Exposure to Phthalates and Cardiovascular Diseases with type 2 Diabetes

Haojie Zhang
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine
Jingsi Chen
Fudan University School of Public Health
Chi Chen
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine
Heng Wan
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine
Yi Chen
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine
Yuying Wang
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine
Wen Zhang
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine
Bo Chen
Fudan University School of Public Health
Yingli Lu
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine
Ningjian Wang (wnj486@126.com)
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine https://orcid.org/0000-0001-9591-6991

Research Article

Keywords: urinary phthalates, type 2 diabetes mellitus, cardiovascular diseases, smoking

DOI: https://doi.org/10.21203/rs.3.rs-226068/v1

Abstract

Purpose Cardiovascular disease (CVD) results in more than half of the mortality and the majority of morbidity in patients with type 2 diabetes. We aim to evaluate the associations of urinary concentrations of phthalate metabolites with CVD in diabetic patients and explore whether CVD risk factors mediate or interact with these associations.

Methods A total of 675 type 2 diabetic participants were enrolled from one community in Shanghai, China in 2018. CVD was defined as a self-reported diagnosis by a physician and included coronary heart disease, myocardial infarction or stroke; it was further reconfirmed in the records from the registration platform. Ten phthalate metabolites were measured in urine.

Results Positive associations were found among the level of monoethyl phthalate and monoisobutyl phthalate and CVD (OR 1.138, 95% CI 1.032, 1.254; OR 1.369, 95% CI 1.049, 1.786, respectively). Monoisobutyl phthalate and monobenzyl phthalate were marginally and positively associated with carotid intima-media thickness and common carotid artery diameter, respectively. None of the CVD risk factors, including HOMA-IR, body mass index, lipid profile or blood pressure, significantly mediated the association between the metabolites and CVD. The conditional indirect effect on CVD was significantly stronger for current smoking and dyslipidemia for monoethyl phthalate and for no statin usage and men for monoisobutyl phthalate.

Conclusion Phthalate exposure was positively associated with CVD in type 2 diabetes. Type 2 diabetic men who are currently smoking, have an uncontrolled lipid profile and are not using statins may be more susceptible to CVD when exposed to phthalates.

Introduction

Type 2 diabetes mellitus has become prevalent throughout the world, especially in China. The estimated prevalence of diabetes among a representative sample of approximately 100 thousand Chinese adults has reached 11.6% (up to 113.9 million Chinese adults) (Xu et al., 2013). Cardiovascular disease (CVD) serves as a major complication in patients with diabetes mellitus (DM) and has resulted in more than half of the mortality and the majority of morbidity in patients with DM (Rydén et al., 2013). Worryingly, 67% of diabetic patients will suffer from some form of CVD in their lifetime (Fox et al., 2008; Nelson et al., 2019), and studies have shown no benefit from intensive glucose-lowering therapy on the prevention of CVD (Duckworth et al., 2009; Group et al., 2008). Therefore, CVD has become the a crucial socioeconomic burden for diabetic patients (Dong et al., 2018), and potential modifiable risk factors are critical for CVD prevention in these patients.

Currently, phthalates are widespread in our daily life. Phthalates are a large group of compounds added as liquid plasticizers in many products, including food packaging, electronic equipment, medications, building materials, and so on (Goodman et al., 2014; Weuve et al., 2010). Phthalates have been found to deteriorate cardiometabolic risk factors such as obesity and diabetes (Dong et al., 2018; Gore et al., 2015). Sun et al. detected significantly strong positive associations between increased exposure to monobutyl phthalate and monoisobutyl phthalate and diabetes risk, and adiposity seemed to be involved in the mediation pathway between them (Sun et al., 2014). In another prospective study, several phthalate metabolites, including monobenzyl phthalate and monobutyl phthalate, were found to be associated with faster weight gain in a dose-response manner (Song et al., 2014). However, few studies have investigated the potential role of phthalate exposure in CVD, especially in type 2 diabetic patients. One study in 2330 Chinese participants from the general population observed no phthalate association with CVD outcome (Dong et al., 2017), and another case-control study found that exposure to di-(2-ethylhexyl)-phthalate and dibutyl phthalates was positively associated with coronary heart disease (Su et al., 2019).

Therefore, we analyzed data from a cross-sectional METAL study (Environmental Pollutant Exposure and Metabolic Diseases in Shanghai, www.chictr.org.cn, ChiCTR1800017573) to evaluate associations between urinary concentrations of phthalate metabolites and CVD and ultrasound vascular measurements. Then, we further used the conceptual models of moderation and mediation effects suggested by Hayes et al. (Hayes, 2018) to explore whether CVD risk factors mediated or interacted with the association between phthalate metabolites and CVD.

Methods

Study population

The 2018 METAL study was performed to investigate the association between exposure to environmental pollutants and diabetic complications in Chinese type 2 diabetic adults. Study participants were recruited from the outpatient clinics of seven communities in Huangpu District and Pudong District, Shanghai, China. Using a simple random sampling method, we invited half of the diabetic patients from the registration platform in each community healthcare clinic. Chinese diabetic citizens ≥18 years old who had lived in their current area for ≥6 months were included. Those with severe communication problems, acute illness, and an unwillingness

to participate were excluded (n=96).

In one of the seven clinics, all 698 registered diabetic individuals who provided urine and urinary phthalates were included. We excluded participants who were missing ultrasound results, urinary creatinine measurements and information on CVD history (n=23). A total of 675 diabetic participants were involved in the final analyses.

The study protocol was approved by the Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the appropriate institutional review committee. Informed consent was obtained from all participants included in the study.

Measurements

A questionnaire about sociodemographic characteristics, medical history, family history, and lifestyle factors was adopted during the interview. The same group of trained and experienced personnel in the SPECT-China study (Wang et al., 2016; Wang et al., 2017) conducted the interviews and clinical examinations, including measurements of weight, height and blood pressure, according to a standard protocol. Body mass index (BMI) was calculated as weight in kilograms divided by height squared in meters squared. Current smoking was defined as having smoked at least 100 cigarettes in one's lifetime and currently smoking cigarettes (Xu et al., 2013). Insulin resistance was estimated by the homeostasis model assessment index of insulin resistance (HOMA-IR): (fasting insulin [mIU/L])*(FPG [mmol/L])/22.5.

Blood samples were obtained between 6:00 am and 9:00 am after fasting for at least 8 h. Blood was refrigerated immediately after phlebotomy, and after two hours, it was centrifuged, and the serum was aliquoted and frozen in a central laboratory. Serum C-peptide was detected by the chemiluminescence method (Abbott i2000 SR, USA). Glycated hemoglobin (HbA1c) was measured by high-performance liquid chromatography (MQ-2000PT, Medconn, Shanghai, China). Fasting plasma glucose and lipid profiles were performed with a Beckman Coulter AU 680 (Brea, USA).

Carotid atherosclerosis in the common, internal, and bifurcation sites of the bilateral common carotid arteries (CCA) was assessed by the same batch of trained sonographers who were blinded to any clinical conditions of the participants with a Mindray M7 ultrasound system (MINDRAY, Shenzhen, China) with a 10-MHz probe (Wang et al., 2019). They were trained by performing a carotid ultrasound on the same patients before the study began to achieve an interobserver

coefficient of variation of less than 10%. As in our previous paper (Wang et al., 2019), the measurement method was based on the consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force (Stein et al., 2008). The bilateral mean value of the carotid intima-media thickness (CIMT) was used for analysis. The CCA diameter was measured between the leading edge of the adventitia-media echo of the near wall and the leading edge of the media-adventitia echo of the far wall, based on an average of the end-diastolic diameter measurements 5-10 mm proximal to the carotid bulb.

Measurement of urinary metabolites of phthalates

We used ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) to determine 10 urinary phthalate metabolites, including monomethyl phthalate (MMP), monoethyl phthalate (MEP), mono-n-butyl phthalate (MnBP), monoisobutyl phthalate (MiBP), monobenzyl phthalate (MBzP), mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP) and mono-2-carboxymethyl-hexyl phthalate (MCMHP). Among them, MMP, MEP, MnBP, MiBP and MBzP are the metabolites of dimethyl phthalate (DMP), diethyl phthalate (DEP), di-n-butyl phthalate (DnBP), di-iso-butyl phthalate (DiBP) and benzyl butyl phthalate (BBP), respectively, and MEHP, MEOHP, MEHHP, MECPP, and MCMHP are all metabolites of dis (2-ethylhexyl) phthalate (DEHP). We also assessed the coexposure by calculating the micromolar sum of DEHP metabolites (ΣDEHP) and calculated the percentage of total ΣDEHP excreted as MEHP, referred to as %MEHP [%MEHP=MEHP /ΣDEHP] (Joensen et al., 2012).

The method has been described in our previous study with a slight modification (Dong et al., 2018). Briefly, 1 mL of the urine sample was thawed, transferred to a 10 mL glass tube, and incubated with β-glucuronidase (*E. coli* K 12; Roche, Mannheim, Germany) at 37 °C for 120 min. The sample was subsequently mixed with 1 mL of aqueous 2% (v/v) acetic acid and 100 μL of internal standard (100 μg/L). The mixture was loaded into a PLS column (Dikma, China; 60 mg/3 mL) previously activated with 2 mL of methanol and 2 mL of aqueous 0.5% (v/v) acetic acid. After sample loading, the column was washed with 2 mL of aqueous 0.5% (v/v) acetic acid. Next, 1 mL of methanol was added to elute the metabolites. Finally, the eluate was passed through a 0.2-μm filter and analyzed (2 μL) by an UPLC-MS/MS system integrated by Waters ACQUITY UPLC H-Class (Waters, USA) coupled with an ABSCIEX QTRAP 6500 (AB Sciex Technologies, Framingham, MA, USA). The analytical column was a Waters ACQUITY UPLC BEH C18 Column (1.7 μm, 2.1x50 mm, Waters, USA).

An internal standard method was used to quantify the target metabolite. For every 20 samples, a procedural blank and two matrix-spiked samples at two different spiking concentrations (5 and 15 ng/mL) were processed. The average recoveries and relative standard deviations (RSDs) of the target metabolites respectively ranged from 86.5% to 123.1% and from 0.5% to 12.5% at 5 μg/L and from 75.3% to 98.4% and from 0.7% to 8.8% at 15 μg/L. Sample concentrations of these metabolites were determined after subtraction of blank values. The limits of detection (LODs) were calculated at a signal-to-noise (S/N) ratio of 3 at concentrations of 0.100, 0.080, 0.006, 0.006, 0.060, 0.006, 0.010, 0.020, 0.020 and 0.040 μg/L of MMP, MEP, MnBP, MiBP, MBzP, MEHP, MEOHP, MEHHP, MECPP and MCMHP, respectively (Supplemental Table 1).

Definitions

Hypertension was assessed by systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥90 mmHg, or a self-reported prior diagnosis of hypertension by a physician. Dyslipidemia was defined as total cholesterol ≥6.22 mmol/L (240 mg/dL), triglycerides ≥2.26 mmol/L (200 mg/dL), LDL-C≥ 4.14 mmol/L (160 mg/dL), HDL-C< 1.04 mmol/L (40 mg/dL), or a self-reported previous diagnosis of hyperlipidemia by a physician, according to the modified National Cholesterol Education Program-Adult Treatment Panel III.

The outcome CVD was defined as a self-reported diagnosis by a physician and included coronary heart disease, myocardial infarction or stroke. The related question in the questionnaire was “Have you ever been told by a doctor or other healthcare professionals that you have coronary heart disease, myocardial infarction or stroke?” The same question was adopted by another large study in Chinese, where the validation rate reached 91.07% (Lu et al., 2014). Then, the self-reported diagnoses were further verified in the registration platform. Present CCA plaque was identified as focal thickening (≥ 1.5 mm) of the artery wall.

Statistical Analysis

Data analyses were performed using IBM SPSS Statistics, Version 22 (IBM Corporation, Armonk, NY, USA). A *P* value <0.05 indicated significance (two sided) unless other values were mentioned. Continuous variables were summarized as medians (interquartile range) and categorical variables as percentages (%). Phthalate metabolite concentrations and urine creatinine-adjusted concentrations were presented as geometric means, percentiles and medians.

First, we performed multiple linear (continuous variable outcome) or logistic (categorical variable outcome) regression models to analyze the associations of urine creatinine-adjusted phthalate metabolites with CVD and vascular measurement. The model was adjusted for sex, age, duration of diabetes, BMI, smoking status, hypertension and dyslipidemia. The concentrations of phthalate metabolites were logarithmically transformed to achieve a normal distribution in the analyses. Backward stepwise regression was used, and the criteria for removal were $P > 0.1$. Bonferroni correction was used to reduce the false discovery rate with multiple comparisons.

Then, we tested the mediation and moderation effects by the SPSS PROCESS macro in an approach with 5000 bootstrap samples (Hayes, 2018). The mediation and moderation models were also adjusted for sex, age, duration of diabetes, BMI, smoking status, hypertension and dyslipidemia if necessary. First, mediation analysis was used to clarify whether exposure X was proposed as influencing outcome Y via an intervening variable M (Figure 1A). In this study, we predicted that “phthalates” impacted “CVD outcome” with “relevant CVD risk factors” as mediator variables. PROCESS was operated using one independent variable (each of the phthalate metabolites), one mediator [HOMA-IR, BMI, systolic blood pressure (SBP), low density lipoprotein (LDL), HbA1c, etc.], and one dependent variable (CVD). Second, in the moderation or interaction analysis, we predicted that exposure to different levels of CVD risk factors and medication usage would moderate the relationship between phthalate metabolites and CVD. PROCESS was operated using one independent variable (each of the phthalate metabolites), one moderator (each CVD risk factor and antidiabetic usage), and one dependent variable (CVD) (Figure 1B).

Results

Characteristics of the diabetic participants (Table 1)

This study recruited 675 type 2 diabetic participants, including 325 men and 350 women. The median age was 68 years (max 99, min 44). The medians of BMI, HOMA-IR and HbA1c were 24.8 kg/m^2 (IQR 22.5, 27.1), 2.68 (IQR 1.77, 4.41) and 7.2% (IQR 6.6, 8.2), respectively. The prevalence of current smoking, hypertension, dyslipidemia and CVD was 19.4%, 86.4%, 72.4% and 40.9%, respectively. Of these, 28.4% and 34.5% of the patients had unilateral and bilateral CCA plaques, respectively. The medians of CCA diameter and CIMT were 7.60 mm (IQR 7.10, 8.20) and 0.80 mm (IQR 0.70, 0.85), respectively.

Table 1
Characteristics of the study participants (n=675).

Characteristic	Results
Men, %	48.1
Age, year	68 (63, 74)
Duration of diabetes, year	10 (5, 16)
BMI, kg/m^2	24.8 (22.5, 27.1)
FPG, mmol/L	7.44 (6.23, 9.14)
HbA1c, %	7.2 (6.6, 8.2)
C-peptide, ng/ml	1.60 (1.17, 2.10)
HOMA-IR	2.68 (1.77, 4.41)
Systolic blood pressure, mmHg	147 (135, 160)
Diastolic blood pressure, mmHg	79 (72, 86)
HDL, mmol/L	1.08 (0.92, 1.27)
LDL, mmol/L	3.17 (2.53, 3.76)
Triglycerides, mmol/L	1.59 (1.16, 2.36)
CCA diameter, mm	7.60 (7.10, 8.20)
CIMT, mm	0.80 (0.70, 0.85)
Unilateral/bilateral CCA plaque, %	28.4/34.5
Current smoker, %	19.4
CVD, %	40.9
Hypertension, %	86.4
Dyslipidemia, %	72.4
The data are summarized as medians (interquartile ranges) for continuous variables or as a percentages for categorical variables. BMI, body mass index; CCA, common carotid artery; CVD, cardiovascular disease; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein; HbA1c, glycated hemoglobin; LDL-C, low-density lipoprotein.	

Urinary concentrations of phthalate metabolites

Supplemental Table 2 provides urinary concentrations and creatinine-corrected concentrations of the determined phthalate metabolites. The metabolites could be detected in all subjects. Among them, MnBP and MiBP had the highest concentrations (141.55 μg/g, IQR 78.77-251.05 and 86.04 μg/g, IQR 53.13-139.41, respectively). MBzP had the lowest concentration (0.92 μg/g, IQR 0.47-1.73). The median ΣDEHP was 0.29 μmol/g (IQR 0.18-0.49).

Association of urinary phthalate metabolites with CVD and vascular measurement (Table 2)

After backward stepwise regression, four metabolites (MEP, MiBP, MECPP and MEHHP) remained as associated with CVD. After Bonferroni correction, significant and marginally significant positive associations were found between the levels of MEP, MiBP and CVD (OR 1.138, 95% CI 1.032, 1.254, P 0.009; OR 1.369, 95% CI 1.049, 1.786, P 0.021, respectively). ΣDEHP and %MEHP were not associated with CVD. To further confirm assess the associations independently, we provided the association between each phthalate metabolite and and CVD and vascular measurement (Supplemental Table 3). MEP and MiBP were still siginificantly associated with CVD. No significant association was observed between phthalate metabolites and CCA plaque. MiBP and MBzP were marginally associated with CIMT and CCA diameter, respectively.

Table 2
Regression analysis of associations between phthalates and CVD and its risk factors

	CVD	*P*	Unilateral CCA plaque	*P*	Bilateral CCA plaque	*P*	CIMT	*P*	CCA diameter	*P*
MEP	1.138(1.032, 1.254)	0.009	-	-	-	-	-	-	-	-
MiBP	1.369(1.049, 1.786)	0.021	-	-	-	-	0.024(0.005, 0.043)	0.014	-	-
MnBP	-	-	-	-	-	-	-0.019(-0.036, -0.002)	0.025	-	-
MBzP	-	-	-	-	-	-	-	-	0.060(0.011, 0.108)	0.017
MEHP	-	-	-	-	-	-	-	-	-	-
MECPP	1.491(0.964, 2.304)	0.072	-	-	-	-	-	-	-	-
MEHHP	0.537(0.340, 0.850)	0.008	-	-	-	-	-	-	-	-

A multiple linear (continuous variable outcome) or logistic (categorical variable outcome) regression model (backward stepwise) was applied to analyze the association of urinary concentrations of phthalate metabolites with cardiovascular diseases and vascular measurement. The model included the following covariates: sex, age, duration of diabetes, body mass index, smoking status, hypertension and dyslipidemia. "–": removed from the linear model. Bold indicates $P<0.05/4=0.0125$ (Bonferroni correction, four metabolites in the model at most). CCA, common carotid artery; CIMT, carotid intima-media thickness; CVD, cardiovascular diseases.

Mediation analysis (Table 3)

After finding that MEP, MiBP, MECPP and MEHHP were associated with CVD outcome, we further performed mediation analysis and tried to understand whether some CVD risk factors were significant mediators. Figure 1A illustrates the model for the mediation effect. "a" indicates the path from phthalates (exposure) to mediators, "b" indicates the path from mediators to cardiovascular diseases (outcome), and "c'" indicates the direct path from phthalates (exposure) to outcome when controlled for mediators.

Table 3
The mediation of metabolic risk factors on the association between phthalates and cardiovascular diseases in type 2 diabetic patients

Effect	MEP	MiBP	MECPP	MEHHP
HOMAIR[1]				
a (exposure-mediator)	0.032(-0.002, 0.066)	-0.049(-0.131, 0.033)	0.056(-0.009, 0.122)	0.054(-0.016, 0.124)
b (mediator-outcome)	0.212(0.001, 0.423)	0.242(0.032, 0.451)	0.225(0.016, 0.435)	0.231(0.022, 0.441)
c' (direct effect)	0.116(0.021, 0.210)	0.255(0.029, 0.480)	0.035(-0.142, 0.212)	-0.049(-0.238, 0.140)
ab (mediated effect)	0.007(-0.002, 0.018)	-0.012(-0.040, 0.006)	0.013(-0.002, 0.039)	0.012(-0.004, 0.041)
BMI[2]				
a (exposure-mediator)	0.071(-0.081, 0.222)	-0.321(-0.687, 0.045)	-0.056(-0.350, 0.237)	0.020(-0.291, 0.331)
b (mediator-outcome)	0.045(-0.002, 0.091)	0.050(0.003, 0.097)	0.047(0.000, 0.093)	0.047(0.000, 0.093)
c' (direct effect)	0.124(0.030, 0.218)	0.246(0.023, 0.470)	0.048(-0.128, 0.224)	-0.046(-0.234, 0.142)
ab (mediated effect)	0.003(-0.003 0.011)	-0.016(-0.048, 0.003)	-0.003(-0.025, 0.011)	0.001(-0.018, 0.018)
LDL[3]				
a (exposure-mediator)	-0.020(-0.057, 0.017)	0.018(-0.072, 0.108)	-0.041(-0.113, 0.031)	-0.015(-0.092, 0.061)
b (mediator-outcome)	-0.461(-0.661, -0.261)	-0.473(-0.672, -0.274)	-0.465(-0.664, -0.266)	-0.467(-0.665, -0.268)
c' (direct effect)	0.131(0.036, 0.226)	0.264(0.038, 0.489)	0.038(-0.141, 0.216)	-0.045(-0.235, 0.145)
ab (mediated effect)	0.009(-0.008, 0.031)	-0.009(-0.050, 0.031)	0.019(-0.015, 0.059)	0.007(-0.032, 0.046)
SBP[4]				
a (exposure-mediator)	0.067(-0.772, 0.906)	1.633(-0.405, 3.671)	1.070(-0.558, 2.697)	1.166(-0.559, 2.891)
b (mediator-outcome)	-0.006(-0.014, 0.003)	-0.006(-0.015, 0.002)	-0.006(-0.014, 0.003)	-0.006(-0.014, 0.003)
c' (direct effect)	0.123(0.029, 0.217)	0.255(0.030. 0.480)	0.061(-0.115, 0.238)	-0.034(-0.222, 0.154)
ab (mediated effect)	0.000(-0.007, 0.006)	-0.010(-0.036, 0.006)	-0.006(-0.025, 0.005)	-0.007(-0.028, 0.005)
HbA1c[1]				
a (exposure-mediator)	-0.033(-0.095, 0.028)	-0.031(-0.180, 0.118)	0.046(-0.073, 0.165)	0.065(-0.062, 0.191)
b (mediator-outcome)	-0.006(-0.122, 0.110)	-0.011(-0.127, 0.105)	-0.014(-0.130, 0.101)	-0.012(-0.128, 0.103)
c' (direct effect)	0.124(0.030, 0.218)	0.246(0.023, 0.469)	0.049(-0.127, 0.225)	-0.045(-0.233, 0.142)
ab (mediated effect)	0.000(-0.005, 0.006)	0.000(-0.010, 0.012)	-0.001(-0.011, 0.007)	-0.001(-0.014, 0.009)

The mediation analysis was applied to identify the mediators in the association between phthalates and cardiovascular diseases. BMI, body mass index; LDL-C, low-density lipoprotein; SBP, systolic blood pressure. Bold indicates *P* <0.05. The results are expressed in a log-odds metric.

[1]The model was adjusted for age, sex, current smoking, body mass index, dyslipidemia, hypertension, and duration of diabetes.

[2]The model was adjusted for age, sex, current smoking, dyslipidemia, hypertension, and duration of diabetes.

[3]The model was adjusted for age, sex, current smoking, body mass index, hypertension, and duration of diabetes.

[4]The model was adjusted for age, sex, current smoking, body mass index, dyslipidemia and duration of diabetes.

Among the four metabolites, MEP and MiBP showed consistently significant direct effects on CVD (Table 3). Moreover, none of the representative risk factors, including HOMA-IR, BMI, LDL and SBP, significantly mediated the association between the four metabolites and CVD, although HOMAIR and BMI seemed to have a marginal mediation effect on these associations (β 0.007, 95% CI -0.002, 0.018; β 0.004, 95% CI -0.002, 0.013). We also tested the mediation effect by other possible CVD risk factors (waist circumference, HDL-C, triglycerides and diastolic blood pressure), and the mediation results remained negative (Supplemental Table 4).

In the sensitivity analyses, we also further adjusted for antidiabetic drug usage, statin usage and antihypertensive drug use, and the mediation results were not significantly changed.

Moderation analysis

Figure 1B shows the model for the modification effect. The bootstrap method was used to evaluate the conditional effects of the phthalate metabolites on CVD. Indirect effects for the binary factors of sex (men or women), current smoking (yes or no), hypertension (yes or no), dyslipidemia (yes or no), BMI (≥25 kg/m^2 or <25 kg/m^2), using anti-diabetic drugs (yes or no) and using statins (yes or no) were examined in the associations between the four metabolites and CVD. As shown in Supplemental Table 5, current smoking, dyslipidemia, sex, hypertension and insulin usage significantly or marginally modified the association between certain phthalate metabolites and CVD (P for interaction <0.1). Then, in the stratification analyses (Figure 2), we observed that the conditional indirect effect on CVD changed according to the moderator category and was significantly stronger for current smoking (OR 1.494, 95% CI 1.125, 1.983 for MEP), dyslipidemia (OR 1.195, 95% CI 1.071, 1.334 for MEP), no statin usage (OR 1.433, 95% CI 1.104,1.859 for MiBP) and men (OR 1.619, 95% CI 1.138, 2.304 for MiBP).

Discussion

In this study of community-dwelling Chinese adults with type 2 diabetes, we reported that urinary phthalate metabolites, especially MEP and MiBP, were positively associated with CVD. CVD risk parameters, including obesity indices, insulin resistance, blood pressure, LDL and HbA1c, did not mediate the relationship between exposure to phthalates and CVD. We also found a synergistic interaction between elevated phthalate exposure (MEP and MiBP) and some factors (current smoking, dyslipidemia, no statin usage and men) for an elevation of CVD risk. Therefore, exposure to phthalates in diabetic patients, especially those currently smoking, with dyslipidemia and not using statins, may be a risk factor for the development of CVD.

A major finding of our study is the positive association between the levels of MEP and MiBP and self-reported CVD outcome in type 2 diabetes, which was consistent in the mediation and moderation analysis. A previous study examined urinary phthalate metabolites with self-reported CVD in 2330 Chinese participants from the general population, and no significant association was observed in both the overall population and in the sex-separated populations, although

sporadic associations between phthalates and hyperlipidemia were found (Dong et al., 2017). However, another case-control study (180 vs 360 participants with and without coronary heart disease) found that participants with greater urinary concentrations of MEHP, MnBP and MiBP had significantly higher odds of coronary heart disease, which reached 2.77, 2.90 and 3.19, respectively, in the higher tertiles (Su et al., 2019). Both studies did not perform analyses on diabetic patients, so we have no idea whether our results would be consistent with theirs, although our MiBP results seem to be consistent with theirs. Thus, further prospective studies are needed to draw a conclusion from this result.

Two study groups investigated associations with ultrasound vascular measurements in different age groups of the population. One study in young adults (mean age 20 years) suggested that MEHP, ΣDEHP and MnBP were strongly associated with a greater CIMT. The mean MEHP for subjects with CIMT ≥0.46 mm was strikingly 6-fold greater than those with CIMT <0.40 mm (16.3 vs. 2.73 μg/g creatinine). In contrast, the other study investigated elderly patients and observed that MMP, MiBP and MBzP were positively related to the echogenicity of the CIMT or plaques (Lind and Lind, 2011; Wiberg et al., 2014). In type 2 diabetes, in addition to MiBP and CIMT, we found a significant association between MBzP and CCA diameter. A larger CCA diameter was associated with incident CVD and mortality in a pooled analyses of four cohort studies including approximately 5000 participants (Sedaghat et al., 2018). Thus, it may be of value to further explore the causal association among MBzP, CCA diameter, and CVD.

Given that people are exposed to mixtures of phthalate metabolites, there may be a benefit to drawing conclusions for subclasses of phthalates rather than for individual chemicals. DEHP is one of the most commonly used phthalates and has been shown to have interaction effects on androgen receptors, PPAR receptors and the aryl-hydrocarbon receptor (Stroheker et al., 2005; Wojtowicz et al., 2019). In our study, we did not find that DEHP was significantly associated with self-reported CVD, plaque and CIMT, though a marginal association with CCA diameter (β 0.088, 95% -0.003, 0.179, *P* 0.057) was found.

In addition to reducing phthalate exposure, it would be meaningful to understand how the prevention of cardiometabolic risk factors could reduce the effects of phthalate exposure on CVD. To understand the role of cardiometabolic risk factors in the association between phthalates and CVD, we conducted mediation analysis. Our results identified that the relationship between phthalates and CVD was mediated by none of the cardiometabolic risk factors tested, which included HbA1c, BMI, waist circumference, lipid profile and blood pressure. Although it seems that BMI and HOMAIR have marginal mediation effects on the pathway between MEP and MiBP and CVD, the effects were rather small. However, we cannot completely deny the possibility that some risk factors may still mediate the pathway, such as HbA1c, lipid profile and blood pressure, because these factors are relatively easily modifiable by medications and some patients had already received these medications in a cross-sectional setting, although adjusting the medication usage did not change the results. We still think obesity indices and insulin resistance should be further investigated to better understand their possible roles in the mediation pathway.

Questions about *how* relate to mediation, but questions about *when* or *for whom* are in the area of moderation analysis. Our results showed some interesting findings in the moderation analyses. A synergistic interaction was found between elevated MEP and MiBP levels and cardiometabolic factors including current smoking, dyslipidemia, no statin usage and men for the elevation of CVD risk. This indicates that type 2 diabetic men who are currently smoking, have an uncontrolled lipid profile and are not using statins may be more susceptible to CVD when exposed to certain phthalates, which contributes to the prevention strategy of CVD in type 2 diabetes. First, male is a risk factor for CVD, and a recent study suggested that a significant inverse association was observed between testosterone in men and MiBP (β = -0.099) (Al-Saleh et al., 2019). Therefore, it is not difficult to understand the possible interaction between sex and MiBP. There is little direct evidence describing how smoking and phthalates interact. We think smoking here may be more likely to reflect an unhealthy lifestyle. For example, the Women's Health Initiative found that a lack of healthy behaviors such as not smoking, a high-quality diet, and moderate physical activity were predictors of urinary phthalate metabolites (Reeves et al., 2019). A Norwegian study also indicated that smoking, consuming food with plastic packaging and eating with hands

were associated with higher levels of phthalate metabolites (Giovanoulis et al., 2016). Third, it is interesting that dyslipidemia was found to be a moderator rather than a mediator. However, it does have similar importance for prevention as mediation. A moderator, here dyslipidemia, is a third variable that can influence the strength of the relationship between MEP and CVD. Reduction of phthalate exposure in diabetic patients with uncontrolled lipid profiles or avoiding the use of lipid-lowering medications (e.g., statins) may minimize the deteriorating effect of MEP and MiBP on CVD.

Phthalates may initiate and regulate some important steps in the development of atherosclerosis. Animals treated with phthalates displayed enhanced cardiovascular reactivity and prolonged blood pressure recovery that often preceded clinical manifestations of atherosclerosis. Alterations in the cardiac gene expression levels of endothelin-1, angiotensin-converting enzyme, and nitric oxide synthase may partly explain these alterations (Jaimes et al., 2017). Additionally, the expression of E-selectin and intercellular adhesion molecules 1 indicated the initiation of atherosclerosis and the expression of matrix metalloproteinase-2 and -9, indicating that the progression of atherosclerosis was significantly upregulated in phthalate-exposed vascular smooth muscle (Shih et al., 2018). Moreover, in diabetic patients, phthalates may elevate serum γ-glutamiltransferase (Dong et al., 2018). γ-Glutamiltransferase may directly participate in the promotion of atherosclerosis by inducing reactive oxygen species (Ndrepepa et al., 2018). The cysteinyl-glycine moiety produced by γ-glutamiltransferase could strongly reduce Fe^{3+} to Fe^{2+}, which leads to the formation of superoxide and hydrogen peroxide (Dominici et al., 2005). Catalytically higher amounts of active γ-glutamiltransferase within cerebral, carotid and coronary artery atherosclerotic plaques also colocalized with oxidized lipids and CD68+ foam cells, providing further direct evidence (Franzini et al., 2009).

This study has several strengths. We used ultra-performance liquid chromatography tandem mass spectrometry-based measurements of urinary phthalate metabolites, resulting in high data accuracy and precision. Moreover, the present analysis, to our knowledge, is the first to use conditional process analysis to demonstrate mediators and moderators of possible CVD risk caused by exposure to phthalates. Our study also had some major limitations. First, the nature of this study is cross-sectional; thus, unmeasured or residual confounding and causal relationships or coincidental phenomena cannot be fully determined. Second, Han Chinese were the ethnic group investigated; thus, the results may not be generalizable to other ethnicities. Third, because of the rapid metabolism of phthalates, urinary concentrations of metabolites can fluctuate within an individual over time (Meeker et al., 2009). The one-time sampling method may result in measurement error and bias the results toward the null. However, some studies have indicated that phthalate levels may be sufficiently stable based on a single first morning void urine measurement (Hoppin et al., 2002).

In conclusion, we reported that urinary phthalate metabolites, especially MEP and MiBP, were positively associated with CVD. Obesity indices, insulin resistance, blood pressure and lipid profile did not mediate the relationship between exposure to phthalates and CVD. We also found modification effects from current smoking, dyslipidemia, no statin usage and men that strengthened the association between phthalate exposure and CVD. These results indicate that type 2 diabetic men who are currently smoking, have uncontrolled lipid profiles and are not using statins may be more susceptible to CVD when exposed to phthalates and for whom reduction of phthalate exposure could therefore be essential.

Declarations

Compliance with Ethical Standards

Funding

This study was supported by National Natural Science Foundation of China (91857117); Science and Technology Commission of Shanghai Municipality (19140902400, 18410722300); the Major Science and Technology Innovation Program of Shanghai Municipal Education Commission (2019-01-07-00-01-E00059); Commission of Health and Family

Planning of Pudong District (PWZxq2017-17); Shanghai JiaoTong University School of Medicine (19XJ11007). The funders played no role in the design or conduct of the study, collection, management, analysis, or interpretation of data or in the preparation, review, or approval of the article.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

DISCLOSURE SUMMARY:

The authors have nothing to disclose.

References

1. Al-Saleh, I., Coskun, S., Al-Doush, I., Al-Rajudi, T., Abduljabbar, M., Al-Rouqi, R., Palawan, H., Al-Hassan, S., 2019. The relationships between urinary phthalate metabolites, reproductive hormones and semen parameters in men attending in vitro fertilization clinic. Sci Total Environ 658, 982-995.
2. Dominici, S., Paolicchi, A., Corti, A., Maellaro, E., Pompella, A., 2005. Prooxidant reactions promoted by soluble and cell-bound gamma-glutamyltransferase activity. Methods Enzymol 401, 484-501.
3. Dong, R., Chen, J., Zheng, J., Zhang, M., Zhang, H., Wu, M., Li, S., Chen, B., 2018. The role of oxidative stress in cardiometabolic risk related to phthalate exposure in elderly diabetic patients from Shanghai. Environ Int 121, 340-348.
4. Dong, R., Zhao, S., Zhang, H., Chen, J., Zhang, M., Wang, M., Wu, M., Li, S., Chen, B., 2017. Sex Differences in the Association of Urinary Concentrations of Phthalates Metabolites with Self-Reported Diabetes and Cardiovascular Diseases in Shanghai Adults. Int J Environ Res Public Health 14.
5. Duckworth, W., Abraira, C., Moritz, T., Reda, D., Emanuele, N., Reaven, P.D., Zieve, F.J., Marks, J., Davis, S.N., Hayward, R., Warren, S.R., Goldman, S., McCarren, M., Vitek, M.E., Henderson, W.G., Huang, G.D., Investigators, V., 2009. Glucose control and vascular complications in veterans with type 2 diabetes. N Engl J Med 360, 129-139.
6. Fox, C.S., Pencina, M.J., Wilson, P.W., Paynter, N.P., Vasan, R.S., D'Agostino, R.B., 2008. Lifetime risk of cardiovascular disease among individuals with and without diabetes stratified by obesity status in the Framingham heart study. Diabetes Care 31, 1582-1584.
7. Franzini, M., Corti, A., Martinelli, B., Del Corso, A., Emdin, M., Parenti, G.F., Glauber, M., Pompella, A., Paolicchi, A., 2009. Gamma-glutamyltransferase activity in human atherosclerotic plaques–biochemical similarities with the circulating enzyme. Atherosclerosis 202, 119-127.
8. Giovanoulis, G., Alves, A., Papadopoulou, E., Cousins, A.P., Schutze, A., Koch, H.M., Haug, L.S., Covaci, A., Magner, J., Voorspoels, S., 2016. Evaluation of exposure to phthalate esters and DINCH in urine and nails from a Norwegian study population. Environmental Research 151, 80-90.

9. Goodman, M., Lakind, J.S., Mattison, D.R., 2014. Do phthalates act as obesogens in humans? A systematic review of the epidemiological literature. Crit. Rev. Toxicol. 44, 151-175.
10. Gore, A.C., Chappell, V.A., Fenton, S.E., Flaws, J.A., Nadal, A., Prins, G.S., Toppari, J., Zoeller, R.T., 2015. EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. Endocr Rev 36, E1-E150.
11. Group, A.C., Patel, A., MacMahon, S., Chalmers, J., Neal, B., Billot, L., Woodward, M., Marre, M., Cooper, M., Glasziou, P., Grobbee, D., Hamet, P., Harrap, S., Heller, S., Liu, L., Mancia, G., Mogensen, C.E., Pan, C., Poulter, N., Rodgers, A., Williams, B., Bompoint, S., de Galan, B.E., Joshi, R., Travert, F., 2008. Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. N Engl J Med 358, 2560-2572.
12. Hayes, A.F., 2018. Introduction to mediation, moderation, and conditional process analysis: a regression-based approach.
13. Hoppin, J.A., Brock, J.W., Davis, B.J., Baird, D.D., 2002. Reproducibility of urinary phthalate metabolites in first morning urine samples. Environ Health Perspect 110, 515-518.
14. Jaimes, R., 3rd, Swiercz, A., Sherman, M., Muselimyan, N., Marvar, P.J., Posnack, N.G., 2017. Plastics and cardiovascular health: phthalates may disrupt heart rate variability and cardiovascular reactivity. Am J Physiol Heart Circ Physiol 313, H1044-H1053.
15. Joensen, U.N., Frederiksen, H., Blomberg Jensen, M., Lauritsen, M.P., Olesen, I.A., Lassen, T.H., Andersson, A.M., Jorgensen, N., 2012. Phthalate excretion pattern and testicular function: a study of 881 healthy Danish men. Environ Health Perspect 120, 1397-1403.
16. Lind, P.M., Lind, L., 2011. Circulating levels of bisphenol A and phthalates are related to carotid atherosclerosis in the elderly. Atherosclerosis 218, 207-213.
17. Lu, J., Bi, Y., Wang, T., Wang, W., Mu, Y., Zhao, J., Liu, C., Chen, L., Shi, L., Li, Q., Wan, Q., Wu, S., Qin, G., Yang, T., Yan, L., Liu, Y., Wang, G., Luo, Z., Tang, X., Chen, G., Huo, Y., Gao, Z., Su, Q., Ye, Z., Wang, Y., Deng, H., Yu, X., Shen, F., Chen, L., Zhao, L., Dai, M., Xu, M., Xu, Y., Chen, Y., Lai, S., Ning, G., 2014. The relationship between insulin-sensitive obesity and cardiovascular diseases in a Chinese population: results of the REACTION study. Int J Cardiol 172, 388-394.
18. Meeker, J.D., Sathyanarayana, S., Swan, S.H., 2009. Phthalates and other additives in plastics: human exposure and associated health outcomes. Philosophical Transactions of the Royal Society B-Biological Sciences 364, 2097-2113.
19. Ndrepepa, G., Colleran, R., Kastrati, A., 2018. Gamma-glutamyl transferase and the risk of atherosclerosis and coronary heart disease. Clin Chim Acta 476, 130-138.
20. Nelson, A.J., Peterson, E.D., Pagidipati, N.J., 2019. Atherosclerotic cardiovascular disease and heart failure: Determinants of risk and outcomes in patients with diabetes. Prog Cardiovasc Dis.
21. Reeves, K.W., Santana, M.D., Manson, J.E., Hankinson, S.E., Zoeller, R.T., Bigelow, C., Hou, L.F., Wactawski-Wende, J., Liu, S.M., Tinker, L., Calafat, A.M., 2019. Predictors of urinary phthalate biomarker concentrations in postmenopausal women. Environmental Research 169, 122-130.
22. Rydén, L., Grant, P.J., Anker, S.D., Berne, C., Cosentino, F., Danchin, N., Deaton, C., Escaned, J., Hammes, H.P., Huikuri, H., Marre, M., Marx, N., Mellbin, L., Ostergren, J., Patrono, C., Seferovic, P., Uva, M.S., Taskinen, M.R., Tendera, M., Tuomilehto, J., Valensi, P., Zamorano, J.L., Zamorano, J.L., Achenbach, S., Baumgartner, H., Bax, J.J., Bueno, H., Dean, V., Deaton, C., Erol, C., Fagard, R., Ferrari, R., Hasdai, D., Hoes, A.W., Kirchhof, P., Knuuti, J., Kolh, P., Lancellotti, P., Linhart, A., Nihoyannopoulos, P., Piepoli, M.F., Ponikowski, P., Sirnes, P.A., Tamargo, J.L., Tendera, M., Torbicki, A., Wijns, W., Windecker, S., De Backer, G., Sirnes, P.A., Ezquerra, E.A., Avogaro, A., Badimon, L., Baranova, E., Baumgartner, H., Betteridge, J., Ceriello, A., Fagard, R., Funck-Brentano, C., Gulba, D.C., Hasdai, D., Hoes, A.W., Kjekshus, J.K., Knuuti, J., Kolh, P., Lev, E., Mueller, C., Neyses, L., Nilsson, P.M., Perk, J., Ponikowski, P., Reiner, Z., Sattar, N., Schächinger, V., Scheen, A., Schirmer, H., Strömberg, A., Sudzhaeva, S., Tamargo, J.L., Viigimaa, M., Vlachopoulos, C., Xuereb, R.G., 2013. ESC Guidelines on diabetes, pre-diabetes, and cardiovascular diseases developed in collaboration with the EASD: the Task Force on diabetes, pre-diabetes, and cardiovascular diseases of the European Society of Cardiology

(ESC) and developed in collaboration with the European Association for the Study of Diabetes (EASD). Eur. Heart J. 34, 3035-3087.

23. Sedaghat, S., van Sloten, T.T., Laurent, S., London, G.M., Pannier, B., Kavousi, M., Mattace-Raso, F., Franco, O.H., Boutouyrie, P., Ikram, M.A., Stehouwer, C.D.A., 2018. Common Carotid Artery Diameter and Risk of Cardiovascular Events and Mortality: Pooled Analyses of Four Cohort Studies. Hypertension 72, 85-92.
24. Shih, M.F., Pan, K.H., Liu, C.C., Shen, C.R., Cherng, J.Y., 2018. Treatment of beta-thujaplicin counteracts di(2-ethylhexyl)phthalate (DEHP)-exposed vascular smooth muscle activation, inflammation and atherosclerosis progression. Regul Toxicol Pharmacol 92, 333-337.
25. Song, Y., Hauser, R., Hu, F.B., Franke, A.A., Liu, S., Sun, Q., 2014. Urinary concentrations of bisphenol A and phthalate metabolites and weight change: a prospective investigation in US women. Int J Obes (Lond) 38, 1532-1537.
26. Stein, J.H., Korcarz, C.E., Hurst, R.T., Lonn, E., Kendall, C.B., Mohler, E.R., Najjar, S.S., Rembold, C.M., Post, W.S., American Society of Echocardiography Carotid Intima-Media Thickness Task, F., 2008. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force. Endorsed by the Society for Vascular Medicine. J Am Soc Echocardiogr 21, 93-111; quiz 189-190.
27. Stroheker, T., Cabaton, N., Nourdin, G., Regnier, J.F., Lhuguenot, J.C., Chagnon, M.C., 2005. Evaluation of anti-androgenic activity of di-(2-ethylhexyl)phthalate. Toxicology 208, 115-121.
28. Su, T.C., Hwang, J.J., Sun, C.W., Wang, S.L., 2019. Urinary phthalate metabolites, coronary heart disease, and atherothrombotic markers. Ecotoxicol Environ Saf 173, 37-44.
29. Sun, Q., Cornelis, M.C., Townsend, M.K., Tobias, D.K., Eliassen, A.H., Franke, A.A., Hauser, R., Hu, F.B., 2014. Association of urinary concentrations of bisphenol A and phthalate metabolites with risk of type 2 diabetes: a prospective investigation in the Nurses' Health Study (NHS) and NHSII cohorts. Environ Health Perspect 122, 616-623.
30. Wang, C., Zhang, W., Wang, Y., Wan, H., Chen, Y., Xia, F., Zhang, K., Wang, N., Lu, Y., 2019. Novel associations between sex hormones and diabetic vascular complications in men and postmenopausal women: a cross-sectional study. Cardiovasc Diabetol 18, 97.
31. Wang, N., Chen, Y., Ning, Z., Li, Q., Han, B., Zhu, C., Chen, Y., Xia, F., Jiang, B., Wang, B., Wang, X., Jensen, M.D., Lu, Y., 2016. Exposure to Famine in Early Life and Nonalcoholic Fatty Liver Disease in Adulthood. J Clin Endocrinol Metab 101, 2218-2225.
32. Wang, N., Wang, X., Li, Q., Han, B., Chen, Y., Zhu, C., Chen, Y., Lin, D., Wang, B., Jensen, M.D., Lu, Y., 2017. The famine exposure in early life and metabolic syndrome in adulthood. Clin Nutr 36, 253-259.
33. Weuve, J., Hauser, R., Calafat, A.M., Missmer, S.A., Wise, L.A., 2010. Association of exposure to phthalates with endometriosis and uterine leiomyomata: findings from NHANES, 1999-2004. Environ. Health Perspect. 118, 825-832.
34. Wiberg, B., Lind, P.M., Lind, L., 2014. Serum levels of monobenzylphthalate (MBzP) is related to carotid atherosclerosis in the elderly. Environ Res 133, 348-352.
35. Wojtowicz, A.K., Sitarz-Glownia, A.M., Szczesna, M., Szychowski, K.A., 2019. The Action of Di-(2-Ethylhexyl) Phthalate (DEHP) in Mouse Cerebral Cells Involves an Impairment in Aryl Hydrocarbon Receptor (AhR) Signaling. Neurotox Res 35, 183-195.
36. Xu, Y., Wang, L., He, J., Bi, Y., Li, M., Wang, T., Wang, L., Jiang, Y., Dai, M., Lu, J., Xu, M., Li, Y., Hu, N., Li, J., Mi, S., Chen, C.S., Li, G., Mu, Y., Zhao, J., Kong, L., Chen, J., Lai, S., Wang, W., Zhao, W., Ning, G., China Noncommunicable Disease Surveillance, G., 2013. Prevalence and control of diabetes in Chinese adults. JAMA 310, 948-959.