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## Research Article

# Hypermethylation in Gene Promoters Are Induced by Chronic Exposure to Benzene, Toluene, Ethylbenzene and Xylenes

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## Abstract

**Background and Objective:** Gas station attendants are occupationally exposed to benzene, toluene, ethylbenzene and xylene (BTEX) compounds and thus more susceptible to the biological effects of this mixture present in gasoline, especially due to the carcinogenicity of benzene. Furthermore, the harmful effects of BTEX exposure may be potentiated by genetic and epigenetic inactivation of critical genes. The objective was to evaluate such gene-BTEX interactions accessing the promoter methylation status of *p14<sup>ARF</sup>*, *p16<sup>INK4A</sup>* and *GSTP1* in peripheral blood leukocyte samples. **Materials and Methods:** The 59 exposed and 68 unexposed participants from Rio de Janeiro, Brazil, were included. The promoter methylation status was accessed by methylation-specific PCR (MSP) and *GSTP1* Ile105Val polymorphism was investigated by PCR-restriction fragment length polymorphism (PCR-RFLP) technique. **Results:** Both *p14<sup>ARF</sup>* and *p16<sup>INK4A</sup>* were significantly hypermethylated in exposed subjects compared to unexposed ( $p = 0.004$  and  $p < 0.001$ , respectively). Additionally, *p16<sup>INK4A</sup>* hypermethylation in the exposed group was correlated with chromosomal abnormalities (CAs) ( $p = 0.018$ ), thus highlighting the influence of the gene-environment interactions on genome instability. Noteworthy, *p16<sup>INK4A</sup>* methylation was significantly associated with miscarriage among female attendants ( $p = 0.047$ ), in which those who reported miscarriage exhibited hypermethylation in at least 2 of the 3 genes analyzed. The *GSTP1* heterozygote genotype, which could affect the metabolism of benzene detoxification, was found in both groups but was more frequent in those occupationally exposed. No significant association was observed between *GSTP1* genotypes and methylation status. **Conclusion:** Together, these findings indicate that gas station attendants with the aforementioned epigenetic and genetic profiles may be at greater risk of occupational BTEX exposure-induced genome instability, which could require concerted efforts to establish more preventive actions and constant biomonitoring in gas station attendants.

**Key words:** Benzene, chromosomal, gasoline, methylation, polymorphism

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**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Benzene, toluene, ethylbenzene and xylene (BTEX) are aromatic hydrocarbons present in gasoline mixture, which are harmful, in special benzene. In Brazil, gas station attendants are occupationally exposed to BTEX, requiring genomic and health monitoring. Epigenetic and genetic biomarkers have been investigated to assess the effects of occupational and environmental exposure to chemical carcinogens on human health risks<sup>1</sup>. Such mechanisms can modulate expression of tumor suppressor genes and contribute to increased genetic instability<sup>2-4</sup>. The cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus located in chromosome 9p21 encodes two tumor suppressor proteins, p14<sup>ARF</sup> and p16<sup>INK4A</sup>, which play a key role in the cell cycle control<sup>5,6</sup>. Genetic and epigenetic inactivation of the *CDKN2A* tumor suppressor gene locus was associated with pathogenesis and progression of numerous neoplastic diseases, such as leukemia and breast cancer<sup>7-9</sup>.

Oxidation of benzene results in benzene oxide, a reactive intermediate whose detoxification is catalyzed by glutathione S-transferases (GSTs)<sup>10</sup>. The GST gene family encodes crucial metabolic enzymes of phase II involved in xenobiotic detoxification processes by promoting the conjugation of an electrophilic compound to the reduced glutathione (GSH), thereby protecting the intracellular environment from oxidative damage<sup>11</sup>. Hence, inter-individual genetic variability in GST genes may modulate their ability to eliminate carcinogenic compounds and contribute to increased susceptibility to DNA damage<sup>12,13</sup>. The GSTP1 enzyme is a cytosolic GST that exhibits genetic variability. The *GSTP1* Ile105Val polymorphism has been widely investigated due to its proven clinical relevance in oncology and pharmacogenetics<sup>14-16</sup>. The *GSTP1* Ile105Val polymorphism involves A to G nucleotide substitution (c.313A>G) in the coding sequence and leads to an amino acid change of isoleucine to valine at codon 105 (Ile105Val), resulting in reduced enzyme activity. Additionally, decreased *GSTP1* gene activity due to promoter hypermethylation has also been reported and may play a role during multistep cancer development<sup>17</sup>.

Therefore, the present study, it was evaluated the relationship between BTEX exposure and epigenetic/genetic changes and their potential influence on the health of gas station attendants. Methylation status in the promoter region of the *p14<sup>ARF</sup>*, *p16<sup>INK4A</sup>* and *GSTP1* genes was investigated in addition to examining the *GSTP1* Ile105Val polymorphism accessing peripheral blood leukocyte samples from BTEX exposed and unexposed populations in Rio de Janeiro, Brazil.

## MATERIALS AND METHODS

The study was carried out in the Department of Pathology, Circulating Biomarkers Laboratory from January, 2011-March, 2019. All the blood tests were carried out in Pedro Ernesto Hospital. The metaphases were taken to the Jena University Hospital, Institute of Human Genetics in Germany. The statistical analysis was performed in the Kennedy Faculties.

**Study population:** A total of 127 subjects were investigated in this study, including 59 gas station attendants (21 females and 38 males) and as control group 68 age-matched volunteers (30 females and 38 males) with no previous history of occupational exposure to BTEX. All participants were recruited at different gas stations located in the northern region of Rio de Janeiro, near the State University of Rio de Janeiro and the eligibility criteria for occupational BTEX exposure included current employment for at least 6 months at gas stations. This study was approved by the Ethics and Research Committee of the Pedro Ernesto University Hospital (CAAE: 34310014.9.0000.5259). Informed consent was obtained for all eligible subjects in the study and the participants were invited to answer a questionnaire about demographic and lifestyle characteristics (age, gender, tobacco smoking, alcohol and illicit drug consumption, etc.), as well as a medical and occupational history. Hematological indices (erythrogram, white blood cell and platelet counts) and biochemical analysis (hepatic and renal parameters) of all participants were performed at the hospital's laboratory.

**DNA methylation analysis:** Five milliliters of peripheral blood (anticoagulant substance EDTA) was obtained from each participant. Genomic DNA was extracted directly from peripheral blood leukocytes by the phenol-chloroform method and stored at -20°C until further analysis. DNA concentration was measured on a nanophotometer (NanoPhotometer® P330, IMPLLEN, Germany). The methylation-specific PCR (MSP) technique to analyze the methylation status in the promoter region of the *p14<sup>ARF</sup>*, *p16<sup>INK4A</sup>* and *GSTP1* genes was performed as previously described<sup>18</sup>.

Briefly, genomic DNA was bisulfate-treated using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA), according to the manufacturer's instructions. Afterwards, bisulfite-treated DNA was amplified for MSP reactions in a thermocycler (Applied Biosystems, CA, USA) using specific

primers for each promoter region of interest. Primers used for *p14<sup>ARF</sup>* and *p16<sup>INK4A</sup>* MSP reactions have been previously described and the thermocycling conditions for both consisted of a predenaturation at 94°C for 10 min, followed by 35 cycles at 94°C for 45 sec, 60°C for 45 sec and 72°C for 1 min, with final extension at 72°C for 7 min<sup>18,19</sup>. For *GSTP1*, primers used have been previously described and similar MSP cycling conditions were carried out, except for the annealing temperature (57°C)<sup>20</sup>. The Universal Methylated Human DNA Standard (Zymo Research, #D5011) was used as methylation positive control. Methylated and unmethylated MSP products were visualized on nondenaturing 10% polyacrylamide gels.

**GSTP1 genotyping:** The *GSTP1* Ile105Val (rs1695; A>G) polymorphism was analyzed by PCR restriction fragment length polymorphism (PCR-RFLP) assay, as previously described<sup>21</sup>. The thermocycling conditions consisted of a predenaturation at 95°C for 3 min, followed by 30 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, with final extension at 72°C for 10 min. Afterwards, PCR products were digested with *BsmAI* (3U) restriction endonuclease enzyme (New England Biolabs, NEB, UK) at 37°C for 2 h and then the fragments were visualized on nondenaturing 10% polyacrylamide gels. The size of digested PCR products for the genotypes homozygous wild-type (Ile/Ile, A/A), heterozygote (Ile/Val, A/G) and homozygous variant (Val/Val, G/G) were 294, 294/234/60 and 234/60 bp, respectively.

**Cytogenetic data:** A previous study conducted determined the frequencies of chromosomal abnormalities (CAs) in gas station attendants using fluorescence in situ hybridization (FISH) technique, in which subjects were divided into 2 groups ( $\leq 10$  CAs per 1,000 metaphases and  $> 10$  CAs per 1,000 metaphases)<sup>22</sup>. Here, cytogenetic data were available for 58/59 attendants from the present study: 29 attendants analyzed in a previous study<sup>22</sup> and the remaining 29 attendants with unpublished data<sup>23</sup>.

**Statistical analysis:** Contingency tables were used to associate the methylation status of each gene (*p14<sup>ARF</sup>*, *p16<sup>INK4A</sup>* and *GSTP1*) in the study groups with the following variables: gender, miscarriage occurrence, lifestyle factors (cigarette smoking status and alcohol consumption) and CAs. Pearson's chi-square ( $\chi^2$ ) or Fisher's exact tests were adopted to test the statistical significance of the association between methylation status and such variables. Associations between the duration of benzene exposure/time of employment (for gas station attendants) and either CAs or gender were evaluated by non-parametric Mann-Whitney test. The data were analyzed with IBM SPSS Statistics Version 20. For all statistical tests, a level of significance of 5% was considered ( $p < 0.05$ ).

## RESULTS

### Sociodemographic characteristics of the participants:

Demographic data of the studied groups are summarized in Table 1. The participants were predominantly male, but similar with respect to age between groups, in which the mean age was 36.3 ( $\pm 11.2$ ) years for exposed and 37.8 ( $\pm 11.9$ ) years for unexposed. The highest percentage of smokers and alcohol drinkers was observed in the group of attendants, with significant difference ( $p = 0.006$  and  $p = 0.004$ , respectively). Among exposed subjects, significant difference was observed in the mean duration of BTEX exposure at gas stations between male and female attendants ( $p = 0.010$ ) (Table 1). It is important to highlight that female attendants are usually young people of reproductive age and therefore the health risk due to BTEX exposure may have a significant impact, requiring special attention and so we compared the occurrence of miscarriage among women in this study. Although the highest frequency of miscarriage has been reported in the attendants, no significant difference between groups was found ( $p = 0.109$ ). Hematological and biochemical parameters were also evaluated in the both studied groups (Table 2).

Table 1: Demographic characteristics of the studied groups

Variables	Unexposed (n = 68)	Exposed (n = 59)	p-value
Duration of BTEX exposure (months) male/female (Mean $\pm$ SD)	NA	118.4 (111.8)/46.5 (39.8)	0.010 <sup>ab</sup>
<b>Cigarette smoking status</b>			
Current or former	12 (17.6%)	24 (40.7%)	0.006 <sup>ab</sup>
Never	56 (82.4%)	35 (59.3%)	
<b>Alcohol consumption</b>			
Yes	25 (36.8%)	37 (62.7%)	0.004 <sup>ab</sup>
No	43 (63.2%)	22 (37.3%)	
<b>Miscarriage experience</b>			
Yes	2 (6.7%)	5 (23.8%)	0.109 <sup>b</sup>
No	28 (93.3%)	16 (76.2%)	

SD: Standard deviation, NA: Not applicable, <sup>a</sup>Mann-Whitney test, <sup>b</sup> $\chi^2$  test, \* $p < 0.05$ : Represents a significant difference

Table 2: Hematological and biochemical parameters evaluated in the study population

Parameters	Control (n = 68)	Mean ± SD	Exposed (n = 59)	Mean ± SD
<b>Hematological parameters</b>				
RBC (million mL <sup>-1</sup> )	4.91	0.54	4.82	0.47
Hemoglobin (g dL <sup>-1</sup> )	14.53	1.98	14.14	1.41
Hematocrit (%)	42.87	4.29	41.62	3.64
WBC (cells μL <sup>-1</sup> )	7939.12	2283.65	7474.19	1750.32
Neutrophil (%)	56.46	12.04	57.30	8.16
Eosinophils (%)	2.21	2.50	2.62	1.90
Basophils (%)	0.56	0.90	0.41	0.34
Lymphocyte (%)	35.25	10.28	32.52	7.48
Monocytes (%)	5.51	2.10	7.25	2.10
Platelet (billion L <sup>-1</sup> )	263.06	57.05	255.90	54.62
<b>Hepatic biomarkers</b>				
AST (U L <sup>-1</sup> )	22.03	6.42	22.05	7.71
ALT (U L <sup>-1</sup> )	22.49	11.78	22.36	11.38
<b>Renal biomarker</b>				
Creatinine (mg dL <sup>-1</sup> )	0.91	0.19	0.86	0.17

SD: Standard deviation

Table 3: Methylation rates of *p14<sup>ARF</sup>*, *p16<sup>INK4A</sup>* and *GSTP1* in the studied group

Characteristics	<i>p14<sup>ARF</sup></i>				<i>p16<sup>INK4A</sup></i>				<i>GSTP1</i>			
	Unexposed (n = 68)		Exposed (n = 59)		Unexposed (n = 68)		Exposed (n = 59)		Unexposed (n = 68)		Exposed (n = 59)	
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
Overall	19	27.9%	32	54.2	12	17.6	31	52.5	31	45.6	21	35.6
	p = 0.004*				p < 0.001*				p = 0.281			
Smokers	1	8.3%	13	54.2	3	25	14	58.3	6	50	9	37.5
	p = 0.011*				p = 0.083				p = 0.499			
Non-smokers	18	32.1	19	54.3	9	16.1	17	48.6	25	44.6	12	34.3
	p = 0.049*				p = 0.002*				p = 0.384			
Alcohol drinkers	5	20	20	54.1	5	20	19	51.4	13	52	13	35.1
	p = 0.009*				p = 0.017*				p = 0.203			
Non-alcohol drinkers	14	32.6	12	54.5	7	16.3	12	55.5	18	41.9	8	36.4
	p = 0.112				p = 0.003*				p = 0.791			

\*p < 0.05: Represents a significant difference,  $\chi^2$  test

***p14<sup>ARF</sup>*, *p16<sup>INK4A</sup>* and *GSTP1* promoter hypermethylation:**

The *p14<sup>ARF</sup>* and *p16<sup>INK4A</sup>* methylation rates presented significant differences between groups, in which aberrant promoter hypermethylation was found, respectively, in 54.2 and 52.5% in the exposed group compared to 27.9 and 17.6% observed in the unexposed group (p = 0.004, p < 0.001, Table 3). Regarding *GSTP1* analysis, no significant difference between exposed and unexposed groups was found (p = 0.281). Methylation rates were also correlated with sociodemographic characteristics of the participants (Table 3). According to lifestyle factors, *p14<sup>ARF</sup>* methylation was significantly prevalent in the group of attendants who were smokers (54.2%) compared to the smokers in the unexposed group (8.3%) (p = 0.011). Interestingly, *p14<sup>ARF</sup>* and *p16<sup>INK4A</sup>* methylation rates among non-smokers were also significantly higher in the exposed group (p = 0.049 and p = 0.002, respectively).

Similarly, both *p14<sup>ARF</sup>* and *p16<sup>INK4A</sup>* methylation rates among the alcohol drinkers were higher in the exposed group (p = 0.009 and p = 0.017, respectively) and *p16<sup>INK4A</sup>* methylation was significantly prevalent in the exposed group among non-alcohol drinkers (p = 0.003).

Among female attendants, we also found that *p16<sup>INK4A</sup>* methylation rates were significantly associated with miscarriage experience (p = 0.047). Furthermore, all the 5 female attendants who reported miscarriage experience showed hypermethylation in at least 2 of the 3 genes analyzed. In contrast, none of the 2 unexposed women had detectable methylation for any of the 3 genes studied. Despite significant longer BTEX exposure in male attendants, this long-term exposure showed no significant differences between genders in methylation rates for any of the 3 genes analyzed.

Table 4: Correlation of chromosomal abnormalities (CAs) with *p14<sup>ARF</sup>*, *p16<sup>INK4A</sup>*, and *GSTP1* methylation rates and with *GSTP1* polymorphism in exposed subjects

Analysis	CAs				p-value
	Yes (n = 25)		No (n = 33)		
	Number	Percentage	Number	Percentage	
<i>p14<sup>ARF</sup></i> methylation	16.00	64.0	15.00	45.50	0.192 <sup>a</sup>
<i>p16<sup>INK4A</sup></i> methylation	18.00	72.0	13.00	39.40	0.018 <sup>*a</sup>
<i>GSTP1</i> methylation	8.00	32.0	12.00	36.40	0.786 <sup>a</sup>
<b>GSTP1 polymorphism</b>					
Ile/Ile	4.00	16.0	9.00	27.30	0.358 <sup>a</sup>
Ile/Val+Val/Val	21.00	84.0	24.00	72.70	
Duration of BTEX exposure (months) (Mean ±SD)	111.12	94.41	73.39	96.18	0.035 <sup>*b</sup>

SD: Standard deviation, \*p<0.05: Represents a significant difference, <sup>a</sup>χ<sup>2</sup> test, <sup>b</sup>Mann-Whitney test

**Combined effect of hypermethylation and CAs:** Cytogenetic data for 58 attendants were available<sup>22,23</sup>, 25/58 (43%) had some type of CAs. The highest frequencies of *p14<sup>ARF</sup>* and *p16<sup>INK4A</sup>* methylation were observed in the group with CAs detected (64 and 72%, respectively) compared to those without abnormalities (45.5 and 39.4%, respectively), in which *p16<sup>INK4A</sup>* methylation revealed a significant association with CAs between these exposed subjects (p = 0.018, Table 4). In contrast, *GSTP1* methylation rate did not differ significantly between groups (p = 0.786). Additionally, the group with CAs had a significantly longer occupational exposure to BTEX compared to those without alterations (p = 0.035).

**GSTP1 Ile105Val polymorphism:** To further investigate the factors that could affect the metabolism of benzene detoxification, the *GSTP1* Ile105Val polymorphism was determined by PCR-RFLP assay. Genotype frequencies of the *GSTP1* Ile105Val polymorphism were not in Hardy-Weinberg equilibrium in both exposed and unexposed populations. Since the homozygous variant genotype (Val/Val) was only detected in the exposed group, the Ile/Val and Val/Val genotypes were combined for the analysis. Overall, an enhanced rate (72%) of individuals carrying the allele variant was found in this study. Although the highest frequency was detected in the exposed group (76 vs. 68%), no significant difference in genotype distribution between groups was observed (p = 0.327). In addition, similar rates (36%) of *GSTP1* methylation were observed between subjects in the exposed group carrying the *GSTP1* Ile/Ile wild-type genotype (5/14) and those carrying the variant allele (16/45). Similarly, 9/22 (41%) of subjects carrying the *GSTP1* Ile/Ile genotype and 22/46 (48%) carrying the variant allele in the unexposed group showed *GSTP1* methylation. Furthermore, the *GSTP1* variant allele was detected in 84% of the subjects in the exposed group who presented CAs and in 72.7% of those without abnormalities. Despite this, the genotypic distribution showed no significant difference (p = 0.358, Table 4).

## DISCUSSION

Epigenetic inactivation by the DNA methylation process may be induced by BTEX exposure and therefore result in increased genomic instability, especially for those individuals occupationally exposed to these compounds such as gas station attendants<sup>2-4</sup>. Both *p14<sup>ARF</sup>* and *p16<sup>INK4A</sup>* are well characterized tumor suppressor genes and their epigenetic inactivation by hypermethylation could be an important molecular event with significant biological implications to trigger different malignancies<sup>7-9</sup>. In the current study, high rates of hypermethylation for both *p14<sup>ARF</sup>* and *p16<sup>INK4A</sup>* (54.2 and 52.5%, respectively) were detected in the blood samples from gas station attendants and this was significantly associated in the exposed subjects compared to those unexposed (27.9 and 17.6%). In concordance with these findings, Xing *et al.*<sup>24</sup> also observed higher *p16<sup>INK4A</sup>* methylation in individuals with clinical manifestation of benzene poisoning compared to a control group. Zhang *et al.*<sup>25</sup> described a gradual increase of *p14<sup>ARF</sup>* and *p16<sup>INK4A</sup>* methylation rates with cumulating working years in the blood samples of workers in a coke oven, an emitting source of polycyclic aromatic hydrocarbons<sup>25</sup>. Altogether findings highlight the effects of occupational BTEX exposure on DNA methylation dynamics in gas station attendants, in which the *CDKN2A* (*p14<sup>ARF</sup>/p16<sup>INK4A</sup>*) locus seems to be a potential target for epigenetic inactivation induced by BTEX.

The current study revealed significant difference in the duration of BTEX exposure between genders. However, the longer occupational exposure time found in male attendants was not accompanied by enhanced prevalence of methylation rates.

As aforementioned, occupational and lifestyle influences seemed to modify gene-specific methylation status in this work. It is important to highlight that the exposed group showed prevalent *p14<sup>ARF</sup>* and/or *p16<sup>INK4A</sup>* methylation rates compared to the unexposed group even among non-smokers

and non-alcohol drinking subjects. These findings could reinforce the potential influence of BTEX exposure on methylation rates even when other factors are excluded. Also interesting, the *p16<sup>INK4A</sup>* hypermethylation phenomenon among the exposed subjects was accompanied with the detection of CAs. It is possible that epigenetic inactivation in crucial genes induced by chronic BTEX exposure could play an important role in increasing genomic instability and mutagenic damage and thus result in CAs in gas station attendants.

Changes in placental DNA methylation levels can be induced by air pollutant exposure and lead to placental dysfunction<sup>26</sup>. Feto-maternal pathophysiology can be potentially affected by epigenetic alterations and result in recurrent pregnancy loss<sup>27</sup>. A previous report revealed a high risk (OR = 4.97) of miscarriage for female gas station attendants compared to the unexposed group<sup>28</sup>. In this present work, *p16<sup>INK4A</sup>* methylation rates were significantly associated with miscarriage among female attendants and all 5 attendants who reported miscarriage experience exhibited hypermethylation in at least 2 of the 3 genes analyzed. In contrast, none of the two unexposed women had detectable altered methylation patterns. Since attendants are outdoors in the workplace and thus exposed to atmospheric pollution in addition to be occupationally exposed to BTEX, prenatal exposure to such factors may have contributed to the methylation detection in attendants. Moreover, CAs are common findings in most miscarriages<sup>29</sup>. Here, cytogenetic data also revealed that 4/5 of these female attendants had a high frequency of CAs (>10 CAs per 1,000 metaphases). Together, increased genomic instability induced by *p16<sup>INK4A</sup>* epigenetic inactivation associated with CAs may have potentially facilitated miscarriages in these exposed women. However, other factors may also have contributed to this occurrence and would require more detailed analysis to establish the direct association between BTEX exposure and miscarriage in such cases.

The *GSTP1* Ile105Val polymorphism has been widely investigated due to its clinical relevance since it can affect the metabolism of benzene detoxification. Genotype frequencies of the *GSTP1* Ile105Val polymorphism were not in Hardy-Weinberg equilibrium in the study population, similar to other Brazilian studies performed in different cities<sup>30,31</sup>. Our results revealed a higher frequency of individuals carrying the *GSTP1* heterozygote genotype (Ile/Val) in both groups. Therefore, this genetic profile could be particularly worrisome for those occupationally exposed to benzene since it can result in lower detoxification activity of this carcinogenic compound and thus modulate the risk of illnesses associated

with BTEX exposure. No significant association was found between *GSTP1* genotypes and methylation status in both exposed and unexposed groups. Some studies have reported genotoxic effects associated with the *GSTP1* polymorphism and benzene exposure. Priya *et al.*<sup>12</sup> found significantly higher micronuclei frequencies in individuals carrying the *GSTP1* heterozygote and homozygous variant genotypes as compared to the wild-type genotype in both gasoline pump workers and control groups in India. Leng's group reported that the *GSTP1* homozygous variant genotype exhibited higher micronuclei frequencies in Chinese coke oven workers at a steel company<sup>32</sup>. In contrast, an Iran study conducted by Nourozi *et al.*<sup>33</sup> found no significant differences in the risk of hematological disorders among *GSTP1* genotypes among petrochemical plant employees occupationally exposed to benzene.

Although key epigenetic and genetic biomarkers have been evaluated to describe gene-BTEX interactions, some limitations of this study include the non-monitoring of the airborne BTEX levels at gas stations and the non-performance of genotoxicity assays. There is no official BTEX monitoring data provided by the Labor Union of Employees at Oil and Gas Derivatives Service Offices of the State of Rio de Janeiro (SINPOSPETRO-RJ) since they do not record these data. The gas stations' managers included in this study reported that routine air monitoring is not mandatory by law.

In Brazil, the concentration of benzene has been reduced to 1 ppm (3.19 mg m<sup>-3</sup>) as determined by the Benzene Legislation (NR 15, Annex 13-A) to prevent the health risks of subjects occupationally exposure to this compound<sup>34,35</sup>. However, biological and genotoxic effects of occupational exposure to BTEX are still observed in lower concentrations of benzene as reported by studies conducted in different cities in Brazil<sup>36</sup>. In a recent study conducted by Amaral *et al.*<sup>36</sup>, the concentrations of BTEX in the air were analyzed in 5 gas stations also in the city of Rio de Janeiro and located in regions similar to those in this study (northern region). The authors reported that even if the genotoxic risk was observed, BTEX concentration values were within the limits established in Brazil. This means that there is no safe concentration limit for benzene exposure when it is chronic.

## CONCLUSION

High rates of epigenetic inactivation by hypermethylation for both *p14<sup>ARF</sup>* and *p16<sup>INK4A</sup>* were found in the exposed group, in which *p16<sup>INK4A</sup>* hypermethylation was correlated with chromosomal abnormalities, thus highlighting the influence of the gene-environment interactions on genome instability.

Also,  $p16^{INK4A}$  hypermethylation was significantly associated with miscarriage among female attendants. More occupational protection and constant biomonitoring is necessary, especially for those subjects with the  $CDKN2A$  ( $p14^{ARF}/p16^{INK4A}$ ) hypermethylation pattern that could be defined as at-risk groups for the harmful effects of BTEX exposure.

### SIGNIFICANCE STATEMENT

This study discover that the promotor of tumor suppressor gene  $CDKN2A$  encoding for the proteins  $p14^{ARF}$  and  $p16^{INK4A}$  are significantly higher methylated in the blood of gas station workers who are occupational exposed to BTEX in Rio de Janeiro, Brazil. No other research was conducted with the gas station workers in Rio de Janeiro assessing the methylation of the tumor suppressor gene  $CDKN2A$  in the case-control method that was carefully constructed. This finding can be beneficial for the health surveillance of these workers. This study will help the researcher to uncover the critical areas of epigenetics and carcinogenesis and that many researchers were not able to explore in the target population. Thus a new theory on the epigenomics effects of the BTEX exposure in the target population may be arrived at.

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