University of Witwatersrand

Polycyclic Aromatic Hydrocarbons: Is there an Association with Squamous Cell Carcinoma of the Oesophagus in South Africa?

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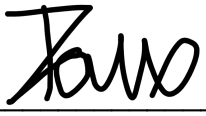
Declaration

I declare that the following research is my own, unaided work. It is being submitted for the Degree of Mastery of Medicine at the University of Witwatersrand, Johannesburg. It is being submitted in the submissive format according to department regulations. It has not been previously submitted for any degree or examination at any other institution.



(Signature of candidate)

14 of September 2022 at JHB

Dedication

To my friends and family - working hard to keep me sane.

Abstract

Background

Oesophageal squamous cell carcinoma (OSCC) is the eight most common cancer diagnosis globally. The disparity in incidence worldwide is poorly understood. The developing world has rising numbers and poor outcomes because of the nature of the disease and late presentation. Polycyclic aromatic hydrocarbons (PAH) have been identified as a potential driver of incidence in other endemic areas. In South Africa, there is a paucity of data on the association of PAH with OSCC.

Objectives

To determine the urine concentration of PAH in patients with newly diagnosed OSCC, compared to controls presenting to an endoscopy clinic. Demographic data were collected to identify potential sources of exposure in both groups.

Methods

A prospective case-control study was performed at the Endoscopy Unit of the Charlotte Maxeke Johannesburg Academic Hospital. Informed consent was obtained from newly diagnosed cases of OSCC and control patients without OSCC. Demographic data were obtained, and a urine specimen was collected. Urine concentrations of 1-hydroxypyrene as an indirect marker for PAH, were measured.

Results and Discussion

The case and control groups were matched for age and gender and no statistical significance was found with respect to demographic characteristics and personal areas of PAH exposure.1-Hydroxypyrene was detected in 9/20 cases and 3/20 control patients ($p=0.035$). Furthermore, on univariate analysis, having a PAH level detected has an OR=4.65 (3.5) of having OSCC. These data suggest an association between PAH exposure in OSCC patients.

Conclusion

This is the first study in South Africa to demonstrate greater presence of PAH exposure in a small number of newly diagnosed OSCC patients. Further research is recommended to determine tissue levels, potential sources, exposure to PAHs and their effects on disease processes.

Acknowledgements

The Endoscopy Suite, Ward 554, CMJAH

Thank you for accommodating me in your space and assisting with easing the recruitment process.

V&M Analytical Toxicology Laboratory Services

The analysis of urine specimens was led by them and done with the utmost professionalism. Thank you for allowing me to observe and assist with the analysis.

Maryn Viljoen, Statistic Consulting

Thank you for the help with the analysis of the dataset.

Table of Contents

List of Tables

List of Abbreviations

CMJAH - Charlotte Maxeke Johannesburg Academic Hospital

DNA - Deoxyribonucleic Acid

HPLC-FLD - High-performance Liquid Chromatography with Fluorescence Detector

OSCC - Oesophageal Squamous Cell Carcinoma

PAH - Polycyclic Aromatic Hydrocarbons

SAS - Statistical Analysis System

SD - Standard Deviation

Introduction

Oesophageal carcinoma remains the eighth most common cancer in the world, with squamous cell carcinoma being the most common type. There are marked variations in incidence in different geographic locations with a 50-fold difference between the highest and the lowest areas [1,2,3]. The asymptomatic nature of early disease leads to late presentation and poor outcomes. This skewed distribution of oesophageal squamous cell carcinoma (OSCC) has identified areas with a disproportionately high incidence. Iran and Iraq, the Linxian area of China, areas within South America and Southern Africa are reported to have the highest incidences in the world and are considered endemic to the disease [1]. Multiple factors have been identified as potential causative factors in the development of OSCC. Smoking of tobacco products leads to a ninefold increased risk for the development of OSCC [3]. Other identified risks include caustic injuries, motility disorders of the oesophagus, the drinking of hot liquids and chewing tobacco [2,3,4]. Multiple studies have linked polycyclic aromatic hydrocarbons (PAH) in not only the development of other malignancies, as well as a specific association with the development of OSCC within some of the above-mentioned endemic areas. [5,6,7]. The role of PAH in the pathogenesis of OSCC is an area of current study.

PAH are well-described organic substances, comprising of carbon and hydrogen atoms, grouped into two or more aromatic rings. How these compounds behave is determined by interactions and reactions to other pollutants like nitrogen oxides and sulphur dioxide [5,6]. The major routes of exposure are from breathing, both ambient and indoor air, ingesting foods containing PAH, cigarette smoking or smoke inhalation from open fireplaces. Food processing methods (drying and frying) and cooking food at high temperatures have been implicated in the formation of PAH [6]. The concern is that with prolonged or high exposure these compounds have carcinogenic properties. The metabolites of PAH bind to cellular proteins and bind DNA causing mutations, setting off a sequence of toxic events. This results in the disruption of biochemical pathways resulting in cellular damage, leading to genetic malformations, tumour development and ultimately cancers [5,8].
PAH measurement can be done in two ways - an internal and external level. The external dose may be measured by checking levels in air and food. The internal dose is determined by levels in bodily fluids [5,6] or from tissue samples. Urinary 1-hydroxypyrene has been proven to be a good biomarker for recent PAH exposure [5]. The compound represents PAH exposure within the last 24-48 hours. PAH-DNA complexes analysed from the tissues

of non-tumoural oesophagus and bladder have been found to be a better marker as the DNA sampling gives a level more consistent with chronic exposure [7,9]. Both have been utilised in previous studies to assess for the possible association between PAH and OSCC.

The Golestan province in Iran has been found to have one of the highest incidences of OSCC in the world. As mentioned, in countries with low incidence, the main causative agents are smoking and alcohol, but this was not confirmed by the findings in the Iranian population [1]. Smoking was much higher amongst males, yet the incidence of OSCC in males and females was similar. A recent study performed in Iran tried to bridge this information gap by testing patients for levels of PAH in people with confirmed OSCC. Non-tumoural tissue (oesophagus) DNA was measured. There were statistically significant higher levels of PAH in patients with confirmed OSCC compared to controls. These findings strengthen the association between PAH and the development of OSCC in endemic areas [10,11].

A further study conducted in Iran confirmed elevated levels of PAH-DNA in patients with confirmed OSCC. Both tumoural and non-tumoural tissues (oesophagus) were tested in patients with confirmed OSCC and in controls without. These levels were not statistically significant in tumoural tissues but were elevated in non-tumoural tissue in patients with confirmed OSCC, compared to levels in the controls. [12].

Mate, a traditional drink in South America, has been implicated in the high incidence of OSCC within their population. A study conducted in Brazil, confirmed elevated urine levels of PAH in patients with recent Mate consumption. Urinary levels of metabolites were significantly raised in the Mate drinking group, compared to those with either less exposure or no exposure [13], proving a link between Mate, or more specifically PAH in Mate, and the development of OSCC [14]. This was further evaluated by comparing other hot drinks. The temperature these were consumed at and the levels of known PAH within them were measured. The levels of PAH were variable within all these drinks and method of preparation played a big part in the concentration of PAH. Temperature played the biggest role. Mate is drunk in many ways and studies have revealed that drinking it at extremely high temperatures can increase the risk of developing OSCC [15,16]. These findings have made countries like Brazil adopt legislation to ensure acceptable and safe levels of PAH in

Mate and in other food sources. Food Safety regulations from the European Union and that of the US are now being instituted in South America [17].

In Bomet county in Kenya, an endemic area for OSCC, a study compared levels of PAH in the urine of people living in Bomet to those of citizens in the USA. These were asymptomatic people without OSCC. The levels of PAH in the Bomet population were significantly higher, providing more evidence of a possible link between PAH and OSCC [18]. The effects of hot drinks were also assessed within this group and found to have increased risk associated with OSCC. [18,19]

South Africa is a society at high risk for the development of OSCC. It is responsible for the second highest cancer-related deaths. The black population has the highest risk with a general reported incidence of 47/100 000 in males and 19/100 000 in females [2]. The risk of alcohol and smoking in the development of OSCC is well known in South Africa. [2] In the Transkei region in the Eastern Cape province of South Africa, a toxin-producing fungus, *Fusarium verticillioides*, has been associated with an increased risk of the development of OSCC [4]. There are no published data on the role of PAH in OSCC in South Africa.

The aim of this study is to determine whether PAH levels in the urine of patients with confirmed OSCC is higher than that of controls. Demographic data and some known risk factors were also assessed.

Methods

Ethics approval was obtained from the Human Research Ethics Committee (Certificate number M190757) from the University of Witwatersrand, Johannesburg, South Africa.

Study Design:

This prospective case-control study was performed from January 2020 to January 2022 at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). Cases were selected based on the clinical suspicion of an oesophageal lesion. This decision was made on clinical suspicion, regardless of previous workup. The questionnaire (see annexure) was completed, and urine collected prior to endoscopy. Biopsies of oesophageal tumours were taken. Once histology confirmed the presence of OSCC, they were formally enrolled as

cases. The controls were selected from patients presenting with no suspicion of malignancy who were scheduled for upper endoscopy. The controls were age and sex matched to make the two groups comparable. The same procedure was followed, and once endoscopy confirmed no lesion, they were recruited as controls. The urine was collected on site at CMJAH, stored in glass containers. Samples were frozen in a -70-degree Celsius freezer within 20 minutes of collection and kept frozen until analysis.

The sample size was determined by convenience sampling. There were no pilot studies to determine a sample size, the numbers were based on analysis of annual presentation to the endoscopy unit. The cases that qualified based on inclusion and exclusion criteria for the duration of the study, were included. Undetected levels of 1-hydroxypyrene were considered negative and any recorded value considered positive.

Inclusion criteria:

Cases: Adult males and females, above the age of eighteen were included. The patients with histologically proven OSCC qualified.

Controls: Patients presenting to the GIT Unit requiring endoscopy without OSCC.

Exclusion criteria:

Patients with previously confirmed OSCC.

Any previous oesophagogastric surgery.

Specimen Analysis:

In the urine of both groups, a determination of 1-hydroxypyrene level was performed using a method of high-performance liquid chromatography (HPLC) with fluorescence detector. Pyrene is present in all PAH mixtures. 1-Hydroxypyrene is formed once pyrene undergoes simple metabolism. This allows the use of 1-hydroxypyrene as an indirect indicator of PAH.

This method allows for the determination of free and conjugated 1-hydroxypyrene in urine. After enzymatic hydrolysis to release the conjugated 1-hydroxypyrene, using sodium acetate and B-glucuronidase/arylsulphate, the analyte was separated from the matrix and concentrated on a reverse phase column. The mobile phase using 40% and 95% methanol in water, with 50% methanol used as diluent. The columns consisted of C18 synergy 4 max-PR 80 A, a guard column and the alternative column of XBrige C18. The flow rate was set at 1ml/L at 40-degrees Celsius. The injection volume as 5.0 mcL and the

wavelengths set for excitation 336nm and emission 338nm respectively. The total runtime was 18 minutes. The fluent was separated by HPLC and the 1-hydroxypyrene determined with fluorescence detection. The final extract was measured against a known standard calibration curve and values expressed in nanogram per milliliter. Any value above 0.0 ng/ml was considered positive and treated as such.

Data Analysis:

Data from the data collection sheets were captured electronically by the researcher in Microsoft Excel©. Further analysis was done by a statistician using SAS Version 9.4©. Descriptive statistics, namely frequencies and percentages, were calculated for categorical data and means, standard deviations or median and percentiles were calculated for numerical data. The Shapiro-Wilke test was used to determine if numerical data followed a normal distribution. The Fisher's exact test was used to analyse continuous variables of age and years spent in urban and rural communities. The Mann-Whitney or T-test was used on data as appropriate. A significance level (α) of 0.05 was used in this study.

Results

Demographic and Lifestyle Information:

A cohort of 40 patients were recruited, of whom 20 subjects were in the case group and a reflective 20 subjects were in the control group. Baseline demographic information was obtained upon recruitment. The aim was to determine potential geographical areas of exposure to PAH. There was no statistical difference in age between the two groups. The case group had a mean age in years of 64.45 (SD 10.59) and the control group had a mean age in years of 63.45 (SD 8.74) (p=0.75). Gender distribution was equal in both groups. (Table 1).

When comparing birthplace, ten of the eleven provinces of South Africa were represented. Seven of the participants were foreign nationals, residing within South Africa. When looking at the time spent in urban areas, the median time spent in years by the case group was 15.50 (IQR 2.50-29.50) and in the control group 20.00 (IQR 0.00-45.00). Once compared, 13 of 40 patients spent more time in urban areas than rural areas. The longer urban exposure concerning for possible increased PAH exposure. This did not reach statistical significance when compared (p=0.50). The small nature of this study and multiple groups did however not make it possible to reach a conclusion on geographic importance.

Table 1: Demographic Statistics

Characteristic	Group		p-value
	Case	Control	
Age in Years (Mean + SD)	64.45 ± 10.5	63.45 ± 8.74	0.75
Gender (Variable, frequency %)			
Male	10 (50%)	10 (50%)	1.00
Female	10 (50%)	10 (50%)	
Birthplace (Variable, frequency %)			
Eastern Cape	2 (10%)	1 (5%)	0.53
Free State	2 (10%)	0 (0%)	
Gauteng	4 (20%)	6 (30%)	
Kwazulu-Natal	2 (10%)	3 (15%)	
Limpopo	3 (15%)	1 (5%)	
Mpumalanga	2 (10%)	2 (10%)	
North West	1 (5%)	3 (15%)	
Western Cape	1 (5%)	0 (0%)	
Foreign African Country	3 (15%)	3 (15%)	
Urban Rural Exposure (Variable, frequency %)			
Urban Predominant	5 (25%)	8 (40%)	0.50
Rural Predominant	15 (75%%)	12 (60%)	

The case group consisted of 40% domestic workers, a profession with exposure to chemicals and potential PAH exposure. When the data were analysed, there was no statistical difference in the considered high-risk group when compared to those performing considered lower risk work (p=0.22). This again is a Type II error and due to small

numbers with multiple groups, and larger studies are required to determine the significance. (Table 2).

Exposure to cigarette smoke is a known driver for elevated levels of PAH. In this study 40% of cases were smokers and 30% of controls. There was a further 50% of cases with a smoker in the home and 45% of controls. Meaning 90% of cases and 75% of controls had some form of smoking exposure. Neither of these had a statistical significance when compared, smokers (p=0.20) and smokers in the home (p=0.23). The use of alcohol did also not have a statistical effect on PAH levels (p=0.21).

Table 2: Occupational and Social History

Characteristic	Group		p-value
	Case	Control	
Occupation (Variable, frequency %)			
Domestic Worker	8 (40%)	3 (15%)	0.22
Factory Worker	1 (5%)	2 (10%)	
Labourer	6 (30%)	4 (20%)	
Miner	2 (10%)	1 (5%)	
Office Work	2 (10%)	8 (40%)	
Homemaker	1 (5%)	2 (10%)	
Smoking (Variable, frequency %)			
Smoker	8 (40%)	6 (30%)	0.21
Smoker in Home	10 (50%)	9 (45%)	0.23
Alcohol Use (Variable, frequency %)	5 (25%)	7 (35%)	0.21

Comparing the cooking methods for a potential risk of PAH exposure, the participants were asked whether most of cooking was done on gas, electric stove or open flame. When looking at exposure to open flame, 30% of cases and 5% of controls reported it. This is a known exposure of PAH. Most cases and controls reported electric cooking at their main

source for food preparation, when comparing the 70% that cooked on electric to those cooking on gas or open flame, there was no statistical significance in favour of other methods of cooking (p=0.08). (Table 3).

Table 3: Cooking Methods

Characteristic	Group		p-value
	Case	Control	
Cooking Method (Variable, frequency %)			
Electric	11	17	0.08
Other (Gas and open flame)	9	3	

Polycyclic Aromatic Hydrocarbon Levels:

The primary aim of this study was to determine if PAH concentrations were elevated in the urine of newly diagnosed cases of OSCC compared to patients without OSCC. The case group had a 45% positivity rate and the control group had 15% of patients testing positive for PAH. This was statistically significant (p=0.03). On univariate regression having a PAH level detected has an odds ratio of 4.63 (3.57) of having OSCC (p=0.04). This indicates the possible association between PAH exposure and OSCC. (Table 4).

Table 4: Polycyclic Aromatic Hydrocarbon Positivity

Characteristic	Group		p-value	p-value (Logistic regression)
	Case	Control		
PAH (Variable, frequency %)				
PAH Positive	9 (45%)	3 (15%)	0.03	0.04
PAH Negative	11 (55%)	17 (85%)		

When comparing the differences in levels in the positive patients, cases had a median of 0.58 (IQR 0.39-1.77) ng/ml and controls had a median of 0.34 (IQR 0.30-0.57) ng/ml. This was an unexpected finding within our cohort. The association between PAH and OSCC

the expectation was higher levels within our case group. The level of 1-hydroxypyrene detected did however not show statistical difference once compared (p=0.24). (Table 5).

Table 5: Polycyclic Aromatic Hydrocarbon Levels

Characteristic	Group		p-value
	Case	Control	
PAH (Median + IQR)			
Level of PAH	0.34 (0.30-0.57)	0.58 (0.39-1.77)	0.23

Discussion

The aim of this study was to identify a possible association between PAH levels in urine in newly diagnosed OSCC patients presenting to an urban hospital in Johannesburg, South Africa. There were more OSCC patients positive for urine PAH than controls thus confirming a possible association. This is in keeping with studies of similar design. They mostly looked at the communities at risk, compared to those considered lower risk, and found elevated levels in the high-risk group. We have now confirmed in a study population where both groups are at risk that regardless of the level, that you have a 4.63 higher likelihood of developing OSCC with a positive PAH level, compared to a negative level. We analysed multiple demographic and social factors to ascertain areas of potential exposure. The research conducted by other studies have proven that smoking of tobacco, occupational exposure and urbanisation lead to high PAH exposure. This study did not find this, and further studies will be needed to explore these factors more in our context and that of PAH.

PAH-DNA from non-tumorous tissue has been analysed in many studies. This method is more costly than assessing 1-hydroxypyrene in urine but has the advantage of assessing chronic PAH exposure [10,11]. The study has confirmed an association, but this would be the next step. Performing more complex analysis on a bigger cohort to not only further establish the association and even causation, but to aid in future therapies. These changes are potentially reversible or targetable if properly identified [20]. The urine 1-hydroxypyrene test is not as reflective for long-term exposure but has provided a positive correlation between PAH and OSCC in the urban setting. This will serve as the starting point to not only understand but manage the association between PAH and OSCC.

Limitations

The recruitment of newly diagnosed cancer cases was slow and therefor sample size is small. The COVID-19 pandemic limited recruitment, as endoscopy services were restricted during peaks from March 2020 to November 2021. The CMAJH Endoscopy Centre was closed from April 2021 to July 2021 because of a hospital fire, further limiting recruitment.

The analysis method, although accurate, reflects short-term exposure to PAH. This implies that if there is a significant lifestyle change prior to recruitment, it could lead to a low level and not necessarily represent chronic or previous exposure, therefor underestimating the effect or potential harm caused by PAH exposure.

Conclusion

This is the first study in South Africa investigating PAH and OSCC. The data from this study proved that even in a small cohort, there was a statistical difference in the presence of urine PAH of newly diagnosed OSCC cases, compared to controls. Demographic data within this group did not make a difference to PAH level or differ amongst the specific groups. This confirms the need for further countrywide studies with larger sample size using non-tumorous PAH-DNA testing, more extensive assessment of sources of PAH exposure and development of local expertise in analysing PAH levels.

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Annexure A (Questionnaire)

Case Number: _____ Biopsy number: _____

Case O Control O

Consent obtained O

Demographics:

Age: ___

Place of birth: _____

Sex: Male O Female O

Places lived: (duration of stay)

1. _____

2. _____

3. _____

Occupational history: (duration)

1. _____

2. _____

3. _____

Smoker Yes O No O

Alcohol consumption: (quantify units a week) _____ units

Traditional beer consumption: Yes O No O

Quantity: _____

Home life:

Smokers in the home: Yes O No O

Indoor cooking: Yes O No O

Methods of cooking:

Gas O

Open flame/Braai O

Electric O

Signature of case/control: _____

Date: _____

Annexure B (Original Protocol)

Polycyclic Aromatic Hydrocarbons: Is there an association with Squamous Cell Carcinoma of the Oesophagus in South Africa?

MMed Protocol

Candidate:	Name:	Niël Roux
	Student Number:	1849053
	Qualification:	MBChB (Stell)
Supervisors:	Adjunct Professor Damon Bizos	
	Professor Geoffrey Candy	

Table of Content

1. Abbreviations

CMJAH - Charlotte Maxeke Johannesburg Academic Hospital

ESCC - Oesophageal Squamous Cell Carcinoma

PAH - Polycyclic Aromatic Hydrocarbons

SA - South Africa

2. Abstract

Oesophageal cancer is the eight most common malignancy worldwide. The predominant types - adenocarcinoma and squamous cell carcinoma - have a very different distribution. The latter having a higher incidence in the developing world [1]. Areas of high incidence have been identified - the Linxian area in China, Iraq and Iran, areas within South America and Southern Africa [1]. Polycyclic aromatic hydrocarbons have been an area of study in these parts, possibly linking it to the development of ESCC. This link has not been made in South Africa. We intend to perform a prospective case-control study at CMJAH to study the possibility of this association in our population.

3. Introduction and Literature Review

Oesophageal carcinoma remains the eighth most common cancer in the world, with squamous cell being the most common type amongst them. The incidence show big differences in geographic location with a 50-fold difference between the highest and lowest [1]. The asymptomatic nature of early disease leads to late presentation and poor outcomes. Clinicians are left with few management options for cure. The distribution of ESCC is skewed, favouring those in the developing world. Areas of a disproportionally high incidence have been identified within this group. These areas are considered endemic to the disease. The Linxian area in China, Iraq and Iran, areas within South America and Southern Africa are the areas with the highest incidence in the world [1]. Multiple factors have been identified as causative factors in the development of ESCC. Smoking and alcohol are considered the main risk factors - these factors are however not exclusive to these areas. Other reasons for the development are therefor being sought. The role of polycyclic aromatic hydrocarbons are an area of current study. These compounds are found in our food, the air we breathe and other environmental sources. Multiple studies are now linking PAH in not only the development of other malignancies,

but with a specific association with the development of ESCC within these endemic areas [2].

In South Africa the risk of alcohol and smoking in the development of ESCC is well known. In the Transkei region in the Eastern Cape province of South Africa - Fusarium moniliforme has also been identified as a large contributor of disease [1,3]. A fungus growing on maize, producing fumonism - a toxin associated in the reduction of nitrates - producing nitrosamines. These compounds are considered carcinogenic [3]. The question is, are polycyclic aromatic hydrocarbons contributing to our burden of disease - like other endemic areas within the developing world?

PAHs are well known organic substances, comprising of carbon and hydrogen atoms, grouped into two or more aromatic rings. How these compounds behave is determined by interactions and reactions to other pollutants [2]. Entry into the body may be via inhalation or ingestion. The main route of exposure is thought to be from inhalation. Ambient air, cigarette smoke and smoke inhalation from other sources all contain these compounds. Various foods are high in PAHs and food preparation and processing may increase PAH levels.Their effect are also enhanced by high temperatures [2,4]. The above makes avoiding these substances near impossible and puts our population at high exposure and possible high risk.

PAHs behave differently depending on the interactions with other pollutants. In ambient air it is in a vapour form - with contributions by temperature and humidity - they react with other pollutants in the atmosphere. The most common reacting substance being emissions from incomplete carbon combustion [2]. The fate of these compounds are then threefold. Deposition into soil, photo-decomposition or long range transport to elsewhere in the

atmosphere [2]. PAH measurement can be done in two ways - an internal and external dose. The external dose can be measured by checking levels in air and food. The internal dose is determined by bodily fluids [2,5]. Urinary 1-hydroxypyrene has been proven as a good biomarker for recent PAH exposure [5]. Naphthalene and phenanthrene are also showing promise as biomarkers for PAH exposure. PAH-DNA has been found to be a good marker - the DNA sampling gives a level more consistent with chronic exposure - with a longer duration of elevated levels [2].

With a great variation in incidence of ESCC worldwide, a province in Iran has been found to have one of the highest in the world [1]. As mentioned, in countries with low incidence the biggest causation is smoking and alcohol, but these numbers did not correlate with the findings in the Iranian population. Smoking was much higher amongst males, yet the incidence comparing males and females was similar. A recent study performed in Iran tried to bridge this information gap by testing patients for levels of PAH in people with already confirmed ESCC. Non-tumoral tissue was used in the testing. Findings confirmed their suspicion - there was statistically significantly higher levels of PAH in patients with confirmed ESCC, compared to those biopsied and tested as controls. These findings possibly drawing a link between PAHs and the development of ESCC [6].

Another study conducted in Iran confirmed elevated levels of PAH-DNA in patients with confirmed ESCC. Both tumoral and non-tumoral tissues were tested in patients with confirmed ESCC and in controls without. These levels were not statistically significant in tumoral tissues, but was indeed elevated in non-tumoral tissue in patients with confirmed ESCC, compared to levels in the controls. Thus solidifying the link between PAH and ESCC [7].

In South America a traditional drink, Mate, has been implicated in the high incidence of ESCC within their population. A study conducted in Brazil, confirmed elevated urine levels of PAH in patients with recent Mate consumption. Urinary levels of metabolites were significantly raised in the Mate drinking group, compared to those with either less exposure or no exposure. Leading to a link between Mate, or more specifically PAHs, and the development of ESCC [8].

In Bomet county in Kenya, an endemic area for ESCC, a study compared levels of PAH in the urine of people living in Bomet to those of citizens in the USA. These were asymptomatic patients. Patients without ESCC. The findings however found elevated levels in the people of Bomet. Providing evidence of the presence of elevated PAH in the population of Bomet, possibly linking it to the increased risk of developing ESCC [9].

South Africa is also a society at high risk for the development of ESCC. The aim of this study is to determine whether PAH levels in the urine of patients with confirmed ESCC is higher than that of the general population.

4. Study Objectives

Primary Objective:

To determine if levels of PAH in the urine of patients with ESCC is higher than controls - making an association between PAH exposure and ESCC.

Secondary Objective:

To Compare demographic data of cases and controls - determining areas of possible PAH exposure in our study population. (Appendix A)

5. Methods

5.1 Study Design

Prospective case-control study

5.2 Site of Study

CMJAH endoscopy unit

5.3 Study Population

Patients presenting to CMJAH endoscopy unit. This can be from local drainage or a basis of referrals. If they require endoscopy they are eligible. This will allow us to either confirm or exclude an oesophageal lesion and enrol patients as either cases or controls. For cases only newly diagnosed cases will be used. The reasoning behind this is that the urine analysis gives a snapshot of acute exposure. Previous diagnosis might have allowed for a change in lifestyle prior to diagnosis - causing an inaccurate level of PAH.

5.4 Sampling

Subjects will be selected from patients presenting to CMJAH endoscopy unit with lesions suggestive of ESCC. Consent will be taken, urine collected and patient information given at first presentation. Once ESCC is histologically confirmed will they be enrolled as cases. The control group will be selected from patients also presenting to the unit with other indications for endoscopy, without the presence of ESCC. If confirmed adenocarcinoma - patients will be enrolled in the control group. Urine will be frozen on collection and stored until analysis. Urine is being used instead of oesophageal biopsies because of costs associated with using biopsies and technical expertise required for biopsy analysis. Estimated sample size of 40-50 controls and 40-50 cases. (funding and time allowing)

Inclusion criteria:

Cases: Adult males and females (above the age of 18)

Histologically proven ESCC

Controls: Other histological types of SCC

Patients presenting to the GIT Unit requiring endoscopy without ESCC

Exclusion criteria:

Previously confirmed ESCC (only new diagnosis on biopsy)

5.5 Measuring Tool

Sample analysis will be performed to determine levels of PAH exposure in both the cases and controls of the study. On-line analysis using 1-hydroxypyrene in urine specimens of both cases and controls. Assays will be performed at the University of Witwatersrand.

5.6 Data Collection

Data sheets will be used to record demographic data of patients and of sample results using MacSheets. Sheet example in Appendix A

5.7 Sources of Bias

All confirmed cases that present will be used. Selection bias is a concern with the control group - this is however needed to make sure the two groups are comparable.

6. Data Analysis

Levels of PAH will be evaluated and compared between groups. Confidence intervals of the urinary concentrations of the PAH metabolites. Baseline and demographic characteristics obtained by the questionnaire (Appendix B) will be compared using the t-test

7. Benefit

The study would ascertain whether there is an association between elevated PAH levels and ESCC in SA. If there is an association, further studies would be warranted to compare different geographical areas and to try identify the source of primary PAH exposure. Positive findings can lead to further study comparing rural and urban communities and lead to programs limiting exposure in high risk communities.

8. Ethics

Clearance for the prospective case-control study will be obtained from the ethics committee of the University of Witwatersrand. The cases and controls will all be thoroughly counselled and consented before enrolment. (Appendix C)

9. Timing

	2018									2019							
	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
Collecting Data	X	X	X														
Literature Review				X	X												
Preparing Protocol						X	X										
Protocol Assessment								X									
Ethics Application								X									
Data Analysis										X	X	X	X	X	X	X	
Writing Up - Thesis																X	
Writing Up - Paper																	X

10. Cost

Funding will be needed for the processing and interpretation of samples. Costs for sample analysis will be R25 000. This will be the only major expense. Stationary and printing costs will be undertaken by the lead investigator. Sampling and analysis cost is to be obtained through MMed fund application, outside grants from non-conflicting sources and personal cost.

11. References

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[7] Marjani, H. A. et al. (2010) 'Investigation of NQO1 genetic polymorphism, NQO1 gene expression and PAH-DNA adducts in ESCC. A case-control study from Iran', Genetics and Molecular Research. doi: 10.4238/vol9-1gmr693.

[8] Lopes, A. B. et al. (2018) 'Urinary Concentrations of Polycyclic Aromatic Hydrocarbon Metabolites in Maté Drinkers in Rio Grande do Sul, Brazil', Cancer Epidemiology Biomarkers & Prevention. doi: 10.1158/1055-9965.EPI-17-0773.

[9] Pritchett, N. R. et al. (2018) ’High Urinary Polycyclic Aromatic Hydrocarbon Concentrations in Bomet County, Kenya, a Region With a High Incidence of Esophageal Squamous Cell Carcinoma’ (no date). doi: 10.1200/JGO.2017.009464.

Appendix A (Datasheet)

Case Number	1	2	3	4	5	6	7
Case							
Control							
Age							
Sex							
Birthplace							
Years of rural life							
Years of urban life							
Occupation							
Smoking status							
Alcohol Units consumed in a week							
Tradional beer intake							
Indoor cooking							
Gas							
Electric							
Fire							
Biopsy Barcode							
Result							
PAH level							

Appendix B (Questionnaire)

Patient Name: _____

Case Number: _____ Biopsy number: _____

Case O Control O

Consent obtained O

Demographics:

Date of birth: _____ Age: ___

Place of birth: _____

Sex: Male O Female O

Places lived: (duration of stay)

1. _____

2. _____

3. _____

Occupational history: (duration)

1. _____

2. _____

3. _____

Smoker Yes O No O

Alcohol consumption: (quantify units a week) _____ units

Traditional beer consumption: Yes O No O

Quantity: _____

Home life:

Smokers in the home: Yes O No O

Indoor cooking: Yes O No O

Methods of cooking:

Gas O

Open flame/Braai O

Electric O

Signature of case/control: _____

Date: _____

Appendix C (Consent)

I, _____, hereby consent to partake in the study. Consenting to the submission of a urine specimen, analysis thereof and the use of my demographic information. Understanding the risks of the procedure and the anonymity of my information.

Signature: _____

Date: _____

Details of Consenter:

Name: _____

Signature: _____

Witness:

1. _____

2. _____

Appendix D (Patient information sheet)

STUDY INFORMATION DOCUMENT

Polycyclic Aromatic Hydrocarbons: Is there an association with Squamous Cell Carcinoma of the Oesophagus in South Africa?

To whom it may concern

We are currently conducting research at CMJAH to help us find out why South African people have a bigger chance to develop throat cancer - compared to other places in the world. This is not routine care for your current condition or complaint. The idea is to collect this extra information to give us more answers and hopefully improve the way we diagnose and treat this condition.

We would like you to take part in this study. Your personal information will be kept confidential and this will not form part of your official hospital records. You have the option to deny participation and your care will not be affected by it.

We will be collecting some of your urine. It will then be frozen and tested at a later stage. We will test it for levels of polycyclic aromatic hydrocarbons. This is something that is found in polluted air, it can be released when we cook our food in certain ways and people who smoke also have higher levels. The study will start in early 2019 and we plan to collect the information of 100 patients.

You will get your scope as planned and urine will be collected at the same time. If you qualify then we will use your urine for the test mentioned. After it has been used it will be discarded ethically. You will also complete a list of questions that will help us to know where you might have been exposed to this substance. This will only take a few minutes and can be done while you wait for your scope.

Your involvement will not have any extra risk to you. Collection of your urine will not be painful and will be done in private. The results will be kept confidential - you will not be informed about the result either.

This study will not have a direct effect on your current treatment, but it may help us with future patients. You consenting to this will play a small part in improving our knowledge and the way we treat and diagnose throat cancer.

Again this is completely voluntary. You are under no pressure to take part in this study. Thank you for reading this information sheet.

If any concerns about this study or the way it is being done, please feel free to contact the HREC chairperson at 011 717 2301.

Regards
Dr N Roux
Lead Investigator
January 2019

Appendix E (PAH Sources)

Dietary:

- Processing and cooking of food
  - Smoking of flame-grilling food
- Crops exposed to ambient air containing high levels of PAH

Tobacco smoke

Opium Use

Occupational exposure

- Coke-oven, Diesel industry

Indoor air pollution

- Smokers
- Indoor cooking method

Environmental air pollution

Annexure C (Ethics Clearance)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr Niel Roux

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M190757

NAME: (Principal Investigator)	Dr Niel Roux
DEPARTMENT:	General Surgery Charlotte Maxeke Academic Hospital Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	Polycyclic Aromatic Hydrocarbons: Is there an association with squamous cell carcinoma of the oesophagus in South Africa?
DATE CONSIDERED:	26/07/2019
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Damon Bizos and Geoffrey Candy
APPROVED BY:	Dr C Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	18/12/2019

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed July and will therefore be due in the month of July each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Annexure D (Plagiarism Report)

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