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Gasoline-station workers in Brazil: Benzene exposure; Genotoxic and immunotoxic effects

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ABSTRACT

Chronic exposure to benzene is a risk factor for hematological malignancies. Gasoline-station workers are exposed to benzene in gasoline, via both inhalation and dermal contact (attendants and managers) or inhalation (workers in the on-site convenience stores and offices). We have studied the exposure of these workers to benzene and the resulting genotoxic and immunotoxic effects. Levels of urinary trans, trans-muconic acid were higher among gasoline-station workers than among office workers with no known exposure to benzene (comparison group). Among the exposed workers, we observed statistically significant biological effects, including elevated DNA damage (comet assay); higher frequencies of micronuclei and nuclear buds (CBMN assay); lower levels of T-helper lymphocytes and naive Th lymphocytes; lower CD4 / CD8 ratio; and higher levels of NK cells and memory Th lymphocytes. Both groups of exposed workers (inhalation and inhalation + dermal routes) showed similar genotoxic and immunotoxic effects.

1. Introduction

In developing countries, such as Brazil [1], occupational illnesses may be neglected and employees may not be informed about the risks of exposure to workplace toxicants. The appreciation of workplace risk (by workers, health professionals, and regulatory agencies) would be greater, were causal links to be established between the chemical exposures and the resulting adverse health outcomes [2]. There are several limitations to making such links: the toxic exposures are often to complex mixtures of substances; social determinants of health are often confounded with the exposures; and disease manifestations may become apparent only long after exposure [3,4].

In Brazil, gasoline-station workers fill fuel directly into motor vehicles; health agencies are concerned about this occupational exposure [1]. Chronic exposure of these workers to substances in the fuels puts them at increased risk of adverse health effects [5]. Job categories at filling stations include not only fuel filling per se, but also managerial work, lubrication, car-washing, and work in the associated convenience stores.

Gasoline is a complex mixture of volatile and flammable

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hydrocarbons derived from petroleum. Aromatic hydrocarbons, such as benzene, toluene, ethylbenzene, and xylene (BTEX), are present in the vapors supplied to, and exhausted from, engines [6]. Among these volatile compounds, benzene is classified as a known human carcinogen (Group 1) by the International Agency for Research on Cancer (IARC), with sufficient evidence for the development of acute myeloid leukemia in exposed workers, and positive associations with chronic myeloid leukemia, chronic lymphoid leukemia, non-Hodgkin's lymphoma, multiple myeloma, and lung cancer [7]. For this reason, the concentration of benzene in gasoline is limited to 1 % (v/v) in Brazil [8]. Occupational exposure to benzene at gas stations normally occurs at levels <1 part per million (ppm); nevertheless, this value is well above the typical environmental concentrations to which the general population is exposed (1−10 parts per billion, ppb) [9]. The use of benzene is controlled worldwide, since it is a mutagen and human carcinogen, with no safe limit of exposure [10,11].

Exposure of workers to fuels occurs mainly by inhalation, dermal, and oral routes. In terms of public health, inhalation exposure is most relevant, since this route provides rapid access to the bloodstream, which distributes toxicants to multiple tissues, especially those with high lipid content, where they may accumulate [12,13]. According to the US Centers for Disease Control and Prevention (CDC), chronic exposure to benzene primarily affects blood cells, with bone marrow being the main target [14]. For long-term exposures, the carcinogenic effect of benzene is already well established, as described in the IARC benzene monograph. Benzene metabolites are genotoxic, causing lesions in stem cells similar to those seen in hematopoietic cancers [7].

Smith et al. [15] identified ten key characteristics of human carcinogens. Among these, evidence strongly indicates that benzene is activated to electrophilic metabolites; induces oxidative stress and causes oxidative damage to DNA; is genotoxic, causing DNA damage and chromosomal changes; is immunosuppressive; and is hematotoxic [7, 15]. Studies show immunosuppression of cells of the acquired immune response [16–18] and innate immunity can also be induced, as observed in studies that evaluated natural killer cells, eosinophils, neutrophils, and monocytes [16,18–21]. Following occupational exposure to benzene, its effects on bone marrow cells can lead to malfunction of the immune system and thus compromise immunovigilance, favoring the development of hematological tumors. A relatively simple way to assess toxic effects on the immune system is to quantify relevant cells by flow cytometry.

The alkaline comet assay primarily assesses recent DNA damage, such as single-strand and double-strand breaks. Because these damages may be quickly repaired [22,23] it is informative to accompany the comet assay with the cytokinesis-block micronucleus (CBMN) assay, which enumerates acentric chromosomal fragments (clastogenic effects) and entire chromosomes (aneugenic effects) that are not included in the main nucleus during cell division [24,25]. We have studied exposure of gasoline station workers to benzene, genotoxic and immunotoxic effects in the workers, and the association between exposure and toxicity.

2. Materials and methods

2.1. Study design and population

The Brazilian National Cancer Institute (INCA) Human Research Ethics Committee (CEP/INCA No. 121/09) approved the study and all participants provided written informed consent.

This is a cross-sectional epidemiological study with the participation of workers from 21 gasoline stations in the city of Rio de Janeiro: 12 stations located in the South Zone and nine stations in the Central region. The sites were chosen for convenience and are not probabilistic. The participants were gasoline station workers in the metropolitan region, age ≥18 years, who had worked at the stations for >6 months, regardless of their job function. Workers exposed to ionizing radiation or chemotherapy in the previous 3 months were excluded. The workers were divided into two categories. Convenience store workers (CSW) (workers in the convenience stores and the administrative sectors of the service stations, located on the gas station premises) are exposed to fuels mainly by inhalation. Filling station attendants (FSA) include gas station attendants and gas station managers, whose work activities include supply, receipt, collection of fuel samples from tanker trucks, and reading the levels of the underground tanks; they may have both inhalational and skin exposure to benzene. Office workers (OW) at the National Cancer Institute José Alencar Gomes da Silva (INCA) and the Federal University of the State of Rio de Janeiro (UNIRIO), without reported occupational exposure to fuels, formed a comparison group.

The numbers of participants in each part of the trial were (Fig. 1): comet assay: 349 (100 OW; 95 CSW; 154 FSA); CBMN assay: 311 (110 OW; 75 CSW; 126 FSA); immunophenotyping assay: 334 (101 OW; 77 CSW; 156 FSA).

All urine samples from workers with creatinine values outside the range recommended by the American Conference of Governmental Industrial Hygienists – ACGIH (0.3–3.0 g/L) were excluded and quantitative analysis of trans,trans-muconic acid (t,t-MA) was not performed [26]. The numbers of samples excluded from each assay for this reason were as follows: comet assay: 1/21, OW group; 13/92, CSW group; 18/152, FSA group; CBMN assay: 1/26, OW; 11/72, CSW; 16/124, FSA; immunophenotyping assay: 3/26, OW; 7/75, CSW; 19/151, FSA; see Fig. 1.

2.2. Sociodemographic and occupational data; sample collection

Participants in this study (OW, CSW, and FSA) belonged to the morning (end of shift, 2 p.m.) or afternoon shift (end of shift, 10 p.m.). They answered a questionnaire regarding socio-demographic, lifestyle, and occupation information. The following variables were selected: sex, age, years working (1−10 y; >10−20 y; >20 y), alcohol consumption (no/ yes), smoking (non-smoker/ ex-smoker/ smoker), living with a smoker at home (no/ yes), working with a smoker (no/ yes).

A trained team administered the questionnaires. Qualified professionals collected blood samples, in vacuum tubes containing sodium heparin (genotoxicity test: 2 × 4 mL) and EDTA (immunotoxicity: 1 × 4 mL). Samples were placed in polystyrene boxes with ice packs and transported to the laboratory. Urine samples were collected in 50 mL Falcon tubes, at the end of the working day, and stored in the same conditions until transport to the laboratory, where they were aliquoted into three 15 mL Falcon tubes (for determination of creatinine and t,t-MA) and then stored at −20 °C.

2.3. trans,trans-Muconic acid analysis

Urinary creatinine was determined in triplicate by the modified Jaffé method [27] with the kit Bioclin K016 (Bioclin), before freezing the samples. Chromatographic analysis was based on the work of Ducos et al. [28] and performed on a Shimadzu HPLC with an isocratic pump, thermostatted chromatographic column (4.2 × 250 mm) Lichrosorb RP 18.5 μm (Merck), UV detector (λ =264 nm); ChemStation software. The mobile phase was 1% acetic acid/ methanol (90/10, v/v), pH 2.72, flow rate 1.0 mL/min (pressure of 80 bar). The oven temperature was 40 °C and the total run time was 12 min. From a standard stock solution of t, t-MA in methanol (100 μg/mL), intermediate solutions were prepared in mobile phase at concentrations 100.0; 50.0; 25.0; 12.5; and 3.12 μg/mL, and used to prepare the working solutions.

The solid phase extraction step required conditioning with 3 mL methanol and 3 mL ultrapure water. Urine, 1 mL, was added, followed by pre-wash with 1% acetic acid, 3 mL. Elution was performed with 10 % acetic acid (pH 2.7) and an aliquot, 20 μL, was injected into the HPLC. Analytical parameters established included linearity, equipment detection limit and quantification limit, biological matrix interference, precision, and recovery.

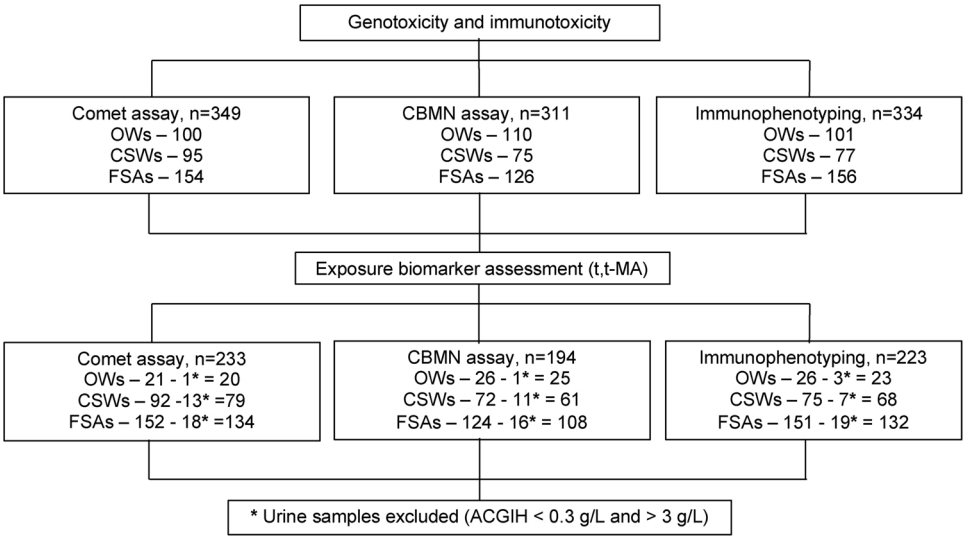

Fig. 1. Flow chart showing numbers of samples in each trial and group of exposure.

2.4. Comet assay

Before carrying out the assay, viability of the blood samples was determined using fluorescein diacetate (30 μg/mL) and ethidium bromide (8 μg/mL) in PBS, as described by Boechat et al. [29], modified. The blood sample (50 μL) was mixed with an equal volume of dye solution, which was then placed on a slide with a coverslip for analysis under a fluorescence microscope. For viability, 50 cells were analyzed, and percentage viability was calculated (viable cells were identified by green fluorescence, while nuclei stained orange indicated dead cells). All samples showed >92.5 % viability and were used in the comet assay.

For the alkaline comet assay, whole blood, 5 μL, was mixed with 0.5 % low-melting-point agarose (LMPA: Sigma-Aldrich) in PBS, 120 μL, at 37 °C and added to slides previously coated with 1.5 % normal-melting-point agarose (Sigma-Aldrich) in PBS. After the LMPA solidified, cells were lysed overnight, protected from light, at 4−6 °C (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% [w/v] N-lauroylsarcosine sodium salt, 1 % [v/v] Triton X-100 and 10 % [v/v] DMSO, pH 10). After lysis, the cells were submitted to alkaline treatment in a horizontal electrophoresis system (Bio-Rad) with alkaline buffer solution, pH > 13 (1 mM Na_2EDTA and 300 mM NaOH) for 20 min, in an ice bath. Next, electrophoresis was performed in an ice bath at 25 V (0.86 V/m) and 300 mA for 20 min. Slides were neutralized in 0.4 M Tris buffer solution, pH 7.5, through three washes, 5 min each, fixed in absolute ethanol for 10 min, and dried at room temperature overnight. The slides were stained with aqueous ethidium bromide (20 μg/mL) and analyzed under a fluorescence microscope (400× magnification) for DNA damage [30,31].

The extent of DNA migration was verified in 150 cells per individual, in three slides, by analyzing 50 cells per slide visually, according to the sizes of the comet tails, in four different classes: Class 0 (absence of tail); Class 1 (small tail); Class 2 (long tail); Class 3 (severely damaged). DNA damage was expressed as a percentage of cells in the four different classes and in an arbitrary unit (AU) according to the formula:

$$AU=[(M0 \times 0)+(M1 \times 1)+(M2 \times 2)+(M3 \times 3)];$$

where M represents the number of cells in each damage class [32].

2.5. Cytokinesis-block micronucleus (CBMN) assay

Whole blood (1 mL) was added to the culture medium: RPMI 1640 medium (Sigma-Aldrich), 5 mL; 20 % fetal bovine serum (Gibco); 4 % phytohemagglutinin (Cultilab). The samples (in duplicate) were kept in culture for 44 h at 37 °C, after which cytochalasin B (Sigma-Aldrich), 4 μg/mL, was added and incubated for a further 72 h. At the end of the culture period, samples were homogenized, centrifuged (800 rpm, 5 min), and supernatants discarded. Hypotonic treatment (KCl 0.075 M) was performed, followed by three fixation steps with methanol and acetic acid (3:1), intercalated by centrifugation (800 rpm, 5 min), and the supernatant discarded. Slides were stained with Giemsa solution, 5 %, for 5 min [24].

Analysis was conducted with an optical microscope, magnification 400× . Evaluation of cytotoxicity followed the recommendations of OECD 487 [33], through the determination of the Cytokinesis-block Proliferation Index (CBPI). The following formula was used: CBPI= (M1 + 2M2 + 3M3)/N; M1, M2, and M3 indicate the number of cells with one, two, or three or more nuclei, respectively; N is the total number of cells analyzed per replica (n = 500). 1000 cells were scored per participant.

Genotoxicity was also evaluated at 400× magnification, counting 1000 cells per replica, corresponding to 2000 cells per participant [24]. Cytogenetic damages evaluated were frequencies of binucleated cells with micronuclei (MN); total micronuclei in binucleated cells (tMN); nucleoplasmic bridges in binucleated cells (NPB); and nuclear buds in binucleated cells (NBUD). The results were expressed as frequency for 1000 cells evaluated.

2.6. Immunophenotyping

Blood samples (50 μL) were stained with monoclonal antibodies (mAb) conjugated to fluorochromes for 30 min at room temperature, in the dark. In all analyses, isotypic controls were used for each fluorochrome tested. After incubation in three antibody panels (Panel 1: CD8-FITC, CD19-PE, CD4-PerCP-Cy5.5, CD3-APC; Panel 2: CD56-PE, CD3-APC; Panel 3: CD45RO-FITC, CD45RA-PE, CD4-PerCP-Cy5.5, CD3-APC), samples were lysed with lysis buffer (BD Biosciences), 2 mL, 10 min, according to the manufacturer's instructions, and washed with Ca^{2+}- and Mg^{2+}-free PBS (Sigma-Aldrich) containing 0.1 % sodium azide. After centrifugation, the cell deposit was homogenized in PBS containing 0.5 % paraformaldehyde. The fixed cells were kept in the dark at 4 °C until acquisition.

Measurements (percentages) of populations of Natural Killer cells (% $CD56^+$), B lymphocytes (%$CD19^+$), T helper lymphocytes (% $CD3^+CD4^+$), T cytotoxic lymphocytes (%$CD3^+CD8^+$), naive T helper lymphocytes (%$CD3^+CD4^+CD45RA^+$), and memory T helper lymphocytes (%$CD3^+CD4^+CD45RO^+$) were done by flow cytometry, using the FACScalibur cytometer (Becton Dickinson, San Jose, CA, USA). For each

participant, at least 10^4 mononuclear cells were analyzed (Cell Quest Pro software; Becton Dickinson, San Jose, CA, USA).

2.7. Statistical analysis

The data obtained were stored in a safe. Statistical Package for the Social Sciences for Windows (SPSS), version 20.0 and GraphPad Prism software, version 4.02 were used for statistical analysis. A descriptive analysis of the characteristics of the study population was carried out based on socio-demographic variables, lifestyle, and occupational variables considering the three exposure groups, through the distribution of frequencies using the Chi-square test. Dispersion measures and central tendency of continuous variables were calculated. The normality of the distribution of continuous variables was analyzed using the Kolmogorov-Smirnov test. In the variables where a normal distribution was not found, the Kruskal-Wallis test with Dunn's multiple comparisons test was used. For parametric data, ANOVA with Bonferroni's multiple comparisons test was used.

For statistical analysis of the genotoxicity data, the percentages of cells in the four different classes of DNA damage and the total number of AU in the comet test were used. In the CBMN assay, the frequencies of MN, tMN, PNB, NBUD, and CBPI were used. The analysis of immunotoxicity used the frequencies of NK, B, T lymphocytes (helper and cytotoxic), naive and memory T helper, in addition to the helper and cytotoxic T lymphocyte ratio (CD4 / CD8 ratio).

To assess the association between exposure variables by work type (FSA, CSW, and OW) and genotoxic effects (comet and CBMN assays), bivariate logistical analyzes were performed. The outcomes (total number of AU (comet assay) and frequency of micronucleated cells, MN (CBMN assay) were categorized using the medians of the comparison group as baseline reference value (comet assay: AU reference category $\leq$6.0; CBMN assay: MN reference category $\leq$3.3). All covariables that were likely to reject an association with the two proposed variables (comet and CBMN assays) <0.20 were tested one at a time in the final models, using unconditional multivariate logistic regression analyzes. The variables sex, age, smoking, alcohol consumption, and working time are often related to genotoxic effects [33–36]; however, after testing insertion one by one, only those that were significant remained ($p \leq 10$ %).

Table 1
Socio-demographic and occupational characteristics of participants.

Variables[1]	Comet assay (n = 349)				CBMN assay (n = 311)				Immunophenotyping (n = 334)			
		Gas station workers[3]				Gas station workers[3]				Gas station workers[3]		
	Office workers[2] n = 100 (%)	Convenience store workers n = 95 (%)	Filling station attendants n = 154 (%)	p	Office workers[2] n = 110 (%)	Convenience store workers n = 75 (%)	Filling station attendants n = 126 (%)	p	Office workers[2] n = 101 (%)	Convenience store workers n = 77 (%)	Filling station attendants n = 156 (%)	p
Age, years[4]	41 (25–68)	29 (20–67)	37.5 (20–70)	**<0.001**	40 (22–68)	29 (20–67)	36 (30–70)	**<0.001**	40 (21–68)	30 (20–67)	36.5 (20–70)	**<0.001**
Sex[5]												
Men	53 (53.0)	27 (28.4)	140 (90.9)	**<0.001**	53 (48.2)	21 (28.0)	116 (92.1)	**<0.001**	50 (49.5)	17 (22.1)	139 (89.1)	**<0.001**
Woman	47 (47.0)	68 (71.6)	14 (9.1)		57 (51.8)	54 (72.0)	10 (7.9)		51 (50.5)	60 (77.9)	17 (10.9)	
Alcohol consumption[5]												
No	36 (36.0)	44 (46.3)	53 (34.4)	0.150	37 (33.6)	35 (46.7)	48 (38.1)	0.200	35 (34.7)	36 (46.8)	57 (36.5)	0.212
Yes	64 (64.0)	51 (53.7)	101 (65.6)		73 (66.4)	40 (53.3)	78 (61.9)		66 (65.3)	41 (53.2)	99 (63.5)	
Smoking[5]												
Non-smoker	69 (69.0)	77 (81.1)	105 (68.2)	**0.030**	79 (71.8)	64 (85.3)	88 (69.8)	**0.043**	73 (72.3)	63 (81.8)	106 (67.9)	0.052
Ex-smoker	22 (22.0)	13 (13.7)	24 (15.6)		21 (19.1)	7 (9.3)	18 (14.3)		20 (19.8)	10 (13.0)	25 (16.0)	
Smoker	9 (9.0)	5 (5.3)	25 (16.2)		10 (9.1)	4 (5.3)	20 (15.9)		8 (7.9)	4 (5.2)	25 (16.0)	
Live with a smoker at home[5]												
No	82 (88.2)	70 (74.5)	122 (79.2)	0.055	91 (87.5)	54 (73.0)	101 (80.2)	**0.050**	83 (86.5)	57 (75.0)	122 (78.7)	0.145
Yes	11 (11.8)	24 (25.5)	32 (20.8)		13 (12.5)	20 (27.0)	25 (19.8)		13 (13.5)	19 (25.0)	33 (21.3)	
Working with a smoker[5]												
No	67 (72.0)	68 (72.3)	85 (55.2)	0.055	77 (74.0)	56 (75.7)	71 (56.3)	**0.003**	72 (75.0)	57 (75.0)	83 (53.5)	**<0.001**
Yes	26 (28.0)	26 (27.7)	69 (44.8)		27 (26.0)	18 (24.3)	55 (43.7)		24 (25.0)	19 (25.0)	72 (46.5)	
Working time (years)[5]												
1–10	70 (70.0)	85 (91.4)	120 (77.9)	**0.003**	70 (68.6)	66 (89.2)	97 (77.0)	**0.012**	68 (70.8)	71 (93.4)	124 (79.5)	**0.002**
>10–20	18 (18.0)	8 (8.6)	20 (13.0)		20 (19.6)	8 (10.8)	19 (15.1)		17 (17.7)	5 (6.6)	14 (9.0)	
>20	12 (12.0)	0 (0.0)	14 (9.1)		12 (11.8)	0 (0.0)	10 (7.9)		11 (11.5)	0 (0.0)	18 (11.5)	

[1] In all variables where the total N of workers is fewer than indicated, they represent workers with answer "*does not know/did not answer*".
[2] Not occupationally exposed to benzene.
[3] Occupationally exposed to benzene.
[4] Variables expressed as Median (min-max).
[5] Variables expressed as absolute and percentage number of individuals. Chi-square test used for comparison between groups in categorical variables ($p \leq 0.05$).
CBMN assay: Cytokinesis-block micronucleus assay.

3. Results

Socio-demographic and occupational characteristics are shown in Table 1. Age, represented by median values (minimum and maximum), was higher for OW (about 40) in the three assays. The groups occupationally exposed to fuels (gas station workers) were composed of slightly younger workers, with the lowest median age about 29 for the CSW group. For all assays, FSA were mostly male, while CSW were mostly female. On the other hand, in the comparison group (OW), the distribution of men and women was more homogeneous in the three assays (Table 1**)**.

Most workers in the three groups, within the three assays, declared themselves to be non-smokers. The majority of workers in all groups in the three assays performed also reported not living or working with smokers. However, the FSA responded more homogeneously ("No"/"Yes") about working with smokers (Table 1). Most workers in the three groups studied had worked in the activity <9 years, for the three assays performed (Table 1).

To assess exposure to benzene, urinary t,t-MA assessment was performed on workers in the two groups occupationally exposed to fuels (CSW and FSA) and in the comparison group (OW). These results are described for the three assays performed (Tables 2–4) with the urinary t,t-MA levels in the groups occupationally exposed to benzene about twice as high as for the OW group. A statistical difference was found between workers exposed to benzene in the comet essay ($p \leq 0.05$), but no statistically significant difference was found between workers in the CBMN and immunophenotyping assays ($p \geq 0.05$).

Table 2 shows the comet assay results. The workers of the FSA and CSW groups had significantly higher DNA damage (Class 1) than the comparison group, with a difference ($p \leq 0.001$). The opposite effect was observed in the number of cells without DNA damage (Class 0): CSW and FSA presented the fewest cells in this category, the OW group more ($p \leq 0.001$). Although Class 2 DNA damage displayed an increasing trend among FSA compared to OW, this was not significant ($p = 0.071$). For total DNA damage in AU, FSA and CSW showed statistically significant differences ($p \leq 0.05$) from OW (Fig. 2).

The results of the CBMN assay are shown in Table 3. The FSA group had significantly the highest frequency of MN with statistical ($p < 0.001$) compared to the CSW and OW groups. The same was observed when CSW were compared to OW. The differences in urinary t,t-MA levels between individuals exposed and not exposed to fuels were not quite statistically significant, and the levels of urinary t,t-MA observed among gas station workers was double those observed among OW.

Table 2
Comet assay results and urinary t,t-MA levels.

Comet assay	Office workers[1] (n = 99)	Gas station workers[2]		p
		Convenience store workers (n = 95)	Filling station attendants (n = 154)	
Class 0	96.00 (88.00–100.0)	94.00 (66.67–100.0)*	94.67 (6.67–99.33)*	**<0.001**
Class 1	4.00 (0.0–11.33)	5.33 (0.0–33.33)*	5.33 (0.0–89.33)*	**<0.001**
Class 2	0.0 (0.0–3.33)	0.0 (0.0–2.00)	0.0 (0.0–13.33)	0.071
Class 3	0.0 (0.0–1.33)	0.0 (0.0–1.33)	0.0 (0.0–2.67)	0.259
T,t-MA[3]	0.05 (<LOD - 0.37)	0.10 (<LOD - 6.20)*	0.10 (<LOD - 3.03)*	**0.012**

Values expressed as median (min-max) percentage of cells in classes of DNA damage in the comet assay.
[1] Not occupationally exposed to benzene.
[2] Occupationally exposed to benzene.
[3] Numbers of participants: Office workers (n = 20); convenience store workers (n = 79); filling station attendants (n = 134). Kruskal-Wallis test with Dunn`s multiple comparison test ($p \leq 0.05$).
* Differ from office worker. **t,t-MA**: trans,trans-muconic acid expressed as median (min-max) of mg/g creatinine. **LOD**: Below detection limit.

Table 3
CBMN assay results and urinary t,t-MA levels.

CBMN assay	Office workers[1] (n = 110)	Gas station workers[2]		p
		Convenience store workers (n = 75)	Filling station attendants (n = 126)	
MN	3.25 (0.50–13.50)	5.00 (1.50–16.00)*	7.00 (0.0–24.00)* #	**<0.001**
NPB[3]	0.0 (0.0–1.50)	0.0 (0.0–1.50)	0.0 (0.0–1.00)	0.568
NBUD	0.0 (0.0–3.50)	0.50 (0.0–3.50)*	0.50 (0.0–4.00)*	**<0.001**
CBPI	1.23 (1.04–1.66)	1.26 (1.09–1.75)	1.26 (1.05–1.96)*	**0.006**
T,t-MA[4]	0.05 (<LOD - 0.47)	0.10 (<LOD - 3.36)	0.10 (<LOD - 3.03)	0.055

Values expressed as median (min-max) frequency of parameters in the CBMN assay.
[1] Not occupationally exposed to benzene.
[2] Occupationally exposed to benzene.
[3] Numbers of participants: Convenience store worker (n = 74).
[4] Numbers of participants: Office workers (n = 25); convenience store workers (n = 61) and filling station attendants (n = 108). Kruskal-Wallis test with Dunn`s multiple comparison test ($p \leq 0.05$).
* Differs from office workers. #Different between Gas station workers. **CBMN assay**: Cytokinesis-block micronucleus assay. **MN**: Frequency of binucleated cells with micronuclei. **NPB**: Frequency of nucleoplasmic bridge in binucleated cells. **NBUD**: Frequency of nuclear bud in binucleated cells. **CBPI**: Cytokinesis-block proliferation index. **t,t-MA**: trans,trans-muconic acid expressed as median (min-max) of mg/g creatinine. **LOD**: Below detection limit.

We also evaluated the frequencies of nucleoplasmic bridge in binucleated cells (NPB), and nuclear bud in binucleated cells (NBUD) (Table 3). There was a statistically significant difference in both exposed groups only for NBUD ($p < 0.001$), compared to the OW group. In the cell proliferation (CBPI) evaluation, the FSA group showed a higher proliferative profile than the comparison group ($p \leq 0.006$).

For total micronucleus frequencies (tMN), a similar pattern was seen as for MN frequency (Table 3). FSA showed a higher frequency (7.75; 0.0–26.00), than CSW (5.50; 1.50–16.50), and OW (3.50; 0.50–15.50). This statistically significant difference was also observed when the FSA group was compared to the group exposed mainly by inhalation (CSW), Fig. 3.

Immunophenotyping showed an increase of NK cells ($CD56^+$) among the FSA, compared to the OW, and CSW, Table 4. For specific immunity cells, a statistically significant decrease in circulating T helper lymphocytes ($CD3^+CD4^+$) was observed in both exposed groups – CSW and FSA– compared to the OW group. A similar change was observed for naive T helper lymphocyte ($CD3^+CD4^+CD45RA^+$) when comparing FSA and OW (Table 4). However, when evaluating the memory T helper lymphocyte ($CD3^+CD4^+CD45RO^+$), the opposite effect was observed, with an increase ($p \leq 0.05$) of these cells both in the CSW group, and in the FSA group, compared to OW.

The levels of urinary t,t-MA in the groups that had blood samples collected for immunophenotyping were similar to those observed in the CBMN assay. The workers exposed to benzene (gas station workers) had about double the levels of urinary t,t-MA as the OWs, with borderline statistical significance ($p = 0.059$) (Table 4).

FSA workers showed a lower CD4 / CD8 ratio than OW ($p \leq 0.05$), but the same was not observed for the CSW (Fig. 4). This immunomodulation may reflect a decrease in T helper lymphocytes or a tendency to increased T cytotoxic lymphocytes, as shown in Table 4, for FSA.

In the genotoxicity tests, multivariate regression analysis (Table 5) indicated a greater chance of genotoxic effect in both groups occupationally exposed to fuels (CSW and FSA), regardless of the genotoxicity test used. The association between types of work and genotoxic effects in comet assay was adjusted for age, while this association in the CBMN assay was adjusted for smoking and age. Other possibly confounding variables, such as length of working, sex, and alcohol consumption, were

Table 4
Immunophenotyping results and urinary t,t-MA levels.

Immunophenotyping	Office workers[1] (n = 101)	Gas station workers[2]		p
		Convenience store workers (n = 77)	Filling station attendants (n = 153)	
$CD56^{+}$[3]	6.90 (0.01–35.52)	8.77 (0.02–21.13)[#]	12.18 (0.22–32.03)[*]	**<0.001**
$CD19^{+}$[4]	7.23 (0.07–19.62)	8.89 (0.93–17.33)	8.36 (2.14–23.52)	0.097
$CD3^{+}CD4^{+}$[5]	58.05 (12.76–84.38)	52.49 (11.04–75.62)[*]	53.08 (12.98–75.91)[*]	**0.003**
$CD3^{+}CD8^{+}$[5]	30.17 (7.58–53.15)	32.20 (6.46–86.62)	33.13 (12.15–78.00)	0.062
$CD3^{+}CD4^{+}CD45RA^{+}$[6]	43.54 (5.41–90.00)	41.70 (4.58–65.88)	39.44 (2.56–78.91)[*]	**0.036**[§]
$CD3^{+}CD4^{+}CD45RO^{+}$[6]	78.80 (1.22–99.42)	83.81 (24.27–98.33)[*]	83.72 (15.61–99.55)[*]	**0.010**
T,t-MA[7]	0.05 (<LOD - 0.47)	0.10 (<LOD - 6.20)	0.10 (<LOD - 1.40)	0.059

Values expressed as median (min-max).
[1] Not occupationally exposed to benzene. [2]Occupationally exposed to benzene.
[3] Numbers of participants: Office workers (n = 100); convenience store workers (n = 72); filling station attendants (n = 149).
[4] Numbers of participants: Office worker (n = 99).
[5] Numbers of participants: Office workers (n = 100).
[6] Numbers of participants: Convenience store workers (n = 72); filling station attendants (n = 152).
[7] Numbers of participants: Office workers (n = 23); convenience store workers (n = 68); filling station attendants (n = 132). Kruskal-Wallis test with Dunn`s multiple comparison test ($p \leq 0.05$).
[*] Differs from office worker.
[#] Different between Gas station workers.
[§] ANOVA with Bonferroni's multiple comparisons test ($p \leq 0.05$). **$CD56^{+}$**: Natural Killer lymphocyte. **$CD19^{+}$**: B lymphocyte. **$CD3^{+}CD4^{+}$**: T helper lymphocyte. **$CD3^{+}CD8^{+}$**: T cytotoxic lymphocyte. **$CD3^{+}CD4^{+}CD45RA^{+}$**: Naive T helper lymphocyte. **$CD3^{+}CD4^{+}CD45RO^{+}$**: Memory T helper lymphocyte. **t,t-MA**: trans,trans-muconic acid expressed as median (min-max) of mg/g creatinine. **LOD**: Below detection limit.

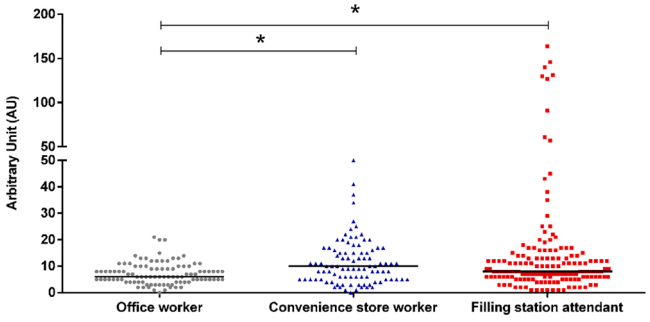

Fig. 2. Scatter chart; comet assay; showing arbitrary unit levels (*Kruskal-Wallis test p = 0.0004, Dunn`s Multiple Comparison test $p < 0.05$).

Fig. 3. Scatter chart; CBMN assay showing total micronucleus found in binucleated cells (*Kruskal-Wallis test $p < 0.0001$, Dunn's Multiple Comparison test $p < 0.05$).

not maintained in the final model because they did not contribute to the magnitude of the associations and because they were not statistically significant after being inserted individually in each model (data not shown).

In the comet assay, CSW and FSA groups had 2.38 and 2.29× higher odds of DNA damage, respectively, than the OW group. The results of the CBMN assay indicated that the CSWs had 3.80× greater chance of presenting binucleated lymphocytes containing micronucleus than the comparison group (OW). In the FSA group, this chance was 7.78× greater than for the OW group (Table 5). The adjustments increased the magnitude of the association in both assays, for both groups of occupational exposure.

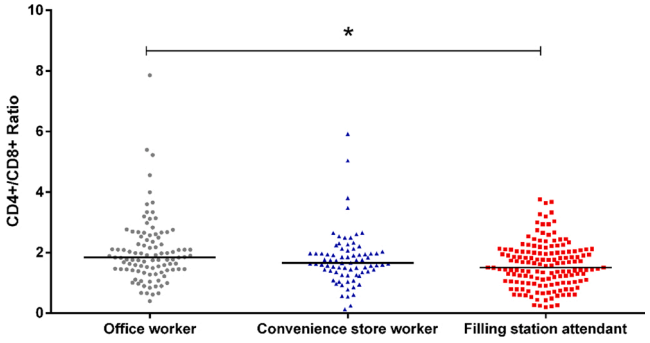

Fig. 4. Scatter chart; CD4/CD8 ratio (*Kruskal-Wallis test p = 0.0025, Dunn post-test $p < 0.05$).

Table 5
Multivariate logistic regression analysis: exposure to fuels and genotoxic effects, adjusted for confounding factors.

Comet assay - AU (n = 349)	OR[1] (CI 95%)	ORadj[2] (CI 95%)	p
Office workers	1.0 (ref.)	1.0 (ref.)	–
Convenience store workers	1.91 (1.07–3.39)	2.38 (1.28–4.43)	**0.006**
Filling station attendants	2.15 (1.28–3.60)	2.29 (1.35–3.90)	**0.002**
CBMN assay - MN (n= 311)	**OR[1] (CI 95%)**	**ORadj[3] (CI 95%)**	**p**
Office workers	1.0 (ref.)	1.0 (ref.)	–
Convenience store workers	3.41 (1.77–6.58)	3.80 (1.85–7.78)	**<0.001**
Filling station attendants	6.88 (3.61–13.09)	7.78 (3.95–15.34)	**<0.001**

[1] OR: *odds ratio* - raw data.
[2] ORadj: OR adjusted for age.
[3] ORadj: OR adjusted smoking and age. Wald test ($p \leq 0.05$). **AU**: reference category of total DNA damage ≤6.0. **MN**: reference category of frequency of binucleated cells with micronuclei ≤3.3. **CI**: Confidence interval.

4. Discussion

Worldwide, cancer is the second leading cause of mortality [38]. Biomonitoring of populations exposed to carcinogens can be an effective preventive tool [39]. The comet and CBMN assays (blood cells) are widely used for human biomonitoring [40,41]. As the immune system

participates in immune surveillance of tumor cells, the identification of immune cells has also been used in biomonitoring studies [16].

The comet assay detected a genotoxic effect among the gas station workers. The two groups of workers (CSW and FSA) gave similar results, indicating that all gas station workers may be at risk, regardless of job category. Similar results (increased damage among workers exposed to benzene) have been reported before [34,42,43], but few studies have included different occupations of workers, who may have been exposed to different levels of benzene or fuels [3,5]. Campos et al. [3], who evaluated gas station workers, fuel quality control analysts, and individuals not occupationally exposed to benzene, observed more DNA damage in exposed than non-exposed workers. This effect was also greater among gas station workers than laboratory analysts. Khisroon et al. [5] found similar results when evaluating filling station attendants, automobile workshop mechanics, and a control group, with a higher frequency of damage among the exposed workers and among mechanics compared to filling station attendants. In that report, trypan blue exclusion was used to evaluate cellular viability, while we used fluorescein diacetate and ethidium bromide. The trypan blue method is not recommended in the comet assay because it evaluates only the integrity of the cell membrane, whereas the test with two dyes also examines the metabolic capacity of the cells [44] and is more sensitive. Also, the number of participants in our study was much higher than in the earlier studies and we evaluated more cells per participant.

Even though the main class of DNA damage detected was the one with the lowest degree (Class 1), the detection of this effect helps to assess risk. Benzene is considered to be a human carcinogen and occupational exposure to oil and petroleum products presents a potential cancer risk due to the presence of benzene [7,45].

Although the alkaline comet assay does not detect mutations, it can detect DNA single-strand breaks (SSB), apurinic/apyrimidinic (AP) alkali-labile sites, intercalation, and strand breaks produced by incomplete excision at repair sites. Strand breaks can be repaired, may be lethal to the cell, or may result in mutations if not repaired [23], which can lead to chromosomal damage associated with human diseases, including cancer. The comet assay detects early and recent responses to genotoxic agents [21] while the CBMN assay detects clastogenic and aneugenic effects [24].

Our CBMN results are in accordance with those in the literature. An increase in MN frequency was observed among gas station workers when peripheral blood samples [43,46] and oral mucosa cells [34,47] were evaluated, as well as in shoe factory workers exposed to benzene [48] and in gas station workers (blood samples evaluated by the micronucleus test without blocking cytokinesis) [49]. Maffei et al. [50] evaluated the effects of environmental exposure to benzene on traffic controllers in Bologna, Italy. That study observed a significant increase in MN among exposed workers [50], in accordance with our data. According to Bonassi et al. [51], an increase in the frequency of MN in a population is associated with increased risk of cancer. Several studies have even considered peripheral blood lymphocyte MN frequency to be a predictive biomarker of cancer [51–56].

The frequency of NBUD, another marker of cytogenetic damage, was higher in those exposed to gasoline than in the comparison group. The nuclear bud represents amplified DNA expelled from the nucleus during the cell division process or even excess DNA resulting from the repair process, or excess chromosome, in the case of aneuploidies [57]. The presence of this marker in gas station workers indicates the presence of DNA damage and chromosomal instability. The literature describes greater frequencies of both MN and NBUD in cancer cases vs. controls [53,56].

In the evaluation of immunotoxic effects, we found that gas station workers had a lower frequency of T helper lymphocyte ($CD3^+CD4^+$) than the comparison group, in accordance with the literature [17,18,58]. The CD4/CD8 ratio was also lower among FSA than OW, an effect also observed in studies of gas station workers in Brazil and India [16,17]. This pattern was also observed among workers in the shoe industry in China [58] occupationally exposed to benzene. The balance of T lymphocytes is important for disease control, and the optimal CD4/CD8 ratio is about 2:1 [59]. Our results indicate an imbalance in this relationship which could contribute to development of infectious diseases and cancer. Another parameter that was less frequent among FSA, compared to the OW group, was naive Th lymphocytes ($CD3^+CD4^+CD45RA^+$).

We observed an increase of innate immunity cells (NK, $CD56^+$) in the FSA compared to OW and CSW, in line with other studies [16,18]. In a case report on two women who worked in gas stations for 4 and 9 years, a low frequency of NK cells was observed, in addition to a history of early miscarriage and the presence of chromosomal aberrations [60].

The increase in the frequency of memory Th lymphocytes ($CD3^+CD4^+CD45RO^+$) among exposed individuals differs from a case-control study of 24 patients with possible benzene poisoning, where the patients had fewer memory cells, compared to the control group [21]. However, in that study, the selected workers (served by the Center for the Study of Occupational Health, CESAT) met these criteria: occupational exposure to benzene; lymphocyte count <4000 and neutrophils <2000; and a decrease in these cells compared to the start of follow-up. In contrast, the workers in our study were had at least 6 months occupational exposure to fuels. The exposed workers who participated in our study may be at earlier stages of immune system damage, presenting an increase in memory Th cells, rather than the decrease reported by Brandão et al. [21].

In previous studies assessing the profile of circulating T lymphocytes, workers exposed to benzene showed decreased frequency of T cytotoxic lymphocyte ($CD3^+CD8^+$) [16,18] or no significant change [17], differing from our data. An increase in the frequency of T cytotoxic lymphocytes ($p = 0.002$) was only observed in a study of workers with a possible diagnosis of chronic benzene intoxication [21]. T cytotoxic lymphocyte and NK cells participate directly in the immune surveillance of cells with tumorigenic potential; thus, their modulation by occupational exposure to fuels may contribute to the development of work-related tumors, such as leukemias and lymphomas, which are more frequent among workers exposed to benzene [7]. These FSA may be in the early stages of cell damage, contributing to the increase in the frequency of these immune surveillance cells (NK and cytotoxic T lymphocytes), which may be "requested" to identify a greater number of cells with tumorigenic potential, unlike the OW, who are not occupationally exposed to gasoline or benzene. If so, this effect might identify workers who are at higher risk for the development of leukemias, lymphomas, and other work-related cancers.

Our data confirm the importance of the airway for damage to the health of persons exposed to fuels. A study based on the US Environmental Protection Agency's Integrated Risk Information System (USEPA-IRIS) was conducted with participants from different areas of Thailand to estimate the risk to human health from chronic exposure to low concentrations of benzene in the air. The study estimated that 70.67 % of workers had a lifetime risk of cancer $>2.2 \times 10^{-6}$, simply due to inhaling benzene vapors. An estimated 51.33 % of workers were at elevated risk of developing other adverse health effects [61].

Urinary metabolites, such as phenol, hydroquinone, t,t-MA, and S-phenylmercapturic acid, can be used to monitor exposure to benzene. Tt-MA is the most widely used biomarker in Brazil, due to its simplicity and its good correlation with environmental levels of benzene. Factors such as exposure levels, smoking, and diet can significantly influence its urinary concentration, especially for low-level exposures ($\leq$0.5 ppm) [62]. In the present study, occupational exposure to benzene was assessed by determining urinary t,t-MA. Brazil Ordinance No. 34/2001 suggests that the level of urinary t,t-MA found in people not occupationally exposed to benzene is up to 0.5 mg/g creatinine. This value reflects environmental exposures due to combustion processes, urban automobile pollution, smoking, and exposure to sorbic acid, a processed food preservative that is metabolized to t,t-MA. Our results show that t,t-MA values observed for those exposed were about twice as high as in

the comparison group, although both values were well below the suggested maximum level [63].

Our recent article on urinary t,t-MA levels in gas station workers [64] is consistent with the present findings. The previous study found that gas station workers showed higher mean values of t,t-MA than did OW, this levels were also higher in the CSW group than in the FSA group, and t,t-MA values were higher in workers from the Downtown region than in the Southern Zone [64]. These data demonstrated that the work environment can directly affect the levels of this exposure biomarker, considering that the working environments of the FSA (open space) and CSW (closed space, in most cases) tend to be very different. The same effect can be seen for gas station workers located in the Downtown vs the Southern Zone of Rio de Janeiro. The downtown area is highly urbanized and more commercial, with tall buildings, old houses, population clusters (such as favelas) and intense vehicular traffic. The Southern Zone has higher real estate values, high infrastructure standards, a large variety of wooded areas and natural landscapes, and a coastal area with numerous beaches [64].

A recent study showed that the relationship between t,t-MA and occupational and environmental exposure to benzene is limited to concentrations <0.5 ppm benzene [62], similar to that observed in gas stations, since the levels of benzene in the air are limited to <1 ppm [9]. However, our results suggest that even low-level occupational exposure to benzene may increase genotoxic risk.

The present study used a cross-sectional design. As expected, the different groups showed significant differences in socio-demographic parameters. Smoking can influence urinary t,t-MA [62,64] and sex, age, smoking, alcohol consumption, and length of working can affect genotoxicity [34–37]. However, adjustments were made during multivariate analysis to account for confounders, suggesting that the associations between occupational exposure and genotoxic effects are real.

5. Conclusions

Our findings reinforce the importance of monitoring occupational exposures, regardless of job function or route of exposure. Elimination of toxic chemicals from work environments is not always possible. Monitoring of exposed workers can help to reduce exposure and to mitigate damage; biological monitoring (e.g., comet and CBMN assays) can be an important tool to assess exposure and possible health effects.

Our study shows genotoxic, immunosuppressive, and immunostimulatory effects among gas station workers and demonstrates the importance of considering different types of exposure to chemical agents during work routines. Workers exposed mainly by inhalation presented genotoxic and immunotoxic effects similar to workers for whom the routes of exposure included both inhalation and dermal routes. The carcinogenic effects of benzene do not have a safety threshold, and possible effects on all employees must be considered, whether they are exposed to this workplace carcinogen directly or indirectly.

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Declaration of Competing Interest

Authors declare no conflict of interest.

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