saudi Arabia.

	mEHP	mEHHP	mEOHP	mECPP	mCMHP	mEP	mBP	mIBP	mCPP	mMP	PA	mBzP	diY	8-OHdG	MDA	8-PGF2α
mEHP	1.0															
mEHHP	0.91**	1.0														
mEOHP	0.93**	1.0**	1.0													
mECPP	0.90**	0.99**	0.98**	1.0												
mCMHP	0.76**	0.90**	0.88**	0.90**	1.0											
mEP	0.67**	0.71**	0.74**	0.72**	0.51**	1.0										
mBP	0.56**	0.73**	0.72**	0.70**	0.63**	0.87**	1.0									
mIBP	0.39**	0.68**	0.63**	0.68**	0.75**	0.54**	0.74**	1.0								
mCPP	0.64**	0.68**	0.68**	0.67**	0.75**	0.64**	0.76**	0.54**	1.0							
mMP	0.58**	0.69**	0.69**	0.70**	0.56**	0.81**	0.87**	0.68**	0.64**	1.0						
PA	0.64**	0.59**	0.61**	0.63**	0.52**	0.79**	0.70**	0.41**	0.72**	0.71**	1.0					
mBzP	0.14	0.29**	0.29**	0.38**	0.32**	0.45**	0.41**	0.62**	0.23*	0.50**	0.47**	1.0				
diY	−0.10	0.07	0.05	0.08	0.17	−0.14	0.09	0.31**	0.01	0.17	−0.20	0.10	1.0			
8-OHdG	0.42**	0.53**	0.49**	0.55**	0.68**	0.44**	0.46**	0.54**	0.54**	0.39**	0.55**	0.43**	−0.03	1.0		
MDA	−0.05	0.18	0.14	0.18	0.33**	0.05	0.27**	0.61**	0.11	0.25*	0.00	0.43**	0.39**	0.28**	1.0	
8-PGF2α	0.26*	0.37**	0.36**	0.38**	0.47**	0.37**	0.47**	0.47**	0.51**	0.43**	0.41**	0.35**	0.14	0.53**	0.23*	1.0
15-PGF2α	−0.09	0.06	0.04	0.13	0.24*	0.07	0.10	0.49**	0.08	0.17	0.14	0.62**	0.16	0.30**	0.52**	0.19

*Correlation is significant at the 0.05 level. **Correlation is significant at the 0.01 level. Non-significant differences are highlighted in grey.

gender, nationality and occupation did not show associations with diabetes. Furthermore, no significant difference was found between men and women for urinary levels of PhMs and OSBs (Table S6).

3.2. Urinary concentrations of PhMs

PhMs were found with detection rates (DRs) of > 90% for mEP, mCPP, mBP, mIBP, mMP, and metabolites of DEHP (Σ_5DEHP: mEHP, mEHHP, mECPP, mCMHP, and mEOHP), 87% for PA, 65% for mBzP, 46% for mPeP, and < 5% for mOP, mIPrP, mCHP, mIDP, mHxP, mHpP, and mINP. Frequent detection of PhMs in urine was linked to extensive exposure to phthalate diesters via personal care products (for LMW: mEP, mBP, mIBP, and mMP) and diet (for HMW: Σ_5DEHP) (Guo et al., 2012; Guo and Kannan, 2013; Kataria et al., 2017; Rocha et al., 2017). PA, formed through the hydrolysis of phthalate monoester metabolites, is a non-specific biomarker of phthalate exposure. Although DEHP is increasingly replaced with di-isononyl phthalate (DINP, the parent compound of mINP) and di-isodecyl phthalate (DIDP, the parent compound of mIDP) (Rocha et al., 2017; Stojanoska et al., 2017), mINP and mIDP were minor compounds found in urine in this study population. Further analysis of data was performed only for analytes with a DR of > 50%.

The urinary concentrations of 12 PhMs with DRs of > 50% were significantly higher in diabetic cases than in controls ($p < 0.01$; Table 1). Among those PhMs that were determined in diabetic cases, mEP was found at the highest concentration (geometric mean [GM]: 310 ng/mL), followed by Σ_5DEHP (127 ng/mL), PA (71.7 ng/mL), mBP (67.0 ng/mL), mIBP (30.9 ng/mL), mMP (11.2 ng/mL), mCPP (2.26 ng/mL), and mBzP (1.30 ng/mL). The non-diabetic control group showed a slightly different rank order of PhM concentrations: Σ_5DEHP (34.1 ng/mL) > mEP (27.3 ng/mL) > mBP (22.5 ng/mL) > mIBP (14.1 ng/mL) > mMP (4.65 ng/mL) > PA (1.14 ng/mL) > mCPP (0.67 ng/mL) > mBzP (0.25 ng/mL). The concentrations of DEHP metabolites and mEP were approximately 10-fold higher in diabetic cases than in controls. Creatinine-adjusted concentrations of PhMs (μg/g creatinine) showed a pattern similar to that of volume-based (i.e., unadjusted) concentrations (ng/mL) in both cases and controls (Table S7). The urinary GM concentrations of PA, mEP, mBzP, Σ_5DEHP, mCPP, mBP, mMP, mIBP, and Σ_{20}PhMs in cases were 63, 11, 5.2, 3.7, 3.4, 3.0, 2.4, 2.2, and 6.5 times higher than those in controls, respectively (Table 1).

In a prospective study of U.S. nurses, T2DM cases contained significantly higher urinary concentrations of DEHP metabolites than controls, but not of mEP, mBP, mIBP and mBzP concentrations (Sun et al., 2014). The GM urinary concentrations of PhMs in our diabetic cases from Saudi Arabia were similar to those reported in a case-only study of Chinese diabetic patients; our case group had relatively higher GM concentrations of mEP (21-fold) and Σ_5DEHP (2.3-fold) but lower concentrations of mMP (3.4-fold) (Duan et al., 2017). In comparison to a study that reported urinary PhMs in a control group (non-diabetic) of the Canadian population (Dales et al., 2018), our controls had relatively lower GM concentrations of mBzP (34-fold), mCPP (3.1-fold), sum of mEHP, mEHHP and mEOHP (2.7-fold), mEP (1.6-fold), and mBP (1.4-fold). The median concentrations of PhMs measured in a general population in Jeddah, Saudi Arabia (Asimakopoulos et al., 2016), fell within the range reported in our study. High concentrations of PhMs, especially PA, in diabetic cases also may be related to the use of phthalates in medications (Stahlhut et al., 2007). Further studies are needed to assess the levels of phthalates in diabetic medications.

3.3. Concentrations of OSBs

The OSBs were found in urine with detection rates (DRs) of > 90% for diY, 8-OHdG and MDA, ~60% for 8-$PGF_{2\alpha}$ and 15-$PGF_{2\alpha}$, and < 50% for 8,15-$PGF_{2\alpha}$ and 11-$PGF_{2\alpha}$. These DRs were similar to those reported earlier for general populations from several countries (Ferguson et al., 2015; Kim et al., 2013; Martinez and Kannan, 2018).

The urinary concentrations of four individual OSBs and Σ_7OSBs were significantly higher in diabetic cases than in controls i.e., 8-OHdG (GM: cases; 4.94, controls; 1.37 ng/mL), 8-$PGF_{2\alpha}$ (0.83, 0.20 ng/mL), 11-$PGF_{2\alpha}$ (0.46, 0.19 ng/mL), 15-$PGF_{2\alpha}$ (0.46, 0.25 ng/mL), and Σ_7OSBs (26.2, 15.4 ng/mL) ($p < 0.01$; Table 1). Similarly, creatinine-adjusted concentrations of diY, 8-OHdG, 8-$PGF_{2\alpha}$ and 11-$PGF_{2\alpha}$ in cases were significantly higher than those in controls (Table S7). No earlier studies have reported urinary OSB concentrations in diabetic cases, and, therefore, this is the first study to document elevated concentrations of OSBs in urine from diabetics.

3.4. PhMs and OSBs

Significant positive correlations were found among the concentrations of 12 PhMs (log-transformed), except between mBzP and mEHP (Table 2). These results suggest a common exposure source for phthalates among the Saudi population. Correlations among the five DEHP metabolite concentrations were the strongest (Spearman $r = 0.76–1.0$), which indicates that their sources originate from the same parent compound (Ferguson et al., 2015; Rocha et al., 2017). DEHP is used mainly as a plasticizer in the production of polyvinyl chloride. Diet is the major source of DEHP exposure (Gao et al., 2016; Guo and Kannan, 2013; Schecter et al., 2013), but medical devices, medications, cosmetics and other PCPs also can contribute to DEHP exposures (Huang et al., 2014). PhMs, other than those of DEHP, presented moderate to weak, but significant, correlations with each other ($r = 0.41–0.87$). LMW phthalates are common additives in PCPs. The concentrations of LMW PhMs, mMP, mEP, mBP, and mIBP were correlated with each other (Asimakopoulos et al., 2016; Holland et al., 2016). Butyl benzyl phthalate, the parent compound of mBzP, is commonly used in floor tiles, traffic cones, and artificial leather (Booker, 2001), suggesting that this compound is used in applications different from those of DEHP and LMW phthalates. A poor correlation between mBzP and other PhMs can be explained by the different applications/sources.

The urinary concentrations of 12 PhMs were significantly correlated with those of 8-OHdG and 8-$PGF_{2\alpha}$ ($r = 0.26–0.68$) (Table 2). This is similar to that reported for a cohort of pregnant women in Puerto Rica and the USA (Ferguson et al., 2014, 2015). A negative relationship was found between urinary mBzP and 8-$PGF_{2\alpha}$ concentrations (Table S2) (Holland et al., 2016). MDA concentrations were significantly and positively correlated with those of mCMHP, mBP, mIBP, mMP, and mBzP in our study. A study from China reported that MDA concentrations were positively associated with 11 PhMs (i.e., mCMHP, mBP, mIBP, mMP, mBzP, mEP, mCPP, mEHP, mEOHP, mEHHP, and mECHP) in a diabetic population (Duan et al., 2017). Another study from Korea showed that MDA concentrations were significantly associated with the sum concentrations of mEHHP and mEOHP in an elderly population (aged ≥60 years) (Kim et al., 2013). DiY concentrations were correlated with those of mIBP, whereas 15-$PGF_{2\alpha}$ concentrations were correlated with those of mCMHP, mIBP, and mBzP. The associations described above did not change appreciably when the urinary concentrations were adjusted for creatinine (Table S8).

3.5. Association of T2DM with PhMs and OSBs

The association between PhM concentrations and the prevalence of diabetes was verified by ORs found in the range of 3.78–276 ($p < 0.05$; Table 3). In comparison to the 1st quartile urinary concentrations, the 3rd quartile concentrations of individual PhMs, Σ_5DEHP and Σ_{20}PhMs showed significantly higher ORs for the diagnosis of diabetes, in both crude and adjusted models. The ORs for PA and most PhMs in the 4th quartile (i.e., mEHHP, mEOHP, mECPP, mCMHP, mEP, mBP, mCPP, and mMP) could not be estimated due to the wide concentration range. Among individual PhMs measured, the rank order of ORs for the diagnosis of diabetes was as follows: mEP ≈ mBP > mEHP > mCPP > mECPP ≈ mEOHP ≈ mEHHP ≈ mIBP ≈ mMP >

Table 3

Odds ratios (95% CI) for the diagnosis of diabetes from urinary concentrations (log (χ + 1); ng/mL) of phthalate metabolites (PhMs) and oxidative stress biomarkers (OSBs) presented in quartiles for a diabetic and non-diabetic population in Jeddah, Saudi Arabia.

Analyte (ng/mL)	1st quartile	2nd quartile	3rd quartile	4th quartile	p-Trend[a]
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	
mEHP	< 0.05–2.27	2.28–4.16	4.17–5.67	5.68–176	
Crude	1.00 (Ref)	**24.0 (2.82, 205)**	**51.0 (5.83, 447)**	**276 (23.4, 3.2e3)**[c]	< 0.01
Adjusted[b]	1.00 (Ref)	**22.0 (2.11, 229)**	**25.5 (2.15, 303)**	**213 (14.8, 3.1e3)**	< 0.01
mEHHP	< 0.02–9.47	9.48–22.3	22.4–29.0	29.1–277	
Crude	1.00 (Ref)	1.78 (0.49, 6.43)	**7.11 (1.99, 25.5)**	nd[d]	< 0.01
Adjusted	1.00 (Ref)	1.55 (0.33, 7.28)	6.53 (0.80, 53.2)	nd	< 0.01
mEOHP	< 0.05–5.28	5.29–13.5	13.6–17.1	17.2–182	
Crude	1.00 (Ref)	2.47 (0.63, 9.63)	**9.92 (2.60, 37.9)**	nd	< 0.01
Adjusted	1.00 (Ref)	nd	nd	nd	< 0.01
mECPP	0.31–10.4	10.5–19.7	19.8–38.2	38.3–415	
Crude	1.00 (Ref)	**4.89 (1.15, 20.8)**	**11.7 (2.77, 49.6)**	nd	< 0.01
Adjusted	1.00 (Ref)	4.04 (0.71, 22.9)	**10.5 (1.39, 79.8)**	nd	< 0.01
mCMHP	0.47–4.89	4.90–7.08	7.09–18.9	19.0–42.4	
Crude	1.00 (Ref)	0.51 (0.14, 1.83)	**3.78 (1.17, 12.2)**	nd	< 0.01
Adjusted	1.00 (Ref)	0.24 (0.04, 1.66)	1.14 (0.13, 9.90)	nd	< 0.01
Σ_5DEHP	2.43–31.7	31.8–68.7	68.8–102	103–1060	
Crude	1.00 (Ref)	1.56 (0.42, 5.78)	**7.56 (2.12, 26.9)**	nd	< 0.01
Adjusted	1.00 (Ref)	1.56 (0.30, 8.09)	**9.06 (1.24, 66.3)**	nd	< 0.01
mEP	< 0.50–32.6	32.7–45.3	45.4–435	436–1250	
Crude	1.00 (Ref)	1.50 (0.23, 9.83)	**276 (23.4, 3.2e3)**	nd	< 0.01
Adjusted	1.00 (Ref)	1.35 (0.15, 12.3)	**725 (8.24, 6.4e4)**	nd	< 0.01
mBP	0.48–26.8	26.9–42.0	42.1–75.8	75.9–316	
Crude	1.00 (Ref)	1.50 (0.23, 9.83)	**276 (23.4, 3.2e3)**	nd	< 0.01
Adjusted	1.00 (Ref)	3.15 (0.29, 33.8)	nd	nd	< 0.01
mIBP	< 0.20–15.0	15.1–21.7	21.8–31.8	31.9–117	
Crude	1.00 (Ref)	2.93 (0.84, 10.3)	**6.00 (1.69, 21.3)**	**46.0 (8.03, 264)**	< 0.01
Adjusted	1.00 (Ref)	1.96 (0.44, 8.80)	**5.92 (1.13, 31.0)**	**28.8 (1.53, 541)**	< 0.01
mCPP	< 0.05–0.69	0.70–1.16	1.17–2.64	2.65–10.7	
Crude	1.00 (Ref)	1.75 (0.37, 8.24)	**38.5 (7.68, 193)**	nd	< 0.01
Adjusted	1.00 (Ref)	1.24 (0.17, 8.99)	**20.9 (2.50, 175)**	nd	< 0.01
mMP	< 2.00–5.10	5.11–7.42	7.43–12.1	12.2–28.8	
Crude	1.00 (Ref)	1.88 (0.52, 6.85)	**6.40 (1.82, 22.5)**	nd	< 0.01
Adjusted	1.00 (Ref)	2.05 (0.34, 12.3)	4.88 (0.81, 29.5)	nd	< 0.01
PA	< 0.10–1.81	1.82–17.2	17.3–93.5	93.6–734	
Crude	1.00 (Ref)	nd	nd	nd	< 0.01
Adjusted	1.00 (Ref)	nd	nd	nd	< 0.01
mBzP	< 0.20– < 0.20	< 0.20–0.40	0.41–2.67	2.68–16.1	
Crude	1.00 (Ref)	nd	1.69 (0.64, 4.48)	**44.0 (5.49, 353)**	< 0.01
Adjusted	1.00 (Ref)	nd	2.51 (0.71, 8.93)	**83.8 (3.67, 1.9e3)**	< 0.01
Σ_{20}PhMs	14.0–126	127–316	317–869	870–3200	
Crude	1.00 (Ref)	4.36 (0.45, 42.1)	**576 (34.0, 9.7e3)**	nd	< 0.01
Adjusted	1.00 (Ref)	7.5 (0.41, 139)	**7.0e3 (39.9, 1.2e6)**	nd	< 0.01
diY	< 0.40–1.39	1.40–1.99	2.00–2.78	2.79–5.86	
Crude	1.00 (Ref)	1.47 (0.43, 4.98)	0.70 (0.22, 2.26)	0.50 (0.15, 1.60)	0.16
Adjusted	1.00 (Ref)	1.55 (0.30, 8.04)	0.22 (0.04, 1.29)	0.10 (0.01, 0.85)	< 0.01
8-OHdG	< 0.40–1.35	1.36–2.47	2.48–5.20	5.21–52.2	
Crude	1.00 (Ref)	0.34 (0.08, 1.54)	**10.9 (2.69, 43.9)**	nd	< 0.01
Adjusted	1.00 (Ref)	0.56 (0.08, 4.12)	**37.9 (2.44, 589)**	nd	< 0.01
MDA	0.76–5.72	5.73–10.4	10.5–18.8	18.9–100	
Crude	1.00 (Ref)	1.00 (0.31, 3.21)	0.41 (0.13, 1.35)	2.77 (0.76, 10.0)	0.25
Adjusted	1.00 (Ref)	0.66 (0.16, 2.71)	0.13 (0.03, 0.69)	0.60 (0.08, 4.19)	< 0.01
8-PGF$_{2\alpha}$	< 0.12– < 0.12	< 0.12–0.72	0.73–1.71	1.72–4.86	
Crude	1.00 (Ref)	2.60 (0.62, 11.0)	**17.3 (4.21, 71.4)**	**7.37 (2.26, 24.0)**	< 0.01
Adjusted	1.00 (Ref)	3.85 (0.65, 22.7)	**13.4 (2.31, 77.8)**	**9.84 (1.88, 51.5)**	< 0.01
15-PGF$_{2\alpha}$	< 0.16– < 0.16	< 0.16–0.33	0.34–0.91	0.92–2.82	
Crude	1.00 (Ref)	nd	1.42 (0.52, 3.87)	**8.67 (2.26, 33.3)**	< 0.01
Adjusted	1.00 (Ref)	nd	1.94 (0.53, 7.06)	**9.00 (1.43, 56.8)**	< 0.01
Σ_7OSBs	3.75–13.8	13.9–20.5	20.6–35.2	35.3–120	
Crude	1.00 (Ref)	1.20 (0.37, 3.88)	1.70 (0.53, 5.47)	**16.3 (3.06, 87.2)**	< 0.01
Adjusted	1.00 (Ref)	0.69 (0.16, 3.05)	1.16 (0.27, 4.95)	4.95 (0.42, 58.1)	< 0.01

[a] Tests for linear trend were performed using the median urinary phthalate metabolite concentration in each quartile as a continuous variable in the model.

[b] Adjusted for age, gender, nationality, smoking status, occupation and creatinine as covariates.

[c] Values with a $p < 0.05$ and ORs > 1 are presented in bold font.

[d] Not reported due to large confidence interval or not calculated.

mCMHP ≈ mBzP. Significant linear trends in ORs for 12 PhMs across the three quartiles of concentrations suggested a significant dose-response relationship between PhM concentrations and diabetes. A few earlier studies reported that urinary concentrations of several phthalates were associated with the prevalence of diabetes (Dales et al., 2018; James-Todd et al., 2012).

Although the measured concentrations of mEHP in the 3rd quartile were lower than those of the other four DEHP metabolites, mEHP

exhibited a significant and consistent positive association with the prevalence of diabetes (ORs: 22–276, p-trends < 0.01) (Table 3). Studies have reported that mEHP was the most potent DEHP metabolite (Chang et al., 2017; Yaghjyan et al., 2016). DEHP, the parent compound of mEHP, was reported to impair metabolic homeostasis of glucose via interference with beta-cell function and insulin resistance, thereby effecting diabetes (Chen et al., 2017; Dales et al., 2018; James-Todd et al., 2012). In contrast, DEHP metabolites, including mEHP, were surprisingly inversely associated with insulin resistance and impaired glucose tolerance in obese and pregnant women (Dirinck et al., 2015; James-Todd et al., 2016). In addition, metabolites of LMW phthalates, especially mEP and mBP, were strongly associated with the prevalence of diabetes (ORs of the 3rd quartile: both 276, p-trends < 0.01). A recent prospective cohort study reported that second-trimester mIBP concentrations were inversely associated with blood glucose level (James-Todd et al., 2018). The disparity in results among epidemiological studies could be attributed to the differences in study population/design (pregnancy, obesity and elderly), diet, sources of exposure, and metabolism of phthalates (Ferguson et al., 2015; Lin et al., 2017).

Compared to the 1st quartile urinary concentrations, ORs for the increased risk for diabetes were significantly higher for the 3rd and/or 4th quartile(s) of 8-OHdG (ORs: 10.9–37.9), 8-PGF$_{2\alpha}$ (ORs: 7.37–17.3), and 15-PGF$_{2\alpha}$ (ORs: 8.67–9.00) in both crude and adjusted models (p-trends < 0.01; Table 3). Each log unit increase in PhM concentrations was significantly associated with increasing levels of 8-OHdG, 8-PGF$_{2\alpha}$, and 15-PGF$_{2\alpha}$. Several studies have reported an association between oxidative stress and exposure to phthalates (Table S2), whereas only three studies related oxidative stress with impaired glucose metabolism and increased insulin resistance, leading to an increased risk of T2DM (Duan et al., 2017; Kataria et al., 2017; Kim et al., 2013). The ORs for the diagnosis of diabetes from diY and MDA urinary concentrations across the four quartiles were statistically non-significant, although a positive link between the concentrations of these two OSBs and diabetic prevalence was found in the adjusted models (p-trends < 0.01; Table 3).

3.6. Mediation of OSBs in the relationship between PhMs and T2DM

Mediation analysis was performed to understand the relationship between urinary PhMs and T2DM through sum concentration of seven OSBs (Table S9) and individual OSBs, as mediator variables (Table S10; Table 4). No significant mediation (i.e., indirect effect with confidence intervals that included zero) was found for the sum concentrations of OSBs. For specific PhMs, mediation analysis was performed only for those PhMs that were significantly and positively correlated with OSBs (Table S10). The results showed that a major proportion of T2DM was

mediated through 8-OHdG followed by 8-PGF$_{2\alpha}$ across all studied PhMs with a DR of > 50% (Table 4). We observed a significant mediation for the relationship between urinary PhMs and T2DM by 8-OHdG, with an estimated mediation range of 25.6% to 105%. The rank order for the mediation effect by 8-OHdG was as follows: mCMHP ≈ mIBP > mEHHP > mECPP > mEOHP > mBP ≈ mCPP > mMP > mEP ≈ mEHP. The existence of a great proportion of the relationship between PhMs and T2DM mediated by 8-OHdG indicates that DNA oxidative pathway is particularly relevant for phthalate induced T2DM. A leading explanation for pathways linked to T2DM was impaired glucose metabolism and increased insulin resistance (Duan et al., 2017; Kataria et al., 2017). Our analysis suggests that phthalate-induced oxidative stress could be origin in this pathway of development of diabetes, which has not been elucidated previously. This has important connotations for further research on environmental impacts in the development of T2DM, because a large number of environmental contaminants can provoke oxidative stress.

4. Conclusions

Our case-control study provides clear evidence of an association between phthalate exposure and oxidative stress, which is related to increased odds of a diagnosis of T2DM. The incorporation of exposure (i.e., urinary phthalate metabolite levels), a mechanistic link (i.e., oxidative stress markers), and adverse outcome (i.e., diabetic cases and controls) is a unique approach, which is similar to the concept of an adverse outcome pathway (AOP) employed in ecotoxicological investigations (Escher et al., 2017). Our findings are sound in both the multiple adjusted and stratified analyses. Significant positive associations were found between urinary concentrations of PhMs (DRs > 50%) and OSBs of DNA, lipid, and protein damage. Positive associations also were found between PhMs/OSBs in the 3rd and/or 4th quartile(s) of urinary concentrations and diabetes, after controlling for confounders such as age, gender, nationality, smoking status, occupation, and creatinine. The rank order of PhMs related with diabetes was as follows: mEP ≈ mBP > mEHP > mCPP > mECPP ≈ mEOHP ≈ mEHHP ≈ mIBP ≈ mMP > mCMHP ≈ mBzP, whereas the rank order of OSBs strongly associated with diabetes was: 8-OHdG > 8-PGF$_{2\alpha}$ ≈ 15-PGF$_{2\alpha}$. Mediation analysis further supported the relationship between phthalate exposure and T2DM, and 8-OHdG emerged as a significant marker linking PhMs and T2DM. Our results support the hypothesis of environmental etiology of T2DM (adverse outcome) with phthalate exposure inducing oxidative stress (a mechanistic link).

Despite the fact that this study adds novel information that links phthalate exposure to oxidative stress and then to T2DM, there are

Table 4

Effect estimates[a] with log-unit increase in exposure and estimated percent mediated by 8-OHdG and 8-PGF$_{2\alpha}$.

	Direct effect (95% CI)	Indirect effect (95% CI)	Estimated percent mediated (%)[b]	Direct effect (95% CI)	Indirect effect (95% CI)	Estimated percent mediated (%)
	8-OHdG			8-PGF$_{2\alpha}$		
mEHP	7.01 (0.947, 13.1)	2.41 (0.892, 39.0)	25.6	8.87 (3.28, 14.5)	0.362 (−0.05, 5.19)	3.92
mEHHP	0.987 (−0.890, 2.86)	4.23 (1.74, 16.7)	81.1	2.47 (0.603, 4.35)	0.377 (−0.003, 1.84)	13.2
mEOHP	1.78 (−0.395, 3.95)	3.48 (1.43, 18.2)	66.2	3.27 (0.840, 5.70)	0.376 (−0.022, 1.98)	10.3
mECPP	1.61 (−0.555, 3.77)	4.03 (1.55, 17.4)	71.5	3.33 (0.879, 5.77)	0.377 (−0.023, 2.05)	10.2
mCMHP	−0.469 (−3.06, 2.13)	10.6 (4.80, 40.1)	105	2.48 (0.480, 4.47)	0.590 (0.009, 2.46)	19.2
mEP	4.68 (2.35, 7.01)	1.81 (0.516, 16.9)	27.8	5.15 (2.51, 7.78)	0.352 (−0.045, 12.3)	6.40
mBP	4.61 (1.18, 8.04)	3.44 (1.04, 72.7)	42.7	6.02 (2.55, 9.49)	0.483 (−3.98, 20.4)	7.43
mIBP	−0.128 (−3.30, 3.04)	8.39 (3.24, 37.5)	102	3.32 (0.070, 6.57)	0.793 (−0.047, 4.07)	19.3
mCPP	9.16 (1.41, 16.9)	6.69 (1.42, 50.0)	42.2	12.0 (4.43, 19.6)	0.082 (−3.10, 3.74)	0.68
mMP	5.86 (1.84, 9.89)	2.67 (0.05, 53.7)	31.3	6.56 (2.83, 10.3)	0.814 (−0.071, 3.74)	11.0
PA	5.9e3 (4.8e3, 6.9e3)	77.5 (−70.9, 183)	1.30	284 (−945, 1.5e3)	−10.3 (−12.9, 8.88)	−3.75
mBzP	29.4 (7.89, 50.8)	10.9 (−177, 990)	27.0	9.83 (4.15, 15.5)	0.181 (−1.24, 1.95)	1.81

[a] The effect is conditional on the level of the covariates of age, gender, nationality, smoking status, occupation, and creatinine.

[b] Percent mediated = indirect effect/(direct effect + indirect effect) × 100.

several limitations that should be taken into account when drawing conclusions. First, a single spot urine sample was analyzed from cases and controls, and the suitability of spot urine to represent long-term exposure to phthalates could be questioned. A review concluded that the analysis of a single spot urine sample may adequately represent long-term exposure, for up to a couple of years, of phthalates in different population groups (Johns et al., 2015). Although the half-lives of PhMs are in the range of 3–18 h, the intraclass correlation coefficients for PhMs ranged from 0.1 to 0.6, with greater stability for LMW than HMW phthalates. Further, creatinine was used as a covariate in regression models, which can minimize the noise in the intra-individual variability of spot urine sample measurements. Second, small sample size limited the power of this study, inducing a null estimation for several ORs. Although we did find consistent positive link between PhM/OSB concentrations in urine and the risk of developing T2DM, further prospective studies with larger sample sizes are warranted for underlying causes. Third, the BMI data were anecdotal and were not included as a covariate in our models. Creatinine-adjusted urinary concentration, as a proxy for BMI, did not change our results in both diabetic cases and controls, indicative of validity of our conclusions. Fourth, this case-control design does not exclude the possibility of an outcome-exposure association (instead of exposure-outcome association). Overall, our findings should be considered as exploratory and warrants further corroboration.

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Notes

The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.01.082.

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