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ORIGINAL ARTICLE

Petrol exposure and DNA integrity of peripheral lymphocytes

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Abstract
Purpose To determine the effect of petrol exposure on DNA integrity in peripheral blood lymphocytes among petrol attendants and a non-exposed comparison population.
Methods This cross-sectional study included 101 fuel station employees and 50 office-based non-exposed workers in Durban, South Africa. Participants were interviewed using a validated questionnaire. Genomic DNA was extracted from peripheral lymphocytes for the benzo(a)pyrene diol epoxide (BPDE)-DNA adduct assay (ELISA), and DNA damage was determined using the comet assay and reported as percentage tail DNA.
Results The exposed ($n = 101$) and non-exposed participants ($n = 50$) varied with regard to age, housing, smoking, and proximity to industry and petrol stations. Among the exposed, the mean duration of employment in the fuel industry was 5.8 years (SD = 4.6), and among those pumping fuel ($n = 75$), the mean metric tons of petrol pumped in the past 12 months per worker was 199.2 (SD = 88.9). The mean percentage tail DNA varied significantly between exposed and non-exposed groups: 23.8 % (SD = 13.3) and 8.1 % (SD = 1.8) ($p < 0.01$), respectively. A significant difference existed between the groups for BPDE-DNA adducts: 30.0 ng/ml (SD = 12.7) and 18.1 ng/ml (SD = 18.2) ($p < 0.0001$), respectively. Regression models, adjusting for cigarette smoking, age, and sex, showed a 16.5 greater percentage tail DNA among the exposed compared to non-exposed (95 % CI 11.8–21.1 %), while the exposed group had a 12.9 ng/ml greater increase in BPDE-DNA adducts has compared to the unexposed (95 % CI 7.2–18.7 ng/ml). Cigarette smoking resulted in almost a 3.5 % increase in percentage tail DNA.
Conclusion Our study adds to the literature that long-term, low-dose exposure to vehicular fuels is likely to result in altered DNA integrity and genotoxicity among petrol attendants. These results strengthen the case that these workers must be afforded appropriate protection to prevent serious adverse outcomes.

Keywords Petrol attendants · DNA damage · Comet assay · BPDE-DNA adduct

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Introduction

Petrol attendants and other workers at fuel-filling stations are at risk for the development of cancers (WHO/IARC 2000). Exposures experienced by such workers include the polycyclic aromatic hydrocarbons (PAH) and other volatile organic compounds. These constituents present an important concern for their carcinogenic potential. In recent years, in South Africa, this risk has increased with the increased concentration of benzene in motor vehicle fuels, particularly with the removal of lead as an anti-knock agent (Graboski 2003).

Leaded petrol had been available in South Africa since the 1920s. In 1996, unleaded petrol was introduced, largely as a result of technological changes to motor vehicles. By

2006, lead in petrol was legally phased out. The removal of this anti-knock agent in fuels meant that alternative fuel technology was necessary to ensure octane rating. This has largely been through the introduction of other metals such as manganese, and increased levels of benzene in the fuels to ensure the octane ratings of the fuel (SAPIA 2008). Currently, South African regulations stipulate a total aromatic level of 50 % by volume (SAPIA 2008). Benzene concentration specifically has risen and is currently about 3 % by volume—although below the legal limit of 5 %, it is about threefold higher than most other industrialized countries.

This higher allowable limit places South African fuel pump attendants at an increased risk of adverse outcomes, particularly as self-service fuel-filling stations are not available in South Africa. These workers are potentially exposed to substantial concentrations of benzene and the other volatile organic compounds (VOCs) such toluene and xylene, as well as the PAHs over their lifetime of employment.

PAHs and the VOCs are associated with DNA damage, while benzene itself is associated with DNA strand breaks, micronuclei and chromosomal aberrations (Angelini et al. 2011). The complex mixtures of exposures in fuel-filling stations, varying durations, and levels of exposure present a challenge to determine the precise individual exposure. As carcinogenic effects are only evident some time later, difficulties in determining dose–response relationships with overt cancer outcomes exist. Some strategies around this have been to measure DNA adducts and assess DNA integrity as possible proxy measures of exposure.

Benzo(a)pyrene is metabolized to benzo[a]pyrene 7,8-diol 9,10-epoxide (BPDE). The latter forms covalent DNA adducts resulting in DNA damage and possible cancer initiation (Lu et al. 2013). BPDE-DNA adducts can be considered as biomarkers of exposure, with some arguing that the albumin adducts cannot be directly involved in carcinogenesis (Wang et al. 2007). Studies have shown important differences in adduct levels between those exposed to PAHs compared to those without exposure (Wang et al. 2007). BPDE-DNA adducts have not been investigated previously among fuel pump attendants.

Comet assays (single-cell gel electrophoresis) are a rapid and reliable test that can detect DNA damage in different exposure circumstances (Collins 2004). The comet assay is more sensitive and specific than micronuclei (MN) detection as a biomarker of effects of genotoxicity, and together with chromosomal analysis (CA), allows for the detection of more specific chromosomal aberrations (Bindhya et al. 2010; DeMarini 2013). The comet assay has been shown to be sensitive in detecting DNA damage due to low levels of benzene exposure (Fracasso et al. 2010). Extensive DNA damage is regarded as a marker of cancer risk (Piperakis et al. 2009). However, the extent of DNA damage as a predictor of cancer is not yet known (Møller et al. 2009).

The aim of this study was to compare DNA integrity between petrol attendant workers and a sample of non-exposed workers, using BPDE-DNA adducts and the comet assay as markers of exposure.

Methods

Ethical approval was received from the University of KwaZulu-Natal's Biomedical Research Ethics Committee (certificate BF110/11). The study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. Signed, informed consent was obtained from all participants. Participation was voluntary, and participants were free to withdraw at any stage.

Sample selection

All employees ($n = 150$) working at the eight selected petrol stations from the urban areas of Durban, South Africa, were invited to participate, provided they met the inclusion criteria. Employees with a history of factors likely to influence the comet assay test, such as undergoing radiotherapy, family history of cancer, leukemia, or recent flu-like illnesses, were excluded from the sample. From these 150, 25 refused to participate, 15 did not meet the inclusion criteria, and a further nine were on leave. Fifty office-based workers were randomly selected from a local tertiary academic institution.

Participant interviews

A previously validated English language questionnaire was modified and piloted before use. This was translated into isiZulu and back translated to ensure consistency. Interviewers were trained in administering the questionnaire. Information was obtained about demographics, occupational, medical and environmental history, history of radiation, infections, genetic or comorbid illnesses, and history of smoking, alcohol intake, diet and exercising, and family history of cancers.

Benzo(a)pyrene diol epoxide (BPDE)-DNA adduct assay

A sample of blood was collected from each participant at the time of the interview. Genomic DNA was extracted from peripheral lymphocytes using an in-house protocol and diluted to 2 μg/ml in cold 0.1 M PBS. A 100 μL aliquot of DNA from each sample and BPDE-DNA standards were added in duplicate to the wells of a DNA High-Binding plate and incubated at 37 °C for 2 h. Each DNA sample including unknown and standard was assayed in duplicate.

After incubation, the DNA solutions were removed and washed twice with 0.1 M PBS. Thereafter, 200 µl of assay diluent was added to each well and incubated [1 h, room temperature (RT)]. Following removal of the assay diluent, 100 µL of the anti-BPDE-I antibody was added to all wells and incubated for 1 h, RT on an orbital shaker. Wells were then rinsed five times with 250 µl of 1X wash buffer with thorough aspiration between each wash. A pre-diluted 1X blocking reagent was added to each well (150 µl) and incubated (1 h, RT) before rinsing three times with 1X wash buffer. A 100 µl aliquot of the diluted secondary antibody–HRP conjugate was added to all wells and incubated (1 h, RT). Wells were then washed five times, and substrate solution (100 µl) was added to all wells and incubated at RT until a color change was observed. The reaction was then stopped by the addition of 100 µl stop solution. The absorbance was measured immediately using a microplate reader (Biotek µQuant) at 450 nm.

DNA damage assay

DNA strand damage was determined using the comet assay as described by Singh et al. (1988). Three slides per sample were prepared. The first layer consisted of 1 % low-melting-point agarose (LMPA, 37 °C), a second layer of 25 µl of whole blood from each sample with 175 µl of 0.5 % LMPA (37 °C), and a third layer of 0.5 % LMPA (37 °C) covered the slides. After solidification, the slides were then submerged in cold lysing solution [2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 1 % Triton X-100, 10 mM Tris (pH 10), 10 % dimethylsulfoxide (DMSO)] and incubated (4 °C, 1 h). DMSO was added to the cell lysis solution to scavenge free radicals. This helps prevent any further DNA damage associated with iron release during lysis of erythrocytes. Following incubation, the slides were placed in electrophoresis solution [300 mM NaOH, 1 mM Na_2EDTA (pH 13)] for 20 min and thereafter subjected to electrophoresis (25 V, 35 min, RT) using a Bio-Rad compact power supply. The slides were then washed three times with neutralization buffer [0.4 M Tris (pH 7.4)] for 5 min each. The slides were stained overnight (4 °C) with 40 µl ethidium bromide (20 µg/ml) and viewed with a fluorescence microscope (Olympus IXSI inverted microscope with 510- to 560-nm excitation and 590-nm emission filters). Images of 50 cells and comets were captured per treatment, and the percentage tail DNA (%TD) were measured using Soft imaging system (Life Science—©Olympus Soft Imaging Solutions v5).

Data analysis

STATA ver13 (StataCorp, Texas) statistical software was used for data analysis. Because of the skewed distribution of the data, nonparametric tests including the Mann–Whitney test, analysis of variance (Kruskal–Wallis rank test), and Spearman correlation analysis were used. Initially, demographic differences were assessed between the exposed and non-exposed groups.

Key variables of interest were DNA damage as measured through the comet assay (the mean %TD for each participant) and the BPDE-DNA adduct assay. The differences in these measures were compared between the exposure groups.

The regression models investigated the following independent variables: age, sex, petrol exposure, cigarette smoking, alcohol intake, positive HIV status, drug intake, residential proximity to highways and other petrol stations, and living in a suburb or informal communities. The final models, in which significant covariates were retained, included the exposure variables such as age, sex, and cigarette smoking and alcohol intake. Several regression models were constructed using different exposure indices: exposed versus non-exposed; high versus low-exposed, and high versus non-exposed. However, only the first is reported here. In addition, smoking and alcohol status were introduced as continuous variables (pack years and units drunk) and as categorical variables (current smoker vs. non-smoker and current drinker vs. nondrinker) into separate models, respectively.

Results

The exposed and unexposed subjects in the study sample varied substantially in respect of key demographic factors (Table 1). The unexposed were older by about 5 years, predominantly female, more likely to be living in an urban home, less likely to be smokers. However, the unexposed lived closer to industrial sources of pollution and closer to petrol stations. Only a small number who had been tested for HIV had tested positive (2 % in each group, respectively)—26 % of unexposed, and 64 % of exposed had never been tested.

Among the exposed grouping, the average duration of employment in the fuel industry was 5.8 years (SD = 4.6), and for those for whom we had data on the volume of petrol pumped in the past 12 months ($n = 75$), the mean metric tons pumped per worker was 199.2 (SD = 88.9) (not shown in tables).

The mean %TD varied significantly between exposed and non-exposed groups: 23.8 % (SD = 13.3) and 8.1 (SD = 1.8) ($p < 0.01$), respectively (Fig. 1), and visible graphically (Fig. 2). No significant difference in %TD was observed for any of the key demographic variables between those exposed and unexposed. A significant difference

Table 1 Demographic factors among exposed and unexposed workers

Variables	Unexposed group ($n = 50$)	Exposed group ($n = 101$)
Age in years [mean (SD)]*	37.2 (9.4)	31.9 (7.9)
Sex [n (%)]*		
Male ($n = 90$)	16 (32.0)	76 (76.3)
Female ($n = 57$)	34 (68.0)	23 (23.7)
Residential area [n (%)]		
Township	21 (42)	72 (71)
Suburb	28 (56)	21 (21)
Informal settlement	1 (2)	4 (4)
Rural	0	4 (4)
Home <1 km from emitting factories [n (%)]*	10 (20)	7 (7.2)
Home <1 km from petrol station [n (%)]*	28 (56)	22 (22.9)
Home <1 km from freeways [n (%)]	25 (35.2)	50 (65.7)
Smoking history [(n (%)]		
Current smokers	6 (12.0)	21 (20.8)
Never smoked	42 (84.0)	65 (63.4)
Ex-smokers	2 (4.0)	15 (14.9)
Cigarette pack years [mean (SD)][a]	1.4 (2.9)	1.9 (4.8)
Alcohol consumption [(n (%)][b]		
Current drinkers	23 (46.0)	37 (36.3)
Ex drinkers*	0 (0.0)	20 (19.8)
Non-drinkers	27 (54.0)	44 (42.6)

* $p < 0.05$

[a] Pack years = number of packs of cigarettes smoked per day (or proportion thereof) × the number of years smoked

[b] Alcohol consumption was defined as the intake of any alcohol containing drinks, irrespective of volume or frequency of intake

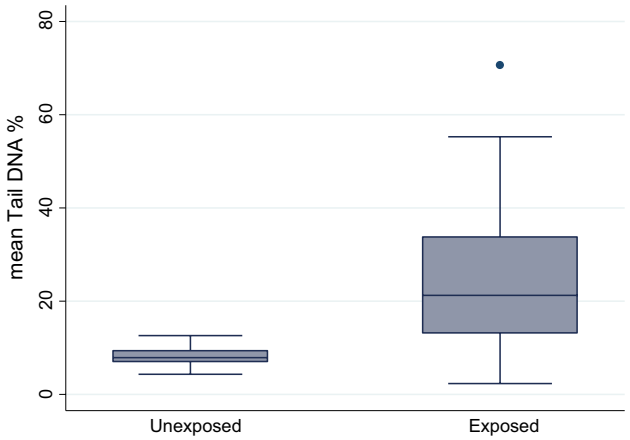

Fig. 1 Mean percent tail DNA among the exposed group ($n = 101$) and unexposed group ($n = 50$)

existed between the groups for mean BPDE-DNA adducts, 30.0 ng/ml (SD = 12.7) and 18.1 ng/ml (SD = 18.2) ($p < 0.0001$), respectively (Fig. 3).

There was a modest correlation between the BPDE-DNA adducts and %TD ($r^2 = 0.2$, $p = 0.01$), with increasing concentrations of adducts associated with increasing comet tail DNA.

To describe the exposure-related difference in %TD, linear regression models were used, adjusting for cigarette smoking, alcohol consumption, age, and sex. Mean %TD was 16.5 % greater in the exposed group (95 % CI 11.8–21.1 %) compared to the unexposed. Similarly, the exposure-related difference in BPDE-DNA adduct was 12.9 ng/ml greater in the exposed workers (95 % CI 7.2–18.7 ng/ml) compared to the unexposed. In both these models, being a cigarette smoker, compared to a non-smoker, resulted in a 3.5 % higher %TD and 5.4 ng/ml greater BPDE-DNA adduct compared to non-smokers (Table 2). Other variables of interest, likely to be associated with DNA damage, such as positive HIV status, drug intake, residential proximity to highways and other petrol stations, and living in a suburb or informal communities, were excluded from the regression model after failing to achieve statistical significance.

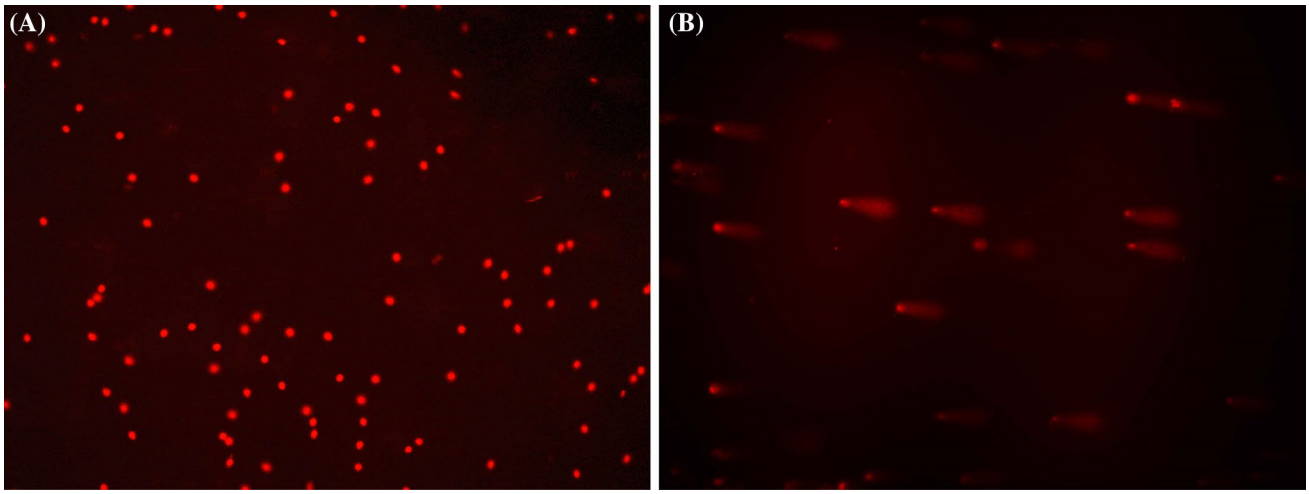

Fig. 2 Comet assay images of non-exposed (**a**) and exposed (**b**) groups (100×)

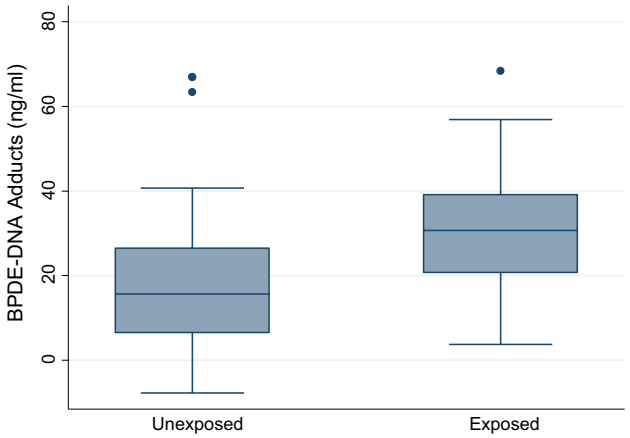

Fig. 3 Mean BPDE-DNA adducts of the exposed group ($n = 101$) and unexposed group ($n = 50$)

Discussion

In this study of 151 petrol attendants and unexposed university staff, we found a modest exposure-related increase in DNA damage, using crude (exposed vs. unexposed) while adjusting for key covariates.

While the findings of increased DNA damage among petrol pump attendants have been described previously (Andreoli et al. 1997; Bukvic et al. 1998; Celik et al. 2003; Navasumrit et al. 2005; Pandey et al. 2008), this is the first study which has investigated the relationship between BPDE-DNA adducts and exposure in this group of workers.

Despite the well-established relationship between benzene exposure and cancer outcomes (McMichael 1988; WHO 2000), petrol pump attendants have low levels of long-term exposure and may thus experience different outcomes. This risk may be impacted with the changing constituents of vehicle fuel (Graboski 2003) and the increase in benzene concentration. There have been inconsistent findings among researchers, with some describing increased DNA damage (Andreoli et al. 1997; Navasumrit et al. 2005; Rekhadevi et al. 2011) and others with no such findings (Bukvic et al. 1998).

Petrol pump attendants experience varying levels of exposure, and in most instances, these are likely to be low exposure. In addition, they are largely in informal or short-term employment. Attempting to characterize exposure–outcome relationships for well-defined clinical entities such as cancers, particularly well-documented outcomes such as hematological cancers, is therefore extremely difficult. Thus, shorter term outcomes need to be considered, and markers of DNA damage is one such option. Benzene, an established hematological carcinogen at high doses, may induce DNA hypomethylation (Fustinoni et al. 2012). The comet assay is a simple method of assessing DNA damage (Speit et al. 2003; Paz-y-Mino et al. 2008), specifically DNA strand breaks in eukaryotic cells (Collins 2004).

Other methods have been used in identifying early DNA damage among fuel pump attendants. Sister chromatid exchange (SCE) and trans, trans-muconic acid, a urinary metabolite of benzene, were found to be significantly higher among petrol-exposed workers, compared to controls, with a chromosomal damage relative risk of 3.0 (95 % CI 1.81–4.98, $p < 0.001$) (Tunsaringkarn et al. 2011). Contrasting findings were reported by other researchers, failing to find relationships between SCE and benzene-exposed workers (Bukvic et al. 1998; Carere et al. 2002; Khalil et al. 1994). Increased frequency of micronuclei and chromosomal aberrations, as alternative markers of DNA damage, have been described in fuel pump attendants

Table 2 Regression models for percent tail DNA and BPDE-DNA adducts

Covariate	Percent tail DNA (coefficient; SE; 95 % confidence interval)	BPDE-DNA adducts (coefficient; SE; 95 % confidence interval)
Exposure group (1 = exposed; 0 = unexposed)	16.5 2.4 11.8–21.1	12.9 2.9 7.2–18.7
Sex (1 = male; 0 = female)	0.02 2.4 −4.6–4.7	−5.8 2.9 −11.5—0.04
Age	0.2 0.1 −0.05–0.4	−0.1 0.1 −0.4–0.2
Smoking status (1 = smoker; 0 = non-smoker)	3.5 2.3 1.0–7.9	5.4 2.8 −0.1–10.9
Alcohol status (1 = alcohol consumer; 0 = non-consumer)	−2.4 2.2 −6.7–1.9	−0.2 2.7 −5.6–5.1

compared to control groups (Celik et al. 2003; Pandey et al. 2008; Rekhadevi et al. 2011).

Accurate exposure assessments among fuel pump attendants are a challenge. Given the informal nature of their work, their multiple responsibilities in the working environment, ranging from direct exposure (pumping fuel) to minimal exposure over the duration of employment or even during a working day, traditional methods of personal sampling may not be adequate unless conducted in repeated cycles. However, the use of biomarkers may provide a more direct measure of exposure. Wang et al. (2007) argue that DNA and protein adducts may be a "biologically effective dose biomarkers of PAHs." The latter argues that protein adducts represent only recent exposure (based on the half-life of albumin) and are not considered to be directly involved in carcinogenesis (Wang et al. 2007).

Using BPDE-DNA adducts, we were able to show an increased frequency among exposed workers, compared to those not exposed, even after adjusting for covariates. We attempted to characterize exposure on the basis of volume of fuel pumped in the past year. However, this did not show any relationship with the comet assay or the adduct formation. This may be reflective of ongoing exposure among those exposed, irrespective of whether pumping fuel or not. The adduct measure may not be the correct biomarker as the causative exposure may be other constituents within the fuel mixture, or other unmeasured non-occupational factors may be implicated.

Some researchers claim that the comet assay may not be the most appropriate for the benzopyrenes because of the latter inducing stable adducts (Genies et al. 2013; Rekhadevi et al. 2010). However, our study found important exposure-related differences in %TD between the exposed and non-exposed groupings. In models adjusting for smoking, sex, and age, increasing exposure was related to increasing DNA strand damage, although this was only marginal. Several studies have reported increased DNA damage assessed through comet assay following PAH exposure (Sanchez-Guerra et al. 2012) and among petrol attendants (Goethel 2014; Navasumrit et al. 2005; Moro et al. 2013) or jet fuel-exposed workers (Krieg et al. 2012), with benzene exposure and traffic police with vehicular exhaust exposure (Prasad et al. 2013; DeMarini 2013). Comet assay was additionally used to investigate DNA damage among workers with low levels of benzene exposure among petrochemical workers and petrol attendants. DNA damage was described among the petrochemical workers and the petrol attendants (Fracasso et al. 2010).

South African workers present a special case for further investigation. The changing constituents of vehicular fuel over the years in South Africa, particularly with the replacement of lead, and the consequent increase in other anti-knock agents suggest that the risks faced by South African petrol pump attendants may differ from those elsewhere. The study by Keretetse et al. (2008) found exposure levels of volatile organic compounds lower than the South African occupational exposure limit. Despite this, the exposed workers had higher levels of DNA damage compared to the unexposed (Keretetse et al. 2008). In addition, infectious diseases are strongly associated with DNA damage (Weitzman and Weitzman 2014). The high prevalence of HIV and other infectious diseases in South Africa confound the association between DNA damage and chemical exposure. In our sample, the levels of HIV infection could not be appropriately described as 64 and 26 % of the exposed and unexposed, respectively, had never had any tests performed. Among those who had been tested, only two percent in each group were positive. This data prevented us conducting detailed analysis or adjusting for HIV in our sample.

The small sample size and the lack of actual exposure monitoring are the key limitations in this study. While the sample size may explain some of the non-statistically significant findings, we believe that the lack of exposure monitoring had limited effect on the findings because of our use of an established biomarker. The strength of our study was our ability to use a biologically useful marker of exposure among a sample of exposed and non-exposed workers, while adjusting for several covariates. Several other factors may influence the DNA effects, apart from exposure to the constituents of petrol. These include those adjusted for in the models, such as cigarette smoking, other environmental exposures such as biomass fuel usage, non-occupational exposures to traffic, and alcohol consumption. Because of the sample size, and the limited variability in these various covariates, we were not able to satisfactorily explore these relationships, despite investigating these in our analysis.

In conclusion, our study adds to the literature which argues that long–term, low-dose exposure to vehicular fuels is likely to result in compromised DNA integrity among petrol attendants. Our findings support that of previous studies which have shown that BPDE-DNA adducts and the comet assay are useful biomarkers of exposure.

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Compliance with ethical standards

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

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