



## Research Paper

# Urinary Phthalates and Leukocyte Telomere Length: An Analysis of NHANES 1999–2002



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## ABSTRACT

The International Agency for Research on Cancer classified the di-2-ethylhexyl phthalate (DEHP) as “possibly carcinogenic to humans”. In vitro studies reported that phthalate exposure resulted in induction of several nuclear transcription factors that are activators of telomerase reverse transcriptase (TERT) and telomerase activity of the human telomerase complex. The objective of this study was to determine whether there is an association between urinary phthalate metabolites [mono-ethyl phthalate (MEP), mono-butyl phthalate (MBP), mono-(2-ethyl)-hexyl phthalate (MEHP), and mono-benzyl phthalate (MBzP)] and leukocyte telomere length (LTL) in the adult population of the National Health and Nutrition Examination Survey (NHANES) 1999–2002 (n = 2472). After adjustment for potential confounders, participants in the 3rd and 4th quartiles of urinary MEHP had statistically significantly longer LTL (5.34%, 95% CI: 1.31, 9.53; and 7.14%, 95% CI: 2.94, 11.63; respectively) compared to the lowest quartile, with evidence of a dose–response relationship ( $p$ -trend = 0.01). The association remained when the analyses were stratified by age groups (20–39 years, 40–59 years, and 60 years and older), and sex. Furthermore, MBP and MBzP were associated with higher LTL in older participants. The age independent association between longer LTL and MEHP (a metabolite of DEHP) might suggest a possible role of MEHP as tumor promoter.

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## 1. Introduction

Phthalates are a group of aromatic chemicals containing a phenyl ring with two attached and extended acetate groups. Phthalate esters are plasticizers used worldwide to add flexibility, longevity, and durability to a multitude of medical, industrial, and consumer products (ATSDR, 2001, 2002; Zota et al., 2014). Low molecular weight phthalates, such as di-n-butyl phthalate (DnBP) and diethyl phthalate (DEP), are largely used in personal care products as aerosol delivery agents, solvents for dyes in nail polishes, enteric coatings for time-release medicines, and in food and food packaging (ATSDR, 2001; Zota et al., 2014). High molecular weight phthalates, such as di-2-ethylhexyl phthalate (DEHP) and butylbenzyl phthalate (BBzP), are the primary plasticizers in polyvinyl chloride (PVC) applications (e.g., building materials and toys); furthermore, they are also used in adhesives and food packaging (ATSDR, 2002; Zota et al., 2014). Because phthalates are not covalently bound to these products, they readily leach out and are thus pervasive in the environment. Humans are widely and ubiquitously exposed to them through ingestion, inhalation, and dermal exposures (ATSDR, 2001, 2002).

Phthalates have been associated with effects on the development of reproductive system of male laboratory animals and their toxicity depends on the chemical structure and timing of exposure: for example, perinatal exposure during to phthalates including BBzP, DEHP, but not other, among them DEP, resulted in the altered sexual differentiation of male rats (Gray et al., 2000). Phthalates are considered endocrine disruptors with anti-androgenic and estrogenic effects on reproductive health and development (National Research Council, 2008). Among the different phthalate compounds, DEHP is associated with liver cancer in rodent models by acting through the peroxisome proliferating pathways. Recently the International Agency for Research on Cancer (IARC) classified DEHP as “possibly carcinogenic to humans” (Group 2B) (IARC, 2013).

Several studies reported that exposure to phthalates resulted in induction of *c-myc* expression both in mouse and human cell line (Yao et al., 2011, 2012; Zhang et al., 2014; Hsieh et al., 2012). Yao et al. (2011) reported the activation of *c-myc* in male mice after exposure to mono-(2-ethyl)-hexyl phthalate (MEHP). Activation of *c-myc* has been reported in primary Sertoli cells from adult male mice exposed to MEHP (Zhang et al., 2014). Moreover, MEHP exposure enhanced tumor progression/metastasis in human testicular embryonal carcinoma cells through *c-myc* induction (Yao et al., 2012). DnBP and BBzP induced proliferation and invasiveness of estrogen receptor-negative breast cancer cells through the activation of *c-myc* (Hsieh et al., 2012).

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Activation of the proto-oncogene *c-myc* is associated with cellular growth and proliferation programs (Dang, 2013) and is therefore an important feature of cancer initiation and maintenance (Gabay et al., 2014). Furthermore, *c-myc* induces the expression of telomerase reverse transcriptase (TERT) and telomerase activity, thus delaying telomere attrition (Daniel et al., 2012; Greenberg et al., 1999). Telomeres are repeating hexanucleotide sequences (TTAGGG) that protect chromosomes against chromosomal end–end fusion and non-reciprocal translocations. During normal DNA replication, the enzyme TERT adds the TTAAGG sequence to the chromosomal ends to compensate for the progressive loss of telomeric sequence during every replication to promote chromosomal stability (Aubert and Lansdorp, 2008). The telomeres shorten with each cell division until they reach a markedly short length, inducing replicative senescence, or irreversible cell growth arrest and apoptosis (Finkel et al., 2007).

Several studies indicate that, independently of chronological age, shorter telomere length (TL) is associated with cardiovascular disease (Haycock et al., 2014), diabetes (Zee et al., 2010), and mortality (Weischer et al., 2012). Longer TL should allow for longer cellular survival, increasing the chance of accumulation of genetic mutations, such as those that promote cancer (Noy, 2009). In contrast, excessive telomere loss may lead to genomic instability and promote carcinogenesis (Blasco, 2005).

Until now, the epidemiological evidence for associations between circulating leukocyte telomere length (LTL) and cancer has been inconsistent and this may be attributed to technical methodology (Cunningham et al., 2013) and the effects that specific cancer types may have on LTL (Gu and Wu, 2013). Shorter telomeres are associated with increased risk for several cancers, including bladder, breast, ovarian, kidney, head and neck, esophagus, stomach, and lung cancer (Wentzensen et al., 2011). However, the meta-analysis stratified by study design reported that the increased cancer risk associated with shorter telomeres was mainly driven by case–control studies (Wentzensen et al., 2011). This finding suggests the possible effects of reverse causation in case–control studies where therapeutic procedures or cancer itself may affect TL. In prospective studies, longer telomeres have been associated with an increased risk of several cancers such as lung cancer (Lan et al., 2013; Seow et al., 2014), melanoma (Han et al., 2009), non-Hodgkin lymphoma (Lan et al., 2009), pancreatic cancer (Lynch et al., 2013), and prostate cancer (Julin et al., 2015). Interestingly, in a 12 years follow-up of 792 normative aging study participants, it was observed a decelerating age-adjusted LTL attrition in cancer cases as they approached diagnosis with significant longer LTL within 4 years pre-diagnosis (Hou et al., 2015). This observation suggests that LTL elongation appears early during cancer development (Hou et al., 2015).

Leukocyte telomere length has been measured in a representative sample of US adults (20 years of age and older) who participated in the National Health and Nutrition Examination Survey (NHANES) from 1999 to 2002. In this study, we examined the potential association of various metabolite compounds of phthalates with LTL. Because of the role of phthalates to induce *c-myc* and promote cellular growth, we hypothesized that phthalate exposure will be associated with longer LTL.

## 2. Methods

### 2.1. Study Population

NHANES is a cross-sectional, nationally representative survey of the non-institutionalized civilian population of the United States conducted by the National Center for Health Statistics, Centers for Disease Control and Prevention (NCHS, CDC) (Johnson et al., 2013). For our study we merged the publicly available files for NHANES cycles 1999–2000 and 2001–2002 using the NCHS recommendations (Johnson et al., 2013). The survey employs a multistage stratified probability sample based on selected counties, blocks, households, and persons within households. NCHS-trained professionals conducted interviews in participants'

homes and extensive physical examinations including blood and urine collection were conducted at mobile exam centers (MECs). All procedures were approved by the NCHS Research Ethics Review Board (Protocol #98-12, <http://www.cdc.gov/nchs/nhanes/irba98.htm>), and all participants provided written informed consent.

### 2.2. Leukocyte Telomere Length (LTL) Measurements

Whole blood DNAs were purified using the Puregene kit protocol (Genra Systems, Inc., Minneapolis, Minnesota) by NCHS. The telomere length assay was performed in the laboratory of Dr. Elizabeth Blackburn at the University of California, San Francisco, using the quantitative polymerase chain reaction method. Briefly, the method measures the ratio of telomere length (T) relative to standard (S) single-copy gene reference DNA, known as the T/S ratio (Cawthon, 2002; Lin et al., 2010). Primers used for amplification of the human telomere (T), *tel1b* and *tel2b*, and for amplification of the standard reference gene (S) human  $\beta$  globin, *hbg1* and *hbg2*, and PCR conditions are described in Lin et al. (2010). Samples were assayed 3 times on 3 different days on duplicate wells, resulting in 6 data points. Control DNA values were used to normalize between-run variability. Runs with more than 4 control DNA values falling outside 2.5 standard deviations from the mean for all assay runs were excluded from further analysis (<6% of runs). Outliers identified for each sample were excluded from the calculations (<2% of samples). Quality control review was conducted by the CDC before linking the LTL data to the NHANES public-use data files. The formula  $3274 + 2413 * (T/S)$  was used to convert T/S ratio to base pairs (bps). The conversion from T/S ratio to bp is calculated based on comparison of telomeric restriction fragment (TRF) length from Southern blot analysis and T/S ratios using DNA samples from the human diploid fibroblast cell line IMR90 at different population doublings ([http://www.cdc.gov/Nchs/Nhanes/2001-2002/TELO\\_B.htm](http://www.cdc.gov/Nchs/Nhanes/2001-2002/TELO_B.htm)).

### 2.3. Urinary Biomarkers

Spot urine samples were collected from study participants and stored at  $-20^{\circ}\text{C}$  for a maximum of one year until analysis was performed by the Division of Laboratory Sciences, National Center for Environmental Health, CDC. In NHANES 1999–2002 seven phthalate metabolites in the urine samples were analyzed: 1) mono-ethyl phthalate (MEP), a metabolite of DEP; 2) mono-(2-ethyl)-hexyl phthalate (MEHP), a metabolite of DEHP; 3) mono-benzyl phthalate (MBzP), a metabolite of BBzP; 4) mono-cyclohexyl phthalate (MCHP), a metabolite of dicyclohexyl phthalate (DCHP); 5) mono-isononyl phthalate (MiNP), a metabolite of di-isononyl phthalate (DiNP); 6) mono-n-octyl phthalate (MOP), a metabolite of di-n-octyl phthalate (DnOP); and mono-butyl phthalate (MBP), which represents the sum of two isomers, mono-isobutyl phthalate and mono-n-butyl phthalate. To avoid bias in estimation among those below the limit of detection (LOD), only the phthalate metabolites that were detected in at least 75% of the samples, such as MEP ( $\geq\text{LOD} = 99\%$ ), MBP ( $\geq\text{LOD} = 99\%$ ), MBzP ( $\geq\text{LOD} = 96\%$ ) and MEHP ( $\geq\text{LOD} = 78\%$ ), were used in our analyses. These compounds were measured by solid phase extraction coupled on-line to high performance liquid chromatography and tandem mass spectrometry. Details of detection and measurement of the urinary compounds are described in the NHANES laboratory method ([http://www.cdc.gov/nchs/data/nhanes/nhanes\\_99\\_00/PHPYPA\\_met\\_phthalates.pdf](http://www.cdc.gov/nchs/data/nhanes/nhanes_99_00/PHPYPA_met_phthalates.pdf), and [http://www.cdc.gov/nchs/data/nhanes/nhanes\\_01\\_02/PHPYPA\\_b\\_met\\_phthalates.pdf](http://www.cdc.gov/nchs/data/nhanes/nhanes_01_02/PHPYPA_b_met_phthalates.pdf)). The reported results for all assays meet the NCEH/DLS quality control and quality assurance performance criteria for accuracy and precision. Urinary concentrations of the phthalates below the level of detection were assigned the limit of detection divided by the square root of two, as recommended by NHANES (Johnson et al., 2013). To account for variation in dilution in spot urinary samples, urinary creatinine was entered into the analyses as an independent variable as suggested by previous studies (Ikeda et al., 2003;

Barr et al., 2005). Urinary creatinine was determined using a Jaffe rate reaction measured with a CX3 analyzer and was entered into the model as a log-natural transformed variable.

#### 2.4. Statistical Analysis

LTL was not normally distributed, thus it was natural log-transformed. Analyses were performed using the weights from the urinary phthalate subsamples as recommended by NCHS. SAS-Callable SUDAAN 10 (Research Triangle Institute, Research Triangle Park, NC) was used to account for the NHANES complex sample design. All tests were two sided, and  $p \leq 0.05$  was the level of significance. Urinary phthalate metabolites were analyzed via quartiles. Quartile cutoffs of the phthalate compounds were based on the weighted distributions of MEP, MBP, MBzP and MEHP in the study population. We ran three models: model 1 was adjusted for urinary creatinine; model 2 was further adjusted for demographic and socio behavioral variables, such as age (continuous), age squared, sex, race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican American, and other), education (less than high-school, high school graduate, some college, and above), alcohol consumption, self-reported smoking status (current, former, or never smoker), serum cotinine (natural log-transformed); model 3 was further adjusted for confounding factors such as body weight status (underweight/normal, overweight and obese), self-reported diabetes, hypertension, self-reported cardiovascular disease (defined as an answer of yes to any of coronary artery disease, angina pectoris, heart attack, stroke, or congestive heart failure on the medical questionnaire) and c-reactive protein, a biomarker for inflammation, since inflammation is associated with telomere length (Rode et al., 2014), as well as phthalate (Ferguson et al., 2011). Further, we performed analyses by age-group stratification (age 20–39 years, age 40–59 years, and age 60 and older). We, also, performed analyses stratified for gender and smoking status. To avoid information bias due to self-reported cigarette smoking, we used both self-reported cigarette use and serum cotinine cutoff to define smoking status (Pirkle et al., 2006): smokers included self-reported current smokers and those with serum cotinine levels  $>10$  ng/mL, and non-smokers included self-reported former and never smokers and those with serum cotinine levels  $\leq 10$  ng/mL.

A sensitivity analysis was conducted using additional adjustment for serum gamma glutamyl-transferase (GGT) a biomarker of oxidative stress which has been associated with phthalate exposure (Ferguson et al., 2011). Oxidative stress has been also associated with telomere length (von Zglinicki, 2002). In our analyses we did not exclude participants with self-reported diagnosis of cancer. However, analyses including, also, as independent variable the self-report diagnosis of cancer (obtained from the medical questionnaire), did not change the estimated phthalate parameters. Since our dependent variable LTL was log-transformed, the results were re-transformed by exponentiation of the  $\beta$  coefficients and presented as percent differences estimated by comparing each of the upper three quartiles to the lowest quartile using the formula  $100 * (e^{\beta} - 1)$ ; statistical tests for linear trends were conducted by modeling quartiles as an ordinal variable using integer values. All models were also run with urinary phthalate metabolites entered as natural log-transformed continuous variables. Moreover, to further characterize the shape of the relationship between MEHP and LTL we used urinary MEHP as restricted cubic spline. We used a modified SAS macro written by Desquilbet and Mariotti (2010) to account for NHANES weight and sample design and the knots used for restricted cubic spline were placed at the 5th, 35th, 65th and 95th percentile as recommended by Harrell (2010).

### 3. Results

The weighted distributions of study population ( $n = 2472$ ) characteristics of the total sample are shown in Table 1. Briefly, the geometric

**Table 1**

Sample size and weighted characteristics of the NHANES 1999–2002 participants 20 years and older.

	ALL	
	n.	Weighted distribution
	2472	
Mono-ethyl phthalate (MEP), (ng/mL), GM (SE)	2467	123.77 (6.14)
Mono-butyl phthalate (MBP), (ng/mL), GM (SE)	2472	20.85 (0.82)
Mono-benzyl phthalate (MBzP), (ng/mL), GM (SE)	2472	8.86 (0.36)
Mono-(2-ethyl)-hexyl phthalate (MEHP), (ng/mL), GM (SE)	2472	3.59 (0.17)
Age (years), GM (SE)	2472	42.83 (0.41)
BMI (kg/m <sup>2</sup> ), GM (SE)	2472	27.53 (0.16)
Serum cotinine (ng/mL), GM (SE)	2472	3.59 (0.54)
C-reactive protein(mg/dL), GM (SE)	2472	0.20 (0.01)
Leukocyte telomere length (T/S ratio), GM (SE)	2472	1.02 (0.02)
<i>Gender</i>		
Men	1192	48.07 (1.29)
Women	1280	51.93 (1.29)
<i>Body weight</i>		
Underweight/normal weight (BMI < 25), % (SE)	767	34.10 (1.29)
Overweight (BMI = 25–<30), % (SE)	879	33.00 (1.24)
Obese, (BMI $\geq 30$ ), % (SE)	826	32.89 (1.29)
<i>Smoking status</i>		
Current Smoker, % (SE)	528	23.72 (1.68)
Former Smoker, % (SE)	643	24.83 (1.15)
Never Smoked, % (SE)	1301	51.45 (1.99)
<i>Alcohol consumption</i>		
No alcohol, % (SE)	877	30.54 (2.30)
1–5 drinks per day, % (SE)	1411	62.15 (2.40)
>5 drinks per day, % (SE)	184	7.31 (0.69)
<i>Education Level</i>		
Less than high school % (SE)	810	20.67 (1.51)
Completed high school % (SE)	562	25.63 (1.51)
More than high school % (SE)	1100	53.71 (1.90)
<i>Race/Ethnicity</i>		
White (non-Hispanic), % (SE)	1273	73.21 (1.85)
Non-Hispanic black, % (SE)	406	9.10 (7.09)
Mexican–American, % (SE)	592	7.20 (0.90)
Other % (SE)	201	10.49 (1.86)
<i>Self-reported diabetes</i>		
No, % (SE)	2264	93.63 (0.49)
Yes, % (SE)	208	6.37 (0.49)
<i>Hypertension</i>		
No, % (SE)	1620	71.86 (1.03)
Yes, % (SE)	796	28.14 (1.03)
<i>Self-reported CDV diseases</i>		
No, % (SE)	2245	92.65 (0.60)
Yes, % (SE)	227	7.35 (0.60)

mean of LTL was 1.02. Women represented approximately 52% of the sample; the geometric mean age of the participants was approximately 43 years old. Obesity prevalence was almost 33%. The prevalence of former smokers and people who had never smoked was approximately 25% and 51%, respectively, and almost 31% of the participants had never consumed alcohol. The geometric mean (GM) of urinary MEP, MBP, MBzP and MEHP was 123.77 ng/mL, 20.85 ng/mL, 8.86 ng/mL and 3.59 ng/mL, respectively, in the study population.

Table 2 shows the results of the multivariable linear regression. Briefly, in crude analyses (adjusting only for urinary creatinine) individuals in the 3rd and 4th quartiles of urinary MEHP had statistically significantly longer LTL compared to the lowest referent quartile (Table 2, Model 1) with evidence of a dose–response relationship ( $p$ -value for trend  $<0.001$ ). Further adjustments for demographics (among them age) and socio-behavioral variables (Table 2, Model 2) and for body weight status

**Table 2**

Percent differences (95% CI) in leukocyte telomere length (T/S ratio) by phthalate exposure, National Health and Nutrition Examination Survey, 1999–2002.

	Model 1	Model 2	Model 3
Mono-ethyl phthalate (MEP)	2467	2467	2449
Q1: ≤42.11 ng/mL	Referent	Referent	Referent
Q2: 42.12–116.10 ng/mL	3.67 (–1.98, 9.53)	2.53 (–1.49, 6.72)	2.43 (–1.39, 6.29)
Q3: 116.11–318.45 ng/mL	4.19 (–1.49, 10.30)	2.02 (–2.96, 7.14)	2.02 (–2.76, 7.04)
Q4: >318.45 ng/mL	1.51 (–4.69, 8.11)	1.51 (–4.02, 7.36)	1.82 (–3.63, 7.57)
<i>p</i> trend	0.20	0.58	0.63
Mono-butyl phthalate (MBP)	2472	2472	2454
Q1: ≤10.40 ng/mL	Referent	Referent	Referent
Q2: 10.41–22.24 ng/mL	–2.27 (–6.85, 2.63)	–0.20 (–4.40, 4.19)	–0.30 (–4.40, 3.98)
Q3: 22.25–42.83 ng/mL	–2.47 (–6.57, 1.92)	–0.70 (–4.40, 3.15)	–1.00 (–4.40, 2.53)
Q4: >42.83 ng/mL	–1.00 (–5.82, 4.08)	0.20 (–4.11, 4.81)	–0.10 (–4.30, 4.19)
<i>p</i> trend	0.46	0.93	0.93
Mono-benzyl phthalate (MBzP)	2472	2472	2454
Q1: ≤4.10 ng/mL	Referent	Referent	Referent
Q2: 4.11–9.58 ng/mL	2.02 (–3.34, 7.68)	1.82 (–2.66, 6.61)	2.53 (–2.27, 7.68)
Q3: 9.58–20.51 ng/mL	0.00 (–5.45, 5.87)	–0.10 (–5.26, 5.23)	–0.05 (–5.07, 5.34)
Q4: >20.51 ng/mL	2.63 (–2.57, 8.11)	1.41 (–3.34, 6.40)	1.41 (–3.34, 6.29)
<i>p</i> trend	0.21	0.28	0.25
Mono-(2-ethyl)-hexyl phthalate (MEHP)	2472	2472	2454
Q1: ≤1.20 ng/mL	Referent	Referent	Referent
Q2: 1.21–3.44 ng/mL	2.84 (–1.29, 7.14)	2.74 (–0.50, 6.18)	2.74 (–0.50, 6.18)
Q3: 3.44–8.04 ng/mL	7.79 (4.08, 11.74)	5.44 (1.51, 9.64)	5.34 (1.31, 9.53)
Q4: >8.04 ng/mL	10.85 (6.72, 15.03)	7.47 (3.46, 11.63)	7.14 (2.94, 11.63)
<i>p</i> trend	<0.001	0.01	0.01

Model 1 = adjusted for urine creatinine; Model 2 = as Model 1 plus adjusted for sex, age (years, continuous), age squared, education (less than high school, high school graduate, some college and above), race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican American, other), alcohol consumption, self-reported smoking status (current, former, or never smoker), serum cotinine (log natural log-transformed). Model 3 = Model 2 plus adjusted for body weight status (underweight/normal, overweight, obese), c-reactive protein (natural log-transformed), hypertension, self-reported diabetes, and self-reported CDV diseases.

and c-reactive protein (Table 2, Model 3) showed decreased parameter estimates for MEHP compared to Model 1, likely driven by the role of aging in telomere attrition and the other covariates such as body weight, but the statistical significant association of MEHP with longer LTL remained. There were no statistically significant associations of the phthalate metabolites MEP, MBP and MBzP with LTL (Table 2).

In complementary analyses, using urinary phthalate metabolites as natural log-transformed continuous variable of the association of increased urinary MEHP level with longer LTL was confirmed (Table 3). With each one-unit of natural log-transformed MEHP unit, there was a 1.71% increase in LTL (Table 3, Model 3). Furthermore, analyses using restricted cubic spline confirmed the dose-relationship between MEHP and LTL (Supplement Fig. 1).

Analyses stratified by age group indicated that the statistically significant association of LTL with MEHP was found in young adults (20–39 years), middle aged (40–59 years) and older adults groups. In the young adult group there was statistically higher LTL percentage change in the 2nd, 3rd and 4th MEHP quartiles compared to the lowest MEHP quartile, with a dose–response trend (Table 4). Both participants in the 4th MEHP quartile were statistically significant associated with higher LTL compared to the referent lowest MEHP quartile, but the dose–response trend was found only in the young adults (Table 4). Moreover, there were positive statistically significant association of the 4th MBP quartile and MBzP quartile compared to their respective lowest referent quartile in the older age group (Table 4). Analyses stratified by sex or by tobacco smoking use, confirmed the association of MEHP with longer

LTL in both sexes and in smokers and non-smokers. (Supplemental Table 1). Sensitivity analyses including GGT yielded results similar to those from the primary analyses (data not shown).

#### 4. Discussion

Several phthalates have been associated with cell proliferation and cancer. In this study we confirmed the hypothesis that phthalate may be positively associated with LTL. The association of MEHP and LTL remained significant after inclusion of several confounder variables. Sensitivity analyses with the inclusion of the information of GGT (data not shown) provided further evidence of the non-spuriousness of the association. The statistical significant positive association, independently from age, remained also when the analyses were stratified among age groups (20–39 years old, 40–59 years and 60 years and older), by sex and smoking. Moreover, a positive association was found in the older age group between MBP and MBzP with longer LTL. Based on the result of the multivariate analyses of this cohort, participants in the third and fourth quartile of MEHP had, on average, longer telomere length (129 and 172 base pair, respectively) compared to those in the lowest quartile, whereas telomere erosion by age leads on average to a loss of 16 bps per year.

Telomeric DNA is lost with each cell division until replicative senescence is reached (Harley et al., 1990; Chiu and Harley, 1997). In the presence of telomerase, telomere length is maintained allowing the cells to escape replicative senescence (Liu et al., 2004). Human *TERT*

**Table 3**

Percent difference (95% CI) in leukocyte telomere length (T/S ratio) by phthalate metabolite exposure, National Health and Nutrition Examination Survey, 1999–2002 by gender and age.

	Model 1	Model 2	Model 3
LN-mono-ethyl phthalate (MEP)	–0.90 (–2.86, 1.11)	–0.30 (–1.98, 1.31)	0.50 (–0.60, 1.71)
LN mono-n-butyl phthalate (MBP)	–1.19 (–3.05, 0.70)	–0.60 (–2.18, 1.11)	–0.10 (–4.30, 4.19)
LN mono-benzyl phthalate (MBzP)	0.70 (–0.50, 1.92)	0.30 (–0.90, 1.51)	0.30 (–1.00, 1.61)
LN mono-(2-ethyl)-hexyl phthalate (MEHP)	2.94 (1.82, 4.08)	1.82 (0.70, 2.94)	1.71 (0.60, 2.94)

Model 1 = adjusted for urine creatinine; Model 2 = as Model 1 plus adjusted for sex, age (years, continuous), age squared, education (less than high school, high school graduate, some college and above), race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican American, other), alcohol consumption, self-reported smoking status (current, former, or never smoker), serum cotinine (natural log transformed). Model 3 = Model 2 plus adjusted for body weight status (underweight/normal, overweight, obese), c-reactive protein (natural log-transformed), hypertension, self-reported diabetes, and self-reported CDV diseases.



**Table 4**

Percent differences (95%CI) in leukocyte telomere length (T/S ratio) by phthalate exposure, National Health and Nutrition Examination Survey, 1999–2002 by age.

	Age 20–39 years	Age 40–59 years	Age ≥ 60 years
Mono-ethyl phthalate (MEP)	n = 890	n = 747	n = 774
Q1: ≤42.11 ng/mL	Referent	Referent	Referent
Q2: 42.12–116.10 ng/mL	–2.18 (–7.96, 3.98)	5.34 (–0.90, 12.08)	3.87 (0.90, 6.82)
Q3: 116.11–318.45 ng/mL	–2.96 (–8.24, 2.74)	3.98 (–1.98, 10.41)	5.44 (–0.80, 11.96)
Q4: >318.45 ng/mL	–3.15 (–10.33, 4.71)	3.77 (–3.44, 11.52)	5.65 (–0.20, 11.85)
p trend	0.76	0.26	0.03
Mono-butyl phthalate (MBP)	n = 890	n = 747	n = 779
Q1: ≤10.40 ng/mL	Referent	Referent	Referent
Q2: 10.41–22.24 ng/mL	1.51 (–3.92, 7.14)	–3.34 (–9.88, 3.77)	1.41 (–4.21, 7.36)
Q3: 22.25–42.83 ng/mL	2.94 (–8.79, 3.56)	–1.29 (–6.95, 4.60)	3.56 (–2.96, 10.52)
Q4: >42.83 ng/mL	–3.82 (–10.51, 3.36)	–0.40 (–7.78, 7.68)	8.00 (1.51, 14.91)
p trend	0.11	0.71	0.046
Mono-benzyl phthalate (MBzP)	n = 890	n = 747	n = 779
Q1: ≤4.10 ng/mL	Referent	Referent	Referent
Q2: 4.11–9.58 ng/mL	6.18 (–9.61, 12.64)	3.15 (–1.98, 8.55)	3.15 (–2.76, 9.31)
Q3: 9.58–20.51 ng/mL	–0.80 (–9.43, 8.65)	–1.78 (–7.78, 4.50)	1.61 (–5.07, 8.76)
Q4: >20.51 ng/mL	–0.40 (10.52, 9.64)	–0.30 (–6.48, 6.29)	7.36 (0.80, 14.34)
p trend	0.93	0.24	0.07
Mono-(2-ethyl)-hexyl phthalate (MEHP)	n = 890	n = 747	n = 779
Q1: ≤1.20 ng/mL	Referent	Referent	Referent
Q2: 1.21–3.44 ng/mL	5.97 (1.71, 10.30)	1.41 (–4.21, 7.36)	0.20 (–3.92, 4.50)
Q3: 3.44–8.04 ng/mL	8.00 (2.02, 14.34)	3.25 (–4.11, 11.07)	3.56 (–2.47, 9.97)
Q4: >8.04 ng/mL	7.68 (2.84, 12.86)	7.36 (0.05, 15.14)	7.68 (0.05, 15.37)
p trend	<0.01	0.08	0.16

Adjusted for urine creatinine, sex, age (years, continuous), education (less than high school, high school graduate, some college and above), race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican American, other), alcohol consumption, self-reported smoking status (current, former, or never smoker), serum cotinine (natural log-transformed), body weight status (underweight/normal, overweight, obese) c-reactive protein (natural log-transformed), hypertension, self-reported diabetes, and self-reported CDV diseases.

(*hTERT*) promoter is the most important regulatory element of telomerase expression and contains numerous binding sites for a variety of transcription factors, including both activators and repressors of *hTERT* (Liu et al., 2004). There are several potential underlying mechanisms that may explain the positive association of MEHP and telomere length through the induction of several transcription factors that act as regulators of *hTERT*.

Previous research has shown that *c-myc* acts as a key regulator of *hTERT* transcription during carcinogenesis via its binding to the E-box (Enhancer Box) and subsequent activation of transcription (Kyo et al., 2008). Therefore, one underlying mechanism for MEHP contribution to telomere length maintenance may be through induction of *c-myc* expression. Yao et al. (2011) using peripubertal (21-day-old) male wild-type C57BL/6J mice exposed to MEHP reported an activation of *c-myc*. Moreover, induction of *c-myc* was, also, reported when primary co-cultures of rat Sertoli cells and rat germ cells were exposed to MEHP (Yao et al., 2011). In a subsequent in vitro study, the same group reported that MEHP exposure enhanced tumor progression/metastasis in human testicular embryonal carcinoma cells (NT2/D1) by induction of *c-myc* (Yao et al., 2012). Activation of *c-myc* after exposure of primary Sertoli cells from adult male mice to MEHP was also reported by Zhang et al. (2014). Similarly, the underlying mechanism for MBP and MBzP contribution to telomere maintenance in the older adults may also be through activation of *c-myc*. Hsieh et al. (2012) reported that dibutyl phthalate (the compound parent of the MBP metabolites) and n-butyl benzyl phthalate (the compound parent of the metabolite MBzP) induce proliferation and invasiveness of estrogen receptor-negative breast cancer cells through activation of *c-myc* (Hsieh et al., 2012).

Another underlying mechanism for MEHP contribution to telomere length maintenance may be through the activation of the P13K/Akt pathways. The P13K/Akt pathway has a central role in cellular immortalization by up-regulating *hTERT* expression and/or by restraining the inactivation of *hTERT* expression (Daniel et al., 2012). Low concentrations of DEHP induce a proliferative effect on human MCF-7 breast cancer cells through activation of the P13K/Akt signaling pathway (Chen and Chien, 2014). Cell proliferation and activation of the P13K/Akt pathway, along with increased expression of AKT, were reported

after exposure of human neuroblastoma cells to DEHP (Zhu et al., 2010; Zheng et al., 2013). Up-regulation of the transcriptional level of P13K and AKT, as well as production of AKT proteins, was reported after exposure of human hepatocellular carcinoma cell lines (Hep3B) to DEHP (Chen et al., 2013). P13kinase/Akt pathway activation during MEHP exposure has been also reported in partially differentiated mouse macrophage cell lines (Bolling et al., 2012).

Based on molecular evidence from several animal cancer models, primarily liver and testis, the International Agency for Research on Cancer (IARC) classified DEHP as “possibly carcinogenic to humans” (Group 2B) in 2012 (IARC, 2013). Also, the National Toxicology Program (NTP, 2014) list DEHP as “reasonably anticipated to be a human carcinogen.” The other phthalate compound that IARC evaluated for carcinogenicity is the BBzP and the IARC working group concluded that BBzP “is not classifiable as to its carcinogenicity to humans (Group 3)” (<http://monographs.iarc.fr/ENG/Monographs/vol73/mono73-9.pdf>).

The role of DEHP in cancer development is based upon animal models, particularly rats. However, the relevance of current animal models to human is questionable since the peroxisome proliferating pathways strongly affected by phthalate in rats may not be relevant in humans (Rusyn and Corton, 2012). Epidemiological studies evaluating exposure to phthalates and cancer are limited. In an age-matched case–control study of breast cancer in women (cases = 233, controls = 221), Lopez-Carrillo et al. (2010) reported a positive association of MEP and an inverse association of MBzP with breast cancer risk. The authors, after adjusting for risk factors and other phthalates, also reported increased odd ratios for breast cancer with urinary concentrations of four DEHP metabolites: MEHP, MEHHP, MEOHP, and MECPP; however, the increased risk was only statistically significant for MECPP (Lopez-Carrillo et al., 2010). In a case–control study of Alaskan native women (75 cases, 95 controls) urinary MEHP was associated with breast cancer (Holmes et al., 2014).

Because of the ability of DEHP and MEHP to induce the expression of *c-myc* and P13K/Akt pathways in human cell lines and the role of these in telomerase maintenances, our findings may be suggestive of a possible action of MEHP as tumor promoter. However, these findings need to be taken with caution since there are several important limitations in the study. Ferguson et al. (2011) reported a positive association

between urinary phthalate metabolites and serum biomarker of inflammation and oxidative stress (CRP and GGT, respectively). MEHP was associated with an increase in GGT, whereas MBZP and MBP were associated with increased CRP (Ferguson et al., 2011). Both oxidative stress and inflammation are associated with shorter LTL (Rode et al., 2014; Jennings et al., 2000). In our analyses, the use of GGT in our models did not affect the statistical significance of our findings (data not shown). The cross-sectional nature of the study limits the inferences that can be made based on the results. A major limitation is the use of single-spot urine measures as an estimate of exposure. Phthalates are rapidly metabolized and excreted and a single exposure measurement may not reflect long-term exposure; however, Hauser et al. (2004) found that a single urine sample may moderately predict the average intra-individual exposure over 3 months exposure with sensitivities ranging from 0.56 to 0.74. Although the strength of our study is that it is based on a nationally representative survey, there could also be other environmental toxicants, since people are exposed to a wide-range of chemicals, that may have had a confounding effect on the associations we observed. Recently, an association of persistent organic pollutants (POPs) with longer LTL using a different subset of NHANES 1999–2004 dataset has been reported (Scinicariello and Buser, 2015; Mitro et al., 2015). Conversely, a study conducted using NHANES 1999–2004 data reported an association of blood cadmium and urinary cadmium with short LTL (Zota et al., 2015).

In conclusion, we found an age-independent association between urinary MEHP and longer LTL after adjusting for several important potential confounders. The finding may be suggestive of the role of MEHP as tumor promoter and further studies to evaluate the effect of MEHP, as well as other phthalate metabolites, on LTL are needed to fully understand the implications of the findings of this study.

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None.

#### Conflict of Interest

The authors report no conflict of interest.

#### Author Contributions

Study concept and design by FS; data acquisition by FS; data analysis by FS and AGF; data interpretation by FS, AGF, RA; manuscript drafting by FS; critical revision of the manuscript for important intellectual content by FS, AGF and RA; study supervision by FS.

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IRB approval: CDC/ATSDR has determined that our research did not meet the criteria for human research as per federal regulation and therefore did not require review.

#### Appendix A. Supplementary Data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2016.02.027>.

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