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Global DNA methylation and low-level exposure to benzene

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DNA methylation; gas chromatography-mass spectrometry; benzene exposure; biomonitoring

Parole chiave
Metilazione del DNA; gas cromatografia-spettrometria di massa; esposizione a benzene; monitoraggio biologico

Summary
Introduction: *Global genomic hypomethylation is a common event in cancer tissues that is frequently observed in hematopoietic malignancies, including leukemia. Benzene, an established leukemogen at high doses, has been suggested to induce hypomethylation based on investigations of DNA methylation in LINE-1 and Alu repetitive elements. Whether global genomic DNA methylation content is reduced in response to benzene exposure is still undetermined.* **Methods**: *We measured global DNA methylation in 78 gasoline station attendants and 58 controls in peripheral blood cells using high-resolution gas chromatography-mass spectrometry. PCR-Pyrosequencing measures of DNA methylation at Alu and LINE-1 repetitive elements, representing a large proportion of methylation in non-coding regions, were also available. Exposure markers included personal airborne benzene, and urinary benzene, t,t-muconic acid (t,t-MA) and S-phelylmercapturic acid.* **Results**: *Mean global DNA methylation was 5.474 (±0.083) %5mC in controls and 5.409 (±0.142) %5mC in exposed participants (p=0.001). All methylation markers were negatively correlated with airborne benzene. Alu and LINE-1 methylation, but not global DNA methylation, were negatively associated with t,t-MA; no association with the other urinary biomarkers was found. Multiple linear regression analysis adjusted for gender and age confirmed the results of correlation analysis and showed a 1.6% decrease in global DNA methylation associated with being gasoline station attendants. Alu and LINE-1 methylation levels were not associated with global DNA methylation.* **Conclusion**: *Our results show that benzene exposure is associated with alterations in both global DNA and repetitive element methylation. Global and repetitive element methylation levels are not correlated in blood DNA, likely representing independent responses to benzene exposure.*

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Riassunto

«Metilazione globale del DNA e esposizione a bassi livelli di benzene». **Introduzione**: *L'ipometilazione genomica globale è un evento comune nei tessuti tumorali, compresi i tumori ematopoietici, tra cui la leucemia. Studi riguardanti la metilazione del DNA negli elementi ripetuti LINE-1 e Alu suggeriscono che il benzene, un accertato leucemogeno alle alte dosi, possa indurre ipometilazione. Rimane da chiarire se il livello di metilazione globale del DNA venga ridotto in risposta ad una esposizione a benzene.* **Metodi**: *La metilazione globale del DNA è stata misurata nelle cellule di 78 addetti alla distribuzione del carburante e 58 controlli mediante gas cromatografia-spettrometria di massa. Erano inoltre disponibili misure di metilazione del DNA, ottenute tramite PCR-Pyrosequencing, negli elementi ripetuti Alu e LINE-1, che sono regioni non codificanti del DNA con un'elevata percentuale di metilazione. L'esposizione personale a benzene aerodisperso e gli indicatori biologici benzene urinario, acido t-t-muconico (t,t-MA) e acido S-fenilmercapturico sono stati misurati come indicatori di esposizione.* **Risultati**: *La metilazione globale del DNA media è risultata 5,474 (±0,083) 5mC% nei controlli e 5,409 (±0,142) 5mC% nei benzinai (p=0,001). Tutti i marcatori di metilazione sono risultati correlati negativamente con il benzene aerodisperso. La metilazione degli elementi Alu e LINE-1, ma non la metilazione globale, è risultata negativamente associata con t,t-MA; non è stata trovata nessuna associazione con gli altri indicatori urinari. L'analisi di regressione lineare multipla, aggiustata per sesso ed età, ha confermato i risultati delle analisi di correlazione e ha mostrato una diminuzione dell'1,6% nella metilazione globale del DNA nei benzinai. I livelli di metilazione di Alu e LINE-1 non sono risultati associati alla metilazione globale del DNA.* **Conclusioni**: *I nostri risultati mostrano che l'esposizione al benzene è associata ad alterazioni sia della metilazione globale del DNA che della metilazione degli elementi ripetuti. Nel DNA di sangue periferico i livelli di metilazione globale e degli elementi ripetuti non sono correlati e probabilmente rappresentano risposte indipendenti alla esposizione a benzene.*

1. Introduction

Methylation at the 5'-C residue of the cytosine located within the dinucleotide CpG, usually referred to as DNA methylation, is the most known epigenetic phenomenon. The proportion of all cytosines methylated in normal human DNA ranges between 4 and 6% (9). Therefore, 5-methylcytosine (5mC) constitutes 0.75-1% of all nucleotide bases (9). CpG-rich regions, known as CpG islands, are usually unmethylated in all tissues and frequently span the 5'-end region (promoter, untranslated region, and exon 1) of a number of genes; in physiological situations methylated CpG islands are found in imprinted genes, X-chromosome gene in females, germline and tissue-specific genes (2, 10). Recently, Irizzary and coworkers (21) have identified sequences located up to 2Kb distant from CpG islands, called CpG island shores, which show distinctive differential methylation in cancer tissues. Because of these findings, it has been argued that DNA methylation plays a key role in chromatin remodeling, in X-chromosome inactivation, and both in the regulation of gene expression, and gene activity (27).

Changes in DNA methylation, i.e. global hypomethylation and/or gene specific hypermethylation, has been described in many pathological processes, typically in cancer; malignant cells are reported having 20-60% less genomic 5mC than normal (10). Such changes have been linked to increased expression of oncogenes, chromosomal instability, reactivation of transposable elements, and loss of imprinting (15, 28, 31).

In hematopoietic tumors, hypermethylation of a number of genes and their promoters were reported having a specific role in the genesis of leukemia (e.i. BCL2, ODC1, LHX2, ER, p16 and TGF-β) (22, 26, 28). Also global hypomethylation has been related to acute leukemia (22, 28, 40) and low global DNA methylation levels has been shown to be a prognosis marker in acute myelogenous leukemia (7). In our recent work, global DNA methylation was dramatically lower in the bone marrow of leukemia patients than in peripheral blood leukocytes of healthy subjects, with median

levels of 3.58 vs. 5.45 %5mC, respectively (35). Part of the global hypomethylation in cancer tissues is accounted for by demethylation of repetitive elements, including Alu and long interspersed nuclear element-1 (LINE-1) retrotrasposons (39, 42); Alu and LINE-1 hypomethylation has been shown to be correlated with the level of global DNA hypomethylation in cancer tissues (39). The determinants of DNA methylation alterations related to cancer are still largely undetermined.

Changes in DNA methylation can be induced by environmental factors such as exposure to heavy metals, organic solvents, particulate matter, and persistent organic pollutants, as reviewed by Baccarelli and Bollati (1). Benzene is a known human carcinogen, classified in Group 1 by the International Agency on Research on Cancer (20), and a ubiquitous pollutant released from industrial emissions, gasoline exhaust fumes, and cigarette smoke. Occupational limit values and guideline values for urban air have been proposed by several regulatory agencies and institutional bodies; for instance, the European Union set an occupational limit value as a time weighted average of 1 ppm (3.2 mg/m^3) (11), and a mean calendar year limit of 5 μg/m^3 in living environments (12).

Several studies showed that benzene causes leukemia in highly exposed individuals (reviewed in 30). More recent work suggested that also exposure to level lower than 10 ppm increases the risk of acute and chronic leukemia (18), and that chronic exposures below 1 ppm interfere with haematopoietic cell proliferation (25) and increase sperm aneuploidy (41).

Exposure to low level airborne benzene in occupationally exposed subjects has been related with a decrease of 5mC located in transposable repetitive elements Alu and LINE-1 in DNA from peripheral blood specimens; these alterations are qualitatively similar, although smaller in size, to those found in haematopoietic malignancies (4). Recently DNA hypomethylation induced by hydroquinone, a metabolite of benzene, has been confirmed in human TK6 lymphoblastoid cells (23). Whether benzene effects are limited to Alu and LINE-1 repetitive elements or produce global genomic hypomethylation has never been investigated.

Aim of this work was to assess global DNA methylation in peripheral blood cells of healthy gasoline station attendants and controls and to relate these findings with exposure to benzene both assessed by personal air monitoring and urinary biomarkers. To measure global DNA methylation a recently developed gas chromatographic-mass spectrometry assay (GC-MS) was applied (35). Because data on methylation at Alu and LINE-1 repetitive elements were available for the same study subjects (4), a further aim of this study was to compare benzene effects on DNA methylation measured using different approaches.

2. Materials and methods

2.1. Study population and sampling protocol

The study subjects were two sub-groups of a previously described population of 415 subjects (17); the first group including 78 gasoline station attendants working in the urban area surrounding Milan, and the second group including 58 subjects working in offices and hospital facilities of the Department of Occupational and Environmental Health of the University of Milan, the latter designated as control group.

The field study was performed in year 2000. A database was available with results from previous work, particularly personal exposure to airborne benzene, assessed by passive sampling for a period of about 5 h (typically from 7:00-8:00 a.m. to 1:00-2:00 p.m.), urinary benzene and *t,t*-muconic acid (*tt*-MA), measured in before sampling (BS) and end of sampling (ES) urine spot samples, urinary S-phenylmercapturic acid (SPMA) determined in ES samples, urinary cotinine, a metrics of cigarette smoking assessed in BS samples (17). Blood samples were collected in heparinized tubes the morning following the day in which ambient monitoring and urine collection were performed. DNA methylation at Alu and LINE-1 repetitive elements was previously measured by pyrosequencing (4).

The two groups of subject included in the present study were selected from the whole popula-

tion included in the previous investigation because of the availability of blood samples for DNA extraction, and to maximize differences in the levels of benzene exposure. For each subject information regarding personal characteristics and lifestyle was collected by a self-administered questionnaire. The study was approved by the Ethical Committee of the University of Milan. Written informed consent was collected by each participant at the study.

2.2. Global DNA methylation by GC-MS

DNA from peripheral blood specimens was obtained using a DNA extraction kit (Promega, Milan, Italy), following the supplier's protocol applied to blood sample stored at -80°C for 9 years. After purity and concentration were measured with an Nanodrop 1000 spectrophotometer (Thermo, Milan, Italy), DNA was dissolved in RNAse-free water (Promega, Milan, Italy) to a final concentration of 40 μg/mL. Aliquots of suitable volume, corresponding to 5 μg of DNA, were kept in 2 mL Eppendorf tubes, and stored at -20°C until analysis.

Global DNA methylation was quantified by GC-MS, as previously described (35). Briefly a suitable volume of DNA solution containing 2.5 μg of DNA was poured in a 2 mL clear glass vial and dried under a gentle stream of nitrogen at room temperature. DNA was hydrolyzed with 88% aqueous formic acid to obtain the free nucleobases and spiked with cytosine-2,4-$^{13}C_2$,$^{15}N_3$ and 5-methyl-$^{2}H_3$-cytosine-6-$^{2}H_1$ as internal standards. Nucleobases were derivatized adding BSTFA as silylating agent in presence of acetonitrile and pyridine. Gas chromatographic separation was performed on a CP-SIL 5 capillary column (30 m length x 0.25 mm internal diameter and 1 μm film thickness, Varian, Leinì, Italy), using a 6890 Series II gas chromatograph, equipped with a 7683 Series autoinjector and interfaced to a 5973 single-quadrupole mass spectrometer (MS) (Agilent, Cernusco sul Naviglio, Italy) with an electron impact source (EI) operating at 70 eV. Signals were acquired in the single ion monitoring mode (SIM) registering the positive ions with m/z 282 for C, and 296 for 5mC. The limit of quantification was 0.031 nmol for C and 0.008 nmol for 5mC. The global DNA methylation was calculated as follows:

$$5mC = \frac{Q_{5mc}}{Q_{5mC} + Q_c} * 100$$

Where Q_{5mC} and Q_C are the molar amounts of 5mC and C in the sample. Each sample was replicated twice, and the average value was used for statistical purpose.

2.3. Statistical analysis

Statistical analysis was performed using the SPSS 17.0 package for Windows (SPSS Inc., Chicago, IL, U.S.A). For benzene in air a value corresponding to one-half of the quantification limit was assigned to measurements below analytical quantification (6 μg/m^3). For urinary benzene and *t,t*-MA the geometric mean of two determinations (BS and ES) was used for statistical evaluations. Subjects with cotinine level below 100 μg/L were arbitrarily classified as "non smokers", while subjects with cotinine level equal to or above of 100 μg/L were classified as "smokers". Statistical analysis was performed on decimal log transformed variables for benzene in air, urinary benzene, *t,t*-MA, SPMA, and urinary cotinine to assure normal distribution; global DNA methylation, Alu and LINE-1 methylation, that were normally distributed, were used untransformed. For global DNA methylation one particularly low value (4.665 %5mC) was dropped as outlier. Student's t-test was applied to compare two groups of independent samples, and Pearson's correlations to test the associations between variables. Multiple linear regression models, adjusted for gender and age, were used to evaluate the effect of benzene exposure indices (air benzene or biomarkers) or exposure group (general population subjects=0, gasoline station attendants=1) on the level of DNA methylation. A p value <0.05 was considered significant.

3. Results

3.1. Study participants

Some characteristics of study subjects are summarized in Table 1. Sixty-nine (88.5%) gasoline station attendants were males, and 9 females (11.5%); mean age was 42 years (from 19 to 75). Thirty-nine (67.2%) controls were males, and 19 were females (32.8%); mean age was 39 years (from 25 to 66). No statistically significant difference between groups was found for age while the preva-

Table 1 - Summary of selected characteristics of the study subjects, exposure markers and DNA methylation indices

	Statistic	General population subjects	Gasoline station attendants	p
N. of subjects	N	58	78	-
Age† (years)	Mean±SD	39±11	42±13 [A]	0.077
Gender				
Female	N (%)	19 (32.8)	9 (11.5)	0.002
Male	N (%)	39 (67.2)	69 (88.5)	
Cigarette smoking				
Smokers (%)	N (%)	21 (36.2)	31 (39.7)	0.675
Cigarettes/die in smokers	Mean±SD	11±9	17±9 [A]	0.047
Urinary cotinine in smokers (μg/L)	Median (5th-95th perc)	536 (198-1578)	798 (287-2393) [A]	0.033
Air benzene (μg/m^3)	Mean±SD	13±18	101±94 [A]	<0.001
	Median (5th-95th perc)	6 (<6-47)	61 (14-301)	
	N valid	58	78	
	% N>LOQ	57%	100%	
Urinary benzene (ng/L)	Mean±SD	398±634	1055±1044 [A]	<0.001
	Median (5th-95th perc)	170 (61-1543)	711 (172-3514)	
	N valid	58	78	
	% N>LOQ	100%	100%	
t,t-MA (μg/L)	Mean±SD	62±73	93±99 [A]	0.009
	Median (5th-95th perc)	32 (<10-258)	54 (<10-301)	
	N valid	57	78	
	% N>LOQ	82%	95%	
SPMA (μg/L)	Mean±SD	5.7±4.1	6.5±4.4	0.347
	Median (5th-95th perc)	5.0 (0.2-12.7)	6.6 (0.2-11.9)	
	N valid	56	65	
	% N>LOQ	100%	100%	
Global DNA methylation (%5mC)	Mean±SD	5.474±0.083[B]	5.409±0.142	0.001
	Median (5th-95th perc)	5.489 (5.314-5.582)	5.419 (5.158-5.614)	
	N valid	58	77	
	% N>LOQ	100%	100%	
Alu methylation (%5mC)	Mean±SD	27.3±3.4	26.4±2.5	0.080
	Median (5th-95th perc)	26.7 (23.1-35.4)	25.8 (23.1-31.1)	
	N valid	58	72	
	% N>LOQ	100%	100%	
LINE-1 methylation (%5mC)	Mean±SD	65.7±5.2 [B]	62.2±6.6	
	Median (5th-95th perc)	64.5 (59.0-74.9)	62.6 (53.5-72.7)	0.003
	N valid	51	61	
	% N>LOQ	100%	100%	

[A] gasoline station attendants higher than general population subjects
[B] general population subjects higher than gasoline station attendants

lence of male subjects was higher in gasoline station attendants than in controls. According to urinary cotinine, 31 gasoline station attendants (39.7%) and 21 controls (36.2%) were classified as smokers. No statistically significant difference between groups was found for smoking status, but smoking intensity, evaluated using both cigarette/day and urinary cotinine was higher in gasoline station attendants.

3.2. Levels of exposure and DNA methylation in gas station attendants and controls

The levels of air benzene, urinary benzene, *t,t*-MA, SPMA and DNA methylation by exposure group are summarized in Table 1. Air benzene was quantifiable in all gasoline station attendants and in 57% of controls, with median levels of 61 and 6 ng/m^3, respectively. Higher levels of air benzene, urinary benzene and *t,t*-MA, but not of SPMA, were detected in gasoline station attendants than in controls. Conversely, lower global DNA methylation was detected in gasoline station attendants relative to controls, with mean levels of 5.409 vs. 5.474 %5mC, respectively (p=0.001). Similarly, gasoline station attendants showed significantly lower LINE-1 methylation (p=0.003), but not Alu methylation (p=0.080), than controls.

While smoking was found to significantly impact all benzene biomarkers (p<0.001 for urinary benzene, *t,t*-MA and SPMA), no influence on DNA methylation was observed (p values from 0.222 to 0.771).

We found a significant association of gender with global DNA methylation with higher level in males than in females, both considering the entire study population (mean 5.460 %5mC in males vs. 5.349 5mC% in females, p <0.001) and dividing the study participants in gasoline station attendants (5.430 5%mC in males vs. 5.254 5mC% in females, p<0.001) and controls (5.513 5%mC in males vs. 5.394 %5mC in females, p<0.001) (figure 1). Considering all subjects, Alu and LINE-1 were only marginally influenced by gender, with males having lower levels than females (Alu 26.5 %5mC in males vs. 28.1 %5mC in females, p=0.058; LINE-1: 63.3 5%mC in males vs. 65.7 %5mC in females and 0.092). After dividing the subjects according to the exposure group, Alu and LINE-1 were similar in male and female gasoline station attendants (Alu: 26.5 5%mC in males vs. 26.3 %5mC in females, p=0.839; LINE-1: 62.3 %5mC in males vs. 61.4 %5mC in females, p 0.720), and significantly or borderline significantly lower in males than in females among controls (Alu: 26.6%mC in males vs. 28.9 %5mC in females, p=0.040; LINE-1: 64.8 %5mC in males vs. 67.8 %5mC in females, p 0.057).

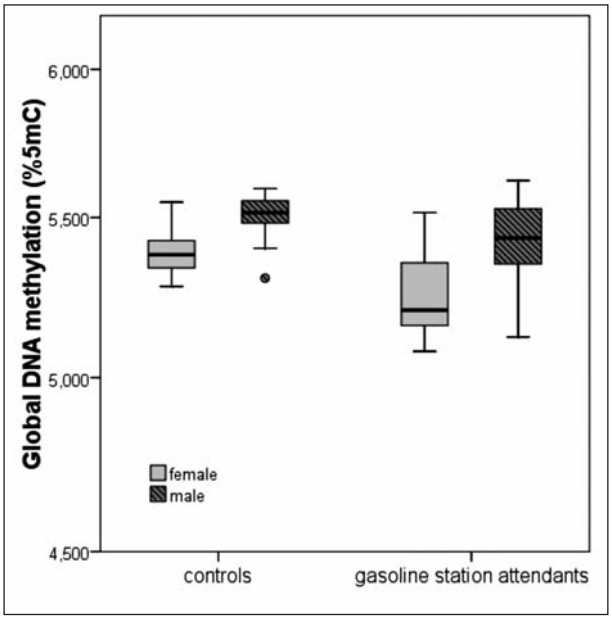

Figure 1 - Global DNA methylation in study subjects divided by exposure group and gender

3.3. Correlations among exposure and DNA methylation markers

Table 2 shows the correlations among exposure and DNA methylation markers, as well as the correlations of these markers with age. Air benzene was positively correlated with urinary benzene and *t,t*-MA, and showed a borderline significant association with SPMA. The urinary biomarkers of benzene exposure were all correlated with each other, as well as with urinary cotinine.

All the DNA methylation markers were negatively correlated with air benzene; the scatter plot

Table 2 - Pearson's correlation analysis between the investigated parameters

		Urinary benzene (ng/L)	t,t-MA (µg/L)	SPMA (µg/L)	Global DNA methylation) (%5mC)	Alu methylation (%5mC)	LINE-1 methylation (%5mC)	Age (year)	Cotinine (µg/L)
Air benzene ($\mu g/m^3$)	Pearson Correlation	0.491**	0.209*	0.156	-0.252**	-0.234**	-0.305**	0.052	0.088
	Sig. (2-tailed)	<0.001	0.015	0.088	0.003	0.008	0.001	<.550	0.308
	N	136	135	121	135	129	112	136	135
Urinary benzene (ng/L)	Pearson Correlation		0.356**	0.407**	-0.053	-0.102	-0.174	0.158	0.545**
	Sig. (2-tailed)		<0.001	<0.001	0.540	0.252	0.066	0.067	<0.001
	N		135	121	135	129	112	136	135
t,t-MA (µg/L)	Pearson Correlation			0.395**	0.029	-0.247**	-0.219*	-0.050	0.410**
	Sig. (2-tailed)			<0.001	0.735	0.005	0.021	0.565	<0.001
	N			121	134	128	111	135	135
SPMA (µg/L)	Pearson Correlation				0.054	-0,015	-0.158	0.104	0.381**
	Sig. (2-tailed)				0.558	0.876	0.123	0.255	<0.001
	N				120	115	97	121	121
Global DNA methylation (%5mC)	Pearson Correlation					0.069	0.152	-0.195*	0.057
	Sig. (2-tailed)					0.438	0.110	0.024	0.515
	N					128	112	135	134
Alu methylation (%5mC)	Pearson Correlation						0.347**	-0.045	-0.065
	Sig. (2-tailed)						<0.001	0.614	0.467
	N						110	129	128
LINE-1 methylation (%5mC)	Pearson Correlation							-0.043	-0.128
	Sig. (2-tailed)							0.649	0.181
	N							112	111
Age (year)	Pearson Correlation								-0.010
	Sig. (2-tailed)								0.904
	N								135

*correlation is significant at the 0.05 level (2-tailed), ** correlation is significant at the 0.01 level (2-tailed)

and the linear regression line between global DNA methylation and air benzene is reported in Figure 2. Interestingly, Alu and LINE-1 methylation levels were also correlated with urinary *t,t*-MA, but not with urinary benzene and SPMA, even if a marginal negative correlation between urinary benzene and LINE-1 was observed (p=0.066). Global DNA methylation, but not Alu and LINE-1 methylation, showed a significant negative association with age. Alu and LINE-1 methylation were positively correlated with each other, but none of them was correlated with global DNA methylation (table 2).

Because of the associations of age and sex with DNA methylation that we identified, we further evaluated the associations of exposure indices with DNA methylation by means of multivariable linear regression models adjusted for gender and age (Table 3). Markers of methylation were considered as dependent variables, while benzene exposure indices (air benzene or biomarkers) or exposure group (general population subjects=0, gasoline sta-

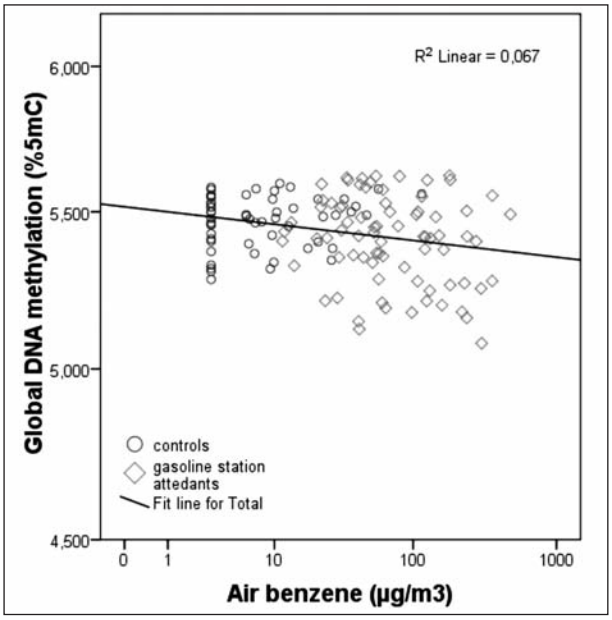

Figure 2. Scatter plot and linear regression line for global DNA methylation vs. air benzene

tion attendants=1) were considered as independent variables.

Considering air benzene as exposure metric, the negative correlation with DNA methylation was confirmed for all indices. In particular for global DNA methylation the model explained 27.4% of the variability: gender was the most relevant predictive factor, with males showing higher levels than females, followed by airborne benzene exposure; a smaller significant effect was found for age. For Alu methylation, the model explained 8.5% of the variability: air benzene was the most significant predictive factor, and gender showed a moderate significant effect; no significant impact of age was found. For LINE-1 methylation, the model explained 10.2% of the variability: only air benzene was a significant predictive factor.

Notably global DNA methylation and LINE-1 methylation, but not Alu methylation, showed a negative correlation also with "exposure group" (general population subjects=0, gasoline station attendants=1). Global DNA methylation, adjusted for age and gender, decreased of 1.6% in gasoline station attendants compared to controls.

Finally, in the model for global DNA methylation, fitting any of the urinary biomarkers available in the present study in place of air benzene, did not give any significant contribution, even if a negative slope was always found. Conversely, in the case of Alu methylation, the introduction of *t,t*-MA in the model showed a negative significant association (p=0.020), and yielded to a slightly increased coefficient of determination for the whole model (R^2=0.092). In the case of LINE-1 methylation, again the introduction of *t,t*-MA in the model showed a negative significant association (p=0.050), even if the model determination coefficient was decreased (R^2=0.064).

4. Discussion

In the present study, personal exposure to airborne benzene in gasoline station attendants and controls was associated with a decrease of global DNA methylation. This finding is in the same direction as our previous findings for DNA methylation of Alu and LINE-1 repetitive elements in a larger sample of this same population (4). This result is also consistent with the majority of previous studies in which a decrease of global DNA methylation was observed following exposure to several environmental pollutants (1). As global DNA hypo-methylation has been consistently demonstrated in leukemia cells, the present work supports the hypothesis that DNA methylation could represent a marker of early biological effects induced by environmental benzene exposure.

The levels of global DNA methylation in human peripheral blood cells detected in this study (about 5.5 %5mC) were comparable with our previous findings (35). These levels are also comparable to some previous studies that reported global DNA methylation in peripheral blood leukocytes between 5.0-6.0 %5mC (14, 37) although other investigations found levels higher than 4.0-5.0 %mC (reviewed in 24). These differences could be attributed to slightly different DNA sources (whole blood/leukocytes/lymphocytes) and/or also different analytical techniques; in fact the majority of liquid chromatography-triple mass spectrometry assays yielded values below 5.0 %5mC (24), while higher values were mostly obtained via GC-MS (35).

Table 3 - Multiple linear regression analysis to evaluate DNA methylation indices (dependent variable) as a function of benzene exposure markers (independent variables), after adjustment for gender and age

Dependent variable	Independent variable	Gender and age adjusted			
		β	SE	*partial r*	*p*-value
global DNA methylation (%5mC)	general population subjects = 0	-0.088	0.019	-0.370	<0.001
Alu methylation (%5mC)	gasoline station attendants = 1	-0.563	0.543	-0.092	0.301
LINE-1 methylation (%5mC)		-3.235	1.209	-0.249	0.009
global DNA methylation (%5mC)	Air benzene (μg/m^3)	-0.00044	0.0001	-0.327	<0.001
Alu methylation (%5mC)		-0.912	0.421	-0.190	0.032
LINE-1 methylation (%5mC)		-2.770	0.926	-0.277	0.003
global DNA methylation (%5mC)	Urinary benzene (ng/L)	-0.020	0.021	-0.080	0.359
Alu methylation (%5mC)		-0.438	0.566	-0.069	0.440
LINE-1 methylation (%5mC)		-2.001	1.242	-0.153	0.110
global DNA methylation (%5mC)	t,t-MA (μg/L)	-0.021	0.021	-0.084	0.340
Alu methylation (%5mC)		-1.270	0.538	-0.208	0.020
LINE-1 methylation (%5mC)		-2.493	1.255	-0.189	0.050
global DNA methylation (%5mC)	SPMA (μg/L)	-0.003	0.019	-0.017	0.856
Alu methylation (%5mC)		0.161	0.510	0.031	0.748
LINE-1 methylation (%5mC)		-1.423	1.104	-0.132	0.201

β=slope; SE=standard error

Although significant, the decrease of global DNA methylation attributable to occupational exposure as gasoline station attendant was as little as 1.6%; this decrease is much smaller than that observed in bone marrow samples from leukemia patients, for which, using the same assay, we determined a median global DNA methylation of 3.58 %5mC in bone marrow cells, with an overall decrease of 38% in comparison with healthy subjects (35).

When predictors of DNA methylation were considered, we found that by introducing the categorical variable "exposure group" instead of the continuous variable "benzene exposure" in the multiple linear regression models, we obtained models that explained a comparable percentage of variability. This suggests the relevance of working as a gasoline station attendant, which entails exposure to a complex mixture of chemicals (gasoline vapors and automotive exhaust fumes), rather than pointing to a specific association with benzene. Consistently, we found no correlation of DNA methylation (all markers) with urinary benzene or SPMA, widely regarded as the most specific biomarkers of benzene exposure.

However, we found significant negative correlations of Alu and LINE-1 methylation, but not global DNA methylation, with *t,t*-MA. This finding may be explained by postulating that benzene effects on DNA methylation are mediated by muconaldehydes, metabolic precursors of *t,t*-MA, possibly involved in benzene toxicity due their strong oxidative action and high reactivity (3). Oxidative stress has been consistently shown to induce DNA de-methylation. In fact, oxidative attack leads to the generation of DNA strand breaks and the formation of modified bases such as 8-hydroxyl-2′-deoxyguanosine and O^6-methylguanine, both reported to interfere with the DNA's ability to act as a substrate for DNA methyltransferases (13). Furthermore, recent studies indicate that benzene in humans is metabolized according to a two-enzyme model, and that the production of *t,t*-MA follows a supralinear kinetics, with a greater than

proportional formation at low exposures, while the same behavior is not observed for SPMA (32, 33). Due to the low benzene exposure in our study subjects, the two-enzyme model could help explain the correlation found for *t,t*-MA and not for SPMA. Taken together, our results suggest an oxidative mechanism involved in Alu and LINE-1 methylation changes induced by benzene, and point to *t,t*-MA as a biomarker associated to oxidative damage.

When tobacco smoking was evaluated as a possible predictor of DNA methylation, no significant association was found, either when considering smoking status or using urinary cotinine as a metric of smoking. Although this finding is consistent with previous observations by us and others (4, 29, 36), it remains hard to interpret; smoking is widely recognized as the main source of benzene body burden in the general population, as well as and also in the investigated gasoline station attendants (17); moreover cigarette smoking is associated with the exposure to thousands of chemicals, many of which are carcinogens and/or induce oxidative stress.

Gender was a significant predictor of global DNA methylation in our data, with females showing a lower level than males. This between-gender difference is consistent with previous observations also showing lower DNA methylation in females compared to males (16, 38). Fuke and coworkers (16) have previously remarked that the lower global DNA methylation level found in female individuals is somewhat surprising considering that X chromosome inactivation in females is mediated by DNA hypermethylation, and suggested that hormonal effects, such as prolactin-induced hypermethylation (34), might account for such difference. Alu methylation showed a weaker association with gender that, however, was opposite in direction (i.e., higher methylation in females). Zhu et al. have also recently shown in a combined analysis of 1,465 individuals from 5 studies that Alu methylation in peripheral blood DNA is higher in females than in males (43). The higher levels of Alu methylation in females has been suggested to depend on the higher density of Alu sequences on the X chromosome (19), which might be hypermethylated in female individuals.

Aging is another predictive factor resulted related to the decrease of global DNA methylation in the present study. From the multiple linear regression analysis we estimated a decrease rate of 0.003% for year in global DNA methylation. Several studies reported an effect of age on DNA methylation (5, 8, 16, 37), and suggested that DNA hypomethylation through aging could contribute to determine age-related chromosome instability (27).

One relevant finding of our study is the lack of correlation that we found of global DNA methylation with either Alu or LINE-1 methylation. Few previous studies are available for comparison: a first work reported good correlations of global DNA methylation in a combined analysis of tissues of different origin, including both normal and cancer tissues (methylation range: 2.88-4.26 %5mC) with Alu, LINE-1 or Sat2 methylation (39). More recently, Choi et al reported no correlation between global DNA methylation (mean levels 3.98 and 4.33 %5mC), and LINE-1 methylation (6) in peripheral blood DNA from 19 breast cancer patients and 18 matched controls. The differences among studies could be attributed to the methylation range width, with significant correlations in the case of a wide range, such as in analysis including both normal and cancer tissues, and a lack of correlation in the case of a narrower range. Cancer cells might undergo profound demethylation of Alu and LINE-1 repetitive elements that parallels and/or contributes to the well-established global hypomethylation commonly observed in normal tissues. We speculate that, in non-malignat tissues from healthy subjects, Alu and LINE-1 are not appropriate estimators of global DNA methylation.

In conclusion, our results offer new evidence of the relationship between environmental exposure to benzene and DNA methylation in humans. The associations we observed with specific exposure biomarkers suggest that benzene-induced oxidative stress is a possible mechanism for DNA de-methylation.

NO POTENTIAL CONFLICT OF INTEREST RELEVANT TO THIS ARTICLE WAS REPORTED

References

1. Baccarelli A, Bollati V: Epigenetics and environmental chemicals. Curr Opin Pediatr 2009; *21:* 243-251
2. Bird AP: CpG-rich islands and the function of DNA methylation. Nature 1986; *321*: 209-313
3. Bleasdale C, Kennedy G, MacGregor JO, et al: Chemistry of muconaldehydes of possible relevance to the toxicology of benzene. Environ Health Perspect 1996; *104* (Suppl 6): 1201-1209
4. Bollati V, Baccarelli A, Hou L, et al: Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer Res 2007; *67*: 876-880
5. Bollati V, Schwartz J, Wright R, et al: Decline in genomic DNA methylation through aging in a cohort of elderly subjects. Mech Ageing Dev 2009; 130: 234-239
6. Choi J-Y, James SR, Link PA, et al: Association between global DNA hypomethylation in leukocytes abd risk of breast cancer. Carcinogenesis 2009; *30*: 1889-1897
7. Deneberg S, Grövdal M, Karimi M, et al: Gene-specific and global methylation patterns predict outcome in patients with acute myeloid leukemia. Leukemia 2010; *24*: 932-941
8. Drinkwater RD, Blake TJ, Morley AA, Turner DR: Human lymphocytes aged in vivo have reduced levels of methylation in transcriptionally active and inactive DNA. Mutat Res 1989; *219*: 29-37
9. Ehrlich M: DNA methylation in cancer: too much, but also too little. Oncogene 2002; *21*: 5400-5413
10. Esteller M: Aberrant DNA methylation as a cancer-inducing mechanism. Ann Rev Pharmacol Toxicol 2005; *45:* 629-656
11. European Commission, 2004. Communication from the Commission to the Council, the European Parliament, the European Economic and Social Committee. "The European Environmental & Health and Action Plan 2004-2010" SEC 729. Brussels, Belgium
12. European Commission, 2008. Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe. European Commission, Brussels, Belgium; 2008. Available at: http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:152:0001:0044:EN:PDF [accessed 12 June 2011]
13. Franco R, Schoneveld O, Georgakilas AG, Panayiotidis MI: Oxidative stress, DNA methylation and carcinogenesis. Cancer Lett 2008; *266*: 6-11
14. Friso S, Choi S-W, Dolnikowski GG, Selhub J: A method to assess genomic DNA methylation using high-performance liquid chromatography/electrospray ionization mass. Anal Chem 2002; *74*: 4526-4531
15. Frühwald MC: DNA methylation patterns in cancer: novel prognostic indicators? Am J Pharmacogenomics 2003; *3*: 245-260
16. Fuke C, Shimabukuro M, Petronis A, et al: Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: an HPLC-based study. Ann Hum Genet 2004; *68*: 196-204
17. Fustinoni S, Consonni D, Campo L, et al: Monitoring low benzene exposure: comparative evaluation of urinary biomarkers, influence of cigarette smoking, and genetic polymorphisms. Cancer Epidemiol Biomarkers Prev 2005; *14*: 2237-2244
18. Glass DC, Gray CN, Jolley DJ, et al: Leukemia risk associated with low level benzene exposure. Epidemiology 2003; *14*: 569-577
19. Grover D, Mukerji M, Bhatnagar P, et al: Alu repeat analysis in the complete human genome: trends and variations with respect to genomic composition. Bioinformatics 2004; *20*: 813-817
20. International Agency for Research on Cancer (IARC): Benzene. In *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans,* Suppl 7. Lyon, France: IARC, 1987
21. Irizarry RA, Ladd-Acosta C, Wen B, et al: The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 2009; *41*: 178-186
22. Issa J-PJ, Baylin SB, Herman, JG: DNA methylation changes in hematologic malignancies: biologic and clinical implications. Leukemia 1997; *11* (Suppl 1): S7-S11
23. Ji Z, Zhang L, Peng V, Ren X, et al: A comparison of the cytogenetic alterations and global DNA hypomethylation induced by the benzene metabolite, hydroquinone, with those induced by melphalan and etoposide. Leukemia 20010; *24:* 986-991
24. Kok RM, Smith DE, Barto R, et al: Global DNA methylation measured by liquid chromatography-tandem mass spectrometry: analytical technique, reference values and determinates in healthy subjects. Clin Chem Lab Med 2007; *45*: 903-911
25. Lan Q, Zhang L, Li G, Vermeulen R, et al: Hematotoxicity in workers exposed to low levels of benzene. Science 2004; *306*: 1774-1776
26. Lyko F, Stach D, Brenner A, et al: Quantitative analysis of DNA methylation in chronic lymphocytic leukemia patients. Electrophoresis 2004; *25*: 1530-1535
27. Mazin AL: Suicidal function of DNA methylation in age-related genome disintegration. Ageing Res Rev 2009; *8*: 314-327
28. Melki JR, Clark SJ: DNA methylation changes in leukaemia. Semin Cancer Biol 2002; *12*: 347-357
29. Moore LE, Pfeiffer RM, Poscablo C, et al: Genomic

DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case-control study. Lancet Oncol 2008; *9*: 359-366
30. National Industrial Chemical Notification and Assessment Scheme (NICNAS), 2001. Benzene. In Priority Existing Chemical Assessment No. 21. Sydney, Australia
31. Paulsen M, and Ferguson-Smith AC: DNA methylation in genomic imprinting, development, and disease. J Pathol 2001; *195*: 97-110.
32. Rappaport SM, Kim S, Lan Q, et al: Human benzene metabolism following occupational and environmental exposures. Chem Biol Interact 2010; *184*: 189-195
33. Rappaport SM, Kim S, Lan Q, et al: Evidence that humans metabolize benzene via two pathways. Environ Health Perspect 2009; *117*: 946-952
34. Reddy PM and Reddy PR: Effect of prolactin on DNA methylation in the liver and kidney of rat. Mol Cell Biochem 1990; *95*: 43-47
35. Rossella F, Polledri E, Bollati V, et al: Development and validation of a gas chromatography/mass spectrometry method for the assessment of genomic DNA methylation. Rapid Commun Mass Spectrom 2009; *23*: 2637-2646
36. Rusiecki JA, Baccarelli A, Bollati V, et al: Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. Environ Health Perspect 2008; *116*: 1547-1552
37. San Romerio A, Fiorillo G, Terruzzi I, et al: Measurement of DNA methylation using stable isotope dilution and gas chromatography-mass spectrometry. Anal Biochem 2005; *336*: 158-163
38. Shimabukuro M, Sasaki T, Imamura A, et al: Global hypomethylation of peripheral leukocyte DNA in male patients with schizophrenia: a potential link between epigenetics and schizophrenia. J Psychiatr Res 2007; *41*: 1042-1046
39. Weisenberger DJ, Campan M, Long TI, et al: Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 2005; *33*: 6823-6836
40. Wilson AS, Power BE, Molloy PL: DNA hypomethylation and human diseases. Biochim Biophys Acta 2007; *1775*: 138-162
41. Xing C, Marchetti F, Li G, et al: Benzene exposure near the U.S. permissible limit is associated with sperm aneuploidy. Environ Health Perspect 2010; *118* (6): 833-839
42. Yang AS, Estécio MR, Doshi K, et al: A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 2004; *32*: e38.
43. Zhu ZZ, Hou L, Bollati V, et al: A predictors of global methylation levels in blood DNA of healthy subjects: a combined analysis. Int J Epidemiol 2010; *online ahead of press doi: 10.1093/ije/dyq154*