UNIVERSITY OF KWAZULU-NATAL

OCCUPATIONAL EXPOSURE AND GENOTOXICITY AMONG ETHEKWINI MUNICIPALITY PETROL ATTENDANTS

By

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Declaration

This research has not been previously accepted for any degree and is not being currently considered for any other degree at any university.

I declare that this Dissertation contains my own work except where specifically acknowledged.

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ABSTRACT

Background

Benzene, a constituent of petrol, is classified as a Group 1 carcinogen. Once benzene enters the human body, its breakdown products, benzene oxide (BO) and 1.4 benzoquinone (BQ), have the ability to interact with DNA and proteins. Hydroquinone (HQ), a metabolite of benzene, has the ability to produce toxicity in the bone marrow once it interacts with phenol. The effects of genotoxicity are seen in a metabolizing gene (*CYP*2E1), detoxification genes (*NQO*1 and *GSTT*1), and in DNA-repair gene (*XRCC*1).

Purpose

To determine whether occupational exposure among eThekwini Municipality petrol attendants is associated with DNA damage.

Methods

This analytic cross sectional study included 151 participants that comprising of 75 high-exposed petrol attendants, 26 low-exposed workers from eight petrol stations within the city of Durban, and 50 office-based controls from University of KwaZulu-Natal. Researcher administered validated questionnaires were used to establish an association between DNA tail length via comet assay and the volume of petrol pumped in the past year, adjusting for various covariates through multivariate modelling.

Results

The median duration of employment in the petroleum industry was 4.5 years (range: 1-14 years) among the 26 low exposed and 5 years (range: 1-27 years) among 75 high-exposed petrol attendants. The median volume of petrol pumped by the 75 petrol attendants was 182 metric

tons in the past year (range: 18-573 tons). The median tail lengths were 60.5µm (range: 18-149) for the high exposed, 89.5µm (range: 24-124) for the low exposed and 56 µm (range: 14-80) for the unexposed. Wilcoxin rank test, showed a statistically significant association between job title and tail length among the exposed and unexposed group. Mann Whitney test showed alcohol consumption to have a significant influence on the level of DNA damage. The multi-variate analysis showed a statistically significant association between job category, smoking, alcohol consumption and comet tail length.

Conclusion

Occupational exposure was associated with an increased comet tail length among the exposed group compared to the unexposed. Cumulative exposure of volume of petrol pumped over one year duration had no significant dose related risk and was not associated with an increase in DNA damage.

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DEFINITIONS

The following definitions have been used in this study and are extracted verbatim from the Southern African Department of Labour (1993), *Occupational Health and Safety Act (Act no 85 of 1993)* and *Hazardous Chemical Substance Regulation 1179 of 1995*.

- OEL-CL or occupational exposure limit - control limit means the occupational exposure limit for a Hazardous Chemical Substance Regulation as listed in Table 1 of Annexure 1 and has a residual risk that may exist and the level set, takes socio-economic factors into account.

- OEL-RL or occupational exposure limit - recommended limit means the occupational exposure limit for a Hazardous Chemical Substance Regulation as listed in Table 2 of Annexure 1 and has been set at a level at which there is no indication of a risk to health listed.

- TWA: 8-hour Time Weighted Averages (TWA) – are average value of exposure over 8 hour shift and 40-hour work week to which nearly all workers may be exposed day after day without harmful effects.

- STEL: Short-Term Exposure Limit is the average concentration to which workers can be exposed for a short period (usually 15 minutes) without experiencing irritation, long-term or irreversible tissue damage, or reduced alertness.

The following definitions have been used in this study and are extracted verbatim from the American Conference of Industrial Hygienists (ACGIH).

- TLV: Threshold Limit Value: the concentration of an airborne substance that represents conditions under which it is believed nearly all workers may be exposed day after day for an eight hour shift without adverse health effects.
- TLV –C: Threshold Limit Value – ceiling (ceiling values - at no time should this exposure limit be exceeded).

From the South African Hazardous Chemical Substances Regulations 1179 of 1995:

- Biological Exposure Index (BEI) is a reference value intended as a guideline for the evaluation of potential health hazards as listed in Table 3 of Annexure 1 of Hazardous Chemical Substances Regulations.
- **Benzene Biological Exposure Indices**

  Determinant total phenol in urine

  - Sampling time: end of shift
  - BEI 50 mg/g creatinine

  Benzene in exhaled air:

  - Determinant mixed exhaled
  - Sampling time: prior to next shift
  - BEI 0.08 ppm
- HCSR or Hazardous Chemical Substance Regulations means any toxic, harmful, corrosive, irritant or asphyxiant substance, or a mixture of such substances for which an OEL is prescribed.

Occupational exposure limits of concentrations of gases and vapours are expressed as follows:

i. In parts per million (ppm), a measure of concentration by volume

ii. In milligrams per cubic metre of air (mg/m^3), a measure of concentration by mass.

iii. In converting from ppm to mg/m^3; a temperature of 25°C and an atmospheric pressure of 101.325 kPa are used. Benzene at 5 ppm TWA OEL-RL is 16 mg/m^3 TWA OEL-RL

iv. Concentrations of airborne particles (fume, dust, etc.) are usually expressed in mg/m^3.

ACRONYMS

AML: Acute myeloid leukaemia

BEI: Biological Exposure Index

BO: Benzo-oxide

BQ: 1, 4-Benzoquinone

BTEX: Benzene, toluene, ethylene and xylene

DSBs: Double-strand breaks

IARC: The International Agency for Research on Cancer

GSTs: Glutathione-S-transferases

GSTM1: Glutathione S-transferase-Mu gene

GSTP1: Glutathione-S-transferase-Pi gene

HCSR: Hazardous Chemical Substances Regulation

HQ: Hydroquinone

MMT: Methylcyclopentadienyl Manganese Tricarbonyl

MPO: Myeloperoxidase

NAT2: N-acetyltransferase 2

NQO1: NAD quinine oxidoreductase 1

OEL: Occupational exposure limit

PEL: Permissible Exposure Limit

PSA: Petrol Station attendants

STEL: Short time exposure limit

SSBs: Single-strand breaks

TLV: Threshold Limit Value

TWA: Time Weighted Average

TTM: Trans-trans-muconaldehyde

VOC: Volatile organic compounds

XRCC1: X-Ray Cross-Complementing Group

CHAPTER 1: INTRODUCTION, HYPOTHESIS AND OBJECTIVES

1.1 Introduction

Prolonged occupational exposure to petrol can be a health hazard due to its acute and chronic effects once it enters the body. Petrol attendants are at risk of exposure through inhalation and dermal routes of entry: inhaling vapour during refuelling; using petrol as a degreaser and washing their hands (1). The standard practice of evaluating health risks from petrol exposure has been to assess benzene, toluene, ethylbenzene and xylenes, (BTEX), as surrogates for exposure to the whole fuel (2). It has been suggested that benzene accounts for the carcinogenic component, and toluene, ethylene and xylene for the non-carcinogenic health risks (2). There is no known data available that has determined the benzene threshold at which DNA damage occurs, (a marker of carcinogenicity) or whether such a threshold exists at all.

Short-term exposure to petrol has been associated with skin and sensory irritation, impacting on the central nervous system (CNS), hence resulting in conditions such as tiredness, dizziness, headache, loss of coordination, respiratory system conditions (throat and nose irritation) and eye irritation (3). The other health effects secondary to short-term exposure to petrol at high concentrations include chemical pneumonitis, cardiac arrhythmia and ventricular fibrillation (3). Researchers have postulated that chronic exposure to benzene, even at low occupational exposure levels, may contribute to the risk of DNA damage, acute myeloid leukaemia or myelodysplastic syndrome, particularly among genetically susceptible individuals (4).
The definition of genotoxicity in an International Agency for Research on Cancer consensus report (IARC 1992) includes both direct and indirect effects on DNA. Genotoxicity is: (i) the

induction of mutation; (ii) indirect surrogate events associated with mutagenesis [e.g. unscheduled DNA synthesis (UDS) and sister chromatid exchange (SCE)] or (iii) DNA damage (e.g. the formation of adducts), which may eventually lead to mutation (5).

In this study, we defined genotoxicity as DNA damage as measured by comet tail length (CTL) (6).

Reports show that exposure to compounds such as toluene, ethylbenzene, or xylene, have not been identified or proven as being carcinogenic in humans (7). Ethylbenzene, according to the IARC, is classified as Group 2B, implying carcinogenicity in animals and possibly in humans. Toluene and xylene have been categorized as not being carcinogenic in humans (8). Benzene is a proven human carcinogen, and classified as a Group 1 carcinogen (9). Observations in humans and animals indicate that exposure to ethylbenzene causes CNS effects as well as irritation of the eyes and respiratory tract that are generally reversible following cessation of exposure (10). Workers exposed to low levels of ethylbenzene in a styrene plant showed no genotoxicity with no increase in sister chromatid exchanges, DNA adduct formation, micronuclei, or DNA single-strand breaks in the peripheral lymphocytes (11).

The nervous system is the critical target of toluene toxicity following acute, intermediate, or chronic inhalation or oral exposure to toluene (12). Workers repeatedly exposed to toluene in the workplace are reported to have an increased incidence of self-reported neurological symptoms, performance deficits in neurobehavioral tests, hearing loss and vision disturbances (eye irritation and reports of colour vision abnormalities) (12).

There is no genotoxic or carcinogenic effect of toluene in humans. The health effects of mixed xylenes, o-xylene, m-xylene, and p-xylene appear to be similar, although the

individual isomers are not necessarily equal in potency with respect to a particular effect (13). Xylene is irritating to the respiratory tract, eyes and skin. Adverse respiratory effects following inhalation of xylene have been observed in human and animals after acute, intermediate and chronic exposure. These include nose and throat irritation, laboured breathing pulmonary congestion, inflammation and oedema (13). Mixed xylenes and the individual xylene isomers have been tested for genotoxicity in a variety of in vitro and in vivo assays with predominantly negative results, indicating that xylenes are non-genotoxic (14).

In many developed countries, petrol stations are self-service. In developing countries, petrol station attendants have been retained. South African petrol garages are reliant on petrol station attendants to sell fuel, lubricants and other automotive products. Hence with cumulative exposure, the petrol attendants are at greater risk of inhaling petrol vapour during refuelling. According to Sasol refinery statistics in 2000, there were 4850 petrol service stations country wide (15). This number has increased frequently over the years, as new outlets are opened. The Sasol report indicated that the majority of petrol stations are in Gauteng province (31%), 19% in Kwa Zulu Natal, 16% in Western Cape and the rest are distributed among the other six provinces in the country. It is estimated that over 55000 people are employed in the petrol retail sector (15). The average petrol service station pumps 250 000 litres of petrol per month, depending on its business. Sasol reported that 80% of petrol stations pumped petrol that ranged from 150 000 to 300 000 litres per month (15).

KwaZulu-Natal province is divided into one metropolitan municipality (the eThekwini Metropolitan Municipality) and ten district municipalities. The district municipalities are in turn divided into fifty local municipalities. Petrol attendants are not provided with personal protective clothing such as gloves or respiratory protective gear while working the 8 to 12

hours per day at the court. It is on the basis of observing lack of control measures such as personal protective equipment within the petrol stations that prompted us to study occupational exposure and genotoxicity among eThekwini municipality.

1.2 Problem statement

The problem is very little is known about possible DNA damage due to the effect of long-term petrol exposure among petrol station attendants in eThekwini and elsewhere in South Africa. Petrol attendants who work in South Africa are exposed to both vapours and contact with petrol through their skin. While the level of benzene in South African petrol is higher than those of the United Kingdom and the United State of America, little is known about the detrimental effects that this has on their DNA.

1.3 Hypothesis

Long term occupational exposure to petrol can cause DNA damage among eThekwini Municipality petrol station attendants.

1.4 Aim

To determine the association between occupational exposure to petrol and DNA damage among petrol attendants at eight eThekwini Municipality petrol stations.

1.5 Specific objectives

- To determine the occupational exposure of petrol through the volume of petrol pumped among the eThekwini municipality petrol attendants in the past year.
- To determine the presence of DNA damage as measured by comet assay
- To determine the association of occupational exposure to petrol and DNA damage as measured by comet assay

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Petrol is a generic term for petroleum fuel which is mainly used for internal combustion engines, its key aromatic hydrocarbons constituents being toluene, xylene, ethylbenzene and benzene. Other compounds found in petrol are manganese, naphthalene, trimethylbenzene and Methyl tert-butyl ether (MTBE). Constituents of petrol, such as toluene, manganese, ethylene or xylene, have not been identified or proven as being either genotoxic or carcinogenic (7). Of these, benzene is currently the only established human carcinogen (16). The acute health effects of working with petrol on a daily basis can be minimised if the chemicals making the petrol composition are within occupational exposure limits, and appropriate health and safety practices are adhered to.

This chapter reviews the literature on the chemistry of petrol, its metabolic pathway and health outcome. It assesses the contributory factors to DNA damage and the different methods such as comet assays that can be used to measure DNA damage.

2.2 Petrol composition and chemistry

Petrol is a complex mixture, made mainly of paraffin, naphthenic, olefin and aromatic hydrocarbons (17). A generic mixture of petrol contains approximately 54% paraffin and isoparaffins (alkanes from C_4 to C_{12}), 36% aromatics (principally benzene, toluene, ethylbenzene, and xylene), 6% olefins (or alkenes), 5% naphthenic hydrocarbons (or saturated cyclic hydrocarbons and <1% of other compounds (18). Petrol has been classified by the International Agency for Research on Cancer (IARC) as a possible carcinogen on the basis of evidence from experimental studies, evidence from human studies, and supportive evidence

from the established carcinogenicity of some of its components, such as benzene and possibly 1, 3-butadiene (19, 20).

As petrol is composed of multiple compounds, there is no precise Material Safety Data Sheet (MSDS) that contains its precise absorption, distribution, as well as metabolism and excretion information data. Petrol is produced from crude oil or synthesized from gas through refining processes. The composition of petrol varies according to the type of crude oil from which it originates as well as the differences in processing techniques and refineries from which it is blended (21). Workers can be exposed to relatively high levels of petrol vapour in petrol service stations (benzene emission of 0.91mg/m3), or to low levels of petrol vapour in the general population (benzene emission of 0.06mg/m3) (22).

The chemistry and production of petrol involves three basic steps: chemical separation (fractional distillation), conversion (cracking and rearranging the molecules) and treatment of the final product (23). In separation, crude oil, typically a mixture of inorganic salt crystals and water is separated from the contaminants (23). The oil is then heated by a large furnace until it becomes part semi-fluid and part vapour. The vapour condenses depending upon its molecular weight. The vapour with lower molecular weight (including kerosene and diesel) separates immediately. In the conversion phase, vapour with higher molecular weight, such as petrol, must go through an additional process to further crack or separate the oil into a greater variety of components or fractions. At the treatment end, companies add a variety of finishing touches or chemicals that will assist in improving the grade of the petrol and its octane level.

2.3 History of petrol in South Africa and regulation of exposure

Leaded petrol was banned in 2006 in South Africa and was replaced by an increase in manganese and aromatics, including benzene (24). The aromatic content of petrol was raised

from 34% to 40% after lead removal to satisfy the Research Octane Number (RON) specifications (24). Petrol is enhanced with toluene and benzene to increase its octane rating (25). The unleaded fuel is reported to contain 5% benzene compared to 2% benzene in leaded petrol (24). The replacement of lead in petrol has represented an important public health measure in South Africa, with documented decreases in blood lead levels in children (26). However, the increase in benzene and manganese is of public concern due to the known health effect of these chemicals in humans.

The Regulations for Hazardous Chemical Substances (Regulation 1179 of 1995) (HCSR) (28) of the Occupational Health and Safety Act No. 85 of 1993 (OHS Act) (27) has established OELs for chemicals such as benzene. The OELs are set to protect workers from the adverse health outcomes associated with exposure to toxic chemicals in the workplace. An OEL defines the maximum average concentration of a chemical in the breathing zone acceptable for a normal eight-hour time weighted average shift. OEL values are intended to be used as guidelines to control potential health hazards and not as evidence of disease.

As genotoxic and cancer effects have been identified at even lower levels, the OEL control limit (CL) for benzene has been extensively revised. The recommended OEL values, range from 0.32 to 16mg.m^3 (0.1ppm to 1ppm) globally (29). In accordance with the HCSR of 1179 of 1995, employers are requested to conduct annual health risk assessments if employees are exposed to hazardous chemicals designated with a control limit. Benzene has an OEL (CL) of 5ppm or 16mg/m^3. Ethylbenzene has an OEL of 435 mg/m^3, toluene 88 mg/m^3 and xylene 435 mg/m^3 (28).

As stipulated by the HCSR, the employer is also expected to ensure that employees are under a medical surveillance programme if they are exposed to hazardous chemical substances that are listed in Table 3 of Annexure 1 of the Regulations, as is benzene. Medical surveillance is a planned programme of periodic examination by an Occupational Medical Practitioner (OMP). Medical surveillance includes biological monitoring (to measure the extent of absorption of a hazardous chemical substance by the employee), a medical screening, within 14 days after a person commences employment and periodic examinations.

In the United States of America (USA), the Occupational Safety and Health Administration standard for benzene is at 3.2mg/m^3 over an eight hour shift. The American Conference of Governmental Industrial Hygienists, although not a legal body, recommend a limit of 1.6mg/m^3 (0.5ppm), while the National Institute for Occupational Safety and Health recommends a limit of 0.3mg/m^3 (0.1ppm). In Europe, the benzene occupational limit is set at 3.2mg/m^3 (1ppm) in eight hours TWA, while in South Africa, it is set at 16mg/m^3 (5ppm) per eight hour TWA. The reason for such a difference in the exposure limits might be due to that, South Africa is a new democracy with the promulgation of the OHS Act in 1993 and the establishment of the HCSR in 1995 following the advent of democracy in 1994 and the promulgation of a range of Acts that improved the lives of its marginalized citizens. Benzene is a chemical with a control limit, which means that a residual health risk may exist, and the OEL takes socio-economic factors into account. South Africa is a developing country that has challenges of poverty and unemployment in comparison to developed countries such as the USA and Europe, and economic viability is an important consideration.

Nigeria has a benzene limit of 6–8% of the content of petrol, this being higher than the 5% in South Africa and the 1% in the USA and Europe (30). This implies that African standards are less stringent than those of developed countries and this may result in adverse health outcomes

for those countries that still have petrol station attendants (PSA) who do not have self-service system.

2.4 Occupational exposure limit of benzene

Studies dating back to 1928 suggested that an association between occupational exposure to benzene and the development of leukaemia (31) may exist. Observations suggest that benzene may contribute to the risk of DNA damage, acute myeloid leukaemia or myelodysplastic syndrome (31). Studies indicate that a causal relation exists between high exposures to benzene and the development of pancytopenia, aplastic anaemia and acute myeloid leukaemia (32, 33). Occupational exposure limits (OEL) are set to protect workers from excessive exposure to toxic chemicals in the workplace. An OEL defines the maximum average concentration of a chemical in the breathing zone acceptable for a normal 8-hour working day for five days a week. The OEL is often accompanied by a short-term exposure limit, which is the maximum average concentration to which workers should be exposed for a short period of time (usually 15 minutes). As the haematotoxic and leukemogenic effects have been identified at ever-lower levels, the OEL for benzene has been extensively revised. Genotoxicity has been reported among employees with low benzene exposures of 1.6 mg/m^3 (34), having a lifetime of exposure. This level is below the occupational exposure limit (OEL) of 16mg/m^3 in South Africa.

The International Programme on Chemical Safety (IPCS) is a joint venture of the United Nations Environment Programme, the International Labour Organisation, and the World Health Organization. In 1991, it formed a task group to evaluate epidemiological studies on occupational exposure to benzene in human. It concluded in 1993 that none of the studies conclusively

showed that an eight hour time-weighted average of 3.2 mg/m^3 (1 ppm) over 40-years working career can be statistically associated with any increase in deaths from leukaemia (35). However, as benzene is a carcinogen, the assumption is made that there is no absolute threshold for its effect, hence any exposure, even at low levels, is associated with some increase in risk, although this may be very small (36).

2.5 Health effects of benzene

Benzene represents 5% of the aromatic content of petrol (32), and is also present in car emissions, cigarette smoke, and is used as an industrial solvent in the workplace. Approximately 50% of inhaled benzene in air is absorbed into the body (37), with the increased incidence of leukemia (cancer of the tissues that form white blood cells) being observed in people occupationally exposed to benzene. The US Environmental Protection Agency (EPA) uses mathematical models, based on human and animal studies, to estimate the probability of a person developing cancer from breathing air containing a specified concentration of a chemical (38). The EPA calculated a range of 2.2 x 10^{-6} to 7.8 x 10^{-6} increase in the lifetime risk of an individual who is continuously exposed on a daily basis to 1x10^{-3} mg/m^3 of benzene in the air over their lifetime. The EPA further estimates that, if an individual were to continuously breathe the air containing benzene at an average of 1.3x10^{-4} to 4.5x10^{-4} mg/m3 per day over his or her entire lifetime, that person would theoretically have one in a million increased chance of developing cancer, compared to an unexposed population.

Inhalation of benzene produces acute toxic effects on the central nervous system in humans. Inhalation of 800–1600mg/m^3 produces vertigo, drowsiness, headache, and nausea (39). Higher concentrations of 4800mg/m^3 cause euphoria followed by giddiness, headache, nausea, staggered gait, and with continued exposure, unconsciousness (39). Short term exposures to

9600mg/m^3 can be tolerated for 0.5–1.0 hours. However, exposure to massive concentrations of 64 000mg/m^3 or higher can be fatal within 5–10 minutes (40). Excessive repeated daily exposure to benzene (>320mg/m^3) results in pancytopenia and aplastic anaemia. Benzene is generally associated with a marked decrease in the number of cells in the bone marrow, (leukocytopenia, thrombocytopenia, granulocytopenia, pancytopenia) resulting in severe clinical manifestations including immunosuppression (40). Daily exposure to benzene at levels less than 96 mg/m^3 results in cytopenia (40). Affected people may display a decrease in white blood cells, potentially resulting in death due to infection, a decrease in platelet count potentially resulting in death due to haemorrhage, or a decrease in red blood cell count (41-43).

Daily exposure longer than 10 years to benzene at 79 mg/mg^3 has a relative risk of 4.2 (95% CI = 1.1-15.9) of developing non-Hodgkin's lymphoma (44). For individuals who were daily exposed to benzene for less than 10 years at levels of 79 mg/mg^3, the relative risk for the combination of acute non-lymphocytic leukaemia and related myelodysplastic syndromes was 7.1 (95% CI= 2.1-23.7) (44). This study reports that the risk of a combination of acute non-lymphocytic leukaemia and related myelodysplastic syndromes are significantly increased among those with more recent benzene exposure, while non-Hodgkin lymphoma was associated with chronic exposure. The European Scientific Expert Group for occupational exposure limits estimates that an occupational exposure limit value of 1.6mg/m^3 per day would reduce the range of best estimated lifetime risks to 0.25–3.3 additional leukaemia cases per 1000 exposed workers (45).

A nested case control study of 10 000 participants was undertaken to investigate whether an excess of lympho-haematopoietic cancers in the Australian petroleum industry was associated with benzene exposure (46). Validated researcher administered questionnaires

were used to enquire about job history, task frequencies, occupational technology use and previous changes. Confirmation of data was done on review of employment record. Retired petrol attendants were followed up and telephone interviews conducted to minimise healthy worker effect. Benzene exposure for each individual petrol attendant was assessed for each job task or category held in the petrol industry. Cumulative exposure (mg/m^3 years) was calculated as the product of the mean benzene exposure in mg/m^3 for each job task and the duration for that specific job in years, summed for all jobs held during the lifetime of employment in the industry.

Overall, all exposure to benzene was less than $16mg/m^3$ at the eight hour TWA, and with cumulative exposure from 0.02 to $162.6mg/m^3$-years (46) with a mean of $16mg/m^3$-years and standard deviation of $22.4mg/m^3$-years. The study showed an association between cumulative exposure to benzene and an increased risk of lympho-haematopoietic cancer. Average benzene concentration (cumulative estimate divided by duration of employment) ranged from 0.03 to $5.4mg/m^3$-years, with a mean of $0.8mg/m^3$-years (46). The significance of this study is that it demonstrates that low dose benzene exposure in petrol can result in cancers.

A study was conducted in Potchefstroom, South Africa, with the aim of evaluating the level of exposure of petrol attendants to petrol volatile organic compounds (VOCs), and its genotoxic effect. The 40 participants comprised of 20 exposed petrol attendants taken from three garages and 20 unexposed individuals matched for age and smoking habits. Petrol attendants were exposed to lower levels of petrol VOCs than the OEL for the individual chemicals (47). The exposure levels were 0.65, 0.73 and 0.31 mg/m^3, for benzene, toluene and xylene, respectively. This study used comet assay visual inspection to determine DNA damage and indicated statistically significant DNA damage that was assessed through comet tail intensity and DNA repair capacity. Significant DNA damage ($P < 0.05$) was seen in the exposed group.

Approximately 75% of the total DNA was still intact or slightly damaged in the unexposed group (47). The group exposed to petrol showed moderate to severe DNA damage (47). The study had a small sample size with the majority of participants (70%) being between the ages of 25 to 35 years, the anticipation being that they would have good DNA repair mechanisms (48). The majority of the subjects were smokers (>55%), thus increasing the likelihood of DNA damage and there was a decreased DNA repair in smokers. Smoking as a confounder was dealt with at the study design stage (with matching of participants on smoking status) (47). Based on direct measurements of benzene in mainstream cigarette smoke, it is calculated that a typical smoker inhales 2 mg benzene daily, compared to 0.2 mg/day for the nonsmoker. Thus, cigarette smoking may be the most important source of exposure to benzene (49)

The comet assay was used to evaluate the DNA status in peripheral blood lymphocytes of 32 petrol pump workers from eight petrol stations in central Chiang Mai, Thailand (50). Thirty control subjects, who had not been exposed occupationally to benzene, were matched to the exposed subjects by gender and age. There were 11 smokers (n=10 in exposed group). The comet assay revealed that DNA damage in the peripheral blood lymphocytes of petrol pump workers was significantly higher than that in the controls. The average tail length in the exposed workers was 5.51μm ± 5.46μm based on 100 cells/individual, whereas the average tail length in the control group was 1.57μm ±1.03μm (50). While the comet assay in the Potchefstroom study among the exposed group was 9.10μm to 15.06μm in comparison to 3.37μm to 6.7μm in the unexposed group (47).

2.6 Metabolites of benzene

When benzene enters the human body, it is first oxidized in the liver to benzene oxide and then further detoxified to phenyl mercapturic acid (51). The rest of the benzene oxide forms phenol, or is converted to catechol and hydroquone (51). Phenol, catechol and hydroquinone are the three principle liver metabolites of benzene; while catechol and hydroquinone are known

carcinogens (52). Further metabolism occurs in the bone marrow where the metabolites are oxidized by myeloperoxidase to benzoquinones. Benzene is excreted in urine and the biomarkers that can be measured in urine are trans-muconic acid and S-phenyl mercapturic acid (53).

Metabolites of benzene such as benzene oxide (BO), 1, 4-benzoquinone (1, 4-BQ), and 1, 2-benzoquinone (1, 2-BQ) produce reactive electrophiles, and are capable of reacting with blood proteins to produce adducts (53). The five benzene metabolites, namely, benzene oxide (BO), 1, 2 and 1, 4 benzoquinone (1, 2-BQ and 1, 4-BQ, respectively), the muconaldehydes, and benzene diolepoxide have the ability to bind to DNA. Researchers have postulated that BO and 1, 4-BQ, bind to DNA and inhibit cell division and DNA synthesis (54). Albumin adducts of BO and 1, 4-BQ have a half-life of 3-4 weeks (55). Therefore they can serve as intermediate term biomarker of benzene exposure (56).

Urinary metabolites of benzene: S-phenylmercapturic acid (S-PMA) and trans muconic acid (tt-MA) or urinary phenol correlate with recent atmospheric exposure with a half-life of 48hrs (57). S-PMA proved a more reliable biomarker than tt-MA for benzene exposures during 12 hours shift work for low-level benzene exposures. S-PMA enabled reliable determination of benzene exposures down to 0.96 mg/m^3 (0.3 ppm), eight hours TWA (57). The correlation found in between personal exposure to benzene and the urinary excretion of both metabolites (S-PMA and tt-MA) was $r = 0.321$, $p = 0.0001$; $r = 0.250$, $p = 0.0025$, for S-PMA and tt-MA, respectively (57). A statistically significant correlation was observed between DNA damage and urine S-PMA for exposed workers. This study indicates that both, urinary S-PMA and DNA damage assessed by the comet assay are both more sensitive to exposure to low levels of benzene, than tt-MA.

In contrast to this study that stated that S-PMA and tt-MA can be reliable measures of low level benzene exposure, the American Conference of Government Industrial Hygienists reported in 2001, that urinary tt-MA and SPMA are recommended metabolites for biological exposure indices for benzene exposure at high levels above the exposure limit of 1 ppm (16mg/m^3) (58). However correlation with benzene exposure at low concentration below exposure limits (16mg/m^3) was poor and unreliable, since the metabolites were non-specific or insensitive in low exposure ranges (58). These findings are supported by researchers that report that current bio monitoring methods such as urinary phenol, S-phenylmercapturic acid, and trans-trans muconic acids were found to be unreliable as analytical methods to detect benzene exposure (54).

Covalent binding of chemicals to DNA with the formation of chemically stable products known as adducts plays a major role in the mode of action of chemical mutagens and carcinogens. For chronic exposure, measuring adducts formed in serum of benzene or its metabolite; 1.4 benzoquinone gives a half-life of 4 weeks and can be used to correlate exposure up to 11 months (57). The metabolites of benzene such as 1,4-benzoquinone (BQ) were found to be the most potent metabolite in induction of oxidative stress formation and DNA damage, followed by 1,2,4-benzene triol (BT) and to a lesser extent, phenol (PH) and trans,trans-muconaldehyde (MD) (59). These findings of DNA damage play an important role in the mechanism of carcinogenesis induced by benzene metabolites (59). Benzene in blood is documented as more specific and sensitive but markedly affected by current/recent exposures rather than the average cumulative exposure (60).

2.7 Individual susceptibility to DNA damage

Susceptibility to benzene toxicity has been related to genetic polymorphisms (61-65). Carcinogenesis is a multistage process that results from the interaction of carcinogenic exposures, genetic traits, and other endogenous factors (64). Involved in these processes are metabolic activation and detoxification of chemical carcinogens, genetic sequences of proto-oncogenes and tumour suppressor genes, and DNA repair genes that affect the DNA repair capacity (65). Biological indicators of genotoxic risk are subdivided into indicators of internal dose, biologically effective dose, and early biological effect (63). Each of these varies widely among individuals and can be associated with increased cancer risk. Two enzymes have been particularly implicated in the mechanism of benzene toxicity: cytochrome P450 2E1 (CYP2E1) and quinone oxido reductase (NQ01). CYP2E1 is responsible for metabolically activating carcinogens and medications in the liver (64). Its genetic coding has now been identified as polymorphic and linked to cancer risk (64). Benzene, which is a leukemogen, has genetic variants in its metabolic pathway that can modulate the risk of genotoxicity and leukaemia following exposure (61). In particular, underactive variants of the NAD (P) H: quinone oxido reductase 1 gene (NQO1) seems to increase the risk of acute myeloid leukaemia (22). CYP2E1 is inducible by benzene therefore alterations in the level of CYP2E1 might influence human health effects (62). The frequency for each of these genetic polymorphisms varies among ethnic and racial groups.

Of the multiple chemicals found in petrol, benzene is the most toxicologically dangerous, as it causes genotoxic, mutagenic and carcinogenic effects. The DNA effect or genotoxic effect can be assessed by comet assay, with the rate of DNA damage being dependent on the DNA repair mechanism. The rate of DNA repair is dependent on cell type, the age of the cell, and the environment (66). DNA lesions have specific DNA-repair mechanisms in the different phases

of the cell cycle. Cells resume cell-cycle progression once damage has been repaired. Failure of DNA to repair itself can result in (i) an irreversible state of dormancy (ii) cell suicide/ apoptosis or (iii) unregulated cell division, which can lead to the formation of a tumour that is cancerous (66). The failure of cells to repair DNA damage effectively can result in chromosome breakage, cell death, onset of cancer and defects in the immune system of higher vertebrates (67). As toluene, ethyl benzene or xylene, have not been identified or proven to be carcinogenic in humans (7), it is assumed that genotoxic effect or DNA damage among petrol attendants is related to benzene exposure.

2.8 Other factors related to DNA damage

Multiple factors can produce DNA damage that can be detected by comet assays. These include age, diet, exercise, gender, viral infection, residential radon exposure, smoking, and season (68). Yet none of these factors are unequivocally associated with permanent DNA damage in the majority of the studies as in general, since single short-term low level exposures are unlikely to cause obvious health problems due to the body's DNA repair mechanism (68). It has been proven in a study that alcohol dependent workers have a positive association of 1,4-BQ-Alb/BO-Alb with daily alcohol intake that results in the induction of cytochrome (CYP 2E1) by ethanol (69). Benzene is estimated to be responsible for approximately one-tenth to one-half of smoking-induced total leukaemia mortality, and up to three-fifths of smoking related acute myeloid leukaemia mortality (70).

DNA damage as a direct result of Haemophilus influenza virus has been reported to occur within 24 hours, followed by complete DNA repair within 48 to 72 hours post infection (71), while exposure to X-rays can cause DNA damage within eight hours (71). DNA repair up to 35% takes place within 20 hours (72) post x-ray exposure and complete repair within 90 hours

(69). An infection such as HIV, which is a RNA virus, does cause DNA damage and is not significant to cause carcinogenicity (73). The University of KwaZulu-Natal researchers investigated the effect of exercise on DNA damage by using comet assays (74). There were non-significant mean DNA strand breaks ($P > 0.05$) (74). In a placebo-controlled study, dietary fruit and vegetable (which are antioxidants) had no effects on the level of oxidative DNA damage (75).

Diesel exhausts fumes and DNA damage:

The exhaust from diesel engines is made up of gases and soot. The gas portion of diesel exhaust is mostly carbon dioxide, carbon monoxide, nitric oxide, nitrogen dioxide, sulfur oxides, and hydrocarbons, including polycyclic aromatic hydrocarbons (PAHs) (76). The soot (particulate) portion of diesel exhaust is made up of particles such as carbon, organic materials (including PAHs), and traces of metallic compounds (77).

In 2012 the IARC concluded that diesel exhaust particulates are carcinogenic to human (Group 1) based on sufficient evidence that their exposure is associated with increased risk of lung cancer (78).

Diesel exhaust particles have been shown to generate reactive oxygen species, which lead to oxidative stress and DNA damage (79). PAH associated with diesel exhaust are genotoxic, forming PAH-DNA adducts and resulting in mutation and DNA strand breakage (79) Occupational studies of railroad workers, heavy equipment operators, and truck drivers have demonstrated a significantly higher-than-normal incidence of death from lung cancer (80,81).

There is limited data both nationally and internationally on the effect of diesel exhaust fumes on DNA damage (especially using comet assay) among petrol station workers.

Based on research on the effect of diesel exhaust emissions in causing DNA damage and lung cancers among other job categories, we deduct that it is highly possible that diesel exhaust fumes would contribute in causing DNA damage among the petrol attendants in our study.

2.9 Comet assay testing for DNA damage

Comet assays are a rapid and reliable test that can detect DNA damage in different exposure circumstances (59). The test is more sensitive and specific than chromosomal analysis (CA) and micronuclei (MN) as a biomarker of effects of genotoxicity and carcinogens (82). After reviewing 45 different reports that compared results of different cytogenetic assays such as CA, MN and SCE with the comet assay, one study concluded that the results of the comet assay were the most reliable and sensitive in detecting DNA damage (83).

The damage detected by comet assay is commonly referred to as strand breaks. DNA damage is better regarded as a marker of effect of genotoxic agents than as an absolute indicator of the likelihood that cancer will occur in an individual. The development of cancer depends on a number of factors, including the extent of DNA damage, antioxidant defences and DNA repair systems. During the body's repair mechanism, DNA repair genes affect the DNA repair capacity and thus may determine an individual's susceptibility to carcinogens (50).

The comet assay provides a measurement of single or double-strand DNA breaks at the level of the single cell (84). The technique involves the evaluation of cells kept in agarose gel (on a microscope slide), submitted to electrophoresis and dyed with ethidium bromide. Cells with damaged DNA form a comet consisting of a head (nuclear matrix) and a tail (formed by DNA fragments). The amount of DNA that has migrated is correlated with the degree of damage (85). Despite a number of disadvantages in its ability to identify the causes of DNA damage, the comet assay is considered a suitable and fast test for assessing DNA-damage.

Advantages of comet assay

The comet assay has a number of advantages which include:

- While not absolutely specific, it is a sensitive method for measuring single and double strand DNA breaks even at low levels of damage (86)
- It is an inexpensive technique, easy to apply and there are no delays in obtaining sample scores. It only requires a small number of cells per sample for analysis (87).

Disadvantages of comet assay

There are multiple environmental, occupational and genetic factors that can result in DNA damage that will be detected by comet assay, and it is therefore difficult to isolate the damage caused by a specific factor. Factors that can affect DNA damage include:

- Environmental factors such as radiation exposure and air pollution can affect the extent of DNA damage (87).
- There is evidence that antioxidants in a diet can increase the resistance of cellular DNA to oxidative attack (88).

A study compared the three different scoring techniques of comet assay, such as visual scoring, automated and semi-automated image analysis when assessing comets in the same set of gels from dose-response experiments with typical DNA-damaging agents (89). All three scoring methods proved capable of detecting a significant level of damage at the lowest concentration of each agent. Visual scoring systematically overestimated low levels of damage compared with computerized image analysis (89). On the other hand, heavily damaged comets were less efficiently detected with image analysis (89). Overall, according to the Bland–Altman analysis, the degree of agreement between the three scoring methods was within acceptable limits (90).

There was however a variation in the basal level of DNA damage among healthy individuals (91).

Reports suggested that control cells with no chemical exposure should in general have a normal level of DNA damage of 10-20% to allow detection of either increased or decreased tail length migration (60). The migration of the tail length that is greater than the control reference point is a measure of increased DNA damage. It has been accepted by researchers that extensive DNA damage is regarded as a marker of cancer risk from DNA damage in mutagenesis and carcinogenesis (92). The greater the DNA damage, the greater the risk of mutagenesis and carcinogenicity (68). However, the exact conclusive extent of DNA damage predicting cancer is not yet known, as it is not possible at the present time to give an estimate of the relative contributions of physiological, environmental, or assay variation to the overall variation of the comet assay (17).

The benzene threshold that causes DNA damage at low level of occupational exposure is unknown. The comet assay analysis of single strand breaks and alkali labile sites reported in a study which showed a significant excess of DNA damage in circulatory lymphocytes of petrol attendants who were occupationally exposed to low benzene levels, compared to an age-matched reference group (93).

Inter-laboratory variability of comet assay measured DNA damage is an important consideration. In a study, 12 laboratories analysed the level of DNA damage in monocyte cells by either visual classification, computer-aided image analysis of pre-made slides, or coded cryo-preserved samples of cells and reference standard cells (calibration curve samples). The reference standard samples were irradiated with ionizing radiation (0–10Gy) (94). All

laboratories detected dose–response relationships in the coded samples irradiated with ionizing radiation (1.5–7 Gy) (94). There was inter-laboratory coefficient of variation (CV) of 28%, indicating a statistically significant difference ($P < 0.05$) (94). The inter-laboratory variation originated from differences in image analysis, whereas the intra-laboratory variation was considerably smaller than the variation between laboratories. Adjustment of the primary comet assay results by reference standards reduced inter-laboratory variation in the level of DNA damage from 47% to 28%.

The comparison of the efficiency of scoring method between visual scoring, automated and semi-automated image analysis when assessing comets in the same set of gels from dose-response experiments with typical DNA-damaging agents was done in Norway. All three scoring methods proved capable of detecting a significant level of damage at the lowest concentration of each agent (95). Overall, the degree of agreement between the scoring methods was within acceptable limits according to a Bland–Altman analysis (95)

2.10 Conclusion

Prolonged occupational exposure to petrol can be a health hazard, as benzene is classified as a Group 1 carcinogen. Petrol is a complex mixture of low-molecular mass compounds, some of which are carcinogenic and genotoxic. In view of the occupational health risk caused by benzene, it is important to determine the level of exposure to benzene and also to evaluate the effect caused by this exposure. Comet assay is a reliable method that will detect DNA damage. The comet assay is extremely versatile, rapid and sensitive, and is used extensively due to its capacity and sensitivity in demonstrating DNA breaks, both single and double strands breaks and alkali-labile sites. Genotoxicity has multi-factorial risk factors age, radiation exposure, smoking, exercise and infections can produce DNA damage that can be detected by comet assays.

Chapter 3

* This section is drafted as a manuscript for submission to the International Archives of Occupational and Environmental Health. The journal requirements are listed below:

Title Page

The title page should include:

- The name(s) of the author(s)
- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide a structured abstract of 150 to 250 words which should be divided into the following sections:

- Purpose (stating the main purposes and research question)
- Methods
- Results
- Conclusions

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Text Formatting

Use a normal, plain font (e.g. 10-point Times Roman) for text

References

Reference in the text by name and year in parentheses

CHAPTER 3: OCCUPATIONAL EXPOSURES AND DNA DAMAGE AMONG PETROL ATTENDANTS

* This section is drafted as a manuscript for submission to the International Archives of Occupational and Environmental Health

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Abstract

Purpose

To determine whether occupational exposure among eThekwini Municipality petrol attendants is associated with DNA damage.

Methods

This analytic cross sectional study included 151 participants that comprising of 75 high-exposed petrol attendants, 26 low-exposed workers from eight petrol stations from within two urban areas in the city of Durban, and 50 office-based controls from University of Kwa Zulu-Natal. Researcher administered validated questionnaires were used to establish an association between DNA tail length via comet assay and the volume of petrol pumped in the past year, adjusting for various covariates through multivariate modelling.

Results

The median duration of employment in the petroleum industry was 4.5 years (range: 1-14 years) among the 26 low exposed and 5 years (range: 1-27 years) among 75 high-exposed petrol attendants. The median volume of petrol pumped by the 75 petrol attendants was 182 metric tons in the past year (range: 18-573 tons). The median tail lengths were 60.5µm (range: 18-149) for the high exposed, 89.5µm (range: 24-124) for the low exposed and 56 µm (range: 14-80) for the unexposed. Bivariate analysis showed alcohol consumption ($p< 0.04$) to have a significant influence on the level of DNA damage. The multivariate analysis showed a statistically significant association between job category, smoking, alcohol consumption and comet tail length ($p<0.05$).

Conclusion

While occupational exposure was associated with an increased comet tail length among the exposed group compared to the unexposed, the volume of petrol pumped was not associated with an increase in DNA damage.

Introduction

Benzene is a proven human carcinogen, and is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC) (1). One of the commonest sources of exposure to benzene is petrol; petroleum derived liquid mixture composed of a variety of chemicals. A generic mixture of petrol consists of approximately 54% paraffin and isoparaffins (alkanes from C4 to C12), 36% aromatics (principally benzene, toluene, ethylbenzene, and xylene), 6% olefins (or alkenes), 5% naphthenic hydrocarbons (or saturated cyclic hydrocarbons) and <1% other compounds (2). Exposure can occur via inhalation, dermal and incidental ingestion from unwashed hands (3). Unleaded petrol in South Africa contains 5% benzene compared to the 2% found previously in leaded petrol (4). The replacement of lead with an increase in benzene and other agents, such as manganese, is of public concern due to the known health effect of these chemicals in humans. According to the IARC, exposures to the constituents of petrol, such as toluene, ethylbenzene, or xylene, have not been identified or proven as being carcinogenic in humans (5). Ethyl benzene is classified as a Group 2B, which is carcinogenic in animal studies (5). Toluene and xylenes have been categorized as not being carcinogenic in humans by the IARC (1).

The South African Occupational Health and Safety Act No. 85 of 1993 (6) has established Regulations for Hazardous Chemical Substances (Regulation 1179 of 1995) (7) that provide occupational exposure limits (OEL) for chemicals such as benzene, at 16mg/m^3 (5ppm) eight

hour time weighted average (TWA). In the United States, the Occupational Safety and Health Administration standard for benzene is 3.2 mg/m^3 in an eight hour shift. The American Conference of Governmental Industrial Hygienists recommends a limit of 1.6mg/m^3 (0.5ppm), and the National Institute for Occupational Safety and Health has a recommended limit of 0.32mg/m^3 (0.1ppm). In Europe, the benzene limit is set at 3.2mg/m^3 (1ppm) as eight hours TWA.

In many developed countries, petrol stations are self-service, with people needing petrol dispensing it themselves. In many developing countries, including South Africa, petrol attendants have been retained, and spend all day working on the forecourt dispensing petrol and providing other services to customers e.g. providing oil and water, and checking car tyre pressure. Petrol attendants are therefore at risk of exposure by inhaling vapour during refuelling, and via contact with their skin when using petrol as a degreaser and washing their hands (1). In Nigeria, the benzene concentration is 6–8% of the content of petrol in comparison to 5% in South African, and 1% in the USA and Europe (8). This suggests that their standards are less stringent than those of developed countries, which could result in adverse health outcomes among the petrol attendants. As South Africa has no self-service petrol stations, petrol station attendants are at higher risk of adverse health outcome than their European and American counterparts who utilize self-service-petrol stations.

Comet assays are a rapid and reliable test that can detect DNA damage in different exposure circumstances (9). The comet assays are more sensitive and specific than Chromosomal Analysis (CA) and Micro Nuclei (MN) as a biomarker of effects of genotoxicity and carcinogenicity (10). Extensive DNA damage is regarded as a marker of cancer risk (11). However, the extent of DNA damage as a predictor of cancer is not yet known (12)

South African petrol attendants are at increased risk of being exposed to petrol. This is despite legislation requiring their station owners to provide a safe and healthy working environment as per Occupational Health and Safety Act of 1993 (6) and the legislation for a medical surveillance programme of workers as per Hazardous Chemical Substance Regulation of 1995 (7), yet this was observed not to be done by petrol station owners. With limited data in South Africa on the impact of exposure to petrol by attendants, the aim of the study was therefore to determine if an association exists between occupational exposure and DNA damage among eThekwini Municipality petrol attendants.

Materials and Methods

Petrol Station and participant selection

A sample of eight petrol stations within the eThekwini Municipality was selected. Two garages were sampled from each of four petroleum companies agreeing to participate. All the stations were selected within the same geographic area in the city, and approximately eight to ten full time petrol attendants worked at each station depending on its location.

A previous South African study of petrol attendants (n= 40) and comet tail length found a mean difference of 8.8 units between exposed and non-exposed (13). Given that the sample of "non-exposed" was likely to have some exposure (workers in the shop and wash bays), and our interest in adjusting for important covariates such as cigarette smoking, age and alcohol consumption, a sample size of n=150 (comprising of exposed and non-exposed) was considered appropriate for this study. All workers (petrol attendants and other employees working in wash bays, cashiers or cooks) at each petrol station who met our inclusion criteria were invited to participate.

A total of 150 employees who worked both night and day shifts at the selected petrol stations were invited to participate. Employees with no history of factors likely to influence the comet

assay test, such as undergoing radioactive therapy, family history of cancer, leukaemia or recent flu-like illnesses, were included in the study. Among the petrol attendants, those with no history of volume of petrol pumped in the last year were excluded. From the 150 employees, 25 employees refused to participate in the study, which resulted in 125 completing the questionnaires. Of these 125, 24 were removed from the study as 15 did not meet inclusion criteria and nine were on leave at the time of taking blood samples. The remaining 101 met the inclusion criteria and participated into the study and consisted of 75 petrol attendants (high exposed) and 26 car washers and shop attendants (low exposed).

For the non-exposed group, 78 office based workers were approached, 28 of whom declined to participate, while 50 met the inclusion criteria and volunteered to participate. All participants had to be over 18 years of age, and had to have worked for at least one year in their respective working environments. All participants were fully informed about the procedures and objectives of this study, and an informed consent form was signed by each person prior to the study starting. Ethical approval for this study was obtained from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal.

Data Collection

A previously validated English language questionnaire (13) was modified and piloted before use. This was translated into isiZulu and back translated to ensure consistency. Interviewers were trained in administering the questionnaire. The questionnaires were administered to the participants during their routine working hours. Information was obtained about their demographics, occupational, medical and environmental history, history of radiation, infections, genetic or co-morbid illnesses, and history of smoking, alcohol intake, diet and exercising and family history of cancers. Blood samples were also taken to enable the comet assay test to be conducted.

The monthly volume of petrol pumped for the preceding 12 months (from period February 2011 to January 2012) was collected. Data on the volume of petrol pumped per year by each participant was retrieved from the petrol station records, as this was maintained electronically at all stations.

Comet assay protocol

All bloods samples for comet tail length were sent to the laboratory within four hours of being obtained, and were analysed by two laboratory workers double blinded to exposure status. The comet assay was performed under alkaline conditions. Aliquots of 25µl heparinised blood were mixed with 175µl low melting agarose (0.5%) in phosphate buffered saline (PBS) and added to frost-ended microscope slides, in triplicate, which had been covered with a bottom layer of 1% agarose. Slides were placed in lysis buffer (2.5M sodium chloride (NaCl), 100mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X, 1M Tris, 10% dimethyl sulfoxide (DMSO); pH 10; 4 ◦C, 1hour). Slides were then equilibrated and denatured in alkali buffer (300mM sodium hydroxide (NaOH), 1mM disodium ethylenediaminetetraacetic acid (Na2EDTA); pH13) for 20 minutes followed by 35 minute electrophoresis (25Volts, 35 min). Images of 25 randomly selected cells stained with ethidium bromide were analysed from each coded slide. Measurements for comet tail migration were made for 25 cells between 3 slides per sample by image analysis (Analysis 5 Software). Parallel evaluation of slides was performed by a second investigator.

This computerized image analyses of DNA damage was carried out in accordance with the standard protocols by Collins (14), with two readers reviewing the image microscopically. In instances of discrepancy in the reading, an experienced third reader was consulted. The comet assay image is composed of pixels, which is a process of segmentation in which the image is divided into two areas: the head and tail of the comet, and the background area (9). To measure

tail length, the numbers of pixels were counted along the x-axis. The computerized image analyser has a geometric calibration, with a known scale of micrometers per pixel (10). The comet tail lengths (measured in micrometers) of the exposed petrol station employees were compared to the tail lengths of the office based employees.

Statistical analysis

Stata version 11 statistical software was used for the statistical data analysis. Standard data quality measures in data capture and analysis were adopted. Non-parametric tests including the Mann Whitney test, analysis of variance (Kruskal Wallis rank test) and Spearman correlation analysis were used, with the level of significance set at 5%. The outcome variable was the comet assay score of tail length. The independent variables were duration of employment and volume of petrol pumped per month up to one year.

The Mann Whitney test was used to compare comet tail length between the three exposure groups. Spearman's correlation analysis was used to determine the correlation between the variables comet assay tail length and the volume of petrol pumped. The Kruskal Wallis rank test was used to test the statistical significance of the difference between the medians of variables studied within the two groups of petrol exposed group (n=101) and the non-exposed group (UKZN office based workers n=50).

The regression models included the following independent variables: age, sex, petrol exposure, cigarette smoking, alcohol intake, current or recent influenza infections, positive HIV status, drug intake and living in a suburb. Several regression models were constructed using different exposure indices: exposed vs. non-exposed; high vs. low-exposed and high vs. 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. non-drinker) into separate models respectively.

Results

Sixty percent of the participants fell in the age group 25–35 years (Table 1). There were no meaningful differences observed between exposure groupings with regard to the various demographic variables. There was a statistically meaningful difference in comet tail length between the exposed group and the non-exposed group of 66 µm (range 23µm - 145µm) vs. 56µm (14µm - 80µm) respectively; $p < 0.05$ (Figure 1). There was no association between the volume of petrol pumped and the comet tail length (Figure 2).

Among those who consumed alcohol, comet tail length was significantly greater among the exposed (72µm) than the unexposed (56µm) (Table 2). There was no statistical association between exposure category and comet tail length when stratified by cigarette smoking, positive HIV status and drug intake. Environmental exposures, such as living near emitting factories, petrol station located within a kilometre from participants' homes and living within a kilometre of the highway, did not contribute to differences between exposure category and comet tail length. (Table 2)

While there was a small but statistically significant correlation with pack years of cigarette smoked ($r^2 = 0.23$, $p<0.05$) and alcohol consumption ($r^2 = 0.18$, $p<0.05$), none of the other continuous variables showed any meaningful correlation. Adjusting for age, alcohol consumption, smoking, drugs intake, current influenza infection, HIV positive status and living in the suburbs, exposed workers had a statistically significant greater comet tail length of 9.2µm (95% CI: 0.26; 18.61) than those without exposure (Table 3). Smokers and current alcohol consumers showed statistically significant longer comet tail lengths when compared to non-smokers and non-alcohol consumers respectively (Table 3).

We ran other models comparing comet tail length between the high exposure petrol attendants (n=75) and the low exposure employees (n=26) whose job activities did not routinely include pumping petrol (data not shown). There was a significant difference in comet tail length

between those with high exposure 57µm (range 23µm - 145µm) compared to those with low exposure 76µm (range 25µm -130µm) with $p<0.05$.

Discussion

This study provided evidence that exposure to petrol resulted in increased DNA damage, although there was no evidence of a dose-response relationship.

Regression models, adjusting for age, gender, infections such as influenza and HIV, drug intake and living in suburbs, showed that there was a statistically significant relationship between comet tail length (a marker of DNA damage) and benzene exposure in petrol. This finding is supported by several other studies (13, 15). Previous studies have shown an effect ranging from an average tail length in the exposed workers was 5.51µm ± 5.46µm with the average tail length in the control group being 1.57µm ±1.03µm (15). In our study, the tail length ranged from 23µm - 145µm with a median of 66 µm in exposed workers to a range of 14µm - 80µm with median of 56µm in unexposed. This was in a counter-intuitive direction. The longer comet tail length in the low-exposure group suggests that car washers and other workers in petrol stations may have some occupational exposures, and are therefore at risk for DNA damage.

The differences seen in our study compared to others could be explained by a higher content of benzene level in petrol in these working environments. Benzene in South Africa has an occupational exposure limit of 16mg/m^3, while in countries such as America and Europe; it ranges from 0.1mg/m^3 to 3.2mg/$m^{3.}$

There was an association between current alcohol consumption, smoking and comet tail length. The regression models suggested that alcohol and smoking have an effect on DNA damage than that of occupational petrol exposure. Given the consistent reporting in the literature about the association of alcohol and smoking with comet tail length, this finding gives credibility to our data, laboratory assessment and modelling.

The interesting finding in our study was the absence of an exposure related association with comet tail length in unadjusted bivariate analyses, which may have been due to the strong con-

founding effect. Although demographically similar, variables such as smoking or alcohol use may have driven the findings in the unadjusted results. There were differences in the smoking results, as there were more current smokers in the exposed group in comparison to the non-exposed group. Although there was similar current alcohol consumption in both groups, there were more ex-drinkers in the exposed than the non-exposed group.

There was no association between the volume of petrol pumped and the comet tail length, for which there could be several explanations. Due to benzene's rapid excretion via exhaled air, this can contribute to lack of association between volume of petrol pumped and DNA damage on comet assay. We hypothesised that a dose-response relationship is likely to exist, but that the annual volume of petrol is not a sensitive indicator. We were not able to find support in the literature for this finding. It is possible that shorter term volumes are more likely predictive of DNA damage, or a more comprehensive exposure metric is necessary. We were not able to describe exposure in such a manner in our study. In addition, gender, HIV status, drug intake and current infection such as influenza had no influence in the level of DNA damage in our study.

The exposure related differences in comet tail length between the exposed and unexposed group in our study, compared with those from other countries, could be explained by a different content of benzene level in petrol. Studies have reported benzene concentrations of between 6-8% in petrol in Nigeria, compared to the 5% in South Africa (8, 13). The environmental benzene level in petrol stations could have been higher than in the petrol stations in South Africa (of less than 16mg/m^3 of benzene), with Swedish measurements during petrol attendants refuelling show concentrations varying from 0.01 to 27 mg/m^3 (16).

The significant difference in comet tail length between those with high exposure 57µm (range 23µm - 145µm) compared to those with low exposure 76µm (range 25µm - 130µm) with $p<0.05$,

Conclusions

In this sample of 101 workers, the different levels of occupational exposure to petrol were related to increased DNA damage, as indicated by comet assay. In addition, there was greater DNA damage among those who smoked cigarettes and consumed alcohol.

The volume of petrol pumped did not show greater DNA damage among the exposed group in comparison to the non-exposed employees. Further research is required to understand the dose response relationship of volume of petrol pumped and the effect of the DNA damage among petrol attendants

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Table 1: Demographic factors among exposed and unexposed workers

Variables	Unexposed group (n=50)	Exposed group (n=101)
Age in years [median(range)]	35 (23-60)	30 (21-57)
Sex (M) [n (%)]	16 (32)	76 (75)
Residential area [n (%)]		
Township	21 (42)	72 (71)
Suburb	28 (56)	21 (21)
Informal settlement	1 (2)	4 (4)
Rural	0	4 (4)
Home <1km from emitting factories [n (%)]	10 (20)	7 (7)
Home <1km from petrol station [n (%)]	28 (56)	22 (22)
Home <1 km from freeways [n (%)]	25 (50)	50 (50)
Duration of employment in years in petroleum industry [median(range)]	0	5 (1-27)
Volume of petrol pumped in metric tons/year [median(range)]	0	163 (0-573)
Influenza infection [(n (%)]	9 (18)	39 (39)
HIV status [(n (%)]		
Positive	1 (2)	2 (2)
Negative	36 (72)	34 (34)
Never tested	13 (26)	64 (64)
Smoking history [(n (%)]		
Current smokers	14 (54)	64 (64)
Never smoked	10 (38)	22 (21)
Ex-smokers	2 (8)	15 (15)
Packs of cigarette per year [median(range)]	1 (0.5-10)	2.5 (0.2-26)
Alcohol consumption [(n (%)]		
Current drinkers	19 (38)	34 (34)
Ex-drinkers	8 (16)	30 (30)
Non-drinkers	23 (46)	37 (37)
Standard drink unit/ day [median(range)]	1 (1-40)	3.2 (1-40)

Table 2: Comparison of median comet tail length (in µm) between the exposed (n=101) and non-exposed group (n=50)

	Median comet tail length (µm)	
Independent Variable	Unexposed group (n=50) [Median (range)]	Exposed group (n=101) [Median (range)]
Sex (M)	61 (24-71)	58 (23-145)
(F)	56 (20-80)	72 (25-136)
HIV status		
Positive	60	79 (48-109)
Negative	60 (14-80)	59 (25-145)
Never tested	56 (24-68)	62 (32-127)
Smoking		
Current smokers	32 (23-82)	54 (25-109)
Never smoked	38 (36-76)	59 (25-143)
Ex-smoker	52 (24-80)	70 (25 -127)
Alcohol		
***Current drinker**	56 (24-80)	72 (34-143)
Ex-drinker	64 (24-66)	53.5 (25-136)
Non-drinkers	56 (14-53)	70 (25-105)
Residing area		
Live in township	59 (24-80)	60 (27-130)
Suburb	56 (20-76)	65 (34-143)
Informal settlement	22	53 (32-83)
Village	0	37 (23-53)
Areas around residence		
Home <1km from emitting factories	51 (38-70)	56 (29-127)
Home <1km from petrol station	50 (14-76)	58 (34-105)
Home <1km from highway	44 (20-71)	56 (25-143)

***p<0.05: Mann Whitney**

Table 3: Multivariate analysis of comet tail length (in µm) and independent variables (n=151)

Characteristics	Coefficient	Standard error	(95%CI)
Demographics			
Age (years)	0.08	0.211	(-0.33; 0.50)
Sex (Male)	-2.77	4.29	(-11.27; 5.71)
Occupational exposure to petrol (Yes)	9.17*	4.77	(0.26; 18.61)
Living in the suburb (Yes)	6.86	4.07	(-1.18 ; 14.92)
Social data			
Pack years of cigarette	2.19*	0.31	(1.57 ; 2.82)
Alcohol consumption (unit/ day)	0.63*	0.28	(0.08 ; 1.19)
Medical data			
HIV positive (Yes)	4.16	11.39	(-18.36 ; 26.69)
Current influenza infection	2.70	3.97	(-5.14 ; 10.56)
Medication intake (Yes)	0.03	3.92	(-7.72 ; 7.79)

***P<0.05** (Model $R^2 = 0.4$)

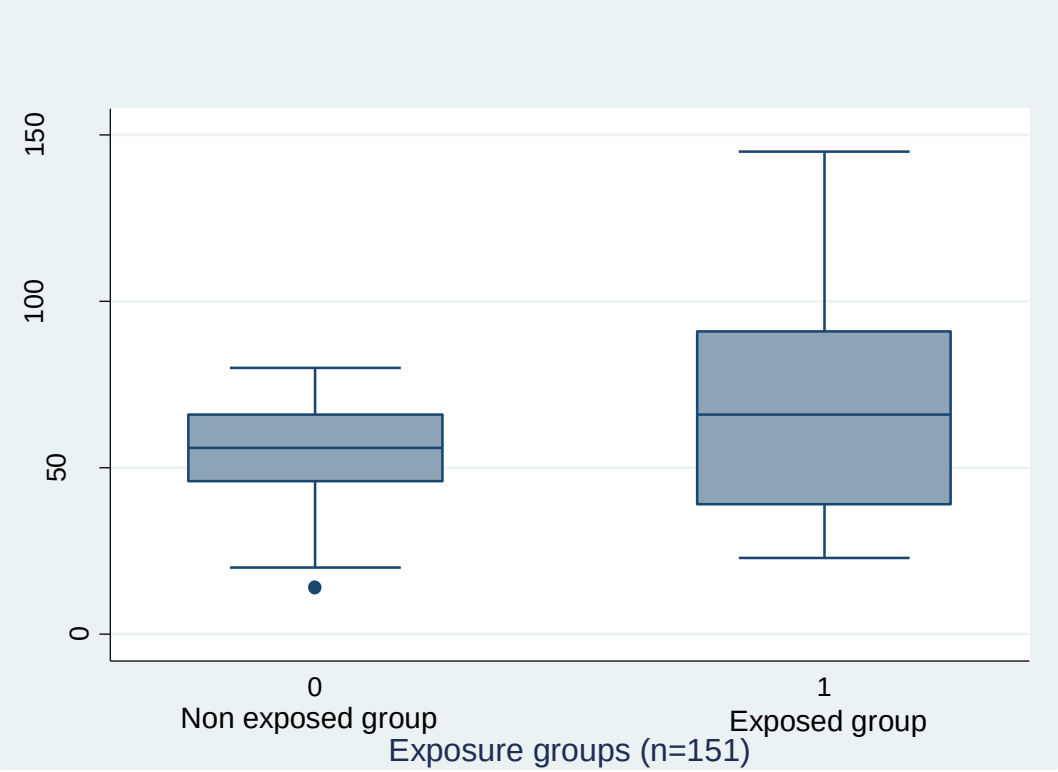

Fig 1: Comparison of the median comet tail length among the unexposed group (n=50) and exposed group (n=101)

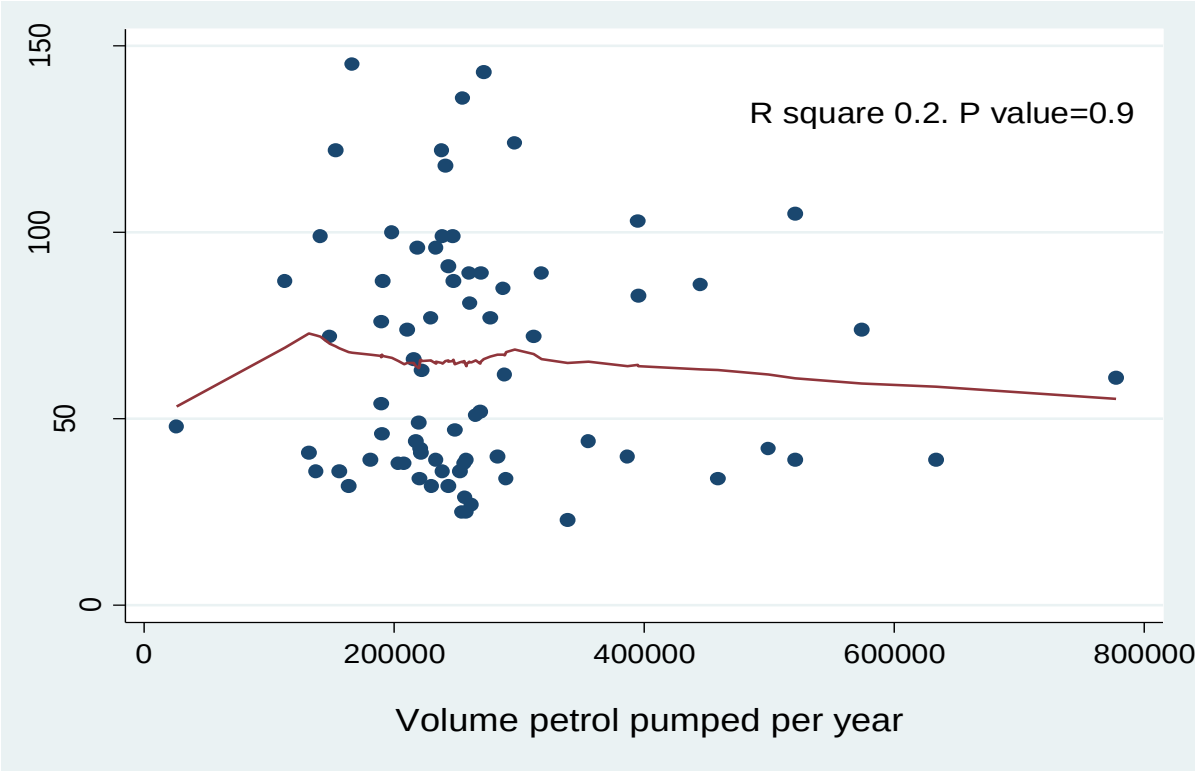

Fig 2: The volume of petrol pumped in the past year in relation to the participants' comet tail length (n=75)

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 Introduction

This chapter presents a discussion of the study results, outlines its strengths and limitations, and argues whether the hypothesis was relevant, makes recommendations for further research, and indicates the study's significance in the field of public health. The aim of the study was to determine the association between occupational exposure to petrol and DNA damage among eThekwini Municipality petrol attendants. The discussion is done with respect to the three objectives:

- To determine occupational exposure through the volume of petrol pumped among petrol attendants at eThekwini petrol stations in the past year
- To determine the presence of DNA damage as measured by comet assay
- To determine the association between occupational petrol exposure and DNA damage as measured by comet assay

Objective 1. *To determine occupational exposure through the volume of petrol pumped among petrol attendants at eThekwini petrol stations in the past year*

The median duration of employment in the petroleum industry was 4.5 years (range: 1 - 14 years) among the 26 low exposed and 5 years (range: 1 - 27 years) among the 75 high exposed petrol attendants. The median volume of petrol pumped by the 75 petrol attendants was 182 metric tons in the past year (range: 18 - 573 tons).

Objective 2. *To determine the presence of DNA damage as measured by comet assay*

The comet tail length between the exposed group and the non-exposed group was 66µm (range 23µm - 145µm) vs. 56µm (14µm - 80µm) respectively; $p < 0.05$ A statistically significant association between exposure category and comet tail length was seen in our data. This is in keeping with other studies that also showed that exposures to benzene

causes an increase in the level of DNA damage in exposed groups in comparison to non-exposed groups (92, 95, 96, 97). However, there was no association between the volume of petrol pumped and the comet tail length

Objective 3. *To determine the association between volume of petrol pumped and DNA damage as measure by comet assay*

Occupational exposure was associated with an increased comet tail length among the exposed group, compared to the unexposed, after adjusting for various confounders. The volume of petrol pumped was not associated with an increase in DNA damage. Our data supports the findings in the literature of benzene exposure related DNA damage that may increase the risk for carcinogenic outcomes.

Overall there were two key findings from this study:

1. The participants who were exposure to petrol were associated with DNA damage as measured through the comet assays
2. No evidence for a dose-response relationship was present, suggesting that increasing cumulative exposure to petrol was not associated with increasing comet tail length over one year of petrol exposure.

The results indicated an association between current alcohol consumption, smoking and comet tail length, with pack years of cigarette smoking contributing to increased comet tail length damage. A cigarette can contain estimated 60-80μm benzene which is estimated to be responsible for approximately one-tenth to one-half of smoking-induced total leukaemia mortality, and up to three-fifths of smoking related acute myeloid leukaemia mortality (70). Alcohol dependent workers had a positive association of 1, 4-Benzo quinone and benzo oxide (metabolites of

benzene) with daily alcohol intake, which result in induction of cytochrome (CYP 2E1) by ethanol (69), and result in greater DNA damage.

Environmental exposures that affect the level of DNA damage included antioxidants, exercise, sunlight and air pollution. These exposures are rarely important determinants in cross-sectional studies (60) as in general, single short-term low level exposures are unlikely to cause obvious health problems, due to the body's DNA repair mechanism (70).

The confounding factors taken into account in this study were age, smoking and alcohol consumption. Factors causing increased risk of DNA damage, such as drug intake (supplements with anti-oxidant properties), HIV positive status or having acute influenza like symptoms, were considered. Environmental exposure, such as living in suburbs with greater car exhaust emissions and living near freeways, petrol station and emitting factories, were adjusted for in the regression modelling.

Age had no significant effect on the level of DNA damage, despite an increased risk of DNA damage and physiologically slowing of the ability for cell to repair and regenerate expected with increasing age. The comet analysis in other studies (68, 93) showed no significant degree of DNA damage in circulating lymphocytes of subjects occupationally exposed to low benzene levels compared to age matched unexposed subjects. We postulate that age had no significant effect on level of DNA damage in our study, as 60% of our participants fell in the age group 25–35 years. With a young group, we expect participants to have good DNA repair mechanism (48). In contrast, an American study comprising 41 individuals (age range, 24–93 years) detected a small effect of age by the comet assay. The study reported a 12% increase in DNA damage among individuals 60 years and older in comparison with individuals below 60 years (98)

Gender, HIV status, drug intake and current infection such as influenza had no influence in the level of DNA damage in our study. The results from a large cross-sectional study of healthy individuals reported that men had more DNA damage than women, displayed as a wider span of DNA damage in the group of men (99). In contrast, there is strong evidence that women have more DNA damage than men (100). However, a German study using repeated measurements did not detect an effect by gender on comet tail length (91).

While it is possible that HIV/AIDS may have an effect on the outcome of DNA damage, this is unlikely as previous studies have reported that HIV, which is a RNA virus did not cause statistically significant DNA damage among petrol attendants (47).

A number of studies have investigated the effect of nutrients, antioxidants, or a combination of antioxidants on the level of DNA damage as assessed by the comet assay in humans (101, 102). No effect of daily supplements was shown on DNA damage. It might be expected that the comet assay would detect more DNA damage in individuals suffering from infectious diseases. However, reported data on health status among petrol attendants were recorded, but the contribution of health status to the variation in statistical analyses was not provided.

4.2 Strengths and Limitations

The strengths of the study

- The researchers' ability to identify a sample of workers with known exposures to benzene, to quantify such exposures and adjust for factors likely to influence DNA damage.
- The comet assay was analysed at an accredited laboratory by two double blinded reviewers that followed international guidelines on comet assay scoring.
- The presence of a non-exposed control group provided a better understanding of the expected median DNA damage among the unexposed workers. This allowed for a better estimation of associations attributable to work related factors.

- It provided additional knowledge and understanding on the health risk of petrol attendants with benzene exposure in South Africa.

Limitations of the study

- This study was limited by the cross sectional study design which was chosen as it is relatively low in cost and quick to conduct. With cross sectional studies, it is impossible to determine the precise temporal relationship between petrol exposure and development of DNA damage.
- The findings suggest that the exposed workers were predisposed to greater DNA damage than the non-exposed group. Consequently, DNA damage via comet assay may have been underestimated.
- It is possible that some petrol attendants may have already developed DNA damage and health outcomes but have left the petrol stations before the study was conducted (healthy worker effect). Funding was not available to follow up previous employees at the participating petrol stations.
- The measure of DNA repair rate among the exposed group could also have been assessed or investigated to determine the rate of genotoxicity, but this was not done in our study due to financial constraints.
- There was a possible misclassification of participants in the low exposed group (car washers and those working in the shop) as they assisted in pumping petrol occasionally, such as once a month during peak hours or during major staff shortage at the petrol station court. This could result in cumulative exposure to benzene.
- The participants who were employed as petrol attendants had a tag specific to their name that recorded the exact amount of petrol pumped per day. While the low exposed group had no tags to help in quantifying how much petrol they assisted in pumping.

- Biological monitoring of exposure to benzene was done, but due to administrative delays these results were not available for statistical analysis at the time of the preparation of this report. Blood was taken and analysed for benzo[*a*]pyrene-DNA adducts. benzo[*a*]pyrene is found in the combustion of petrol and is one of the most potent carcinogens to which humans are frequently exposed (103, 104). A metabolic pathway involving cytochrome P450 and epoxide hydrolase converts benzo[a]pyrene to benzo[a]pyrene diol epoxides (BPDEs) (105). Recent studies suggest that DNA polymerases facilitate either carcinogenic or mutagenic bypass of BPDE adducts (106).
- Biological exposure methods, such as urinary metabolites of benzene: S-phenylmercapturic acid (S-PMA) and trans muconic acid (tt-MA) or urinary phenol, could have been used to correlate with recent atmospheric exposure with a half-life of 48 hours (55). For long-term exposure, measuring adducts formed in serum of benzene or its metabolite-1.4 benzoquinone could have been used, as it gives a half-life of four weeks and can be used to correlate exposure up to 11 months (57).

  No active personal sampling or environmental monitoring was done at the petrol stations. Environmental exposure data was replaced with the use of volume of petrol pumped by each individual petrol attendant over a year.

4.3 Public Health Significance

Benzene has long been recognized as a carcinogen in petrol, and recent concern has centred on the effects of continuous exposure to low concentrations of benzene, both occupationally and environmentally. As low benzene levels are associated with increased DNA damage, there is a need for further research among petrol exposed workers. Benzene genotoxicity can accumulate to carcinogenicity with continuous increased DNA damage, and the opportunity to enforce occupational protection measures as to decrease benzene risk to employees is therefore paramount. South Africa should aim to reduce the amount of

benzene in petrol. Studies have reported genotoxicity at benzene doses below the South African occupational exposure limit. Genotoxicity has been reported among employees with low benzene exposures of 1.6 mg/m^3 (34). South African petrol attendants are at greater exposure since, petrol station attendants have been retained while many developed countries, petrol stations are self-service in an attempt to reduce exposure and adverse health effect.

4.3.1 Health risk assessments

In accordance with the South African Hazardous Chemical Substances Regulations (HCSR) of 1179 of 1995 (28), employers are expected to conduct annual health risk assessments if their employees are exposed to hazardous chemicals designated with a control limit (CL). Benzene has an OEL (CL) of 16mg/m^3. However, the enforcement of legislation is limited, and needs to be strengthened to protect the health of workers.

4.3.2 Medical surveillance

Petrol attendants are seldom subjected to pre-employment medical examination or provided with regular medical assessments to detect potential serious health outcomes as a result of benzene exposure. As stipulated by the HCSR, the employer is also expected to ensure that employees are under a medical surveillance programme if they are exposed to hazardous chemical substances that are listed in Table 3 of Annexure 1 of the Hazardous Chemical Substances Regulations. Benzene is a substance listed in Table 3. We noted that although these mandatory commands were legislated for employers, this was however not implemented in all the participating garages, and there was no enforcement of the legislation by the Department of Labour Inspectorates.

4.4. Recommendations

The following recommendations are made as a result of this study

- Petrol station owners should conduct health risk assessments in their petrol stations.
- Petrol station owners should make sure that health regulations are implemented, with employees being provided biennial medical surveillance programme.
- There should be more stringent hierarchy of control measures in place, that include provision of personal protective equipments.
- The Department of Labour should be more stringent in its audit of petrol stations, as to review that employers are compliant with legislations that are designed to assist in protecting workers health and safety.

  Further research is required to understand the dose response relationship of volume of petrol pumped and duration of employment in petroleum industry's effect on DNA damage among petrol attendants.

4.5 Conclusions

With over 55 000 attendants dispensing petrol across South Africa, the implications for their health, with respect to the effect of benzene on their DNA, are significant. The results of this study indicate a statistically significant association between job exposure and DNA damage. It is difficult to separate the impact of the petrol from the other factors that could also contribute to DNA damage. The number of people likely to be affected would probably increase substantially if those who are no longer employed in the industry (healthy worker effect), due to health problems, age or redeployment, are included. Current legislation appears to be adequate in protecting these workers, but its implementation remains problematic, to the detriment of those affected. The hypothesis that long-term occupational exposure to petrol can cause DNA damage among eThekwini Municipality petrol station attendants was found to be

valid. On the bases of our study finding, the genotoxic effects of benzene exposure among petrol attendants are better understood. These suggest an increased risk of carcinogenic outcomes in such workers, although the study was not specifically investigating cancer. Hence with all public health issues, an important consideration is to protect the petrol attendants who may well not be aware of the health implications of their employment conditions.

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CHAPTER 5: APPENDICES

ANNEXURE A: Informed consent

Occupational benzene exposure and genotoxicity among petrol attendants study

Information and consent sheet

1. Title of research project

Occupational benzene exposure and genotoxicity among petrol attendants

2. Name of the researchers

Dr Mpho Makwela– MMed Occupational

Prof. Rajen Naidoo – PhD

3. Purpose of the research

The purpose of our study is to establish if there is a relationship between eThekwini Municipality petrol attendants' workers health and the petrol which they work with.
The chemical we are most concerned about and will be studying is benzene and this forms part of petrol and has been proven to cause cancer. This study will try and find a link between the amount of petrol pumped, number of cars filled, protective clothing provided and the extent of poor health and the body's ability to repair itself. eThekwini Municipality is selected because it is easily accessible to the researcher.

4. Description of the research project

If you agree to participate, the informed consent will be for:

- **Researcher administered questionnaire** which will enquire about your work and past medical history.

- **Blood samples** will be taken from you via finger prick for checking cells damage caused by petrol. We will check for damage to the body cells through a technique called comet assay that will confirm the presence or absence of damage to the body.
- **Environmental assessment** of your work place, will include the collection, monitoring and checking of the chemical benzene (which is a component of petrol) levels in the air:

5. Duration of participation of the subject in the study

- There will be three visits to your work place; the first session will be interview by the researcher to explain the research protocol. This session will take a total of up to one hour.
- The second session will last about 1 hour and will be for the researcher to administer the questionnaire
- The last session to the work place will be to collect blood samples from you and air monitoring that was left to accumulate for a period of one month.

6. Risks and discomforts of the research

The equipment that will be left in your work station is an air monitor that might produce a low noise which might take time for the people in the work place to get used to. This type of equipment will be set for 24 hour collection of environmental information and will be left at the petrol station for a period of 1 month. Other equipment to be used will course no discomfort or risks to the other employees.

7. Measures to be taken to minimize risks and discomforts:

Not applicable

8. Expected Benefits to You or the Others

- Knowledge on how petrol can cause damage to the human body.

- You will be provided with results and explanation about the damage and the body's ability to mend itself.
- You will be informed about the importance of the outdoor air measurements and their effect on status of your health.
- Health officers in the area will have an idea about the outdoor environment and how it affects the health of the petrol attendants working in this area.

9. Costs to subject resulting from participation in the study

There are no costs to the participants since we will come to your work place to provide the questionnaires, take blood samples and measure outside air and chemicals. No health costs of the comet assay will be charged.

10. Payments to subject for participating in the study

You will receive no financial benefits from participating in this study.

11. Confidentiality of information collected

You will not be identified in any reports on this study. The records will be kept confidential to the extent provided by law. Only researchers on this study may have access to your results.

12. Management of physical injury

Not applicable

13. Availability of further information

If significant new knowledge is obtained during the course of this research which may relate to your willingness to continue participating, you will be informed of this knowledge. Also, you may contact the Department of Occupational and Environmental Health which is based at the University of Kwa-Zulu Natal (contacts at the end) for answers to further questions about the research, your rights, or any injury you may feel is related to the study.

14. Voluntary nature of participation

Your participation in this project is voluntary. Subsequent to your consent, you may refuse to participate in or withdraw from the study at any time without penalty or loss of benefits to which you may otherwise be entitled.

15. Documentation of the consent

One copy of this document will be kept together with our research records on this study. A second copy will be given to you to keep.

16. Consent of the petrol attendant

I have read [or been informed] of the information given above. I understand the meaning of this information. Dr/ Mr /Ms _____has offered to answer any questions I may have concerning the study. I hereby consent to participate in the study.

PETROL ATTENDANT'S NAME

_____ _____

Printed Name Consenting signature

PERSON OBTAINING CONSENT

_____ _____

Printed Name Signature

DATE: _____

CONTACT DETAILS OF DOEH ADMINISTRATOR FOR REPORTING COM-

PLAINTS /PROBLEMS:

Department of Occupational and Environmental Health,

Room 320 George Campbell Building, UKZN,

Queen-Mary Avenue

Durban

4000

Tel: +27 (0)31 260 4471/4071

Fax: +27 (0)31 260 4663

Administrator: Nhlanhla Ntshangase

CONTACT DETAILS OF BREC ADMINISTRATOR OR CHAIR FOR REPORTING COMPLAINTS /PROBLEMS:

Biomedical Research Ethics,

Research Office, UKZN,

Private Bag X 54001,

Durban,

4000

ANNEXURE B : Consent Document

Consent to Participate in Research

Greeting: Good-day; Sanibonani; Middag

Title of research project

Occupational benzene exposure and genotoxicity among petrol attendants in eThekwini municipality

Name of the researchers

Dr Mpho Makwela– MMed Occupational

Prof. Rajen Naidoo – PhD

You have been asked to participate in a research study that will look at the effect of working with petrol and the impact on the health of the petrol station workers.

You have been informed about the study by the field worker in the language of your choice.

You have been informed about no available compensation or medical treatment if injury occurs as a result of study-related procedures;

You may contact the researcher Dr Makwela at 031260 4471 or 073205094 any time if you have questions about the research or if you are injured as a result of the research.

You may contact the **Biomedical Research Ethics Office** on **031-260 4769 or 260 1074** if you have questions about your rights as a research participant.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to stop at any time.

If you agree to participate, you will be given a signed copy of this document and the participant information sheet which is a written summary of the research.

The research study, including the above information, has been described to me orally. I understand what my involvement in the study means and I voluntarily agree to participate. I have been given an opportunity to ask any questions that I might have about participation in the study.

_____ _____

Signature of Participant **Date**

_____ _____

Signature of Witness **Date**

(Where applicable)

_____ _____

Signature of Translator **Date**

(Where applicable)

ANNEXURE C: Questionnaires

EThekwini Municipality Petrol Attendants Occupational Exposure; Pilot Questionnaire

All of this information is confidential, and available ONLY to the Research team

The purpose of this questionnaire is to collect information about petrol attendant's exposures in their work place and identify possible impact to their health. If there are questions one does not want to answer, please alert the interviewer and they will be skipped. All the responses are confidential and will not be shown to anyone outside the research team without written informed consent. If the employee wishes to stop the interview, they can do so and reschedule with the interviewer for another convenient time

Administrative Details

1. **Date of interview**

------/------------/---------

Day Month Year

2. **Employee Study Identification Number----------**

3. **Preferred language of interview** □

   i. Zulu ☐

   ii. English ☐

Demographic Details

4. **Age** (in years)----------

5. Gender

i. Male ☐

ii. Female ☐

6. Your physical address

i. Name of Suburb/township/village:----------

7. Place of residence is it near (1km away from)

i. ☐Emitting industrial Factory Yes ☐ No ☐ Yes/No

ii. ☐Petrol stations Yes ☐ No ☐

iii. ☐Highways Yes ☐ No ☐

iv. Others: Specify----------

Occupational Details

8. Job title (please tick)

i. Petrol attendant ☐

ii. Cashier ☐

iii. Others: Specify----------

9. Date of appointment ------/----------/-----

Day Month Year

10. Name of workplace petrol station:----------

11. Address/ location of petrol station:----------

12. Number of years working in the petroleum industry including other petrol stations---

----

13. Hours per shift per day (in past week)

i. 8 hours ☐

ii. 12 hours ☐

iii. 16 hours ☐

iv. 24 hours ☐

v. Others (specify) ☐

14. Provide details of all jobs you have ever held. Start with the current job

Workplace	**Job title**	**Exposures**	**Number of years worked**
1			
2			
3			
4			
5			

15. Are you provided with workplace personal protective equipment (PPE) in your current job?

i. Yes ☐

ii. **No** ☐

What are the type of Personal Protective Equipment provided?----------

---

16. When are they used: choose one:

i. Always ☐

ii. Specific task ☐

iii. During emergency ☐

Leisure Time

17. Were you involved in the following activities in your leisure time in the past week

a. **Car mechanics**

i. Yes ☐

ii. No ☐

b. Burning coal

i. Yes ☐

ii. No ☐

c. Painting

i. Yes ☐

ii. No ☐

Medical History

19. Do you have a history of cancers (leukaemia)?

iii. Yes ☐

iv. ☐

20 a. Have you had (H-influenza) flue-like symptoms in past 1 week (such as coughing, nasal congestion, sore throat and fever lasting less than a week?)

i. Yes ☐

ii. No ☐

b. If you have ever tested for HIV in your life, what was the outcome of the definitive results?

i. Tested positive ☐

ii. Tested negative ☐

iii. Never tested ☐

iv. Choosing not to disclose ☐

c. Others infectious disease(specify) ----------

21 .Do you have family history of cancers/ leukaemia?

i. Yes ☐

ii. No ☐

22. Do you have childhood history of cancers?

i. Yes ☐

ii. No ☐

iii. Provide details----------

Medication and Drug History

23. Have you recently in the past month used anti-cancer drugs?

i. Yes ☐

ii. No ☐

24. Have you used any x-rays in past week?

i. Yes ☐

ii. No ☐

25. Provide the details:----------

26. List all drugs been taken in past week and specify for what

i.----------

ii.----------

27. Provide details on how long you have been taking the drug ----------

Social History

28. Do you smoke cigarette?

i. Yes ☐

ii. No ☐

iii. Ex-smoker ☐

29. If you answered yes to question 28, mark what you smoke and indicate the amount per day

Content	**Amount smoked per day**
Cigarette	
Tobacco (zoll)	
Snuff	
Pipe	
Others, please specify	

30. When did you start smoking (year)?----------

31. If you answered question 28 as ex-smoker, when did you stop smoking (year)?----------

----

-

32. Do you drink alcohol?

i. Yes ☐

ii. No ☐

iii. Ex-drinker ☐

33. If the answer is yes to question 32, when did you start drinking alcohol (year)?----------

----

34. If yes, mark the type of alcohol you mainly use:

Content	**Amount drank in past week**	**Amount drank in past month**
Traditional beer (homemade) per glass (250ml)		
Tlokwe per glass (250ml)		
Beer (commercial) per can (340ml)		
Spirits per tot (50ml)		
Wine per glass (250ml)		
Others, please specify		

35. If you answered as ex drinker in question 32, when did you start consuming alcohol (year)?----------

36. If you answered as ex drinker in question 32, when did quit drinking alcohol (the year)----------

37. Do you do physical exercise?

i. Yes ☐

ii. No ☐

38. If yes, mark the type of exercise and specify the hours per day/per week in the past month:

Exercise regimen	**Hours per day**	**Hours per week**
Gymnasium exercise (e.g. weights)		
Training for long distance running (e.g. gym or road)		
Swimming		
Cycling		
Others, please specify		

39. Do you take any vitamin supplements weekly?

i. Yes ☐

ii. No ☐

ANNEXURE D: Comet Assay Protocol

Aliquots of 25µl heparinized blood were mixed with 175µl low melting agarose (0.5% in PBS) and added to frost-ended microscope slides, in triplicate, which had been covered with a bottom layer of 1% agarose. Slides were placed in lysis buffer (2.5M NaCl, 100mM EDTA, 1% Triton X, 1M Tris, 10% DMSO; pH 10; 4 ◦C, 1hour). Slides were then equilibrated and denatured in alkali buffer (300mM NaOH, 1mM Na2EDTA; pH13) for 20min followed by 35 min electrophoresis (25Volts, 35 min). Images of 50 randomly selected cells stained with ethidium bromide were analysed from each coded slide. Measurements for comet tail migration were made for 50 cells between 3 slides per sample by image analysis (Analysis5 Software). Parallel evaluation of slides was performed by a second investigator.

Apoptosis - Detection of phosphatidylserine externalisation

An Annexin-V FITC apoptosis detection kit (Roche, Johannesburg, South Africa) was used to detect externalised phosphatidylserine (PS) on peripheral blood mononuclear cells. Briefly, 1 million cells were transferred to polystyrene cytometry tubes and stained with 5 µL of both the Annexin-V FITC and propidium iodide (PI) components by incubation in the dark for 15 min at room temperature. Thereafter, the samples were supplemented with 400 µL of Annexin-V Binding Buffer (1×). Labelled cells were detected by flow cytometry (FACS Calibur, BD Biosciences). Data was collected for 50 000 events per sample and analysed with FlowJo 7.1 software (Tree Star Inc., Ashland, USA).

ANNEXURE E: UKZN Postgraduate Education Committtee

Ethical Approval

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

24 October 2011

Professor R Naidoo
Department of Occupational and Environmental Health
Nelson R Mandela School of Medicine

Dear Professor Naidoo

PROTOCOL: "Occupational benzene exposure and genotoxicity among eThekwini municipality petrol attendants in 2011." Student: MH Makwela, student number: 200267443 (Occupational and Environmental Health)

The Postgraduate Education Committee ratified the approval of the abovementioned study on 11 October 2011.

Please note:

- The Postgraduate Education Committee must review any changes made to this study.
- The study may not begin without the approval of the Biomedical Research Ethics Committee.

May I take this opportunity to wish the student every success with the study.

Yours sincerely

M. Adhikari

Professor M Adhikari
Dean's Assistant: MMed Programme
Postgraduate Education and Research Committee

CC. Dr MH Makwela

Biomedical Research Ethics Committee
Westville Campus

Postgraduate Education Administration,
Medical School Campus
Postal Address: Private Bag 7, Congella, 4013, South Africa
Telephone: +27 (0) 31 260 4745 Facsimile: +27 (0)31 260 4723 Email: Jantjies@ukzn.ac.za Website: www.ukzn.ac.za

ANNEXURE F: UKZN Biomedical Research Ethics Committtee

Ethical Approval

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
RESEARCH OFFICE
BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Westville Campus
Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 260-4609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/ResearchEthics/BiomedicalResearchEthics.aspx

01 November 2011

Dr. Mpho Makwela
Department of Occupational and Environmental Health
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

Dear Dr Makwela

PROTOCOL: Occupational benzene exposure and genotoxicity among eThekwini Municipality petrol attendants in 2011. REF: BF110/11

The Biomedical Research Ethics Committee (BREC) has considered the abovementioned application.

The study was provisionally approved by a quorate meeting of BREC on 12 July 2011 pending appropriate responses to queries raised. Your responses dated 28 October 2011 to queries raised on 28 October 2011 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 01 November 2011.

This approval is valid for one year from **01 November 2011.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/ResearchEthics11415.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The following Committee members were present at the meeting that took place on 12 July 2011:

Professor D Wassenaar	Chair
Professor V Rambiritch	Pharmacology
Professor S Collings	Psychology
Professor D J Pudifin	Medicine
Dr T Hardcastle	Surgery - Trauma
Dr U Govind	Private Pract. - Gen. Practitioner
Dr S Paruk	Psychiatry
Dr H Dawood	Medical School
Mr C Schembri	Legal Advisor
Prof L Puckree	External - DUT
Dr R Green-Thompson	Obstetrics and Gynaecology

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

PROFESSOR D R WASSENAAR
Chair: Biomedical Research Ethics Committee

Annexure G: Additional data that was analysed from the study

Table 1: The number of participants per petrol station

Petrol station	Number of participants
1	24
2	18
3	13
4	11
5	10
6	9
7	8
8	8
Total	101

Table 2: Correlation of comet tail length with independent variables (n=101)

Independent Variable	R ho	P Value
Age	0.04	0.059
Duration of employment in petroleum industry in yrs	0.002	0.97
Volume of petrol pumped in litres/year	0.009	0.91
Pack years of cigarette smoking	0.23	0.0092
Standard unit alcohol consumed	0.18	0.042

Table 3: Model 1: Unadjusted and adjusted analysis of comet tail length (in µm) and independent variables (n=151); **exposed group (n=101) and non exposed group (n=50).**

	Unadjusted			**Adjusted for sex, HIV positive, Current flu infection, Living in suburb and intake of medication**		
	Coefficient	**Standard error**	**95% CI**	**Coefficient**	**Standard error**	**95% CI**
Age*	0.47	0.24	-0.002 - 0.9	0.56	0.25	0.05 – 1.08
Job exposure*	0.87	3.20	-17.21 - -4.54	12.7	3.58	-19.85 -- -5.67
Pack years* of smoking	2.65	0.26	2.13 – 3.18	2.66	0.27	2.12 – 3.20
Alcohol consumption (unit/ day)*	9.04	4.64	-18.21 – 0.13	9.46	4.81	-18.98 – 0.04
Volume of petrol pumped	7.68	0.000014	-0.00002 – 0.00003	5.05	0.000016	-0.000027 – 0.00038

***P<0.05** (Model $R^2 = 0.4$)

Table 4: Model 2: Unadjusted and adjusted analysis of comet tail length (in µm) and independent variables (n=101); high exposed group (n=75) and low exposed group (n=26).

	Unadjusted			Adjusted for sex, HIV positive, Current flu infection, Living in suburb and intake of medication		
	Coefficient	**Standard error**	**95% CI**	**Coefficient**	**Standard error**	**95% CI**
Age*	0.74	0.33	0.07 – 1.42	0.77	0.35	0.075 – 1.48
Job exposure	10.18	7.16	-24.40 – 4.03	11.34	7.51	-26.27 – 3.58
Pack years of smoking*	2.67	0.30	2.06 – 3.28	2.62	0.31	2.00 – 3.24
Alcohol consumption (unit/ day)*	1.84	0.36	1.26 – 2.56	1.84	0.373	1.10 – 2.58
Volume of petrol pumped	0.00002	0.00002	-0.00065 – 0.00013	0.000026	0.000021	-0.000084 – 0.00015

***P<0.05** (Model $R^2 = 0.5$)