



# BPA exposure is associated with non-monotonic alteration in *ESR1* promoter methylation in peripheral blood of men and shorter relative telomere length in peripheral blood of women

Z. Awada<sup>1</sup> · F. Sleiman<sup>1</sup> · A. Mailhac<sup>2</sup> · Y. Mouneimne<sup>3</sup> · H. Tamim<sup>2</sup> · N. K. Zgheib<sup>1</sup>

Received: 31 July 2017 / Revised: 1 December 2017 / Accepted: 29 December 2017 / Published online: 12 April 2018  
© Nature America, Inc., part of Springer Nature 2018

## Abstract

The aim of this study was to evaluate the potential association of urinary Bisphenol A (BPA) levels with estrogen receptor alpha (*ESR1*) promoter % methylation and relative telomere length in a sample of 482 participants. Urinary BPA concentration was measured using organic phase extraction followed by high performance liquid chromatography mass spectroscopy. Peripheral blood *ESR1* promoter % methylation and relative telomere length were measured using direct bisulfite sequencing and real-time polymerase chain reaction, respectively. The mean  $\pm$  SD urinary BPA concentration adjusted for urinary creatinine was  $2.90 \pm 4.81$  ( $\mu\text{g/g}$  creatinine) with a median of  $1.86 \mu\text{g/g}$  creatinine (min–max: <LOD–69.85). There was a potentially non-monotonic relationship between adjusted urinary BPA concentrations and *ESR1* promoter % methylation in men. As a matter of fact, for the lowest tertile of *ESR1* promoter % methylation, the OR and 95% CI of the middle and highest tertiles of urinary adjusted BPA were 2.54 (1.01–6.39) and 1.64 (0.55–4.86) when compared to the lowest BPA tertile, respectively. After adjustment for potential confounders, similar results remained in men and appeared in the whole cohort. As for relative telomere length, there was a significant trend whereby higher adjusted urinary BPA concentrations were significantly associated with shorter relative telomere length in females. For instance, for the shortest relative telomere length tertile, the OR and 95% CI of the middle and highest tertiles of urinary adjusted BPA were 2.91 (1.38–6.16) and 3.19 (1.57–6.49) when compared to the lowest BPA tertile, respectively. This trend remained significant after adjustment for potential confounders.

**Keywords** Bisphenol A · Estrogen receptor alpha · relative telomere length

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1038/s41370-018-0030-4>) contains supplementary material, which is available to authorized users.

✉ H. Tamim  
hani\_t@hotmail.com  
htamim@aub.edu.lb

✉ N. K. Zgheib  
nk16@aub.edu.lb

<sup>1</sup> Department of Pharmacology and Toxicology, Faculty of Medicine, American University of Beirut, Beirut, Lebanon

<sup>2</sup> Clinical Research Institute, Faculty of Medicine, American University of Beirut Medical Center, Beirut, Lebanon

<sup>3</sup> Kamal A. Shair Central Research Science Laboratory, Faculty of Arts and Sciences, American University of Beirut, Beirut, Lebanon

## Introduction

Bisphenol A (BPA) is a synthetic estrogen-like compound that is used in the production of polycarbonates and epoxy resins. BPA is available in a wide variety of consumer products, such as can packaging, bottles, dental fillings, and thermal receipt paper [1]. BPA exposure has been linked to several health hazards such as cardiovascular diseases, endocrine disturbances, developmental problems, and carcinogenesis [2–5]. Large biomonitoring studies reported that greater than 90% of the US [6] and Canadian participants [7] had detectable urinary BPA concentrations of  $\geq 0.1 \mu\text{g/l}$ . Besides, BPA and its conjugates were detected in urine samples of different populations in Europe and Southeast Asia [8–12]. A recent study in Lebanon showed that mean urinary BPA level  $\pm$  SD in adults residing in Greater Beirut area was  $3.67 \pm 4.75 \mu\text{g/l}$  [13], which was consistent with levels reported in other populations [12, 14–16].

BPA is believed to be a weak estrogen as its binding affinity to the classical estrogen receptor alpha (ESR1) is approximately 1000–10,000 folds less than estradiol [17]. However, this low affinity does not translate into negligible biological activity, since more recent studies observed that BPA could induce estrogen-like effects that are equal or even stronger than that of estradiol [18]. BPA was associated with ESR1 overexpression [19], which may be mediated through a decrease in *ESR1* promoter methylation. Few human studies reported that high BPA levels were associated with decrease in global DNA methylation [20, 21], but the association between BPA and *ESR1* promoter methylation has not been investigated yet.

Human telomere reverse transcriptase (*hTERT*), a gene encoding the catalytic subunit of telomerase enzyme that is involved in elongating telomeres, contains an estrogen response element (ERE) which is enhanced by the activated ESR1 [22–24]. One in vitro study showed that BPA increases *hTERT* expression in HeLa (human cervical carcinoma), H1299 (human lung carcinoma), and MCF-7 (human breast cancer) cell lines [25]. However, the association between BPA and relative telomere length has not been investigated yet. Noteworthy, an increase in telomerase expression was not always associated with increase in relative telomere length. Although telomerase overexpression has been observed in 80–95% of many cancer types [26, 27], short telomeres were associated with some cancers such as colon, prostate, and ovarian cancers and chronic lymphoblastic leukemia [28, 29], whereas long telomeres were associated with others, such as melanoma and hepatic cancer [28].

The aim of this study was to evaluate the potential association of urinary BPA levels with *ESR1* promoter % methylation and relative telomere length in peripheral blood. We hypothesized that since BPA has been shown to be associated with increase in ESR1 expression [19], this may be potentially through decreasing *ESR1* promoter % methylation, which may increase *hTERT* expression and alter relative telomere length. We hence expect that high BPA levels are associated with a decrease in *ESR1* promoter % methylation and a change in relative telomere length.

## Methods

### Participants and measurements

This study utilized data from an available database of a cross-sectional community-based study of a representative sample of 501 Lebanese adults (age > 18 years) residing in Greater Beirut that were recruited between February and June 2014. Exclusion criteria included pregnancy, dialysis, mental disability, and employment in a plastic or any other

chemical company. The original and current studies were approved by the Institutional Review Board (IRB) of the American University of Beirut. Data collection entailed a face-to face interview with anthropometric measurements and blood and urine withdrawal. See Mouneimne et al. [13] and Zgheib et al. [30] for details.

First morning spot urine samples were collected in glass jars and stored at  $-20^{\circ}\text{C}$  until analysis. Urinary BPA concentration was measured by organic phase extraction followed by high performance liquid chromatography mass spectroscopy as we recently presented [13]. Sample extracts were run on a 1100 LC/MSD TrapXCT instrument with electrospray ionization and autosampler from Agilent (Santa Clara, CA). Separation by chromatography was performed with the C18 column using a buffered acetonitrile mobile phase. The extracted ion chromatographs of BPA and internal standard 2,2-bis-(4-hydroxyphenyl) butane (BPB) were analyzed by the LC/MSD trap software 5.2 (Agilent, Santa Clara, CA) at 227 *m/z* and 241 *m/z* respectively. Blood and urinary creatinine concentrations were measured by the Jaffe rate method (Cobas 6000, Roche). To adjust for urine volume, the BPA concentration was divided by urinary creatinine ( $\mu\text{g/g}$ ).

Whole blood was drawn into an EDTA tube and stored at  $-80^{\circ}\text{C}$  for future DNA isolation. Total DNA was extracted using a Qiagen kit (Qiagen, USA) as per manufacturer guidelines and stored at  $-20^{\circ}\text{C}$  until analysis. Measurement of *ESR1* promoter % methylation at 5 CpG sites in a CpG island that starts in the promoter, 91 bp upstream of the transcription starting site, and extends into exon 1 was performed using direct bisulfite sequencing as we previously detailed [31]. The five sites lie between the 152,128,305 and 152,128,536 genomic coordinates of the *ESR1* NC\_000006.11 reference sequence. Raw sequence data were visualized using Finch TV v1.4.0 software and analysed using epigenetic sequencing methylation (ESME) 3.2.5 software. Peripheral leucocytes relative telomere length measurement was carried out using a quantitative real-time polymerase reaction as we previously described [30]. Samples were run in triplicates along with standards. Two randomly chosen DNA samples were included in every run as reproducibility controls. A no-template control was also included.

### Statistical analysis

Data were entered into SPSS version 23.0 (IBM, USA). Categorical variables are presented as number and percent, whereas continuous ones are presented as mean and standard deviation ( $\pm\text{SD}$ ). Analyses were performed on the total sample and stratified by gender, as we have previously shown that females in our cohort had significantly higher urinary BPA levels when compared to males [13].

**Table 1** Characteristics of participants

		Total (N = 482)	Males (N = 170)	Females (N = 312)	P-value
Age (years)	Mean ± SD	44.9 ± 14.8	40.8 ± 15.9	47.1 ± 13.8	<0.0001
Waist circumference (cm)	Mean ± SD	95.6 ± 15.6	98.1 ± 13.6	94.2 ± 16.5	0.006
Body mass index (Kg/m <sup>2</sup> )	Mean ± SD	29.0 ± 5.8	28.1 ± 5.3	29.6 ± 6.0	0.008
<30	N (%)	281 (58.3)	109 (64.1)	172 (55.1)	0.06
≥30	N (%)	201 (41.7)	61 (35.9)	140 (44.9)	
Smoking					
No	N (%)	124 (25.7)	28 (16.5)	96 (30.8)	0.002
Ex	N (%)	47 (9.8)	21 (12.3)	26 (8.3)	
Current	N (%)	311 (64.5)	121 (71.2)	190 (60.9)	
Alcohol					
No	N (%)	390 (80.9)	99 (58.2)	291 (93.3)	<0.0001
Yes	N (%)	92 (19.1)	71 (41.8)	21 (6.7)	
Glomerular filtration rate (ml/min)	Mean ± SD	104.1 ± 16.7	103.6 ± 17.2	104.3 ± 16.6	0.77
Diabetes					
No	N (%)	414 (85.9)	150 (88.2)	264 (84.6)	0.27
Yes	N (%)	68 (14.1)	20 (11.8)	48 (15.4)	
Hypertension					
No	N (%)	310 (64.4)	107 (62.9)	203 (65.3)	0.61
Yes	N (%)	171 (35.6)	63 (37.1)	108 (34.7)	
Dyslipidemia					
No	N (%)	370 (76.8)	139 (81.8)	231 (74.0)	0.06
Yes	N (%)	112 (23.2)	31 (18.2)	81 (26.0)	
Urinary BPA levels adjusted for urinary creatinine (µg/g creatinine)	Mean ± SD	2.9 ± 4.8	1.9 ± 2.1	3.5 ± 5.7	<0.0001
Relative telomere length	Mean ± SD	1.4 ± 0.8	1.5 ± 0.97	1.4 ± 0.77	0.81
<i>ESRI</i> promoter % methylation	Mean ± SD	5.3 ± 9.9	4.5 ± 9.2	5.7 ± 10.3	0.18

Similarly to previous analyses [13], BPA urinary levels were adjusted for urinary creatinine (µg/g creatinine) and categorized into tertiles based on statistical grounds. Relative telomere length was categorized into tertiles and into two categories also based on statistical grounds. As for *ESRI* promoter % methylation, and because 50% of the sample had a 0 % methylation level, we divided our sample into three categories: C1 being the zero methylation level, and C2 and C3 being the rest of the samples split in half. In addition, and knowing that the sensitivity of methylation analysis by direct bisulfite sequencing is suboptimal [31], we also analyzed *ESRI* promoter % methylation as two categories, one for samples that were ≤5% methylated (C1) and one for those that were >5% methylated (C2). Finally, a regression analysis was attempted with adjusted BPA urinary levels and relative telomere length or *ESRI* promoter % methylation as continuous variables.

Association between *ESRI* promoter % methylation or relative telomere length categories and other categorical variables (gender, body mass index-BMI, smoking, and alcohol intake) was assessed using the chi-square test,

whereas ANOVA test was used for the association with continuous variables (age, waist circumference-WC, and BMI). Multinomial logistic regression was then carried out for the association between BPA and *ESRI* promoter % methylation and relative telomere length. Multivariate analysis was performed to adjust for potentially confounding variables: those that showed statistical significance ( $P < 0.05$ ) at the univariate analysis and those that were considered clinically important though not statistically significant. Results of the multinomial regression are presented as odds ratio (OR) and 95% confidence interval (CI). Dose-response relationship was assessed by carrying out trend analyses where the  $P$ -value was used to indicate statistical significance.

Of note that, and in order to better visualize potential trends, the primary analysis was run with BPA urinary levels, relative telomere length and *ESRI* promoter % methylation as tertiles, while the rest of the analysis was secondary and hence presented in the supplementary tables as OR (95% CI) with a  $P$ -value for trend or  $\beta$  (95% CI), as applicable.

**Table 2** Associations with estrogen receptor 1 (*ESR1*) promoter % methylation

	All			Males			Females			
	0	0.01–6.21 (N = 126)	>6.21 (N = 122)	0	0.01–6.21 (N = 44)	>6.21 (N = 33)	0	0.01–6.21 (N = 82)	>6.21 (N = 89)	
	(N = 234)			(N = 93)			(N = 141)			
Age	40.6 (14.2)	47.4 (14.9)	50.4 (13.7)	36.8 (14.8)	43.3 (16.3)	48.7 (15.1)	43.2 (13.2)	49.6 (13.7)	51.0 (13.2)	<0.0001
Gender										
Male	93 (39.7)	44 (34.9)	33 (27.0)	0.06						
Female	141 (60.3)	82 (65.1)	89 (72.9)							
Waist circumference	94.5 (16.1)	96.1 (16.7)	97.0 (13.3)	0.32	97.2 (12.2)	101.5 (13.8)	92.7 (18.1)	95.5 (17.1)	95.4 (12.7)	0.36
Body mass index	28.5 (5.8)	29.2 (5.8)	29.8 (5.7)	0.13	28.1 (4.8)	28.6 (5.6)	28.9 (6.3)	30.0 (5.6)	30.2 (5.7)	0.16
Body mass index										
<30	149 (63.7)	67 (53.2)	65 (53.3)	0.07	59 (63.4)	21 (63.6)	90 (63.8)	38 (46.3)	44 (49.4)	0.02
≥30	85 (36.3)	59 (46.8)	57 (46.7)		34 (36.6)	12 (36.4)	51 (36.2)	44 (53.7)	45 (50.6)	
Smoking										
No	56 (23.9)	31 (24.6)	37 (30.3)	0.32	17 (18.3)	4 (12.1)	39 (27.7)	24 (29.3)	33 (37.1)	0.36
Ex	18 (7.7)	15 (11.9)	14 (11.5)		9 (9.7)	6 (18.2)	9 (6.4)	9 (11.0)	8 (9.0)	
Current	160 (68.4)	80 (63.5)	71 (58.2)		67 (72.0)	23 (69.7)	93 (66.0)	49 (59.8)	48 (53.9)	
Alcohol										
No	179 (76.5)	107 (84.9)	104 (85.2)	0.06	47 (50.5)	23 (69.7)	132 (93.6)	78 (95.1)	81 (91.0)	0.55
Yes	55 (23.5)	19 (15.1)	18 (14.7)		46 (49.5)	10 (30.3)	9 (6.4)	4 (4.9)	8 (9.0)	

**Table 3** Association of BPA with estrogen receptor 1 (ESR1) promoter % methylation

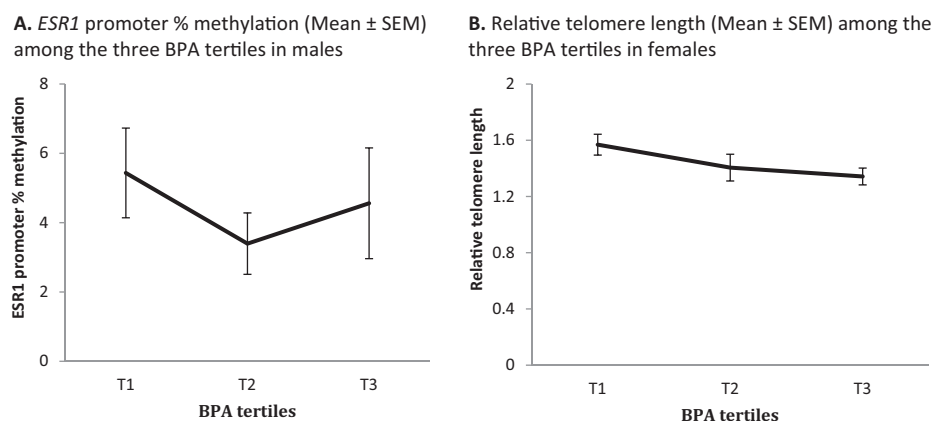
	All		Males		Females	
	ESR1 (C1) (N = 234)	ESR1 (C2) (N = 126)	ESR1 (C1)(N = 93)	ESR1 (C2)(N = 44)	ESR1 (C1)(N = 141)	ESR1 (C2)
<i>BPA adjusted for creatinine</i>						
Unadjusted univariate						
T1	Reference	Reference	Reference	Reference	Reference	Reference
T2	1.69 (0.98–2.92)	1.19 (0.63–2.26)	2.54 (1.01–6.39)	1.58 (0.55–4.50)	1.37 (0.69–2.73)	1.07 (0.47–2.44)
T3	0.94 (0.55–1.60)	1.30 (0.72–2.34)	1.64 (0.55–4.86)	1.58 (0.48–5.24)	0.87 (0.45–1.65)	1.39 (0.67–2.85)
<i>P</i> -value for trend	0.85	0.36	0.19	0.35	0.60	0.34
Adjusted multivariate						
T1	Reference	Reference	Reference	Reference	Reference	Reference
T2	1.85 (1.05–3.25)	1.23 (0.65–2.35)	2.63 (1.01–6.88)	1.62 (0.56–4.65)	1.54 (0.75–3.14)	1.10 (0.48–2.52)
T3	1.31 (0.75–2.30)	1.47 (0.80–2.68)	1.90 (0.60–5.98)	1.72 (0.51–5.80)	1.21 (0.61–2.39)	1.51 (0.72–3.17)
<i>P</i> -value for trend	0.33	0.21	0.13	0.30	0.63	0.25

ESR1 categories: C1: 0; C2: 0.01–6.21; C3: >6.21 (reference)

BPA creatinine adjusted tertiles: T1: <1.26; T2: 1.26–2.44; T3: >2.44

Multivariate model is adjusted for: Age, gender (for the among all model only), BMI and alcohol

**Fig. 1** Plots of *ESR1* promoter % methylation (**a**) and relative telomere length (**b**) among the three tertiles of urinary adjusted BPA concentrations in males and females respectively.



## Results

### Participants' characteristics

Blood was available for 497 participants of whom 15 had decreased renal function (estimated glomerular filtration rate-GFR < 60 ml/min). These were excluded from analysis and the final sample size was hence 482.

As shown in Table 1, the mean  $\pm$  SD age of the participants was 44.9 years ( $\pm$ 14.8), and 64.7% were females with significantly higher urinary BPA levels when compared to males. More than half of the participants were current smokers (64.5%) while 19.1% were current alcohol drinkers, with these behaviors being significantly higher in males. Only eight had been previously diagnosed with cancer, four of which were breast.

### Urinary BPA levels

The limit of BPA detection was 0.1  $\mu$ g/l (ppb) based on three times the signal to noise ratio. The internal quality control relative error was 4.68% with BPA and BPB concentrations of 125  $\mu$ g/l and 133  $\mu$ g/l (ppb), respectively. The mean  $\pm$  SD urinary BPA concentration was 3.71  $\pm$  4.83 ( $\mu$ g/l) with a median of 3.11  $\mu$ g/l (min–max: <Limit of detection LOD –59.71) and the one adjusted for urinary creatinine was 2.90  $\pm$  4.81 ( $\mu$ g/g creatinine) with a median of 1.86  $\mu$ g/g creatinine (min–max: <LOD –69.85).

### *ESR1* promoter % methylation and relative telomere length

For *ESR1*, there was a high and significant correlation between the methylation percentage at the five individual

**Table 4** Associations with relative telomere length (RTL)

	All			Males			Females			
	<1.06 (N = 159)	1.06 – 1.433 (N = 161)	>1.43 (N = 162)	<1.06 (N = 58)	1.06 – 1.43 (N = 57)	>1.43 (N = 55)	<1.06 (N = 101)	1.06 – 1.43 (N = 104)	>1.43 (N = 107)	
			P-value						P-value	
Age	47.8 (14.3)	44.3 (15.1)	42.7 (14.8)	45.2 (15.9)	39.0 (15.8)	38.0 (15.4)	49.3 (13.1)	47.1 (14.0)	45.0 (13.9)	0.09
Gender										
Male	58 (36.5)	57 (35.4)	55 (33.9)							
Female	101 (63.5)	104 (64.6)	107 (66.0)							
Waist circumference	98.8 (14.5)	95.6 (17.6)	92.5 (14.0)	99.9 (12.5)	98.5 (14.5)	95.8 (13.8)	98.1 (15.6)	94.0 (19.0)	90.8 (13.8)	0.005
Body mass index	29.9 (5.7)	28.9 (5.7)	28.3 (5.8)	28.6 (4.5)	28.1 (6.3)	27.5 (4.9)	30.7 (6.2)	29.3 (5.3)	28.7 (6.1)	0.05
Body mass index										
<30	87 (54.7)	93 (57.8)	101 (62.3)	39 (67.2)	37 (64.9)	33 (60.0)	48 (47.5)	56 (53.8)	68 (63.5)	0.06
≥30	72 (45.3)	68 (42.2)	61 (37.6)	19 (32.8)	20 (35.1)	22 (40.0)	53 (52.5)	48 (46.1)	39 (36.4)	
Smoking										
No	39 (24.5)	43 (26.7)	42 (25.9)	9 (15.5)	9 (15.8)	10 (18.2)	30 (29.7)	34 (32.7)	32 (29.9)	0.70
Ex	19 (11.9)	9 (5.6)	19 (11.7)	11 (19.0)	3 (5.3)	7 (12.7)	8 (7.9)	6 (5.8)	12 (11.2)	
Current	101 (63.5)	109 (67.7)	101 (62.3)	38 (65.5)	45 (78.9)	38 (69.1)	63 (62.4)	64 (61.5)	63 (58.9)	
Alcohol										
No	126 (79.2)	126 (78.3)	138 (85.2)	32 (55.2)	31 (54.4)	36 (65.4)	94 (93.1)	95 (91.3)	102 (95.3)	0.51
Yes	33 (20.7)	35 (21.7)	24 (14.8)	26 (44.8)	26 (45.6)	19 (34.5)	7 (6.9)	9 (8.6)	5 (4.7)	

CpG positions and the average of the five, so the average of the five was used in the analysis. Concerning reproducibility, *ESRI* promoter % methylation levels were significantly correlated [ $R$  of 0.81 ( $P < 0.001$ )] in 32 sample repeats [31]. As for relative telomere length, the intra-assay geometric mean of the coefficients of variation for the telomere and single copy gene Ct values were less than 1% for both with a mean  $\pm$  SD of seven different assays of  $0.92 \pm 0.17\%$  and  $0.58 \pm 0.11\%$  for telomere and the single copy gene, respectively. As for inter-run reproducibility, there was a significantly high correlation between the relative telomere length of 18 samples that were run on two different occasions ( $R = 0.88$ ;  $P < 0.0001$ ); in addition, the inter-assay geometric mean of the coefficient of variation was 6.49% [30]. Mean  $\pm$  SD *ESRI* promoter % methylation and relative telomere length were:  $5.28 \pm 9.94$  and  $1.43 \pm 0.84$  with a median of 0.20 (min–max: 0–89.60) and 1.28 (min–max: 0.16–10.28), respectively.

### Effect of BPA exposure on *ESRI* promoter % methylation

As seen in Table 2, age was significantly associated with higher *ESRI* promoter % methylation for the whole cohort and within males and females separately. There was a trend for lower BMI to be associated with less *ESRI* promoter % methylation, and this was significant within females. Current alcohol intake was also associated with less *ESRI* promoter % methylation but this was not significant. At the univariate multinomial regression, trend analysis did not reveal any significant results though it appears that, and in men only, there is a potentially non-monotonic relationship

between adjusted urinary BPA concentrations and *ESRI* promoter % methylation. This is shown with the lowest *ESRI* % methylation OR (95% CI) being 2.54 (1.01–6.39) for the middle urinary adjusted BPA tertile when compared to the first (reference) urinary adjusted BPA tertile, and 1.64 (0.55–4.86) for the highest urinary adjusted BPA tertile (Table 3). This is illustrated in Fig. 1a that shows a lower, though not significant, mean  $\pm$  standard error of the mean (SEM) of *ESRI* promoter % methylation in tertile 2 of the urinary adjusted BPA level when compared to tertiles 1 and 3 (U shaped curve) in males. After adjustment for potential confounders, similar results with the second BPA tertile remained in men [OR (95% CI): 2.63 (1.01–6.88)] and appeared in the whole cohort [OR (95% CI): 1.85 (1.05–3.25)] (Table 3). No significant associations were found in women even after adjustment for menopausal status (data not shown). Of note that no significant results appeared when BPA urinary levels and *ESRI* promoter % methylation were analyzed as continuous variables. Concerning the association analysis with *ESRI* promoter % methylation as two categories, there were no significant results except for the adjusted multivariate model whereby a potentially non-monotonic relationship also appeared in the whole cohort, though the trend was not significant (Supplementary Table 1).

### Effect of BPA exposure on relative telomere length

As seen in Table 4, age was significantly associated with shorter relative telomere length whereby the lowest relative telomere length tertile group had mean age of 47.8 years as compared to 42.7 years for the highest relative telomere

**Table 5** Association of BPA with relative telomere length (RTL)

	All		Males		Females	
	RTL (T1) ( $N = 159$ )	RTL (T2) ( $N = 161$ )	RTL (T1) ( $N = 58$ )	RTL (T2) ( $N = 57$ )	RTL (T1) ( $N = 101$ )	RTL (T2) ( $N = 104$ )
<i>BPA adjusted for creatinine</i>						
Unadjusted univariate						
T1	Reference	Reference	Reference	Reference	Reference	Reference
T2	1.62 (0.94–2.79)	1.11 (0.65–1.90)	0.80 (0.35–1.85)	0.80 (0.34–1.85)	2.91 (1.38–6.16)	1.37 (0.68–2.73)
T3	1.56 (0.90–2.67)	1.09 (0.64–1.85)	0.44 (0.16–1.21)	0.51 (0.19–1.37)	3.19 (1.57–6.49)	1.54 (0.81–2.92)
<i>P</i> -value for trend	0.12	0.74	0.12	0.19	0.002	0.18
Adjusted multivariate						
T1	Reference	Reference	Reference	Reference	Reference	Reference
T2	1.62 (0.93–2.81)	1.12 (0.65–1.90)	0.83 (0.35–1.94)	0.80 (0.34–1.87)	2.85 (1.34–6.10)	1.36 (0.68–2.71)
T3	1.51 (0.87–2.62)	1.08 (0.64–1.84)	0.40 (0.14–1.13)	0.50 (0.18–1.36)	2.97 (1.45–6.09)	1.49 (0.78–2.84)
<i>P</i> -value for trend	0.15	0.76	0.10	0.18	0.005	0.22

RTL tertiles: T1:  $<1.06$ ; T2:  $1.06 - 1.43$ ; T3:  $>1.43$  (reference)

BPA creatinine adjusted tertiles: T1:  $<1.26$ ; T2:  $1.26-2.44$ ; T3:  $>2.44$

Multivariate model is adjusted for: Age, gender (for the among all model only), and WC

length tertile group ( $P = 0.007$ ). In addition, higher WC and BMI as markers of obesity were significantly associated with shorter relative telomere length in the whole group and in females (WC: 98.8 for the lowest relative telomere length tertile vs. 92.5 for the highest relative telomere length tertile,  $P = 0.001$ ; BMI: 29.9 for the lowest relative telomere length tertile vs. 28.3 for the highest relative telomere length tertile,  $P = 0.03$ ). There were no significant associations between BPA and relative telomere length except within the female sub-cohort whereby higher urinary BPA concentrations that were adjusted for urinary creatinine were significantly associated with shorter relative telomere length. This association remained significant in the multivariate multinomial regression: OR (95% CI) with the first relative telomere length tertile being 2.85 (1.34–6.10) and 2.97 (1.45–6.09) for the second and third urinary adjusted BPA tertiles, respectively. Moreover, results of trend analysis were consistent with those of logistic regression (Table 5). This is illustrated in Fig. 1b that shows a decrease (though not significant) in mean  $\pm$  SEM relative telomere length over the three tertiles of urinary adjusted BPA concentrations in females. A similar, though not significant trend was found with the second relative telomere length tertile (Table 5). Of note that no significant results appeared when BPA urinary levels and relative telomere length were analyzed as continuous variables. Concerning the association analysis with relative telomere length as two categories, significant results appeared in the whole and female sub-cohort, the trend was however not significant (Supplementary Table 2).

## Discussion

This study investigated the potential association of BPA exposure with *ESR1* promoter % methylation and relative telomere length. It showed that there is a potentially non-monotonic relationship between BPA exposure and *ESR1* promoter % methylation in the male sub-cohort, and that high BPA levels are associated with shorter relative telomere length within the female sub-cohort only. The predictors of *ESR1* promoter % methylation and relative telomere length were in line with those reported in the literature. As such and consistent with previous studies [32–34], relative telomere length was inversely associated with age, WC, and BMI in the whole cohort and in females. Moreover, *ESR1* promoter % methylation was directly associated with age and BMI, which is similar to previous reports of age-dependent increase in *ESR1* methylation % in various human epithelial tissues such as prostate tissues and colonic mucosa [35–37]. The consistency of our findings with previous data validates the methods applied in this study to measure *ESR1* promoter % methylation and relative telomere length.

## Effect of BPA exposure on *ESR1* promoter % methylation

Epigenetic effects of BPA have been first demonstrated in the Agouti (*ASIP*) gene model whereby maternal exposure to BPA shifted the coat color distribution of agouti mouse offsprings toward yellow through decreasing the DNA methylation of a retrotransposon located upstream of the Agouti gene [38]. This was followed by a number of recent data on DNA methylation and the developmental effects of BPA. For instance, animal studies suggested that exposure to BPA in utero and in early postnatal period may impair brain development, sexual differentiation and behavior through disruption of epigenetic programming of certain genes [39–45]. Neonatal BPA was also associated with a decrease in global DNA methylation in the testis of rats and a change in *DNA methyltransferase (DNMT)* expression [43, 45–47]. Similarly, BPA exposure was associated with a decrease in global DNA methylation in spermatozoa samples of male factory workers and salivary samples of pre-pubescent girls [20, 21]. The potential association between BPA and *ESR1* promoter methylation has been previously studied in rat models whereby neonatal BPA exposure was found to be associated with increased *ESR1* promoter methylation [41]. The inconsistency with our results may be attributed to the differences in study design (human vs. animal) and sample type (blood vs. testes). Besides, it is unclear why analysis showed a significant association in the men sub-cohort only. This prompts a need for more studies to validate our results and to clarify whether the association between BPA and *ESR1* promoter methylation is gender-specific.

Studies evaluating the association between BPA exposure and the *ESR1* gene expression also showed inconsistent results. For instance, urinary BPA levels were associated with mRNA expression of estrogen receptor 2 (*ESR2*) and estrogen related receptor alpha (*ESRRA*) but not with mRNA expression of *ESR1*, estrogen related receptor beta and gamma (*ESRRB* and *ESRRG*), and androgen receptor (*AR*), in peripheral blood leukocytes of 96 adult men from the INCHIANTI population [48]. However, in high risk donor breast epithelial cells (HRBEC) and ER + breast cancer cells (T47D), BPA was associated with increased *ESR1* protein expression and decreased *ESR2* protein expression and thereby increasing the *ESR1/ESR2* ratio, which had been reported in clinical studies to predict the progression of breast hyperplasia to malignancy [19]. Besides, low-dose prenatal BPA exposure significantly altered *ESR1* and *ESR2* mRNA in the hypothalamus and amygdala of neonatal rats of both sexes [49]. The inconsistencies in these results may be attributed to differences in study design (in vitro vs. in vivo) and sample types (leukocytes vs. brain vs. breast). Unfortunately, we did not

measure *ESRI* mRNA and protein expression in our samples. It is possible that the lower *ESRI* promoter % methylation leads to higher *ESRI* expression, albeit the change in *ESRI* promoter % methylation was not large across the tertiles.

The potentially non-monotonic relationship between BPA exposure and *ESRI* promoter % methylation did not come as a surprise since hormones and endocrine disruptors are known for their non-monotonic dose-response effect that is attributed to different pathways triggered by various doses of the same chemical [50]. Concerning BPA, non-monotonic dose-response relationships have been previously observed with some outcomes such as release of prolactin, cardiomyocyte contractility, structural modifications of mammary terminal end buds, and modification of prostate adenocarcinoma cell proliferation index [50, 51]. Prenatal BPA exposure was also associated with non-monotonic alterations in *ESRI* mRNA expression in the hypothalamus of rats [49]. More studies are warranted to clarify and explain the mechanisms behind our results and to understand their clinical significance and implications.

### Effect of BPA exposure on relative telomere length

Few investigators studied the effect of estrogen and estrogen-like compounds on relative telomere length and *hTERT* transcription [25, 52–54]. One study in mice showed that estrogen deficiency resulted in decrease in cell proliferation, inhibition of *hTERT* expression and shortening of relative telomere length in the adrenal gland; and these were restored after estrogen administration [53]. However, another study showed that estrogen-like chemicals were associated with telomere length reduction. For instance noble rats, when exposed to diethylstilbestrol (DES), exhibited different changes in their mammary glands, whereby cell cycle alterations were reported along with increased cell proliferation and reduction in telomere length. As for BPA, MCF-7 cells treated with BPA and DES showed dose-dependent increase in telomeric associations that have been implicated in increased genomic instabilities [54]. Treatment with BPA and estrogen was associated with increased expression of *hTERT* in MCF-7 cells [25], which decreases apoptosis through inhibition of telomere shortening [55]. Taken together, relative telomere length seems to be determined by factors that increase telomere length such as increased telomerase expression, and by factors that decrease telomere length such as increased cellular proliferation, with the dominating factor determining the direction of change in relative telomere length. We have recently shown on the same cohort of Lebanese people from Greater Beirut area that short relative telomere length is associated with obesity, hypertension, and sleeping difficulties [30], findings that are in line with studies in various

ethnic groups [56–59]. Our study results together with the findings in the literature suggest that relative telomere length may play a role in the BPA-associated health hazards. Nevertheless, more studies are required to confirm and explain our findings.

### Limitations

This is a cross-sectional study design, hence it is limited by the potential variability of BPA levels over time and the inability to assess for the longitudinal association of BPA with *ESRI* promoter % methylation and relative telomere length. Besides, although there was an association between short relative telomere length and *ESRI* promoter hypermethylation with older age, there were no significant associations between relative telomere length and *ESRI* promoter % methylation in the whole cohort and per gender stratification even after adjustment for age (data not shown), which suggest that these alterations could be mediated through independent mechanisms. However, this could also be attributed to the sample type that is peripheral blood, as a possible link could have been detected in estrogen-sensitive tissues such as breast or ovarian tissues. In addition, and knowing that methylation levels of CpG islands may not greatly change in response to weak levels of exposures such as those reported herein with BPA, it is possible that stronger results could have appeared had we analyzed regions that are more dynamic (and hence potentially more sensitive to environmental exposure) such as enhancers. Besides, the study may be limited by the sample size whereby a larger sample size could potentially be enriched for hypermethylated *ESRI* promoter (>50%).

### Conclusion

This is the first study to show that BPA exposure is associated with non-monotonic alteration in *ESRI* promoter % methylation in males and shorter relative telomere length in females. These results suggest that the adverse health effects of BPA may partially occur through altering *ESRI* promoter methylation and shortening of relative telomere length. This study however requires replication and further investigations to include the measurement of telomerase and *ESRI* RNA and protein expression levels and evaluation of changes in *ESRI* promoter methylation and relative telomere length in estrogen-sensitive tissues.

**Acknowledgements** This work was supported by the American University of Beirut Faculty of Medicine seed grant to Dr. Hani Tamim and Research grant to Dr. Nathalie K. Zgheib as well as a Lebanese National Council for Scientific Research (LNCSR) PhD scholarship award to Miss Zainab Awada.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

- Chen D, Kannan K, Tan H, Zheng Z, Feng YL, Wu Y, et al. Bisphenol analogues other than bpa: environmental occurrence, human exposure, and toxicity—a review. *Environ Sci Technol*. 2016;50:5438–53.
- Lang IA, Galloway TS, Scarlett A, Henley WE, Depledge M, Wallace RB, et al. Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. *JAMA*. 2008;300:1303–10.
- Melzer D, Rice NE, Lewis C, Henley WE, Galloway TS. Association of urinary bisphenol a concentration with heart disease: evidence from NHANES 2003/06. *PLoS ONE*. 2010;5:e8673.
- Seachrist DD, Bonk KW, Ho SM, Prins GS, Soto AM, Keri RA. A review of the carcinogenic potential of bisphenol A. *Reprod Toxicol*. 2016;59:167–82.
- Richter CA, Birnbaum LS, Farabolini F, Newbold RR, Rubin BS, Talsness CE, et al. In vivo effects of bisphenol A in laboratory rodent studies. *Reprod Toxicol*. 2007;24:199–224.
- Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003–2004. *Environ Health Perspect*. 2008;116:39–44.
- Bushnik T, Haines D, Levallois P, Levesque J, Van Oostdam J, Viau C. Lead and bisphenol A concentrations in the Canadian population. *Health Rep*. 2010;21:7–18.
- Yang M, Kim SY, Lee SM, Chang SS, Kawamoto T, Jang JY, et al. Biological monitoring of bisphenol a in a Korean population. *Arch Environ Contam Toxicol*. 2003;44:546–51.
- Arakawa C, Fujimaki K, Yoshinaga J, Imai H, Serizawa S, Shiraishi H. Daily urinary excretion of bisphenol A. *Environ Health Prev Med*. 2004;9:22–6.
- Matsumoto A, Kunugita N, Kitagawa K, Isse T, Oyama T, Foureman GL, et al. Bisphenol A levels in human urine. *Environ Health Perspect*. 2003;111:101–4.
- Ouchi K, Watanabe S. Measurement of bisphenol A in human urine using liquid chromatography with multi-channel coulometric electrochemical detection. *J Chromatogr B Anal Technol Biomed Life Sci*. 2002;780:365–70.
- Covaci A, Den Hond E, Geens T, Govarts E, Koppen G, Frederiksen H, et al. Urinary BPA measurements in children and mothers from six European member states: overall results and determinants of exposure. *Environ Res*. 2015;141:77–85.
- Mouneimne Y, Nasralla M, Zgheib NK, Nasreddine L, Nakhoul N, Ismail H, Abiad M, Koleilat L, Tamim H. Bisphenol A urinary levels, its correlates and association with cardiometabolic risks in Lebanese urban adults. *Environ Monit Assess*. 2017;189:517.
- Zhang Z, Alomirah H, Cho HS, Li YF, Liao C, Minh TB, et al. Urinary bisphenol A concentrations and their implications for human exposure in several Asian countries. *Environ Sci Technol*. 2011;45:7044–50.
- Ye X, Kuklennyk Z, Needham LL, Calafat AM. Quantification of urinary conjugates of bisphenol A, 2,5-dichlorophenol, and 2-hydroxy-4-methoxybenzophenone in humans by online solid phase extraction-high performance liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem*. 2005;383:638–44.
- Calafat AM, Kuklennyk Z, Reidy JA, Caudill SP, Ekong J, Needham LL. Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. *Environ Health Perspect*. 2005;113:391–5.
- Lapensee EW, Tuttle TR, Fox SR, Ben-Jonathan N. Bisphenol A at low nanomolar doses confers chemoresistance in estrogen receptor-alpha-positive and -negative breast cancer cells. *Environ Health Perspect*. 2009;117:175–80.
- Gao H, Yang BJ, Li N, Feng LM, Shi XY, Zhao WH, et al. Bisphenol A and hormone-associated cancers: current progress and perspectives. *Medicine*. 2015;94:e211.
- Dairkee SH, Luciani-Torres MG, Moore DH, Goodson WH 3rd. Bisphenol-A-induced inactivation of the p53 axis underlying deregulation of proliferation kinetics, and cell death in non-malignant human breast epithelial cells. *Carcinogenesis*. 2013;34:703–12.
- Kim JH, Rozek LS, Soliman AS, Sartor MA, Hablas A, Seifeldin IA, et al. Bisphenol A-associated epigenomic changes in pre-pubescent girls: a cross-sectional study in Gharbiah, Egypt. *Environ Health*. 2013;12:33.
- Miao M, Zhou X, Li Y, Zhang O, Zhou Z, Li T, et al. LINE-1 hypomethylation in spermatozoa is associated with Bisphenol A exposure. *Andrology*. 2014;2:138–44.
- Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science*. 1994;266:2011–5.
- Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, et al. The RNA component of human telomerase. *Science*. 1995;269:1236–41.
- Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR. Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science*. 1997;276:561–7.
- Takahashi A, Higashino F, Aoyagi M, Kyo S, Nakata T, Noda M, et al. Bisphenol A from dental polycarbonate crown upregulates the expression of hTERT. *J Biomed Mater Res B Appl Biomater*. 2004;71:214–21.
- Hiyama E, Hiyama K, Yokoyama T, Shay JW. Immunohistochemical detection of telomerase (hTERT) protein in human cancer tissues and a subset of cells in normal tissues. *Neoplasia*. 2001;3:17–26.
- Carey LA, Hedican CA, Henderson GS, Umbricht CB, Dome JS, Varon D, et al. Careful histological confirmation and microdissection reveal telomerase activity in otherwise telomerase-negative breast cancers. *Clin Cancer Res*. 1998;4:435–40.
- Pellatt AJ, Wolff RK, Torres-Mejia G, John EM, Herrick JS, Lundgreen A, et al. Telomere length, telomere-related genes, and breast cancer risk: the breast cancer health disparities study. *Genes Chromosomes Cancer*. 2013;52:595–609.
- Zhang C, Chen X, Li L, Zhou Y, Wang C, Hou S. The Association between Telomere Length and Cancer Prognosis: Evidence from a Meta-Analysis. *PLoS ONE*. 2015;10:e0133174.
- Zgheib NK, Sleiman F, Nasreddine L, Nasrallah M, Nakhoul N, Isma'el H, et al. Short telomere length is associated with aging, central obesity, poor sleep and hypertension in Lebanese individuals aging and disease 2017. <http://www.aginganddisease.org/EN/10.14336/AD.2017.030610>
- Akika R, Awada Z, Mogharbil N, Zgheib NK. Region of interest methylation analysis: a comparison of MSP with MS-HRM and direct BSP. *Mol Biol Rep*. 2017;44:295–305.
- Cui Y, Gao YT, Cai Q, Qu S, Cai H, Li HL, et al. Associations of leukocyte telomere length with body anthropometric indices and weight change in Chinese women. *Obesity (Silver Spring)*. 2013;21:2582–8.
- Martens UM, Brass V, Sedlacek L, Pantic M, Exner C, Guo Y, et al. Telomere maintenance in human B lymphocytes. *Br J Haematol*. 2002;119:810–8.
- Kim S, Parks CG, DeRoo LA, Chen H, Taylor JA, Cawthon RM, et al. Obesity and weight gain in adulthood and telomere length. *Cancer Epidemiol Biomark Prev*. 2009;18:816–20.

35. Post WS, Goldschmidt-Clermont PJ, Wilhide CC, Heldman AW, Sussman MS, Ouyang P, et al. Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. *Cardiovasc Res.* 1999;43:985–91.
36. Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nat Genet.* 1994;7:536–40.
37. Kwabi-Addo B, Chung W, Shen L, Ittmann M, Wheeler T, Jelinek J, et al. Age-related DNA methylation changes in normal human prostate tissues. *Clin Cancer Res.* 2007;13:3796–802.
38. Chao HH, Zhang XF, Chen B, Pan B, Zhang LJ, Li L, et al. Bisphenol A exposure modifies methylation of imprinted genes in mouse oocytes via the estrogen receptor signaling pathway. *Histochem Cell Biol.* 2012;137:249–59.
39. Jang YJ, Park HR, Kim TH, Yang WJ, Lee JJ, Choi SY, et al. High dose bisphenol A impairs hippocampal neurogenesis in female mice across generations. *Toxicology.* 2012;296:73–82.
40. Zhang HQ, Zhang XF, Zhang LJ, Chao HH, Pan B, Feng YM, et al. Fetal exposure to bisphenol A affects the primordial follicle formation by inhibiting the meiotic progression of oocytes. *Mol Biol Rep.* 2012;39:5651–7.
41. Doshi T, Mehta SS, Dighe V, Balasiner N, Vanage G. Hypermethylation of estrogen receptor promoter region in adult testis of rats exposed neonatally to bisphenol A. *Toxicology.* 2011;289:74–82.
42. Doshi T, D'Souza C, Dighe V, Vanage G. Effect of neonatal exposure on male rats to bisphenol A on the expression of DNA methylation machinery in the postimplantation embryo. *J Biochem Mol Toxicol.* 2012;26:337–43.
43. Anderson OS, Nahar MS, Faulk C, Jones TR, Liao C, Kannan K, et al. Epigenetic responses following maternal dietary exposure to physiologically relevant levels of bisphenol A. *Environ Mol Mutagen.* 2012;53:334–42.
44. Wolstenholme JT, Taylor JA, Shetty SR, Edwards M, Connelly JJ, Rissman EF. Gestational exposure to low dose bisphenol A alters social behavior in juvenile mice. *PLoS ONE.* 2011;6:e25448.
45. Monje L, Varayoud J, Luque EH, Ramos JG. Neonatal exposure to bisphenol A modifies the abundance of estrogen receptor alpha transcripts with alternative 5'-untranslated regions in the female rat preoptic area. *J Endocrinol.* 2007;194:201–12.
46. Hiyama M, Choi EK, Wakitani S, Tachibana T, Khan H, Kusakabe KT, et al. Bisphenol-A (BPA) affects reproductive formation across generations in mice. *J Vet Med Sci.* 2011;73:1211–5.
47. Abdel-Maksoud FM, Leasor KR, Butzen K, Braden TD, Akingbemi BT. Prenatal exposures of male rats to the environmental chemicals Bisphenol A and di(2-ethylhexyl) phthalate impact the sexual differentiation process. *Endocrinology.* 2015;156:4672–83.
48. Melzer D, Harries L, Cipelli R, Henley W, Money C, McCormack P, et al. Bisphenol A exposure is associated with in vivo estrogenic gene expression in adults. *Environ Health Perspect.* 2011;119:1788–93.
49. Cao J, Rebuli ME, Rogers J, Todd KL, Leyrer SM, Ferguson SA, et al. Prenatal bisphenol A exposure alters sex-specific estrogen receptor expression in the neonatal rat hypothalamus and amygdala. *Toxicol Sci.* 2013;133:157–73.
50. Myers P, Hessler W Does 'the dose make the poison?' Extensive results challenge a core assumption in toxicology. *Environ Health News.* 2007 <http://www.ourstolenfuture.org/NewScience/lowdose/2007/2007-0525nmdrc.html>
51. Lagarde F, Beausoleil, Belcher SM, Belzunces LP, Emond C, Guerbet M, et al. Non-monotonic dose-response relationships and endocrine disruptors: a qualitative method of assessment. *Environ Health* 2015;14:13.
52. Sarkar P, Shiizaki K, Yonemoto J, Sone H. Activation of telomerase in BeWo cells by estrogen and 2,3,7,8-tetrachlorodibenzo-p-dioxin in co-operation with c-Myc. *Int J Oncol.* 2006;28:43–51.
53. Bayne S, Jones ME, Li H, Pinto AR, Simpson ER, Liu JP. Estrogen deficiency leads to telomerase inhibition, telomere shortening and reduced cell proliferation in the adrenal gland of mice. *Cell Res.* 2008;18:1141–50.
54. Roy D, Colerangle JB, Singh KP. Is exposure to environmental or industrial endocrine disrupting estrogen-like chemicals able to cause genomic instability? *Front Biosci.* 1998;3:d913–21.
55. Zhang X, Mar V, Zhou W, Harrington L, Robinson MO. Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev.* 1999;13:2388–99.
56. Al-Attas OS, Al-Daghri NM, Alokail MS, Alkharfy KM, Alfadda AA, McTernan P, et al. Circulating leukocyte telomere length is highly heritable among families of Arab descent. *BMC Med Genet.* 2012;13:38.
57. Lee M, Martin H, Firpo MA, Demerath EW. Inverse association between adiposity and telomere length: the Fels longitudinal study. *Am J Hum Biol.* 2011;23:100–6.
58. Fitzpatrick AL, Kronmal RA, Gardner JP, Psaty BM, Jenny NS, Tracy RP, et al. Leukocyte telomere length and cardiovascular disease in the cardiovascular health study. *Am J Epidemiol.* 2007;165:14–21.
59. Brouillette S, Singh RK, Thompson JR, Goodall AH, Samani NJ. White cell telomere length and risk of premature myocardial infarction. *Arterioscler Thromb Vasc Biol.* 2003;23:842–6.