AN ASSESSMENT OF GASTRIC CANCER ASSOCIATED FACTORS AND STRATEGIES FOR EARLY CASE DETECTION IN PATIENTS SEEN AT THE UNIVERSITY TEACHING HOSPITAL IN LUSAKA, ZAMBIA

By

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APPROVAL

The University of Zambia has approved this thesis by Violet Jolezya Kayamba as fulfilling the requirements for the award of Doctor of Philosophy in Gastroenterology.

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Examiner 2

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Examiner 3

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Supervisor

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Co-Supervisor

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DECLARATION

I, Violet Jolezya Kayamba, confirm that the work presented in this thesis is my own.

It has been done in accordance with the University of Zambia PhD thesis guidelines and it has not been submitted elsewhere for a degree at this or another University. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature: ……..……

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ABSTRACT

Gastric cancer is one of the leading causes of cancer related mortality globally. It carries a very poor prognosis with a one-year survival rate of less than 15%. The main objective of this study was to investigate environmental, biological and dietary factors associated with gastric cancer, and to explore the potential of using blood in gastric juice for detection of gastric mucosal lesions through pre-endoscopy screening.

The study was conducted at the University Teaching Hospital, in Lusaka, Zambia. It was a case-control study of patients with histologically confirmed gastric adenocarcinoma (GA) or gastric premalignant (GP) lesions as cases, and those without either as controls. Questionnaires were used to collect data on basic characteristics and associated risk factors. Biological characteristics were measured in gastric juice, blood, urine, and gastric biopsies. This was done using enzyme-linked immunosorbent assay, high-performance liquid chromatography, immunofluorescence, *in situ* hybridisation, urine and pH test strips. A multiplex serological assay was used to quantify antibodies to thirteen immunogenic *Helicobacter pylori* proteins. Study data were analysed in STATA version 15 (College Station, TX, USA). Graphs were prepared in both STATA and Graphpad prism version 7.

Included for analysis in this study were 388 patients, 92 (24%) of whom had gastric tumours seen during endoscopy. Results showed that gastric cancer had similar occurrence in both sexes (OR 1.1; 95% CI 0.5-1.9), and 18/92 (20%) of them were below the age of 45 years. GA disproportionately affected rural (OR 2.9; 95% CI 1.5-5.3) and poor (OR 4.2; 95% CI 1.9-9.1) people. The proportion of Epstein-Barr virus (EBV) associated GA was 11% by *in situ* hybridisation, and it was similar between HIV infected and uninfected patients (OR 1.5; 95% CI 0.02-22). Evidence of microsatellite instability using immunofluorescence for MutL homolog 1 was observed in 63% of GA. Patients regularly exposed to biomass smoke were more likely to have GA, (p=0.001) and to exhibit evidence of oxidative stress to DNA, (p=0.03). The odds of GA in patients with history of regular consumption of processed meat was 7.0; 95% CI 1.4-34. In patients taking green vegetables daily, the odds were 0.2; 95% CI 0.1-0.5. The median estimated 24-hour sodium excretion of 19 g (IQR 14-24 g) by the Tanaka method. Aflatoxin M1 was present in the urine of 61% of the patients, with a median; 18 ng/mg creatinine (IQR 1.7- 40) ng/mg creatinine, while 96% had ochratoxin A in their blood median; 0.1 ng/ml (IQR 0.2-0.6 ng/ml). Being *Helicobacter pylori* (*H. pylori*) seropositive (determined by the presence of at least four antibodies) was not associated with either GA (OR 1.1; 95% CI 0.5-3.3) or GP (OR 1.9; 95% CI 0.4-17.6). Antibodies to CagA (p=0.0007), VacA (p=0.0006), HcpC (p=0.0006) and Omp (p=0.03) were significantly higher in active gastric inflammation than in GA. Overall, there was no association between *H. bilis* or *H. hepaticus* seropositivity and GA or GP. Serological response to four EBV antigens was not associated with GA. The presence of blood in gastric juice was associated with gastric cancer (OR 6.7; 95% CI 2-35), with a case detection sensitivity of 91% and a specificity of 41% and an area under the receiver operating characteristic curve of 0.8; 95% CI 0.7-0.9.

There was a high proportion of early onset and microsatellite unstable GA, disproportionately affecting poor rural residents. Of the infectious risk factors evaluated, *H. pylori* was only associated with active gastric inflammation, but not GA or GP. EBV was absent in most of the tumours and HIV showed no influence on gastric carcinogenesis. Environmental and dietary risk factors showed greater influence on GA than the infectious agents. Testing for blood in gastric juice had high sensitivity but low specificity for gastric cancer detection. Data from this thesis can be used to further analyse specific risk factors for each gastric cancer subtype and explore the pathophysiological mechanisms involved.

Key words: gastric cancer, gastric premalignant lesions, risk factors, biomass smoke, mycotoxins, gastric juice, Zambia

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ABBREVIATIONS

1-OHP	1-hydroxypyrene
8-OHdG	8-Hydroxydeoxyguanosine
CAG	Chronic atrophic gastritis
CI	Confidence interval
CISH	Chromogenic *in situ* hybridisation
DNA	Deoxyribonucleic acid
EBER	Epstein-Barr Encoding Region
EBV	Epstein-Barr Virus
ELISA	Enzyme Linked Immunosorbent Assay
GA	Gastric adenocarcinoma
GIM	Gastric intestinal metaplasia
GP	Gastric premalignant lesions
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
IF	Immunofluorescence
IQR	Inter quartile range
MFL	Median fluorescent intensity
MLH1	MutL homolog 1
NAG	Non-atrophic gastritis
NCD	Non-communicable diseases
NHRA	National Health Research Authority
OGD	Oesophagogastroduodenoscopy
OLGA	Operative Link for Gastric Assessment
OLGIM	Operative Link for Gastric Intestinal Metaplasia

OR	Odds ratio
PBS	Phosphate buffered saline
PNA	Peptide Nucleic Acid
ROC	Receiver operating characteristic
SSA	sub-Saharan Africa
TBS	Tris-buffered saline
UK	United Kingdom
USA	United States of America
UTH	University Teaching Hospital
UNZABREC	University of Zambia Biomedical Research Ethics Committee
WHO	World Health Organisation

GLOSSARY OF KEY WORDS

Active inflammation	Presence of acute inflammatory cells in the stomach
Aflatoxins	Type of toxin produced by fungi
Biomass smoke	Smoke produced by the combustion of any organic matter
Chronic inflammation	Presence of chronic inflammatory cells but without acute inflammatory cells in the stomach
Gastric adenocarcinoma	Type of gastric cancer characterized by glandular features
Gastric atrophy	Loss of gland in the stomach mucosa
Gastric cancer	Malignant neoplasm of the stomach with the potential to metastasize to distant body structures
Gastric dysplasia	Presence of abnormal stomach cells, with anomaly of growth and differentiation
Gastric inflammation	Presence of inflammatory cells in the stomach
Gastric intestinal metaplasia	Presence of goblet cells in the stomach mucosa
Gastric juice	Fluid found in the stomach during endoscopy
Gastric mucosa	Inner lining of the stomach
Gastric premalignant lesions	Presence of either atrophy, intestinal metaplasia or dysplasia in the stomach
Gastric tumour	Any growth or mass in the stomach
Ochratoxins	Type of toxin produced by fungi

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CHAPTER 1: INTRODUCTION

1.1 Background

There is evidence of increasing non-communicable disease (NCD) occurrence in sub-Saharan Africa (SSA) but this has been hidden under epidemics of infectious disease (Naghavi et al., 2010). In Zambia, it was estimated that 23% of all deaths in 2016 were due to NCDs, which include cancer (ZNHSP, 2017). Zambia is a landlocked, lower-middle income country located in sub-Saharan Africa with a total land area of 743,390 km^2. It has a population of 18,014,127, with 41.7 % of the inhabitants living in urban areas. The median age in Zambia is 17.2 years (Worldometers, 2019).

Gastric cancer is a malignant tumour arising from any part of the stomach cardia, fundus, corpus or antrum (Figure 1.1). It has the potential for invasive growth or metastasis to regional and distant organs (Stewart et al., 2003).

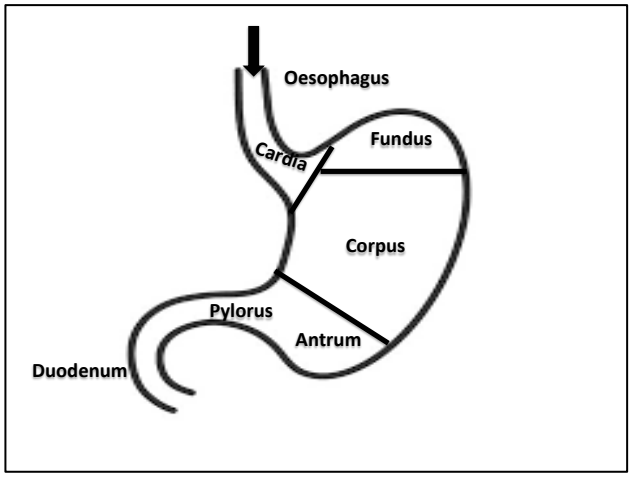


Figure 1.1 Anatomical parts of the stomach

The major type of gastric cancer is adenocarcinoma accounting for more than 90% of the cases (Peleteiro et al., 2012). Other types include gastrointestinal stromal tumours, lymphomas, neuroendocrine tumours, squamous cell and gastric Kaposi's sarcoma. Gastric adenocarcinoma (GA) develops in multiple steps along the Correa pathway (Correa et al., 1975). The major trigger is thought to be *Helicobacter pylori*

(*H. pylori*) infection which results in non-atrophic gastritis, chronic atrophic gastritis (CAG), gastric intestinal metaplasia (GIM), dysplasia and then cancer. CAG, GIM and dysplasia are therefore, known as gastric premalignant (GP) lesions. In addition, other bacterial, environmental and host immune factors are thought to influence the progression of these lesions (Correa et al., 2007) rendering the development of gastric cancer multifactorial.

The gold standard for gastric cancer diagnosis is histology of gastric tissue obtained either endoscopically or surgically. Endoscopic diagnosis is done by direct visualisation of the gastric mucosa during a process called oesophagogastroduodenoscopy (OGD). The development of GP lesions is patchy, and therefore multiple biopsies from different areas of the stomach are usually required to identify these lesions (Figure 1.2).

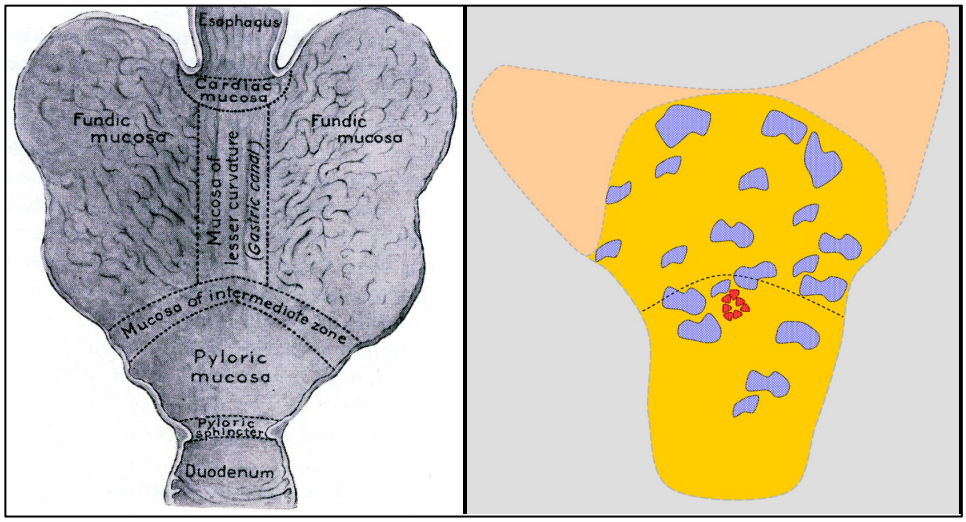


Figure 1.2: Image of the gastric mucosa showing the patchy development of gastric premalignant lesions

*The blue areas are the gastric premalignant lesions while the red spots are an example of where gastric adenocarcinoma could start developing. Adopted from Graham et al., 2008

OGD is a widely used technique with very high specificity but low sensitivity for detection of early gastric cancer or premalignant lesions. Innovative strategies such confocal laser endomicroscopy, narrow band imaging, magnifying endoscopy with blue laser have been developed to enhance the sensitivity of OGD (Kimura-Tsuchiya

et al., 2017; Zuo et al., 2017; Yoshimizu et al., 2018; Kayamba et al., 2018). However, these new techniques are expensive and therefore, cannot be widely applied in poor-resource countries such as Zambia.

The study was conducted at the University Teaching Hospital (UTH) in Lusaka, Zambia. UTH is the largest referral hospital in the country with a full time endoscopy unit with the capacity to carry out both upper and lower diagnostic and therapeutic procedures. Patients seen at UTH are referred from all the ten provinces of Zambia.

Risk factors explored in this study were based on previous observations in Zambia (Fernando et al., 2001; Kayamba et al., 2013; Asombang et al., 2014; Kayamba et al., 2015) that:

- the occurrence of gastric cancer was increasing in young adults;
- there was a high occurrence of early onset gastric cancer;
- the patient outcomes were poor and
- gastric cancer was not associated with HIV infection or the virulence factor CagA.

With a paucity of knowledge on gastric cancer from SSA, this study was designed to explore probable unique factors that could explain these preliminary observations. The study was also set out to identify the parts of Zambia GA patients come from, understand their socio-economic status and identify modifiable risk factors associated with GA as a strategy for risk reduction. In addition, information was collected on referral time frames and a strategy was tested for early case detection. Standard laboratory techniques including enzyme-linked immunosorbent assay, high-performance liquid chromatography, *in situ* hybridisation and immunofluorescence were used to identify various exposures and classify GA subtypes.

This study provides an overview of factors associated with gastric cancer in Zambia, and additionally proposing a strategy that could potentially enable early gastric cancer diagnosis.

1.2 Statement of the problem

The outcome of gastric cancer in Zambia is very poor with a mortality of 87% in the first year of diagnosis (Asombang et al., 2014). Data from Tanzania, another low-resource country showed an overall five-year survival of 6.9% (Mabula et al., 2012). The figures from developed countries are slightly better but the overall outcomes are still not good with a one-year survival of 41% and a five-year survival of 19% in the UK (Cancer Research UK, 2015) and a five-year survival of 24% in the USA (SEER data, 2015). The major contributor to poor outcomes is late diagnosis, as most of the cancers are already advanced at the time of diagnosis and therefore, only suitable for palliative care. Factors leading to delayed gastric cancer diagnosis in Zambia are currently only speculative, as the exact point of delay has not been elucidated. There is no programme directed at early gastric cancer diagnosis in Zambia due to lack of information on which individuals are at risk to allow for targeted screening. The best available method for detecting gastric cancer involves use of endoscopic evaluation on asymptomatic individuals. This method is not only impractical, but it is unaffordable and requires a great deal of material and human resource, both of which are largely unavailable in most parts of the country. It is therefore, very difficult to identify individuals with gastric premalignant lesions or early gastric cancer.

A worrying trend of increasing early-onset gastric cancer cases in Zambia was recently reported (Kayamba et al., 2015) but reasons for this observation have not been established. *Helicobacter pylori* (*H. pylori*) infection is the single most important risk factor for gastric cancer (IARC, 1994) but previous work from Zambia did not find any association with its virulence factors, particularly cytotoxin-associated gene A (Fernando et al., 2001; Kayamba et al., 2013). It is very clear that *H. pylori* infection alone cannot explain gastric carcinogenesis (Amieva et al., 2016).

Gastric cancer is most prevalent among elderly patients throughout the world, but in Zambia close to 22% of the cases occurs below the age of 45 years (Kelly et al., 2008; Kayamba et al., 2013). This proportion is much higher than that of the UK (4%), the USA (6.2%), or Italy (8.5%) (Santoro et al., 2007; Cancer Research UK, 2015; SEER data 2015). Among African Americans the proportion is slightly higher at 10% (Wu et al., 2006). This figure is however, still significantly lower than in Zambia. In Korea, a country with the highest incidence rates in the world, gastric cancer is relatively rare before the age of 30 years (Shin et al., 2011). Using endoscopic records and age specific population figures from the Zambia 2010 census of population and housing report (CSO, 2012), the incidence of gastric cancer in Zambia was estimated and compared to those of the USA and UK. The comparison showed that the incidence of gastric cancer in Zambia is higher in the younger age groups (Figure 1.3).

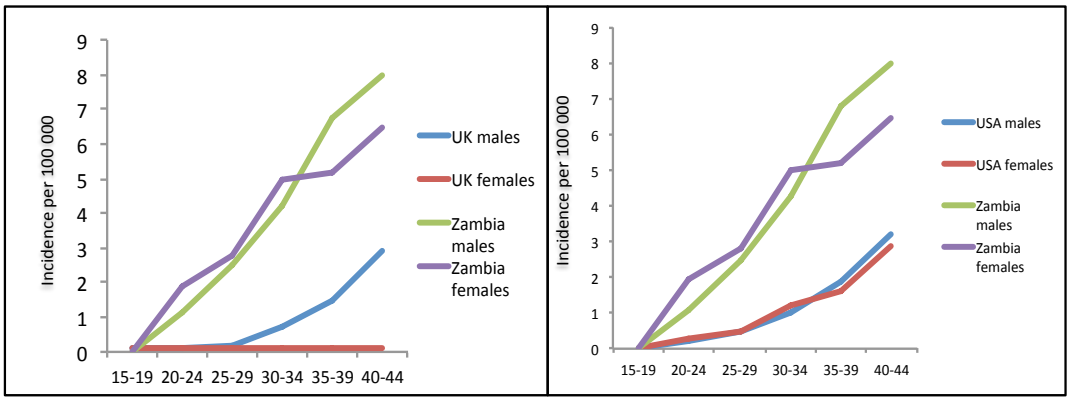


Figure 1.3: Comparison of the estimated gastric cancer incidence in Zambia with the United Kingdom and the United States

Similar findings have been reported from Tanzania (Mabula et al., 2012). There is also evidence that the age at first presentation for gastric cancer differs depending on the ethnicity. In the USA the largest proportion of younger patients being Hispanic and the older gastric cancer patients among Caucasians (Yang et al., 2011). The currently established gastric cancer risk factors cannot fully explain its occurrence in young adults.

Exposure to potential environmental carcinogens is wide spread and it is not known which exposures contribute to gastric cancer development. The paucity of information on gastric cancer in Zambia and other countries of the sub Saharan Africa make it difficult to formulate risk reduction and early detection strategies.

1.3 Study justification

This study was developed on a basis of preliminary information in order to develop relevant, practical and testable hypotheses of gastric carcinogenesis in Zambia. Reported in this thesis are some environmental, dietary and biological factors influencing the development of gastric cancer in Zambia. This information is vital for identification of high-risk individuals among Zambians. Modifiable risk factors if mitigated, could have an impact on the growing numbers of gastric cancer cases. The study also demonstrated that some gastric cancer risk factors reported by investigators from other parts of the world do not necessarily apply to Zambian patients.

In addition, this study identified a simple and affordable technique that could be used to detect gastric mucosal lesions early in centres that do not have endoscopic facilities. This has the potential to improve patient outcomes.

This study was also able to describe histopathological and molecular classifications of gastric cancer, information that could be used in formulating specific therapeutic options for affected individuals. The study has provided exploratory data needed for future long-term and more robust studies on gastric cancer in Sub-Saharan Africa. Knowledge and understanding of the demography and risk factors for gastric cancer has contributed information for better medical practice and policy formulation aimed at reducing the health burden resulting from gastric cancer.

In future, using the insights described in this study, it will be possible analyse specific risk factors for each subtype of gastric cancer and explore the exact pathophysiological mechanisms involved.

1.4 Conceptual framework

A conceptual framework was developed based on the Correa model of gastric carcinogenesis and adopted it from that published by Conteduca et al. (2013). According to the Correa model, gastric cancer develops through a series of gastric pathological changes. Following infection with *Helicobacter pylori,* the stomach develops acute then chronic inflammation. This may subsequently progress to atrophic gastritis, intestinal metaplasia, dysplasia and then gastric cancer as shown in Figure 1.4.

To this framework, this study has added on the role of direct and indirect carcinogenic factors, and at the same time describing factors that have the potential to counteract these processes.

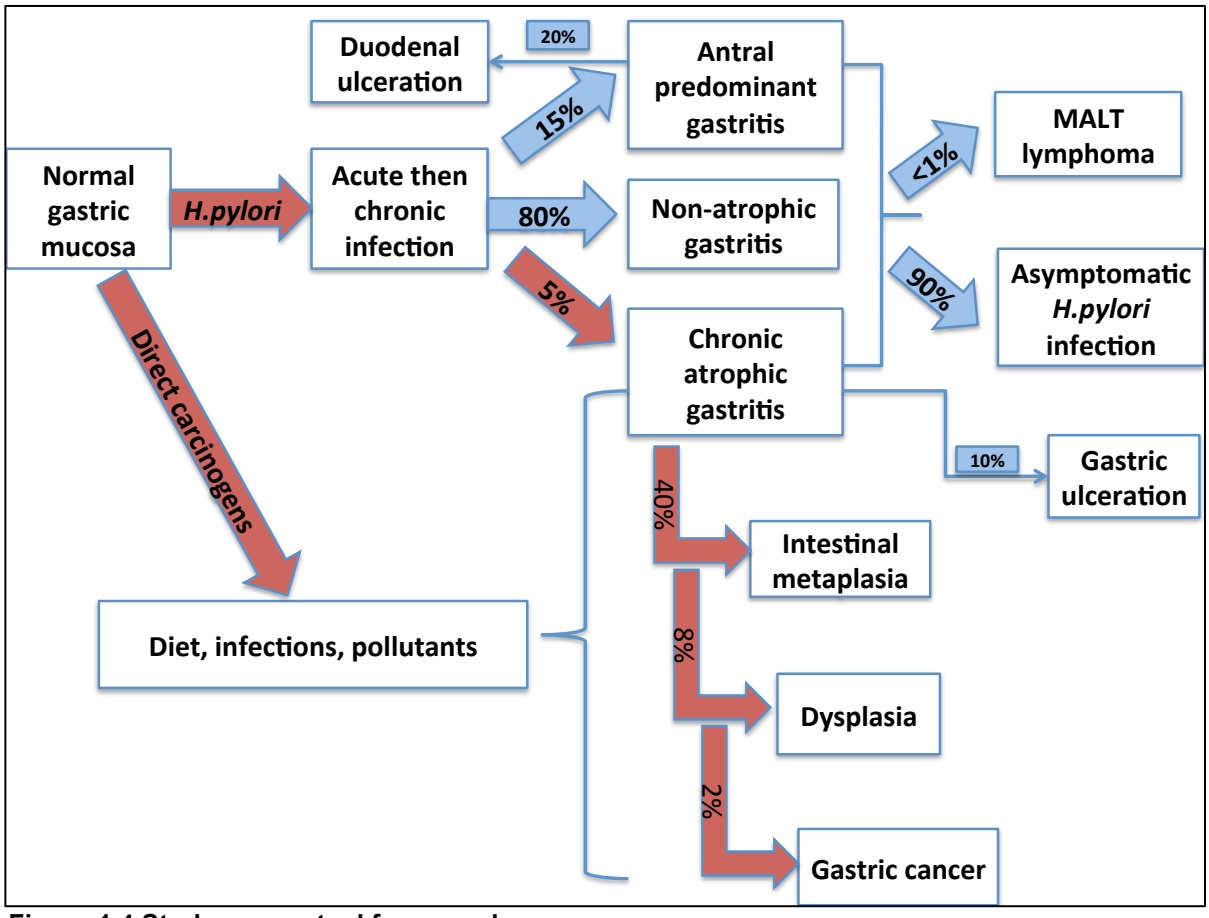


Figure 1.4 Study conceptual framework

*Arrows shown in red depict parts of the framework investigated in this study (the final outcome being cancer with infections, diet and environmental factors being the risk factors)

1.5 Research questions

There were two research questions for this study;

1. Which environmental, dietary and biological factors are associated with gastric cancer in patients seen at the University Teaching Hospital in Lusaka, Zambia?
2. Can detection of blood in gastric juice be used to identify patients with gastric mucosal lesions in need for endoscopic evaluation?

1.6 Study objectives

1.6.1 General objective

The general objective of the study was to investigate environmental, dietary and biological factors associated with gastric cancer and to test the utility of gastric juice as a diagnostic strategy.

1.6.2 Specific objectives

i. To describe the socio-demographic, clinical and histopathological features of gastric cancer patients

ii. To evaluate the association between gastric cancer and the following risk factors;
    a. Biomass smoke exposure and oxidative stress to DNA
    b. Consumption of vegetables and fruits
    c. Consumption of processed and unprocessed meat
    d. Estimated salt intake and excretion
    e. Dietary exposure to aflatoxins and ochratoxins
    f. Human Immunodeficiency virus infection
    g. *Helicobacter pylori* infection
    h. Epstein-Barr virus infection

iii. To investigate the utility of blood in gastric juice as a marker of gastric mucosal lesions

1.7 Organisation of the thesis

Chapter 1: This chapter introduces the study subject and gives a synopsis of the scope of the study and major findings. This chapter also sets out the study justification, major and specific objectives of this study. It also gives an outline of the conceptual framework.

Chapter 2: In the second chapter, a detailed outline of literature that was reviewed is presented, with a particular focus on aspects related to this study. The chapter presents the current understanding of gastric cancer and its related risk factors. It also shows the knowledge deficit particularly in the Zambian context.

Chapter 3: This chapter describes methods that were used to collect data. It outlines in detail the endoscopic and laboratory procedures employed.

Chapter 4: The first part shows an overview of the patients enrolled in the study. This part of the results focuses on describing the basic characteristics of the participants. It also outlines the anatomical, histological and molecular classification of gastric adenocarcinoma and its premalignant lesions. The second results section focuses on the dietary, environmental and biological gastric cancer risk factors that were evaluated. The last section gives an overview of flow of gastric cancer patients through the referral system in Zambia. It demonstrates probable reasons for delayed gastric cancer diagnosis. It subsequently reports on the utility of a simple method for detection of gastric mucosal lesions in centres that do not have endoscopy.

Chapter 5: In this chapter, the study findings are discussed and the implications of the findings brought into context. The discussion ends with a section on study limitations.

Chapter 6: Conclusions drawn from the study findings are presented in this chapter. In addition, recommendations to various stakeholders are outlined, ending with a note on future work.

CHAPTER 2: LITERATURE REVIEW

2.1 Epidemiology of gastric cancer

Gastric cancer is the fifth most common cancer globally and the third leading cause of cancer related deaths. In 2018 over a million new cases were recorded, with an estimated 783,000 deaths representing one in every twelve deaths globally (Bray et al., 2018). Gastric cancer is twice as common in men than women globally (Khazaei et al., 2016). The Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) project of the International Agency for Research on Cancer (IARC), a specialized agency for the World Health Organisation (WHO) provides estimates by cancer site and sex using the best available data in each country and several methods of estimation (Ferlay et al., 2015; Antoni et al., 2016). The maps shown in Figures 2.1 and 2.2 depict estimated age-standardised gastric cancer incidence rates published by GLOBOCAN in 2018 (Farley et al., 2018). The highest gastric cancer incident rates are in Eastern Asia, particularly Japan, Mongolia and Korea. Korea has the highest rate in the world with age-standardised incidence rates of 76.8 per 100,000 for men and 37.9 per 100,000 for females (Kweon et al, 2018).

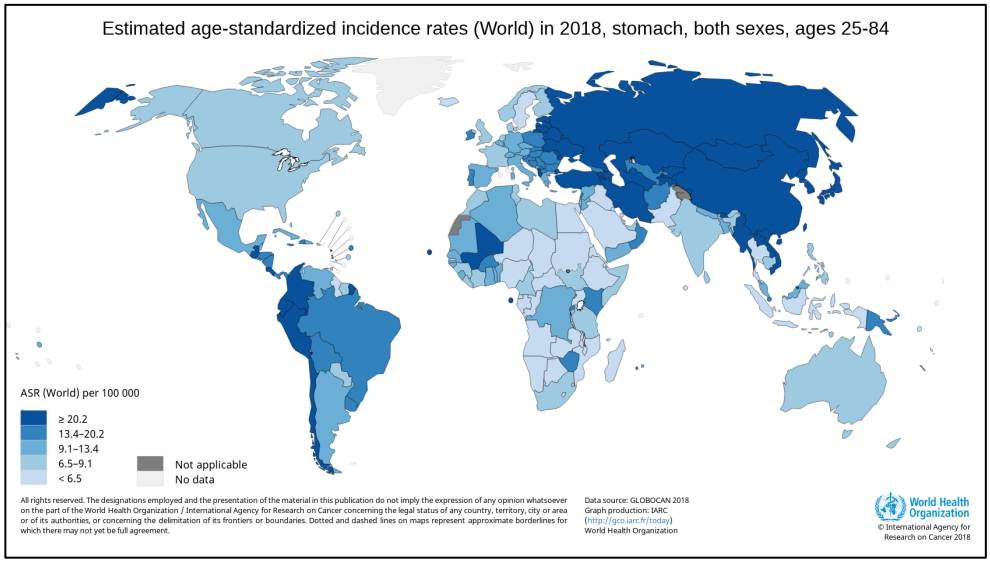


Figure 2.1: World age-standardized estimates of incidence rates for gastric cancer. Source: Farley et al., 2018.

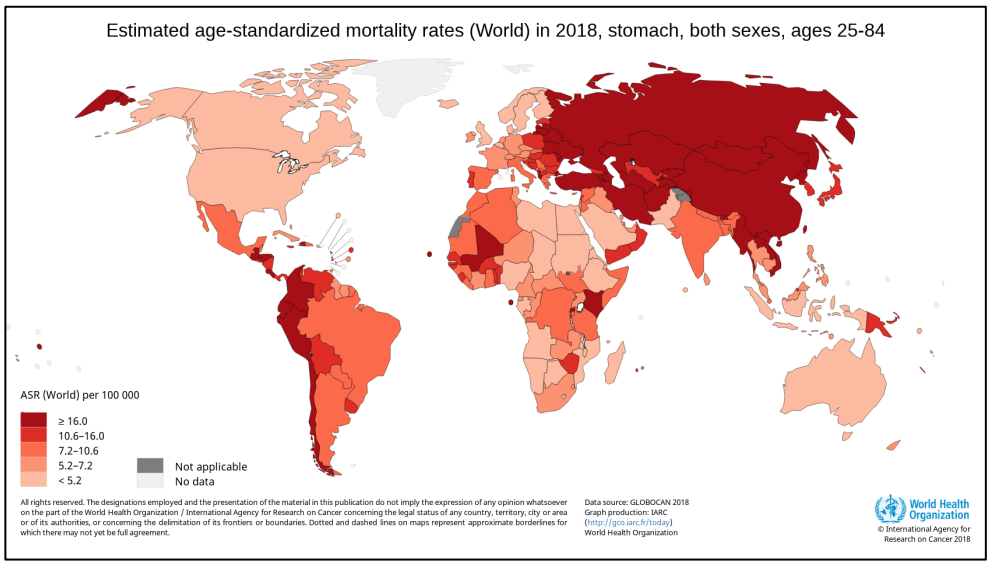


Figure 2.2: World age-standardized estimates of mortality rates due to gastric cancer. Source: Farley et al., 2018.

Data on gastric cancer from sub-Saharan Africa are scarce (Asombang et al., 2012) mainly due to a paucity of population-based registries in this region (Laryea et al., 2012) and limited diagnostic capacity (McFarlane et al., 2001). The predicted percentage increase in cancer incidence in low and lower-middle income countries by 2030 is 82% and 70% respectively. Current GLOBOCAN estimates of gastric cancer for Africa are varied, ranging from less than 5.2 per 100,000 in some countries such as Angola to more than 20.2 per 100,000 in Mali (Figures 2.1 and 2.2). Cancer is an increasing problem in Africa partly due to the ageing and growing population as well as increased exposure to cancer causing agents (Parkin et al., 2014). Despite this increase it receives relatively little public health attention most of which is given to major communicable diseases such as the Human Immunodeficiency virus (HIV), tuberculosis and malaria.

According to GLOBOCAN 2018 estimates, gastric cancer is the tenth most common cancer in Zambia and the ninth most common cause of cancer related deaths. It has an incidence of 3.0 per 100,000 per year with a mortality rate of 3.1 per 100,000 per year (Farley et al., 2018). However, these are estimates obtained by modeling, using incidence: mortality ratios derived from cancer registry data in neighbouring countries.

Gastric cancer case detection is limited due to the limited diagnostic capacity in Zambia.

2.2 Clinical features and diagnosis of gastric cancer

Ancient Egyptians in early as 3000 BC were the first to describe gastric cancer in the papyrus manuscripts and hieroglyphic inscriptions. Throughout the Middle Ages, cancer continued to torment mankind, with doctors believing that once gastric cancer got established it was best left alone (SEER, 2018). One of the most famous cases was that of the French military and political leader, Napoleon Bonaparte, who died in 1821 due to gastric cancer diagnosed at post-mortem. Gastric cancer is, therefore, not a new disease but there has been gradual improvement in the understanding of its development and clinical presentation. In current literature, gastric cancer typically refers to gastric adenocarcinoma, which is the most common type (Peleteiro et al., 2012). Other types are gastrointestinal stromal tumours, lymphomas, neuroendocrine tumours, squamous cell and gastric Kaposi's sarcoma, but for these specific names are usually mentioned when they are being referred to.

Gastric cancer is an aggressive disease and it is frequently diagnosed late. With the diagnosis of advanced gastric cancer, the utility of surgery is limited, leaving the treatment options to neo-adjuvant chemotherapy and radiotherapy with poorer survival outcomes (Zheng et al., 2015; Coghlin et al., 2015; Fontana et al., 2015). Therefore, late gastric cancer diagnosis is one of the major contributors to poor outcomes (Lang et al., 2013). Early detection of gastric cancer is made difficult by the lack of specific symptomatology. Some of these symptoms include unintentional weight loss, abdominal pain, nausea, dysphagia, melaena or haematemesis and early satiety (Cancer Research UK, 2015). Many of these symptoms manifest in late disease, as early gastric cancer is usually asymptomatic. Periodic endoscopic surveillance is the most effective way for early diagnosis of gastric cancer (González et al., 2012) but there are no cheaper tools that can be employed to determine high-risk patients requiring endoscopic evaluation. Population

based endoscopic screening is carried out in countries with high gastric cancer rates such as Korea and Japan as early detection of gastric cancer using screening programmes improves its outcome (Chung et al., 2012; Gong et al., 2014).

There is evidence that countries with gastric cancer screening programmes have much higher rates of early cancer detection than those that do not (Kim et al., 2015). However, these screening programmes are very expensive and impractical in resource-poor countries such as Zambia and they require repeated endoscopic evaluations on a large number of people. For example, the National Cancer Control Committee of Korea recommends that individuals over the age 40 years undergo gastric cancer screening via either the upper gastrointestinal series or endoscopy every 2 years (Kim et al., 2014). The Zambian and many other health care systems are unable to support such an elaborate programme; even those that could afford it would not have large enough gastric cancer cases to justify it. Population coverage of these screening programmes can be a challenge if not well organized. In Chile, the gastric cancer programme was reported to have coverage as low as 14% (Latorre et al., 2015). The programme in Korea, however, is better organized and it has been shown to be available to patients of all socio-economic classes (Jung et al., 2015). Other non-invasive methods of identifying individuals with increased risk of having gastric cancer have been considered. Faecal occult blood testing is a well-established strategy for colorectal cancer screening but not for gastric cancer. There is not enough evidence to recommend the use of faecal occult blood in screening for upper gastrointestinal cancers (Nakama et al., 2000; Allard et al., 2010).

2.3 Classifications of gastric cancer

Gastric cancer has two well-recognized classification systems, the Lauren classification that subdivides it into intestinal and diffuse types and the World Health Organization system dividing it into papillary, tubular, mucinous (colloid), and poorly cohesive carcinomas (Lauren et al., 1965; Lin X et al., 2015).

2.3.1 Intestinal type gastric cancer

The intestinal type of gastric cancer develops through a series of histological changes known as the Correa cascade. According to the cascade, *Helicobacter pylori (H. pylori)* infection leads to the development of non-atrophic gastritis (NAG), which may later become chronic atrophic gastritis (CAG). Gastric intestinal metaplasia (GIM) then ensues leading to dysplastic changes and then cancer (Correa et al., 1975). These are collectively known as gastric premalignant (GP) lesions. Progression from one stage to another is thought to take several years. However, diagnosing these lesions histologically does not give a practical approximation of gastric cancer risk.

In 2008, an international group of pathologists established the Operative Link for Gastritis Assessment (OLGA) for reporting gastritis in terms of stage (Rugge et al., 2008). According to the OLGA staging system, the histological phenotypes of gastritis are arranged along a scale of progressively increasing gastric cancer risk form the lowest (stage 0) to the highest (stage 4). This staging is done on at least five gastric biopsies taken as follows: two from the distal antrum (one lesser and the other greater curvature), two from the body or corpus (one lesser and one greater curvatures) and one from the incisura angularis (Dinis-Ribeiro et al. 2012). In each biopsy, atrophy is scored as a percentage of atrophic glands on a four-tiered scale. No atrophy is 0%, and given a zero score, mild atrophy is one to 30% and given a score of one, moderate atrophy is 31 to 60% and is given a score of two. Severe atrophy is more than 60% and is given a score of three. The scores from the antrum (including the incisura angularis) and the corpus are then plotted onto the OLGA table to determine the respective stage (Table 2.1). Stages lower than three have very low risk for gastric cancer development. Stages three and four are thought to carry a high enough gastric cancer risk to justify endoscopic surveillance (Rugge et al., 2008).

Table 2.1 Staging frame for the Operative Link for Gastritis Assessment. Source: Rugge et al., 2008.

Atrophy Score		Corpus			
		No Atrophy (score 0)	Mild Atrophy (score 1)	Moderate Atrophy (score 2)	Severe Atrophy (score 3)
Antrum	No Atrophy (score 0) (including *incisura angularis*)	STAGE 0	STAGE I	STAGE II	STAGE II
	Mild Atrophy (score 1) (including *incisura angularis*)	STAGE I	STAGE I	STAGE II	STAGE III
	Moderate Atrophy (score 2) (including *incisura angularis*)	STAGE II	STAGE II	STAGE III	STAGE IV
	Severe Atrophy (score 3) (including *incisura angularis*)	STAGE III	STAGE III	STAGE IV	STAGE IV

One major drawback of the OLGA staging system for gastric cancer risk assessment is the low interobserver agreement even among experienced pathologists (el-Zimaity et al., 1996; Chen et al., 1999; Offerhaus et al., 1999). Gastric Intestinal Metaplasia (GIM), however, has much better interobserver agreement resulting in more reproducible results. Therefore, the Operative Link for Gastritis Intestinal Metaplasia (OLGIM) was developed. Similar to the OLGA, the presence of GIM was classified as follows: zero (absent), one (mild), two (moderate) and three (severe) (Capelle et al., 2010). The OLGIM staging system is then applied (Table 2.2) and those with either stage three or four are at high risk for gastric cancer and required endoscopic surveillance. One limitation of the OLGIM staging system is that it does not take into account the presence or severity of CAG, which is also a known risk factor for gastric cancer. There have been no data from Africa describing the CAG and GIM using either the OLGA or the OLGIM staging systems for gastric cancer risk assessment. However, progression of the lesions along the Correa cascade can also be influenced by other bacterial, environmental and host immune factors (Correa et al., 2007) rendering the development of gastric cancer multifactorial.

Table 2.2 Staging frame for the Operative Link for Gastric Intestinal metaplasia. Source: Capelle et al., 2010.

Antrum (includes *incisura angularis*)	Corpus			
IM Score	**No IM (score 0)**	**Mild IM (score 1)**	**Moderate IM (score 2)**	**Severe IM (score 3)**
No IM (score 0)	**STAGE 0**	**STAGE I**	**STAGE II**	**STAGE II**
Mild IM (score 1)	**STAGE I**	**STAGE I**	**STAGE II**	**STAGE III**
Moderate IM (score 2)	**STAGE II**	**STAGE II**	**STAGE III**	**STAGE IV**
Severe IM (score 3)	**STAGE III**	**STAGE III**	**STAGE IV**	**STAGE IV**

2.3.2 Diffuse type gastric cancer

This type of gastric cancer is hereditary and is characterised by the development of diffuse (signet ring) gastric cancer at a young age. The most common mutation associated with diffuse gastric cancer are truncating mutations in *CDH1*, the gene encoding for E-Cadherin (Cancer.Net, 2017). Other genetic syndromes associated with gastric cancer include the Lynch syndrome, Juvenile polyposis syndrome, Peutz-Jeghers syndrome, and familial adenomatous polyposis. Previous research from Zambia showed that less than 20% of gastric cancers were of the diffuse type (Asombang et al., 2013).

2.3.3 The Cancer Genome Atlas

With the limitations of the usefulness of histopathological gastric cancer classification types, a large consortium of scientists used modern genomic technologies to perform comprehensive genomic annotation of 295 gastric tumours in The Cancer Genome Atlas **(**TCGA) for gastric cancer first published in 2014 (TCGA 2014). Among the techniques used in this classification was Gene (mRNA) Expression, Sequencing of All

Coding Genes (exome), Copy-Number Analysis (SNP arrays), MicroRNA Expression (miRNA-seq), DNA Methylation (450K arrays), Protein Phosphorylation Analysis (RPPA) and low-pass whole genome sequencing on a subset. From this extensive work, they were able to sub-divide gastric cancer it into four major subtypes: Epstein-Barr Virus (EBV)-positive tumours, microsatellite unstable tumours, genomically stable tumours, and tumours with chromosomal instability (TCGA, 2014). It is now well accepted that gastric cancer is a heterogeneous disease as these analyses have demonstrated its molecular complexity (Figueiredo et al., 2015).

Classification based on molecular properties has the potential of influencing therapeutic options for precision treatment of gastric cancer patients, resulting in better outcomes. In this study, the occurrence of EBV-positive and microsatellite unstable gastric cancer was evaluated.

2.3.3.1 EBV-positive gastric cancer

EBV is a DNA oncogenic virus present in about 90% of the global population (Balfour et al., 2015). It is associated with nasopharyngeal and gastric cancers, follicular dendritic cell tumours/sarcomas, Burkitt's lymphoma, lymphomatoid granulomatosis, pyothorax-associated lymphoma and HIV associated lymphomas (Maeda et al., 2009). About 9% of gastric cancer is attributed to EBV infection (Tang et al., 2012; TCGA, 2014; Gulley et al., 2015) and TCGA found that these tumours exhibit a higher prevalence of DNA hypermethylation (TCGA, 2014). EBV-positive gastric tumours showed a strong predilection for phosphatidylinositol 3-kinase, catalytic subunit alpha (*PIK3CA*) mutation with a potential for therapeutic importance (Zhang et al., 2014). Clinically, this gastric cancer subtype shows a low rate of lymph node metastasis conferring a slightly better prognosis (van Beek et al., 2004) with a lower number of differentially expressed genes (Carmago et al., 2014; Figueiredo et al., 2015). In Zambia, the proportion of EBV-positive gastric cancer remains unknown and EBV-positive cancer is more common among younger gastric cancer patients (Carmago et al., 2011).

2.3.3.2 Microsatellite unstable gastric cancer

Microsatellite unstable gastric tumours show elevated mutation rates, including mutations of genes encoding targetable oncogenic signaling proteins (TCGA, 2014). The instability is related to loss of function of mismatch repair (MMR) genes. Function of MMR mechanism is mainly due to *MLH1* silencing by promoter hypermethylayion resulting increased number of mutations (Choi et al., 2016). There is growing evidence of differential response of microsatellite unstable tumours to chemotherapy (An et al., 2012; Kim et al., 2015) with promising favourable response to immunotherapy (Le et al., 2015). TCGA did not include samples from Africa and therefore the molecular classification of gastric cancer in this continent remains unknown.

2.4 Risk factors for gastric cancer

Gastric cancer results from genetic and acquired risk factors, although the exact pathogenesis is still not clearly understood. There are some risk factors that have been described in many populations, although the evidence of actual causation is unsatisfactory due an incomplete understanding of the exact pathogenic mechanisms. Established biological risk factors for gastric cancer include advancing age, pernicious anaemia, sporadic gastric adenoma, familial adenomatous polyps, hereditary non-polyposis colon cancer, Li Fraumeni syndrome and blood group A (Stomach Cancer Types, 2015). Proposed environmental and lifestyle risk factors include low consumption of fruit and vegetables, cigarette smoking, salty smoked foods, heavy alcohol intake and possibly red and processed meat, haem iron and obesity (Ladeiras-Lopes et al., 2008; Pourfarzi et al., 2009; Gonzalez et al., 2010; Baroudi et al., 2014; Fang et al., 2015). Other risk factors are exposure to radiation and working in rubber or coal industries.

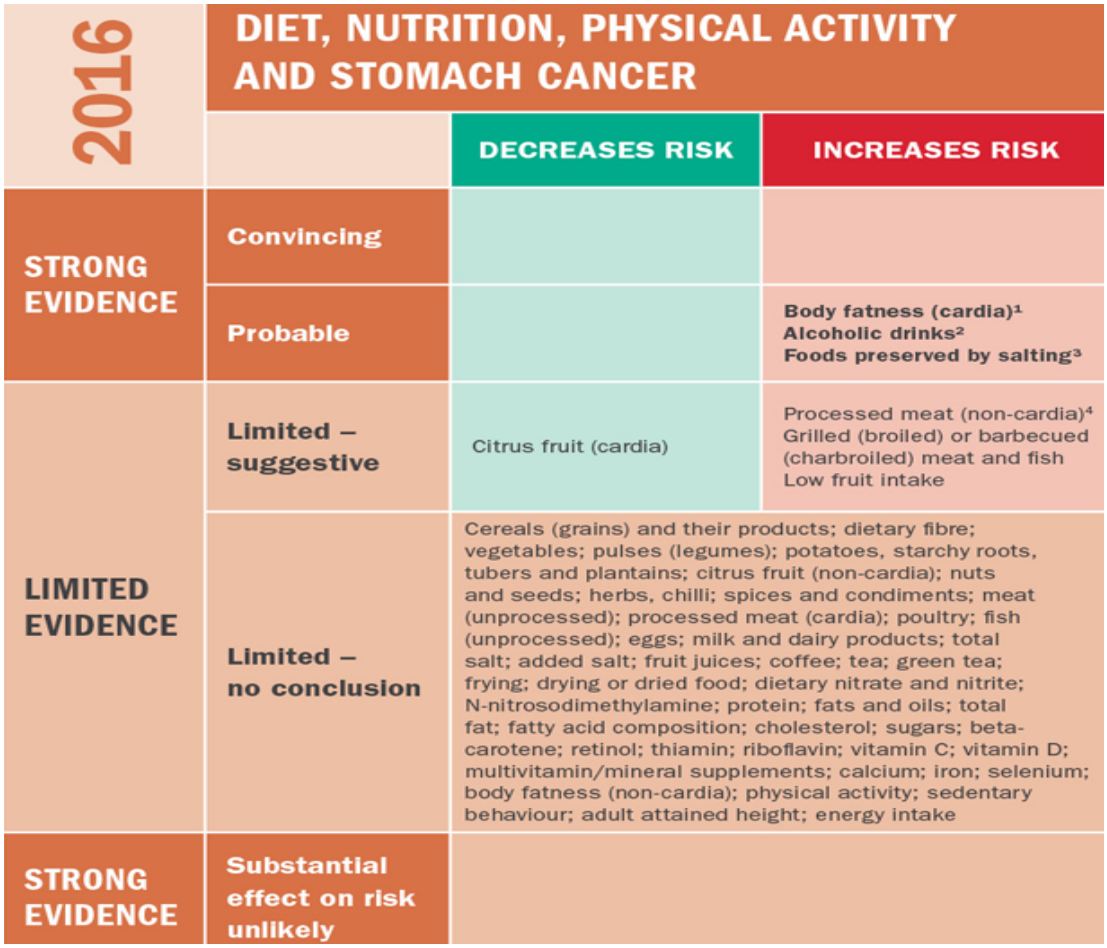
2.4.1 Gastric cancer and nutrition

Several researchers have investigated the role of nutrition in the development of cancer. It is now clear that diet modulation affects the risk of developing gastric cancer, but most data have been obtained from observational studies as opposed to clinical trials (Abnet et al., 2015). With the onset of industrialization, many African communities have experienced drastic changes in lifestyles. In Zambia, this has lead to rapid shifts in dietary habits moving towards the consumption of refined foods, some of which are known to be carcinogenic (IARC Monographs, 2015). Despite these efforts, most of the data on diet and gastric cancer are inconsistent. Depicted in Figure 2.3 is an extract from the 2016 Diet and Cancer Report by the World Cancer Research Fund International. None of the food types considered had strong evidence and convincing evidence for either increasing or decrease gastric cancer risk (Figure 2.3).

Studies have been conducted to test the assertion that micronutrient supplementation can prevent gastric cancer demonstrated by a study in Linxian, China (Blot et al., 1993). In this study, 29,584 individuals were followed over 5 years with half of them having been randomized to vitamin and mineral supplementation. By the close of the study the incidence of gastric cancer was significantly lower in the intervention group (RR = 0.79; 95 % CI = 0.64-0.99). However, subsequent randomized cancer prevention trials have mainly been negative. For example, a large US observational study involving 490,593 participants and an eleven-year follow-up showed no benefit in the use of vitamin and mineral supplementation for prevention of gastrointestinal malignancies (Dawsey et al., 2014). Earlier Plummer et al (2007) also failed to demonstrate the benefit of micronutrient supplementation on gastric cancer prevention and so did You et al (2006).

Similarly, there is evidence that a high intake of foods containing antioxidants such as fruits and vegetables is associated with a reduced risk of developing gastric cancer (Pourfarzi et al., 2009; Gonzalez et al., 2010; Asombang et al., 2013; Baroudi et al., 2014). However, a meta-analysis involving 32,758 gastric cancer cases out of 6,316,385

participants revealed that fruit intake but not vegetables was protective against gastric cancer (Fang et al., 2015).



2016	DIET, NUTRITION, PHYSICAL ACTIVITY AND STOMACH CANCER		
		DECREASES RISK	INCREASES RISK
STRONG EVIDENCE	Convincing		
	Probable		Body fatness (cardia)[1] Alcoholic drinks[2] Foods preserved by salting[3]
LIMITED EVIDENCE	Limited – suggestive	Citrus fruit (cardia)	Processed meat (non-cardia)[4] Grilled (broiled) or barbecued (charbroiled) meat and fish Low fruit intake
	Limited – no conclusion	Cereals (grains) and their products; dietary fibre; vegetables; pulses (legumes); potatoes, starchy roots, tubers and plantains; citrus fruit (non-cardia); nuts and seeds; herbs, chilli; spices and condiments; meat (unprocessed); processed meat (cardia); poultry; fish (unprocessed); eggs; milk and dairy products; total salt; added salt; fruit juices; coffee; tea; green tea; frying; drying or dried food; dietary nitrate and nitrite; N-nitrosodimethylamine; protein; fats and oils; total fat; fatty acid composition; cholesterol; sugars; beta-carotene; retinol; thiamin; riboflavin; vitamin C; vitamin D; multivitamin/mineral supplements; calcium; iron; selenium; body fatness (non-cardia); physical activity; sedentary behaviour; adult attained height; energy intake	
STRONG EVIDENCE	Substantial effect on risk unlikely		

Figure 2.3 Evidence of dietary risk factors for gastric cancer. Source: WCRF, 2016.

Antioxidants are thought to reduce oxidative damage to DNA, lipids and proteins, reducing proliferation and angiogenesis and therefore reducing the chances of developing cancer (Harvie et al., 2014). A meta-analysis conducted by Li et al (2014) showed that higher dietary intake of anti-oxidant vitamins but not blood levels was associated with a lower gastric cancer risk.

Other food types investigated for gastric cancer risk are red and processed meat. Red meat typically refers to unprocessed mammalian muscle meat (including minced meat), such as beef, pork, lamb or goat meat. Processed meat is that which has been transformed through salting, curing, fermentation, smoking or application of other flavour

enhancing or preservation techniques (Bouvard et al., 2015). Several investigators have shown that consumption of red and processed meat increases the risk of developing gastric cancer (Palli et al., 2001; Bahmanyar et al., 2006; Aune et al 2009; Gonzalez et al., 2010; Zhu et al., 2013; Wie et al., 2014; Song P et al., 2014) although the evidence is not entirely conclusive as various confounders were not well accounted for in most studies that have reported this observation (Bouvard et al, 2015). In addition, other investigators have failed to demonstrate the influence of meat consumption on gastric cancer (Keszei et al., 2012; Wang et al., 2012). The EPIC study, with 521,457 participants from 10 European countries, showed that red or processed meat intake was associated with non-cardia gastric cancer but not cardia gastric cancer, suggesting that not all types of gastric cancer could be influenced by the intake of meat (Gonzalez et al., 2006). The intake of poultry and high-fat dairy foods has also been linked to gastric cancer by some investigators (Navarro Silvera et al., 2008; Pourfarzi et al., 2009).

Processed meat was recently reported as a grade one carcinogen by the International Agency for research on cancer, principally because of its role in the development of colorectal cancer but there was no conclusive evidence for gastric cancer (IARC Monographs, 2015). Some of the reasons suggested for the meat being carcinogenic include the presence of haem iron (Jakszyn et al., 2012; Ward et al., 2012) and the production of carcinogenic compounds when preparing the meat for consumption. Meat processing can result in the formation of N-nitroso-compounds and polycylic aromatic hydrocarbons. High temperature cooking can also lead to the production of carcinogenic heterocyclic aromatic amines (Alaejos et al., 2011; Alomirah et al., 2011). Dietary nitrosamines in processed meat might be responsible for the increases risk of gastric cancer (Larsson et al., 2006). In a study that involved 494,978 participants O'Doherty et al (2012), found no association between dietary fat intake and gastric cancer.

According to the Zambian National Food Nutrition Commission report the consumption of meat in rural communities is very low as the diet is predominantly vegetarian (NFNC 2014). With the high cost of meat, many rural communities are unable

to afford it (White et al., 2015). In urban areas there is a mixed picture with the lower socio-economic communities having less access to meat than the elite.

2.4.2 Salt and salty food intake

Human beings have been using large amounts of salt to preserve food for many centuries but the health implications of high salt intake have just been recently described (D'Elia et al., 2014). The exact mechanism by which salt increases the risk of gastric cancer is not clear, but some authors have suggested that high salt concentrations up-regulate the expression of *H. pylori* CagA leading to the development of gastric cancer (Loh et al., 2007). To evaluate the association between cancer and salt, Park et al (2014) analysed the 24-hour urine sodium in 19,083 Koreans. Their findings showed that gastric cancer was significantly associated with increased 24-hour urine sodium excretion. In addition, several other investigators have studied the influence of salt on gastric cancer risk. Some of these studies are as tabulated below (Table 2.3). However, none of these studies were conducted in Africa.

Most of these studies clearly support the role of salt intake in gastric cancer, but the same cannot be said about intestinal metaplasia, which is a premalignant lesion. The plausible explanation for this difference could be that salt intake influences gastric cancer development after intestinal metaplasia has already occurred, or that gastric cancer does not necessarily develop from intestinal metaplasia. The third and most probable explanation is that there have not been enough studies evaluating the association between intestinal metaplasia and salt intake. One aspect that remains unclear is whether it is the salt itself or the high salt containing foods that is carcinogenic.

Table 2.3: Studies investigating the role of high salt intake on the development of gastric cancer

Study	Country	Article type	Salt influence on gastric cancer	Salt influence on gastric intestinal metaplasia
Woo et al., 2014	Korea	Meta-analysis	Yes	-
Park et al., 2014	Korea	Cross-sectional	Yes	-
D'Elia et al., 2014	Italy	Review	Yes	-
Gonzales et al., 2013	Spain	Review	Yes	-
Lin et al., 2014	China	Case-control	Yes	-
Bonequi et al., 2013	Several	Meta-analysis	Yes	-
Ge et al., 2012	Several	Meta-analysis	Yes	-
Zhong et al., 2012	China	Case-control	Yes	-
D'Elia et al., 2012	Several	Meta-analysis	Yes	-
Woo et al., 2011	Korea	Review	Yes	-
Zhang et al., 2011	China	Case-control	Yes	-
Park et al., 2011	Korea	Cross-sectional	Yes	-
Yang et al., 2011	China	Case-control	Yes	-
Peleteiro et al., 2011	Portugal	Case-control	Yes	-
Wen et al., 2010	China	Case-control	Yes	-
Pintalhao et al., 2010	Portugal	Cross-sectional	-	No
Dias-Neto et al., 2010	Several	Meta-analysis	-	No
Takachi et al., 2010	Japan	Cohort	No	-
De Stefani et al., 2009	Uruguay	Case-control	No	-
Wang et al., 2008	China	Cross-sectional	Yes	-
Sjödahl et al., 2008	Norway	Cohort	No	-
Kurosawa et al., 2006	Japan	Cohort	No	-
Strumylaite et al., 2006	Lithuania	Case-control	Yes	-
Tokui et al., 2005	Japan	Cross-sectional	No	-
Tsugane et al., 2004	Japan	Cohort	Yes	-
Lee et al., 2003	Korea	Case-control	Yes	-
Ye et al., 1998	China	Case-control	Yes	-
Ward et al., 1999	Mexico	Case-control	Yes	-
Cohen et al., 1997	Several	Review	No	-
Joossens et al., 1996	Several	Cross-sectional	Yes	-

2.4.3 Mycotoxins

Currently, one of the most insidious challenges to combat in food safety is

mycotoxin contamination (Adebo et al., 2015). Mycotoxins are small molecular weight compounds produced by some filamentous fungi or moulds. Under suitable temperatures and humidity, they may contaminate various foods (Zain et al., 2011). Aflatoxins are produced by fungi *Aspergillus sp* and are known to contaminate peanuts, maize and some spices while Ochratoxins mainly contaminate cereals (wheat, barley and oats), coffee and grape berries. They can also contaminate maize (Reddy et al., 2010). Other commonly consumed foods such as cassava do not support the growth of these fungi and are therefore spared (Adjovi et al., 2014). Fungal proliferation and toxin production are enhanced by storage of improperly dried grain and nuts under hot, humid and unsanitary conditions (Obuseh et al., 2011).

Aflatoxins are classified as grade one carcinogens by the IARC due to the evidence of their role in the development of cancer of the liver (IARC Monographs, 2015). Exposure to mycotoxins is a public health problem in many parts of sub-Saharan Africa (Yard et al., 2007; Shirima et al., 2013; Ezekiel et al., 2014) and exposure to these toxins is almost ubiquitous in some rural populations (Asiki et al., 2014). Maize samples from 18 sub-Saharan countries were tested for the regulatory limits of contamination. Results showed that 47% were contaminated by aflatoxins (Probst et al., 2013). Table 2.4 below summarizes African studies, which looked at mycotoxin contamination of various food types. Only those that reported the percentage of contamination are included in the table. It shows that aflatoxin contamination is widespread in the commonly consumed foods.

In Zambia, Mukanga and colleagues sampled pre-harvest maize from Lusaka, Central and Southern provinces and showed that contamination was between 3-18% for aflatoxins (Mukanga et al., 2010). More recently, Kachapulula et al., (2017) analysed maize and groundnut samples from 27 districts in Zambia. Levels above the regulatory limits for Zambia were found in as much as 58% of groundnut and 20% of the maize samples. In addition, they were able to demonstrate increased levels with poor grain storage (Kachapulula et al., 2017). In another Zambian study, aflatoxins were found in dried fish and edible insects (Kachapulula et al., 2018). There are few data on ochratoxin

exposure in Zambia or Africa as a whole.

Table 2.4: Published data on mycotoxin contamination of various substances from across the African continent from 2010 to date.

	Country	Substance Tested	Percentage Contamination	Above Regulatory Limit
Kachapulula et al., 2017	Zambia	Maize	Upto 20.0	Yes
		Groundnuts	Upto 58.0	Yes
Kumi et al., 2014	Ghana	Home-made porridge	58.3	Yes
Mutina et al., 2015	Kenya	Maize	4.0	Yes
Ezekwesili-O et al 2014	Nigeria	Herbal medication	18.6	-
Kilonzo et al., 2014	Kenya	Maize	45.0	-
Mupunga et al., 2014	Zimbabwe	Peanut butter	91.0	Yes
Oluwafemi et al 2014	Nigeria	Cow milk	75.0	Yes
Ali et al., 2014	Sudan	Powered milk	50.0	Yes
Elbashir et al., 2014	Sudan	Sorghum	38.1	-
Kamika et al., 2014	South Africa	Peanuts	35.0	Yes
	Congo DR	Peanuts	75.0	Yes
Kayode et al., 2013	Nigeria	Maize snacks	62.5	-
El Marniss et al. 2012	Morocco	Milk	8.0	Yes
Daniel et al., 2011	Kenya	Maize	16.0	Yes
Elshafie et al., 2011	Sudan	Peanut butter	100	-
Ghali et al., 2010	Tunisia	Sorghum, spices, nuts	34.4	-
Abbès et al., 2012	Tunisia	Cow's milk	60.7	Yes
Riba et al., 2010	Algeria	Wheat	56.6	-
Idris et al., 2010	Sudan	Sesame oil	43.8	-
		Groundnut oil	3.8	-
Tchana et al., 2010	Cameroon	Eggs	45.2	-
		Cow's milk	15.9	-
Kimanya et al., 2008	Tanzania	Maize	18.0	-
Mwihia et al., 2008	Kenya	Maize	35.5	Yes

The role of mycotoxins in gastrointestinal cancers such as gastric cancer is not fully investigated. Researchers from Korea reported an increased risk for gastric cancer among persons exposed to aflatoxin B1 (Eom et al., 2013) but the mechanism by which this occurs remains obscure. One proposed mechanism is that aflatoxins exert inhibitory effects on DNA synthesis and repair and also induce oxidative stress (Alpsoy et al., 2011). Mycotoxins have also been shown to influence the availability of micronutrients, some of

which might have cancer protective properties. In a study form Ghana, aflatoxins were found to correlate with decreased serum levels of vitamins A and E (Tang et al., 2009). Obuseh et al (2011) also reported evidence of aflatoxins modifying plasma micronutrients resulting in deficiencies despite adequate intake.

Ochratoxin A is the most potent type of ochratoxin, and has been linked to epidemic nephrotoxicity. Ochratoxins have also been shown to produce renal tumours, (Reddy et al., 2010). There is very little data on gastric disease due to ochratoxin exposure but recently Jia et al., (2016) reported that long-term exposure to Ochratoxin lead to malignant transformation of gastric epithelial cells.

There is no study that has reported an association between gastric cancer and these two mycotoxins. With the rising cases of gastric cancer in Zambia, it is imperative that the role of these carcinogens be evaluated, as this could potentially be a preventable risk for the cancer.

2.4.4 Exposure to biomass smoke

The development of gastric cancer can also be influenced by environmental factors. Gastric cancer is more common among the poorer sections of most communities (Uthman et al., 2013; Lagergren et al., 2015) and this cannot be completely attributed to *H. pylori* infection. Chinese investigators showed that gastric cancer is more common in rural than urban communities (Liu et al., 2014). The five-year survival for gastric cancer is also lower in developing countries, suggesting that there could be some environmental agents in these poor communities that are less pronounced in more affluent ones (Lambert et al., 2012; Global Burden of Disease, 2015). One such factor is daily exposure to biomass smoke. Biomass fuel is obtained from animal or plant materials including wood, charcoal, dung or crop residue and has been linked to several other cancers (Kayamba et al., 2017). Complete combustion of biomass fuel produces carbon dioxide and water. When combustion is incomplete (as is usually the case), carbon monoxide, formaldehydes, acrolein, benzene and polycyclic aromatic hydrocarbons (PAH) are

released. The exact products of incomplete combustion depend on the type of biomass fuel being burnt, temperature of the fire and wind conditions. Some carcinogenic products such as PAH found in cigarette smoke are also present in biomass some as illustrated in Table 2.5 (Kayamba et al., 2017).

Table 2.5: Carcinogenic substances present in both cigarette and biomass smoke. Source Kayamba et al,. 2017.

Group	Substance	IARC Group	Year of classification
Polycyclic hydrocarbons	Benz[a]anthracene	2A	1987
	Benzo[a]pyrene	1	2012
Aldehydes	Formaldehyde	1	2012
	Acetaldehyde	2B	1999
Aromatics	Benzene	1	2012
Inorganic toxins	Arsenic	1	2012
	Beryllium	1	2012
	Nickel	2B	2012
	Cobalt	2B	1991
	Lead (inorganic)	2B	1987

PAHs are organic compounds chemically comprised of two or more benzene rings but it is difficult to ascribe health effects to specific PAHs in epidemiological studies as most exposures are to mixed PAHs (Shimada et al., 2006). Particularly, epidemiological evidence of the role of PAH in gastric carcinogenesis is contradictory (Cocco et al., 1996). The most studied PAH is benzo(a)pyrene, designated a group 1 human carcinogen by the International Agency for Research on Cancer, (IARC monograph). PAHs are highly lipophilic and hence easily absorbed with high bioavailability when ingested or inhaled. They acquire carcinogenicity after xenobiotic-metabolizing enzymes convert them to highly reactive metabolites capable of attacking intracellular proteins and DNA. These enzymes include cytochrome P450 (CYP), epoxide hydrolase, glutathione transferase, UDP-glucuronosyltransferase, sulfotransferase, NAD(P)H quinone oxidoreductase 1, and aldo-keto reductase (Shimada et al., 2006). These enzymes are not only found in the liver but the intestinal mucosa as well (Autrup et al., 1982) and are regulated by a

cytosolic receptor, the aryl hydrocarbon receptor which senses PAHs (Diggs et al., 2011 and Korashy et al., 2006). In the stomach, the protective mucus layer may limit the activity of xenobiotic-metabolizing enzymes on PAHs but external factors such as *H. pylori* infection predispose to hypochlorhydria compromising this protection. Tatemichi et al., (1999) were able to demonstrate increased expression of *CYP1A1* and *CYP1A2* in gastric intestinal metaplasia with subsequent PAH metabolism. This is clearly a mechanism through which exposure would lead to cancer. Further supporting evidence was shown in a case-control study from China in which they showed that levels of 1-hydroxypyrene glucuronide, a biomarker of PAH exposure was higher in gastric cancer patients than controls (Liao et al., 2014).

A further mechanism by which PAH could be associated with gastric cancer is through the prostanoid synthesis pathway, which depends on cyclooxygenase-2 (COX-2) as a rate-limiting enzyme. COX-2 is implicated in *H. pylori* induced gastric carcinogenesis (Cheng et al., 2013) and benzo[a]pyrene a PAH, has been shown to induce COX-2 expression in epithelial cells (Kelley et al., 1997). It is well established that disease manifestations such as cancer are not just dependent on the genotype but also the epigenotype, which can alter gene expression without affecting the genetic sequence. Environmentally induced epigenetic changes are therefore cardinal in understanding carcinogenesis and PAHs have been implicated in epigenetic modifications (Upham et al., 1998).

About 77% of the African population uses solid fuel for cooking (Figure 2.4). The use of these fuels is much less common in regions with high gross domestic product (Bonjour et al., 2013).

In Zambia, 98.1% of the rural and 73.0% of the urban population rely on these solid fuels for cooking (Central Statistical Office of Zambia, 2014). Data from the Zambia Demographic and Health survey also show that there has been an increase in the use of charcoal from 25% in 2007 to 37% in 2013-2014.

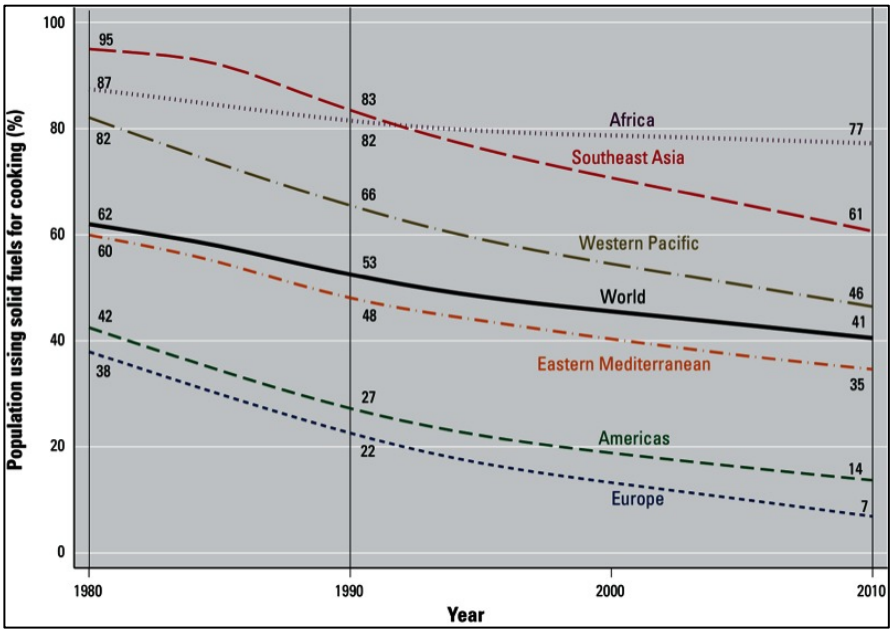


Figure 2.4: Regional trends for the percentage of population using solid fuels as the main cooking fuel in low- and middle-income countries, 1980–2010. Countries are grouped by WHO region and income category. Source: Bonjour et al., 2013.

In the urban areas, 62% of the households have access to electricity but only 26.9% consistently use it for cooking. Table 2.6 summarizes the sources of energy used for cooking in Zambian households. These data show that there is a large proportion of Zambians exposed to wood smoke on a daily basis and there is an urgent need to understand the health implications of this exposure.

Table 2.6: Percentage distribution of cooking sources in Zambia. Source: Demographic and Health Survey, 2013-2014.

	Urban	Rural
Electricity	26.9	1.8
Charcoal	67.2	15.6
Wood	5.8	82.0
Straw/shrub/grass	0.0	0.3
Animal dung	0.0	0.2

Exposure to wood smoke has been associated with oesophageal cancer in studies carried out in Kenya (Patel et al., 2013), Brazil (Mota et al., 2013), South Africa (Wang et al., 2013; Li et al., 2010; Dandara et al., 2006) and Zambia (Kayamba et al., 2015). There is no conclusive evidence of increased gastric cancer risk with wood smoke exposure although this association was suggested by a small study from Peru (Chirinos et al., 2012). In addition, it remains unknown which elements of wood smoke are carcinogenic as the combustion products of wood differ depending on the type of tree being burnt (Fine et al., 2001).

2.5 Infectious agents and gastric cancer

2.5.1 *Helicobacter pylori*

Helicobacter pylori (*H. pylori*) is an important risk factor for non-cardia gastric cancer (Huang et al., 1998; Eslick et al., 1999; Correa et al., 2007). *H. pylori* is a Gram negative, helical, microaerophillic bacterium believed to infect almost half of the world's population. It is usually acquired during childhood and if untreated, persists throughout lifetime of the host, causing chronic gastritis. However, most infected individuals do not develop clinical symptoms, perhaps as a result of the co-evolution between *H. pylori* and *Homo sapiens*. Close to 50% of the global population is infected with *H. pylori* but only 1% develop cancer (Kusters et al., 2006). This suggests that despite being a grade one carcinogen, there are other factors playing a role in the development of gastric cancer. There are great variations in the prevalence of *H. pylori* globally, with Africa having the highest prevalence (Hooi et al., 2017). There are also great variations in predominant strains isolated from different regions of the world (Yamaoka et al., 2008). These variations are thought to contribute toward differences in *H. pylori* related disease manifestation. For example, gastric cancer is not necessarily more common in regions with the highest *H. pylori* prevalence. The prevalence of *H. pylori* in Korea (the country with the highest gastric cancer incidence) is 56% (Yim et al., 2007). It is therefore apparent that the prevalence of *H. pylori*, though it is an important contributor to

pathogenesis, does not directly correlate with the incidence of gastric cancer.

Varying host responses are believed to influence the development of *H. pylori* associated disease including genetics, immune responses and the relationship of the host to specific bacterial virulence factors such as cytotoxin-associated gene A (CagA) (Lang et al., 2016; Vaziri et al., 2018). With these large variations in *H. pylori* associated gastric cancer risk, characterization of bacterial diversity is crucial for identification of high-risk populations and informing decisions about management of *H. pylori* infection both at individual and population level. A selection of immunogenic *H. pylori* antigens identified by multiplex serology assay was developed to quantify antibodies against different antigens (Michel et al., 2009). Table 2.7 shows some of the *H.pylori* specific antibodies that can be measured using this multiplex serology assay.

This assay has not yet been used on samples from Africa, despite the high *H. pylori* prevalence in this region. In Zambia, the prevalence of *H. pylori* (determined using commercially available antibody kits), among healthy community volunteers is 81% with no evidence of an association of CagA expression and gastric cancer (Fernando et al., 2001; Kayamba et al., 2013).

The *H. pylori* test-and-treat strategy has been proposed for populations with high gastric cancer incidence (Venerito et al., 2015). In Zambia, the use of *H. pylori* antibodies to identify these high-risk individuals would not be discriminating enough due to the high seroprevalence, and *H. pylori* serology is of minimal clinical value in clinical practice in Zambia. However, the rationale of using antibody titres and pepsinogens could be helpful but it is yet to be validated (Kishiwa et al., 2015). There is evidence from a local study that using the GastroPanel, (*H. pylori*, pepsinogens and gastrin-17) poorly predicts gastric premalignancy in HIV infected Zambian patients (Kayamba et al. 2018).

Table 2.7: Full names and actions of thirteen *H. pylori* antigens quantified by the multiplex serology assay. Source: Gao et al., 2009.

Antigen	Full name	Action
GroEL	Chaperonin HSP60	Assist protein folding
UreA	Urease alpha subunit	Hydrolysis of urea to NH_3 and CO_2
HP0231	Hypothetical protein	Not clear
Nap	Neutrophil activating protein A	Immune modulator
HP0305	Hypothetical protein	Not clear
HpaA	Putative neuraminylactose-binding hemagglutinin homolog	Lipoprotein- adhesion?
CagA	Cytotoxin associated gene A	Augments inflammation
HyuA	Hydantoin utilization protein A	Arginine and proline metabolism
Catalase	Catalase	Catalyzes H_2O_2 to H_2O and O2
VacA	Vacuolating cytotoxin A	Disrupts tight junctions
HcpC	Helicobacter cysteine-rich protein C	Trigger immune response
Cad	Clinnamyl alcohol dehydrogenase	Catalyse dismutation of Benz aldehyde to benzyl alcohol and benzoic acid
Omp	Outer membrane protein	stabilization of aggregates, receptors, porin

2.5.2 *Helicobacter hepaticus* and *Helicobacter bilis*

Discovered in 1992, *H. hepaticus has* been associated with cholecystitis, cholelithiasis and gallbladder cancer (Falsafi et al., 2013). It has also been linked to chronic hepatitis (Hamada et al., 2009). *H. hepaticus* is a spiral bacterium with a bipolar-sheathed flagellum which unlike those of *H. pylori*, are distributed throughout the bacterial chromosome (Falsafi et al., 2013). *H. bilis* a fusiform bacterium with three to 14 multiple bipolar sheathed flagella and periplasmic fibers wrapped around the cell (Fox et al., 1995). It was first isolated in mice but has now been associated with hepatobiliary disease in humans as well (Zhou et al., 2013). The role of these two bacteria species in gastric disease is yet to be established.

2.5.3 Human immunodeficiency virus (HIV) and gastric cancer

Sub-Saharan Africa bears over two-thirds of the global Human Immunodeficiency

Virus (HIV) burden with the prevalence among adults in Zambia being 11.8%. The estimated number of people living with HIV infection is 1.1 million (AVERT, 2019).

Figure 2.5 shows the prevalence of HIV infection in Zambia by stratified by province, with Lusaka and the Copperbelt provinces having the highest figures.

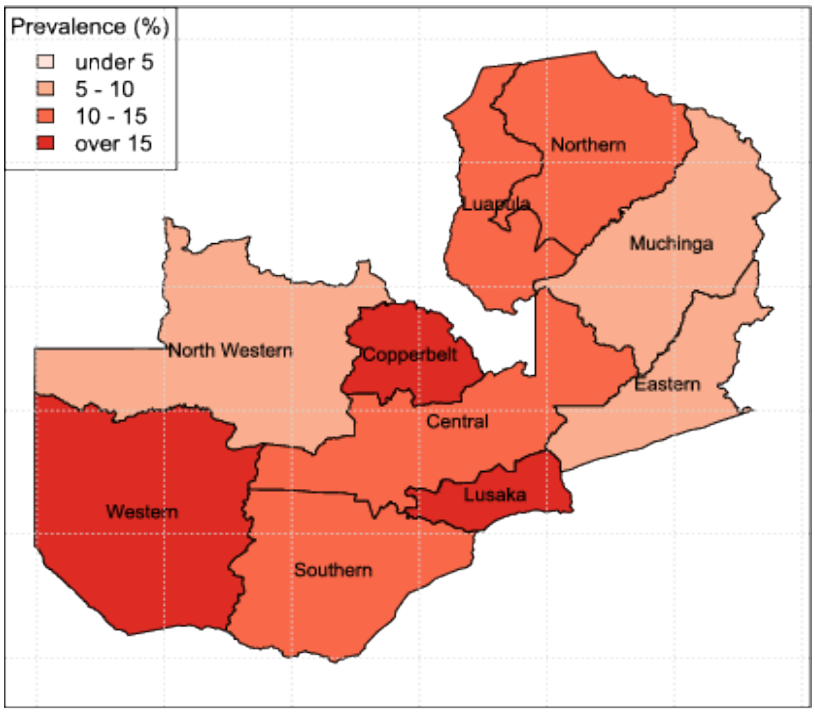


Figure 2.5: Prevalence of HIV infection in Zambia, by province. Source: Demographic and Health Survey, 2013-2014.

There is no evidence of an association between HIV infection and gastric cancer (Kayamba et al., 2013; Jensen et al., 2017). However, HIV infected individuals are more likely to have active EBV infection than those who are uninfected (Jenson et al., 1999; Kayamba et al., 2016). EBV is associated with about 9% of gastric adenocarcinoma (TCGA, 2014). In addition, persistent HIV viraemia and immune activation favours the onset of EBV-related malignancies and many malignant diseases that arise in the setting of HIV infection tend to present at more advanced stage with shorter survival time (Minhas et al., 2010; Yong-xi Zhang et al., 2011; Petrara et al., 2012). The influence of HIV infection on the development of EBV-positive gastric cancer has not been investigated.

2.6 Screening for gastric cancer

The outcome of gastric cancer is much better when diagnosed early (Saito et al., 2013). Early gastric cancer diagnosis is a challenge in low-resource settings as endoscopy is expensive, invasive and requires trained personnel. In addition, ordinary white light endoscopy with histology, which is the gold standard for gastric cancer, has low sensitivity for detection of early gastric lesions (Zhang et al., 2016). There are several innovative strategies being developed to enhance the sensitivity of endoscopic biopsies such as confocal endomicroscopy, narrow band imaging, magnifying endoscopy with blue laser among others (Yoshimizu et al., 2018; Kimura-Tsuchiya et al., 2017; Kayamba et al., 2017; Zuo et al., 2017), but application of these techniques is not possible in many parts of Africa. Less invasive strategies being evaluated make use of easily obtained samples such as blood, urine and saliva. Some of these include circulating tumour cells (Kang et al., 2017), cytokines (Sánchez-Zauco et al., 2017), and tumour markers (Chen et al., 2017). More recently, there have been reports of promising gastric cancer biomarkers detected in gastric juice, particularly long non-coding RNA (Yang et al., 2016; Shao et al., 2014), micro RNA (Yu et al., 2013) and tryptophan metabolites (Choi et al., 2016). However, many of these strategies employ molecular and highly technical approaches that are not currently feasible in poor resource settings with scanty sources of electricity and clean water. Therefore, cheap, less invasive and technically simpler methods are urgently needed for early gastric cancer detection in Africa. There is a need for a simple technique, preferably with a high negative predictive value that would enable clinicians in these low-resource rural settings to determine which individuals need to travel to more specialized centers for endoscopy.

CHAPTER 3: METHODS

3.1. Study design

This was a case-control study, but the analysis was conducted at two levels, first with gastric cancer as cases and second with premalignant lesions as cases. The full set of controls (defined as patients without GA, GP or any other type of gastric cancer) was used for both comparisons. Gastric premalignant lesions included chronic atrophic gastritis (CAG), gastric intestinal metaplasia (GIM) and dysplasia.

3.2. Study site

It was conducted at the Gastroenterology Unit of the University Teaching Hospital (UTH), in Lusaka, Zambia. With a population of over 16 million and an annual growth rate of 2.9 percent (Zambia in Figures, CSO 2018), Zambia has a total of ten provinces. It is a lower-middle income sub-Saharan African country with a limited number of referral health care centres. UTH is the largest referral hospital located in Lusaka, the capital city of Zambia with patients referred for specialist care from all provinces of the country.

UTH has the largest full-time gastroenterology unit in the Zambia with two paediatric, one surgical and three medical gastroenterologists. The unit offers both diagnostic and therapeutic upper and lower endoscopies augmented by weekly specialist clinics for patient follow-up.

3.3. Study population

The study population was all patients referred for diagnostic oesophagogastroduodenoscopy (OGD). They were approached for recruitment when they attended for the procedure.

The inclusion criteria were patients above the age above 18 years, referred for diagnostic OGD with having given full written consent to participate. Excluded were those

with prior history of treatment for gastric cancer, history of ingesting a caustic substance and unwillingness to have an HIV test.

3.4. Patient recruitment

All consenting adults above the age of 18 years were considered for recruitment. Prior to enrolment, the study was explained to each participant and those willing to participate signed a written consent (see Appendix 1).

3.5 Sample size calculation

To calculate the sample size, a ratio of one gastric cancer case to two independent healthy controls per case was used. Due to paucity of pilot data on gastric cancer in Zambia, previously published oesophageal cancer data on exposure to wood smoke was used for the estimates, as the effects are assumed to be very similar (Kayamba et al., 2015). These data indicated that the probability of biomass smoke exposure among healthy controls was 0.38, (a proportion similar to the ZDHS estimates of charcoal use in Zambia which is 0.37). If the true odds ratio for gastric cancer in biomass smoke exposed relative to unexposed individuals was 1.7, 58 gastric cancer cases and 116 healthy controls would be needed to be able to reject the null hypothesis that this odds ratio equals 1 with probability (power) 0.9. The type 1 error probability associated with this test of the null hypothesis was 0.05. To allow for study dropouts and incomplete data, an additional 10% was added to the sample size resulting in a sample size of 64 cases and 128 controls. To achieve the desired sample size, convenience sampling was used.

3.6. Study procedures

3.6.1 Oesophagogastroduodenoscopy and study questionnaire

The study was explained to the participants and their questions answered. All patients came to the Gastroenterology Unit having starved overnight by not taking food or drink for at least seven hours. The OGD was then carried out following routine pre-procedural formalities as determined by standard of care. On entering the stomach, the

biopsy channel was flushed and gastric juice aspirated using a 10 ml syringe. A complete evaluation of the mucosa was then carried out and six biopsies taken from any gastric lesions (both malignant and benign). To assess for gastric premalignant lesions two biopsies each were taken from the antrum, incisura and body. Biopsies were then fixed in formalin for histopathological analysis. For the first 80 patients recruited, an extra biopsy was taken from the antrum for *Helicobacter pylori* testing using HelicotecUT®Plus rapid tests (Strong Biotech Corporation, Taipei, Taiwan). 10 ml of blood (two aliquots of 5 mls each) and a sample of urine were collected from each participant. From the blood, plasma and serum was extracted. Plasma, serum and urine samples were kept frozen at -80^{O}C. Interviews were then conducted using a structured questionnaire to collect demographic characteristics and risk factor details. A food frequency questionnaire was also employed to collect information about food and salt intake (see Appendix 1).

3.6.2 Laboratory procedures

These procedures were employed to achieve specific objectives one and two.

3.6.2.1 Histopathology and case ascertainment

Two experienced pathologists performed histopathological examination of the formalin fixed gastric biopsy samples at the UTH Pathology Laboratory. Using standard methods the histopathological diagnoses were made to determine the presence of cancer, premalignant lesions or non-atrophic gastritis. For the classification of CAG and GIM, the operative link for gastric assessment (OLGA) and the operative link for gastric intestinal metaplasia (OLGIM) were employed to assess the gastric cancer risk (Ruggae et al., 2005; Capelle et al., 2010). Patients with histologically confirmed adenocarcinoma were categorized as cases. Also included, as cases were those with clearly visible gastric tumours whose only available biopsies showed high-grade dysplasia. Those with CAG, GIM or low-grade dysplasia were grouped together as having premalignant lesions.

Patients classified as controls had either non-atrophic gastritis (NAG) or normal histology. NAG was classified as either active or chronic based on the presence of acute inflammatory cells. For each patient a global diagnosis was made based on the most severe histological diagnosis. An experienced histopathologist (Dr Aaron Shibemba) evaluated the biopsies and a second pathologist with specific expertise in gastric premalignant lesions (Dr Blanca Piazuelo) provided the final classification of premalignant lesions (Dixon et al.,1996).

3.6.2.2 Determination of the prevalence of Epstein Barr Virus (EBV) in gastric tumours

Epstein-Barr encoding region (EBER) chromogenic *in situ* hybridisation (CISH) technique was used on gastric tumour biopsies, which had been fixed in formalin and embedded in paraffin. *In situ* hybridisation allows for the detection of EBV DNA without losing understanding of the morphological architecture of the tissue. This was done using a commercially available kit: EBV CISH Detection Kit, Master Diagnostica, Granada, Spain. The digoxigenin-labeled probe used in the kit was a mixture of 5 RNA oligonucleotides complimentary to type 1 and 2 EBER of EBV created for the detection of the presence of EBV infected cells in latency. In addition, hybridisation signals with EBV infections could also be produced. The manufacturer's instructions were adhered to strictly (details of the procedure are in the Appendix)

3.6.2.3 Immunofluorescence staining for MutL homolog 1 (MLH1)

For immunofluorescence (IF), the following protocol was followed:

Dewaxing and hydration

Formalin-fixed paraffin-embedded biopsies mounted onto poly-lysine slides were placed in a 60^{o}C oven over night. They were then quickly placed in xylene twice for five minutes each. Hydration was then done in absolute ethanol for twice for three

minutes each and then 70% ethanol for a minute. The slides were subsequently rinsed in distilled water.

Antigen retrieval

For assay optimization, two alternatives were initially tried for antigen retrieval. A citrate based H-3300, pH 6.0, and a Tris based, H-3301, pH 9.0 (both Vector Laboratories, Burligame, California). They were each diluted at 1:100. The diluted antigen retrieval buffer (ARB) was pre-heated in a microwave at high for 4 minutes. The slides were then placed in the ARB for ten minutes, reheated and incubated for another ten minutes. The slides were then washed with either TBS or phosphate base saline (PBS) three times for three minutes each.

Permeabilisation

The slides were then incubated in either 0.2% Tween 20, detergent (Sigma-Aldrich, St Louis, USA), 0.1% Triton X100 ((Sigma-Aldrich, St Louis, USA) for three minutes. They were then washed with either TBS or PBS X3 respectively to remove the detergent.

Blocking

Blocking was done using goat serum diluted in with TBS or PBS at a 20% concentration for 30 minutes.

Staining

The tissue was then incubated with primary antibodies (anti-MLH1 antibody ab92312 Abcam, Tokyo Japan). Optimization dilutions in 20% goat serum were done at 1:500 Incubation with primary antibodies was done overnight at $4^{o}C$. The slides were then washed with either TBS or PBS four times for five minutes each. Conjugated secondary antibody, goat anti-rabbit ab150077, Tokyo Japan diluted in 20% goat serum at 1:50 was used in all cases. The tissues were incubated at RT for 1 hour and then washed in either TBS or PBS four times for five minutes each.

Mounting

A drop of mounting shield was then added and coverslips placed over the tissue. The slides were then kept at 4^{o}C overnight and read the following day. After optimization testing for MLH1, citrate based ARB diluted 1:100, PBS, 0.2% Tween, 1:500 primary antibody and 1:50 secondary antibody were chosen as optimal conditions. For each run, positive and negative controls were included. Used as positive controls were samples identified during optimization and for the negative controls the tissues were incubated in 20% goat serum instead of primary antibody.

3.6.2.4 Analysis of urinary 1-hydroxypyrene (1-OHP), creatinine and sodium

Frozen urine samples were sent to the Lancet laboratories in Lusaka, Zambia. Following standardized procedures, High Performance Liquid Chromatography (HPLC) was used to measure the amount of 1-OHP (µg/g creatinine).

Briefly, 1-Hydroxypyrene was measured by HPLC with fluorescence detection after enzymatic hydrolysis of the conjugates. The Phenolic Compound stock standard (Clincal^{R}) ref. no. 9925 was used as the calibrator to make up standards of concentration from concentrations of 0.55 to 3.00 µg/l, in deionized water. To all samples, calibrators and controls (600 µl) add β-glucuronidase enzyme mix (300 µl) and mix, the enzyme mix was prepared by adding β-glucuronidase (50 µl) to 0.1M sodium acetate buffer pH 5 (5 ml). All samples, controls and calibrators were incubated at 37^{o}C at 4 hours followed by analysis on the HPLC system.

Two certified reference controls (Clinchek^{R}) ref. no's. 8923 and 8924, level 1 and 2 were used as controls and run after the calibration and after every ten samples. The percentage recovery of the two certified reference controls Clincheck Level 1 and 2 were 97% and 95%, respectively. The limit of quantitation was 0.052 µg/l. A "Waters" system HPLC was used, with a Binary Pump (1525), Autosampler (717), multi and a fluorescence detector (2475). The mobile phase consisted of Methanol: Water ratio (3:1). The volume injected was 100 µl, and separation was

performed on a Phenomenex SphereClone 3µm ODS (2) 80Å 100 x 4.6 mm column. The excitation wavelength was set at 242 nm and the emission wavelength at 388 nm. The flow rate was 1ml/min for 5 minutes. Using the Cobas Integra 400 Plus, levels of sodium and creatinine were measured in the spot urine samples collected.

To calculate the estimated 24-hour urine sodium excretion, the Tanaka (Tanaka et al., 2002) and Kawasaki (Kawasaki et al., 1991 and Kawasaki et al. 1993) formulae were used.

Tanaka method

This method is based on the patient's weight, age and height for both sexes. To estimate the 24-hour urine sodium (Na24h) from a spot sample, the 24-hour creatinine excretion (CrPr24h) is calculated as follows:

CrPr24h (mg) = [(14.89 x weight, kg) + (16.14 x height, cm) (2.04 x age, years)] – 2,244,45.

The Na24h (mEq) excretion is then estimated as *NaUr (mEq) = 21.98 x [Na casual urine, mEq/L/(Cr casual urine, mg/dL x 10)] x CrPr24 h (mg).*

Kawasaki method

This method is also based on the patient's weight, age and height but the formulae are different for each of the sexes.

For males the estimation for CrPr24h is:

CrPr24h (mg) = [(15.12 x weight, kg) + (7.39 x height, cm) (12.63 x age, years)] – 79.9.

For females the estimation for CrPr24h is:

CrPr24h (mg) = [(8.58 x weight, kg) + (5.09 x height, cm) (4.72 x age, years) – 74.95.

The Na24h (mEq) excretion is then estimated as *NaUr (mEq) = 16.3 X (√ [(Na casual urine (mEq/L)/(Cr casual urine mg/dL X 10)] x CrPr24h (mg)) x (CrPr24h)].*

3.6.2.5 Analysis of 8-Hydroxydeoxyguanosine

Urine levels of 8-Hydroxydeoxyguanosine (8-OHdG) were measured using enzyme linked immunosorbent assay (ELISA). Commercial kits were obtained from MyBioSource (Cat. No MBS704914, San Diego, USA) and samples tested following the manufacturer's instructions. Briefly, samples were allowed to thaw, centrifuged and duplicates of 50µl samples and standards were placed in antibody-coated wells with 50µl HRP-conjugate. The wells were incubated at 37^{o}C for one hour, washed and then subsequently 50µl each of Substrates A and B added as instructed by the manufacturer. The after a 15 minute incubation at 37^{o}C, the reactions were stopped with 50µl stop acidic stop solution. Optical density was read using a microplate reader at 450nm. Results were corrected for creatinine excretion and reported as ng 8-OHdG per mg of creatinine.

3.6.2.6 Analysis of urinary aflatoxin M1

For measurement of urine levels of aflatoxin M1 ELISA kits were obtained from Helica Biosystems (Cat. No 991AFLM01U-96, Santa Ana, USA). Briefly as the per manufacture's instructions, frozen urine samples were allowed to thaw, diluted (1:20) and 100 µl placed in antibody-coated wells, followed by a one hour incubation at room temperature. After serial washings, 100 µl each of the conjugate, substrate reagent and stop solutions were added and separated by 15-minute incubations. The optical density was read using a microplate reader at 450nm. Values were corrected for creatinine to obtain ng of aflatoxin M1 per mg of creatinine.

3.6.2.7 Analysis of serum Ochratoxin A

Measurements of aflatoxin A were done on serum using ELISA kits from Helica Biosystems (Cat. No 991OCH01MS-96, Santa Ana, USA). Measurements were done with strict adherence to the manufacturer's instructions. In brief, each thawed sample was mixed with methanol at 1:4 ratio centrifuged and the supernatant used for testing. 200µl of assay diluent was mixed with 100µl of the sample followed

by a 30-minute incubation. After washing with PBS-Tween, incubation was done with 100µl conjugate for 30 minutes followed by the substrate reagent for 10 minutes, a reaction which was stopped after 10 minutes. Using a microtiter plate reader at 450nm, the optical density was measured for each well and a dose-response standard curve constructed. Results were reported as ng per ml of serum.

3.6.2.8 Multiplex assay to test for Epstein-Barr virus, *Helicobacter pylori, bilis and hepaticus* antibodies

Multiplex serology assay was used to test for 13 *H. pylori* (Urea, Catalase, GroEl, NapA, CagA, Hp0231, VacA, HpaA, Cad, HyuA, Omp, HcpC, and HP0305), three *H. bilis*, four *H. hepaticus* and four EBV antibodies (early antigen, viral capsid antigen, Epstein-Barr nuclear antigen and BZLF1-encoded replication activator protein). These were recombinantly expressed as glutathione-S-transferase-tag fusion proteins and affinity-purified on fluorescently labelled glutathione-casein coupled polystyrene beads (Luminex Corp., Austin, Tx, USA).

A mixture of the antigen-loaded beads allowed the simultaneous detection of antibodies (IgG/IgA/IgM) against the selected antigens in one reaction. A luminex flow cytometer (Luminex Corp., Austin, Tx, USA) quantified the amount of bound serum antibodies by detection of a reporter fluorescent (Streptavidin-R-phycoerythrin) on each bead set and the output was the median fluorescence intensity detected on at least 100 beads per type. Cut-offs were defined by visual inspection of percentile plots at the approximate inflection point as described for other antigens (Migchelsen et al., 2017, Teras et al., 2015, Micheal et al., 2008). Overall *H. pylori* sero-positivity was defined as being positive to at least four of the included 13 *H. pylori* proteins.

The multiplex serology testing was done at the German Cancer Research Centre in Heidelberg, Germany. A material transfer agreement was granted by National Health Research Authority to facilitate shipping of samples to Germany.

In addition, IgG antibodies against whole-cell *H. pylori* were quantitatively measured in U/mL with Enzyme-Linked Immunosorbent Assay (ELISA), by Abcam (ab108736), Cambridge, UK. Manufacturer's instructions were strictly followed. 30 U/mL was used as the cut-off for *H. pylori* seropositivity as previously published (Kayamba et al., 2013).

3.6.2.9 Testing for Human Immunodeficiency virus (HIV)

Serum samples were tested for the presence of HIV antibodies using Uni-Gold™ rapid diagnostic kits (Trinity Biotech, Wicklow, Ireland).

3.6.3 Gastric pH testing and determination of blood in gastric juice

Procedures included in this section were used to achieve objective three.

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3.6.3.1 Gastric pH testing

Using commercially available test pH Test Strips, (Sigma Chemical Company St Louis, USA) gastric pH was tested on the aspirated juice. The remaining aliquot of gastric juice was used to test for the presence of blood.

3.6.3.2 Testing for blood in gastric juice

Urinalysis reagent strips (ACON laboratories San Diego, USA) were used to determine the presence of blood in gastric juice. These strips test for the qualitative and semi-quantitative of analytes such as blood in urine with the ability to detect free haemoglobin as low as 0.018-0.06 mg/dL or 5-10 erythrocytes per µL. The test for blood is based on peroxidase-like activity of haemoglobin resulting in colour changes ranging from orange to green to dark blue, which is read manually.

Depending on the colour change, the presence of blood was recorded on an ordinal scale as 1, 2 or 3 plus (+). Samples with 2+ or 3+ were considered to have a high intensity of blood. As gastric juice has much lower pH than urine, preliminary

experiments were conducted to determine the influence of pH on detection of blood using these strips.

Table 3.1: Test results for urine strips tested at various pH levels

pH	Colour change without blood	Colour change with blood
0	Yes	Yes
1	Yes	Yes
2	Yes	Yes
3	No	Yes
4	No	Yes
5	No	Yes
6	No	Yes
7	No	Yes
8	No	Yes
9	No	Yes
10	No	Yes
11	No	Yes
12	Yes	Yes
13	Yes	Yes
14	Yes	Yes

*Used in these experiments were hydrochloric acid (HCl) and sodium hydroxide (NaOH) to make solutions of various pH levels and these were tested with or with blood added

Hydrochloric acid and sodium hydroxide was used to prepare solutions with a pH of 0 to 14. Colour change of the urine strips was then checked at each pH level, with and without blood added (Table 3.1).

Solutions with pH less than 3 or greater than 11 showed a colour change in the absence of blood. In subsequent experiments, therefore samples with pH less than 3 were diluted as follows:

1. Gastric juice with pH 2.5; 1:10 dilution resulting in pH 3.5, (n=9)
2. Gastric juice with pH 2; 1:10 dilution resulting in pH 3, (n=20)
3. Gastric juice with pH 1.5: 1:100 dilution resulting in pH 3.5, (n=29)

4. Gastric juice with pH 1: 1:100 dilution resulting in pH 3, (n=7)

Data were analysed by both including and then excluding these diluted samples. All samples were then re-analysed at 1:10 and 1:100 dilutions.

3.7 Data analysis

Collected data were entered into an excel spread sheet and later exported to STATA version 15 (College Station, TX, USA). For preparation of graphs, GraphPad prism version 7 (GraphPad Software, San Diego, California, USA) was also used. All continuous variables were first checked for normality using the Shapiro-Wilk test. Means and standard deviations were used to summarise normally distributed continuous variables while medians and interquartile ranges were used for skewed variables. Two-way analyses were employed to look for associations between outcomes and the exposures of interest considering potential confounders. The Fisher's exact test was used for small numbers less than five in at least one cell, and the Chi square was used for larger numbers. Odds ratios were therefore computed with 95% confidence intervals. Non-parametric trend tests were used to assess odds ratios for ordered outcomes. The Mann-Whitney U (for paired variables) and Kruskal-Wallis tests (for more than two variables) were used to determine associations between various outcomes continuous variables. Where applicable, Dunn's correction for multiple comparisons was employed. To test for correlations, the Spearman's rank correlation coefficient was used, as data being tested for correlations were all non-parametric**.** In all cases, a two-sided *P* value less than 0.05 was considered statistically significant. In addition, stepwise unconditional logistic regression employed to access the relative contributions of different exposure variables. To evaluate the utility of blood in gastric juice for mucosal lesion detection, sensitivity, specificity and area under the receiver operating characteristic curve were calculated.

3.8 Ethical considerations

3.8.1 Consent

The study participants all gave informed consent to participate in this study and they could withdraw from the study at any given time without any compromise to the level of care. The participant's information was treated with the highest confidentiality and information was obtained solely for the purposes of the study. All the questionnaires were kept under lock and key, and only the principal investigator had full access.

3.8.2 Benefits and potential risks to the patients

Study participants were enrolled from among those already referred for OGD evaluation. Those participating in the study had the additional benefit of being screened for gastric premalignant lesions from the biopsies obtained. All study patients were treated in accordance with the best available standard of care. No adverse events resulting from participation in this study were recorded. However, potential risks were considered at all times. When taking biopsies, there was a minor risk of bleeding or infection at the affected site. However, this risk was very small and estimated to occur in about 1:10000. There was no indication for using local anesthetic, as mucosal biopsies were completely painless. Care was taken by prior explanation to the patients and warning them before the actual prick. The possibility of introducing infection at the puncture site was curtailed by thoroughly cleaning with methylated spirit.

3.8.3 Ethical approval

The University of Zambia Biomedical Research Ethics Committee (reference number 005-03-16) and the National Health Research Authority granted ethical approval for this study. The study was conducted in accordance with the guidelines for good clinical research.

CHAPTER 4: RESULTS

This chapter is divided into three sections. The first section describes clinical and histopathological characteristics of gastric cancer and premalignant lesions. The second section focuses on the risk factors while the third section reports results for early detection of gastric cancer.

4.1 Socio-demographic, clinical and histopathological characteristics of gastric cancer and its premalignant lesions (objective one)

4.1.1 An outline of patient enrolment into the study

Included for analysis in this study were 388 patients, 92 (24%) of whom had gastric tumours seen during endoscopy (Figure 4.1). Of those with gastric tumours, eight were excluded due to lack of confirmatory histopathology reports. Twelve had other types of gastric cancer, including eight with squamous cell or unclassified carcinomas, two with gastric stromal tumours, one with non-Hodgkin's lymphoma and one with a "haematolymphoid tumour". Of 72 confirmed gastric cancer cases, there were 68 patients with adenocarcinoma (GA) and four patients with high-grade dysplasia (carcinoma *in situ*), which for clinical purposes and the purposes of this study are classified hereafter as gastric CANCER CASES. Among those without gastric tumours, 35 had gastric premalignant lesions (GP): eight with chronic atrophic gastritis (CAG) and 27 with gastric intestinal metaplasia (GIM). These are hereafter referred to as PREMALIGNANT CASES. Patients without histological evidence of either premalignant lesions or cancer were used as CONTROLS (n=244).

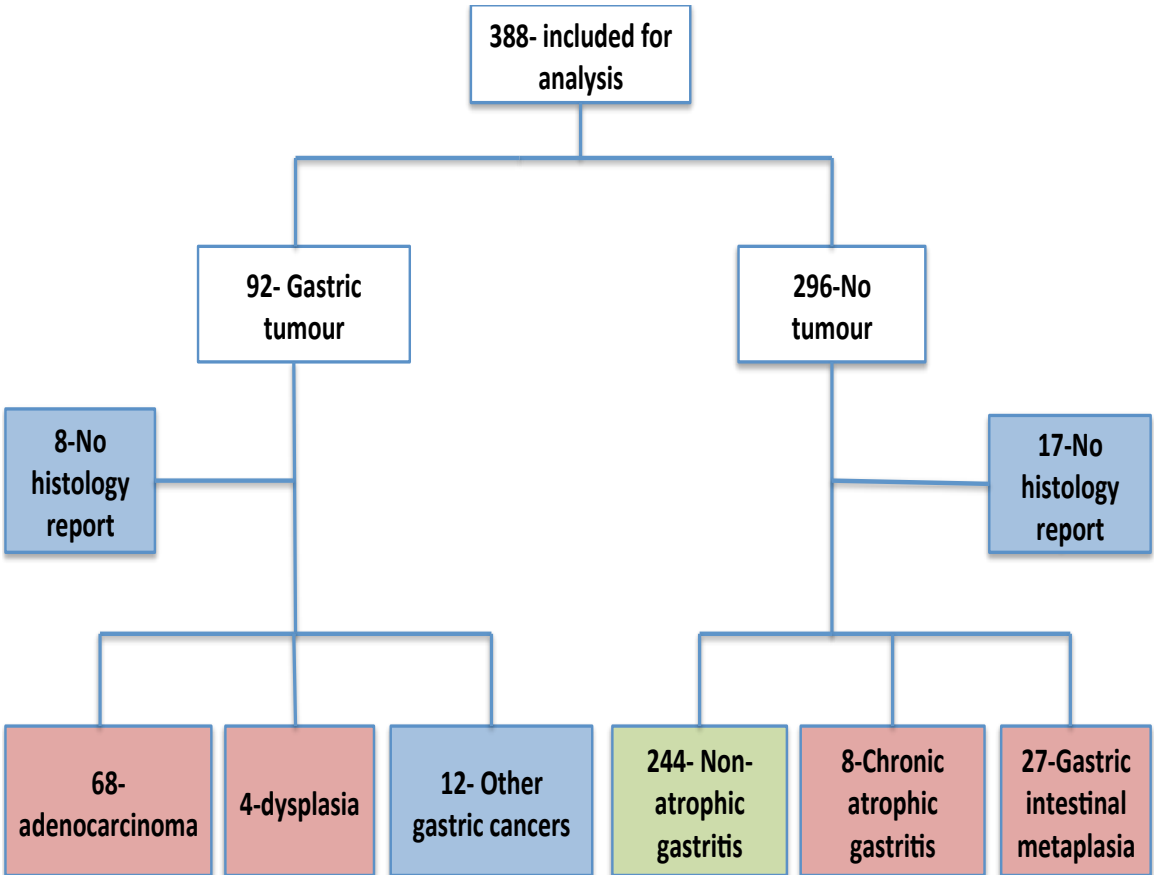

Figure 4.1: Flow chart showing the enrolment of patients

*Shaded in pink were the cases, green were the controls and blue were patients left out of the final analysis.

4.1.2 Basic characteristics of enrolled patients

The map showing each patient's permanent residence was representative of the Zambian population distribution, with higher densities in the centre of the country along the line of rail (Figure 4.2). Of 92 patients with gastric tumours, 46 (50%), and of 296 without tumours, 204 (69%) were Lusaka residents. Lusaka is the capital and most populated city in Zambia.

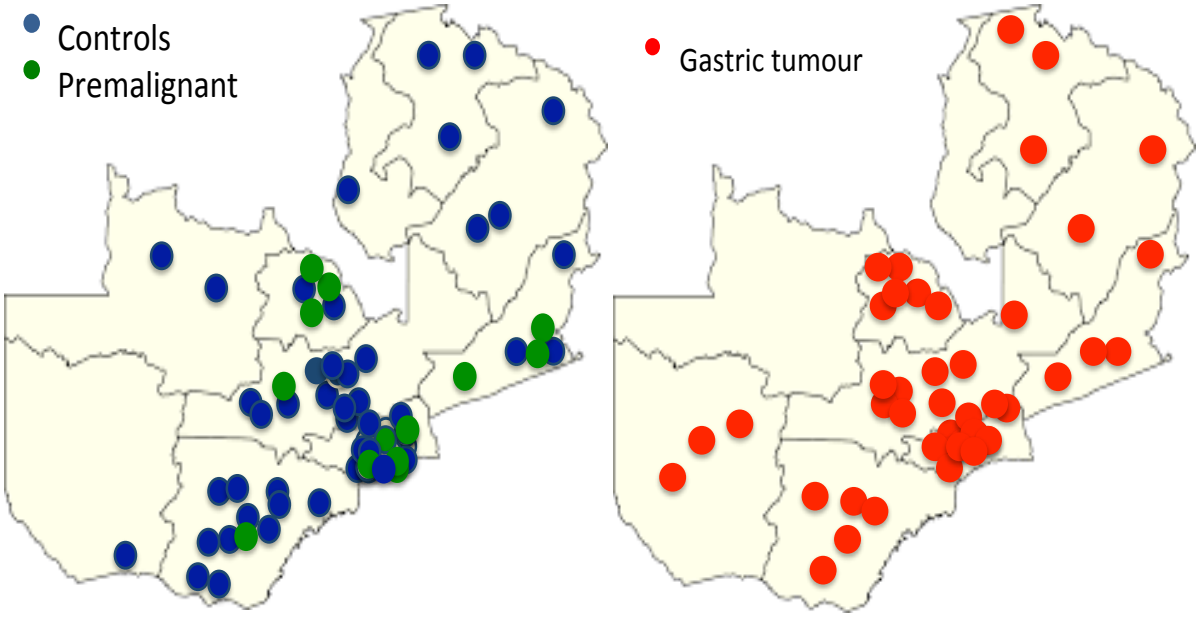

Figure 4.2: Permanent residence of enrolled patients stratified by histological diagnosis

*Each dot represents a single patient and some dots are superimposed

Patients with gastric tumours were significantly older than those without. The median age was 58 years (IQR 60-70 years) for patients with gastric tumours and 48 years (IQR 40-63 years) for those without (p=0.0002; Table 4.1). The age difference was therefore adjusted for in subsequent analyses. Thirty-eight percent of the gastric tumour patients were from rural areas compared to 20% of the controls (p=0.0002; Table 4.1). The median body mass index (BMI) for patients with gastric tumours was 18 kg/m^2, lower than that of patients without gastric tumours (median 25 kg/m^2). A history of cigarette smoking or alcohol consumption was equally frequent in both groups (Table 4.1)

Table 4.1: Clinical and demographic characteristics of patients with or without gastric tumours seen during endoscopy

	Gastric tumour n=92	No tumour n=296	OR (95% CI)	*P* value
Female	49 (53%)	158 (53%)	1.0 (0.6-1.6)	0.98
Age				
Less than 30 years	1 (1%)	11 (4%)		
30-44 years	17 (18%)	103 (35%)		
45-59 years	32 (35%)	96 (32%)		
60 years and above	42 (46%)	86 (29%)	-	**0.0002**
Resident in rural area	35 (38%)	58 (20%)	2.6 (1.5-4.4)	**0.0002**
Body mass index, median (IQR)	18 (16-22)	25 (21-28)	-	**0.0001**
Married	53 (58%)	181 (61%)	0.7 (0.5-1.4)	0.55
Educational level attained				
None	17 (19%)	23 (8%)		
Primary	33 (36%)	72 (24%)	-	
Secondary	27 (29%)	119 (40%)		
Tertiary	15 (16%)	82 (28%)		**0.0001**
No employment	35 (38%)	78 (26%)	1.7 (1.0-2.9)	**0.03**
Family history of gastric cancer	2 (2%)	7 (2%)	0.9 (0.1-4.9)	1.00
History of smoking				
Current	7 (8%)	17 (6%)	1.5 (0.5-4.0)	0.44
Ever	13 (14%)	29 (10%)	1.6 (0.7-3.4)	0.19

*Significance testing was performed using Fisher's exact, Chi square or Kruskal-Wallis tests.

4.1.3 Presenting symptoms

The major presenting symptoms were compared between those with or without gastric tumours. 48/92 (52%) of patients with tumours presented with abdominal pain, while 14/92 (15%) and 12/92 (13%) presented with vomiting and blood loss (either haematemesis or melaena), respectively (Figure 4.3). These patients did not have abdominal pain. Symptoms associated with having a gastric tumour were vomiting [OR 3.2; 95% CI 1.6-6.6, p=0.0005] or dysphagia [OR 9.9; 95% CI 2.8-43, p<0.0001]. In comparison with the controls, patients with abdominal pain were less likely to have a gastric tumour [OR 0.5; 95% CI 0.3-0.9, p=0.01].

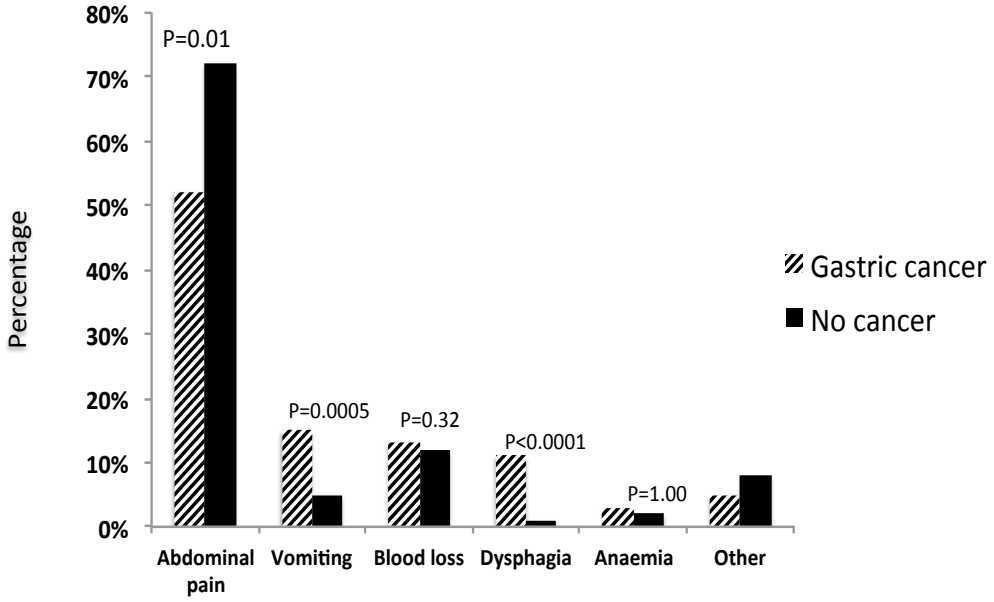

Figure 4.3: Presenting symptoms and major reasons for oesophagogastroduodenoscopy examination compared between patients with gastric tumours and those without

4.1.4 Gastric adenocarcinoma

4.1.4.1 Basic characteristics of patients with gastric adenocarcinoma

Table 4.1 shows a comparison of basic characteristics of patients with gastric adenocarcinoma (GA) and controls. Patients with GA were significantly younger than the controls and were more wasted with a median BMI of 18 kg/m^2. In addition, they were likely to reside in rural areas and have lower educational attainment. The proportion of patients taking alcohol or cigarette smoking was similar in the two groups (Table 4.2).

Table 4.2: Basic characteristics of patients with or without gastric adenocarcinoma

	GA n=72	Controls n=244	OR; 95% CI	*p value*
Female	39 (54%)	128 (52%)	1.1 (0.5-1.9)	0.89
Age				
Less than 30 years	1 (2%)	10 (4%)		
30-44 years	10 (14%)	89 (37%)		
45-59 years	24 (33%)	86 (35%)		
60 years and above	37 (51%)	59 (24%)	-	**0.0001**
Resident in rural area	28 (39%)	45 (18%)	2.9 (1.5-5.3)	**0.0004**
Body mass index, median (IQR)	18 (16-21)	25 (21-28)	-	**0.0001**
Married	40 (56%)	151 (62%)	0.8 (0.4-1.4)	0.41
Educational level attained				
None	13 (18%)	16 (7%)		
Primary	27 (37%)	57 (23%)		
Secondary	20 (28%)	98 (40%)		
Tertiary	12 (17%)	74 (30%)	-	**0.0001**
No employment	26 (36%)	58 (24%)	1.8 (1.0-3.3)	**0.048**
Family history of gastric cancer	1 (1%)	6 (2%)	0.6 (0.01-4.7)	1.00
History of smoking				
Current	7 (10%)	12 (5%)	2.3 (0.7-6.7)	0.21
Ever	12 (17%)	23 (9%)	2.0 (0.9-4.6)	0.08
History of alcohol intake	12 (17%)	58 (24%)	0.6 (0.3-1.3)	0.21

*Significance testing was performed using Fisher's exact, Chi square or Kruskal-Wallis tests.

4.1.4.2 Anatomical location of gastric adenocarcinoma

The location of the GA was determined during endoscopy. Sixty-five percent (47/72) of the tumours were located in either the body or the antrum (referred to as distal gastric tumours).

Thirty-five percent (25/72) were proximal tumours located either in the fundus or gastroesophageal (GE) junction (Figure 4.4).

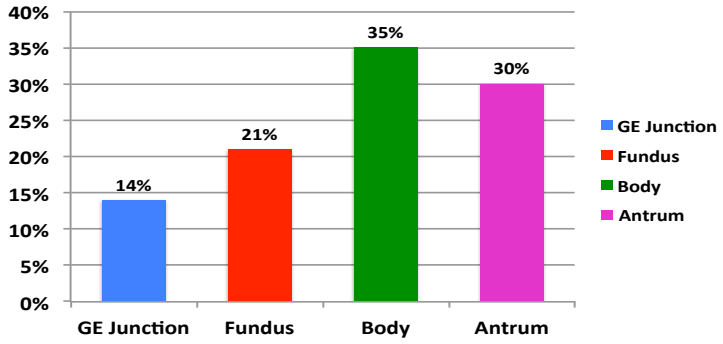

Figure 4.4: Anatomical location of gastric adenocarcinoma As seen during oesophagogastroduodenoscopy

4.1.4.3 Histological and molecular classification of gastric adenocarcinoma

4.1.4.3.1 Lauren classification

Using the Lauren classification, close to two-thirds of the tumours were of the intestinal type (Figure 4.5). None of the diffuse type GA was located at the gastro-oesophageal junction. Only 4/11 (27%) of the diffuse type cancers were early onset. Grouping GA by the Lauren classification was similar regardless of tumour location (p=0.32) or age (p=0.38).

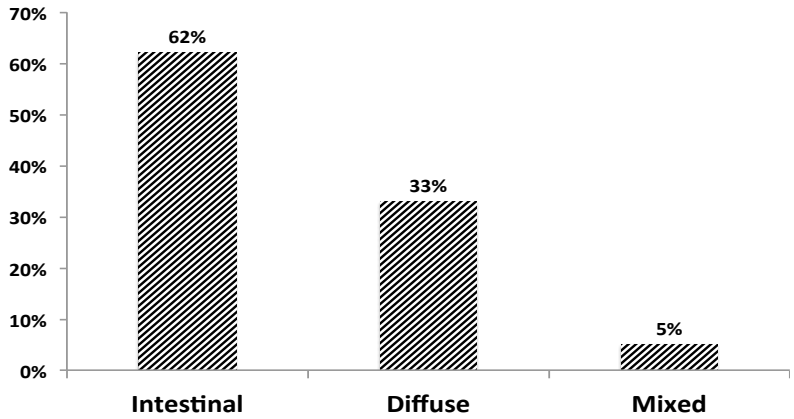

Figure 4.5: Lauren classification of gastric adenocarcinoma

4.1.4.3.2 Epstein-Barr virus associated gastric adenocarcinoma

A total of 57 GA paraffin blocks were available for Epstein-Barr encoding region (EBER) *in situ* hybridisation. Of these, 13 either did not have enough tissue for

analysis or had no visible tumour tissue in the examined sections. Therefore, complete results were available for 44 samples. Of these, 5(11%) were EBV positive (Figure 4.6). The presence of EBV was similar regardless of the type of GA, its anatomical location, patient age or sex (p=0.31, p=1.00, p=0.54 and p=0.22 respectively).

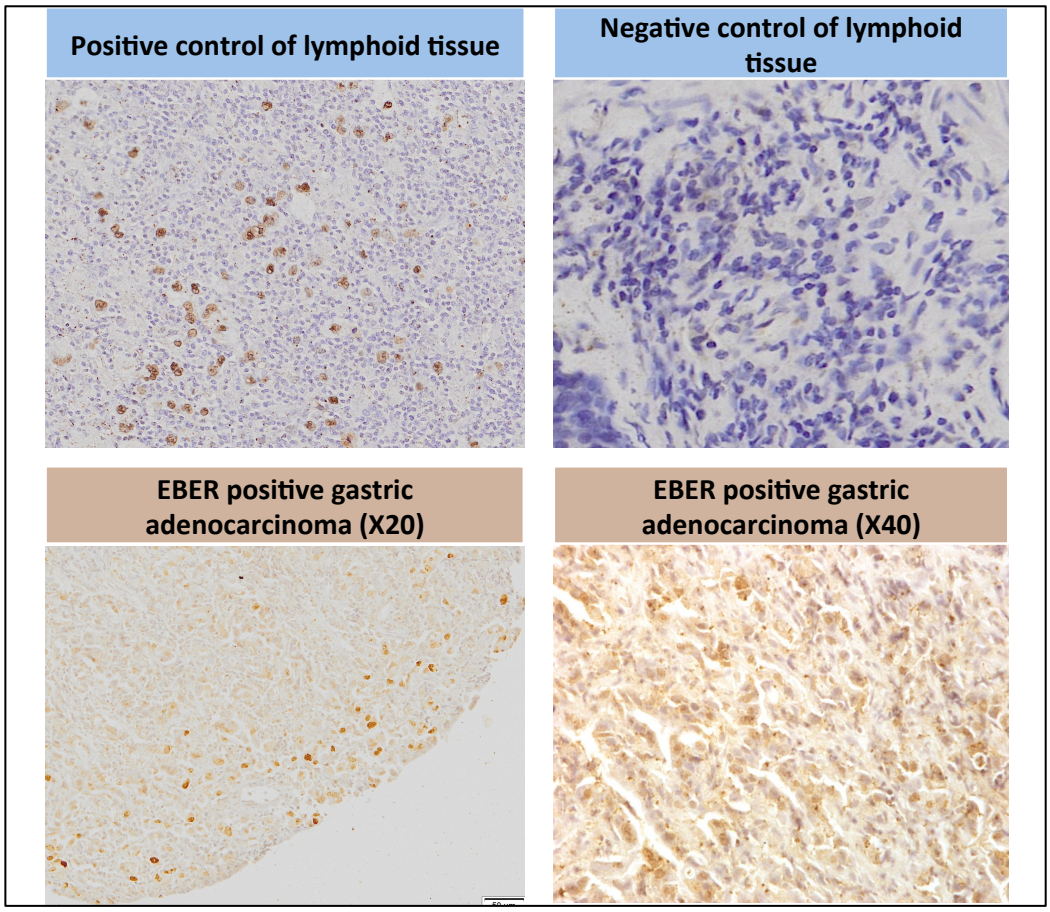

Figure 4.6: Epstein-Barr encoding region (EBER) *in situ* hybridisation images

4.1.4.3.3 Microsatellite unstable gastric adenocarcinoma

The occurrence of microsatellite unstable GA was analysed using immunofluorescence staining for the loss of MLH1 (Figure 4.7). Fluorescent nuclear staining was seen on slides positive for MLH1. Of the GA biopsies successfully stained, 17/46 (37%) showed positive nuclear staining, and 29/46 (63%) showed

evidence of microsatellite instability. Patients above the age of 45 years were more likely to have microsatellite unstable tumours [OR 15; 95% CI 1.5-72, *p*=0.007].

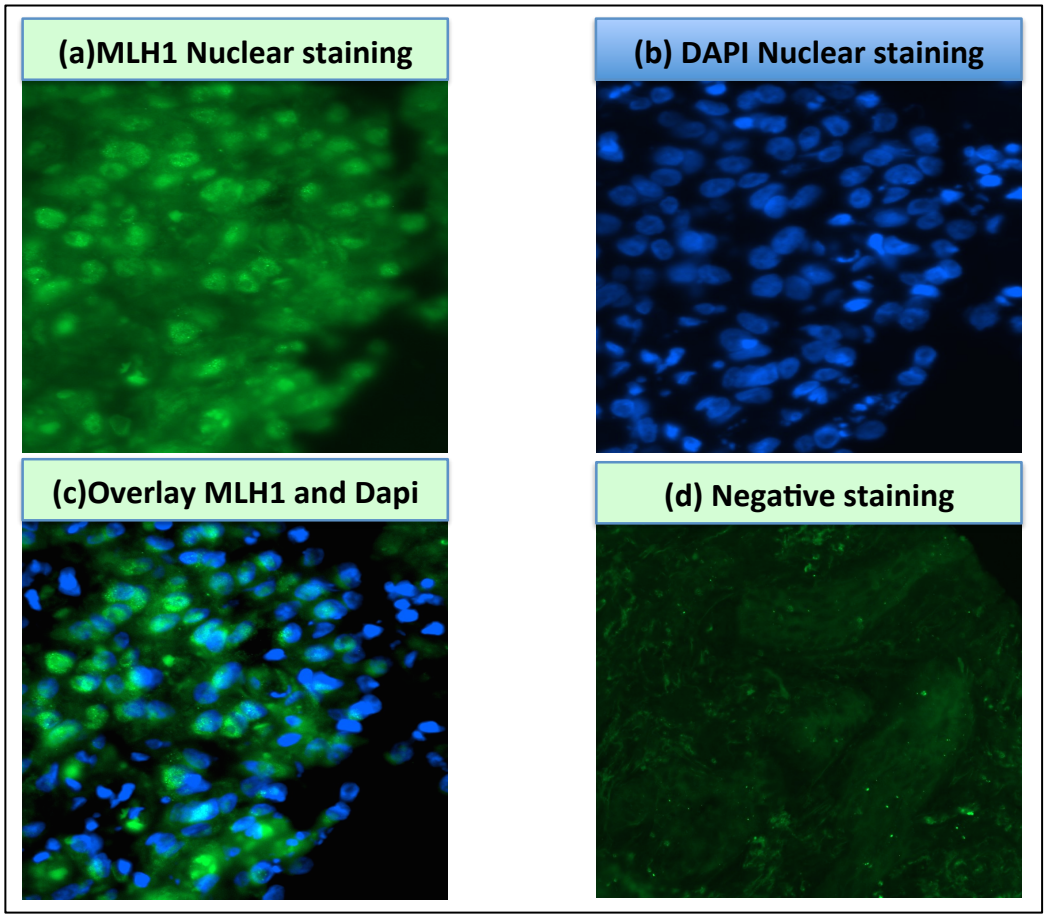

Figure 4.7: Immunofluorescence staining for MLH1; (a) positive staining for MLH1, (b) 4,6-diamidino-2-phenylindole (DAPI) nuclear staining, (c) Overlay of (a) and (b), (d) negative control.

4.1.5 Gastric premalignant lesions

4.1.5.1 Basic characteristics of patients with gastric premalignant lesions

GP lesions were identified in 35/296 (12%) of patients without gastric tumours. These patients were compared to the same set of controls used for GA above, and only age was significantly different (Table 4.3). Unlike for GA, educational attainment for GP patients was not significantly different from the controls. But similar to GA, alcohol consumption or cigarrate smoking did not influence the occurrence of GP.

Table 4.3: Basic characteristics of patients with or without gastric premalignant lesions

	Premalignant lesions n=35	Controls n=244	OR; 95% CI	P
Female	17 (49%)	128 (52%)	0.9 (0.4-1.9)	0.72
Age				
Less than 30 years	0 (0%)	10 (4%)		
30-44 years	10 (29%)	89 (37%)		
45-59 years	6 (17%)	86 (35%)		
60 years and above	19 (54%)	59 (24%)	-	**0.005**
Resident in rural area	8 (23%)	45 (18%)	1.3 (0.5-3.2)	0.50
Body mass index, median (IQR)	26 (23-28)	25 (21-28)	-	0.37
Married	24 (71%)	151 (62%)	1.5 (0.6-3.6)	0.45
Educational level attained				
None	1 (3%)	16 (7%)		
Primary	13 (37%)	57 (23%)		
Secondary	15 (43%)	98 (40%)		
Tertiary	6 (17%)	74 (30%)	-	0.13
No employment	15 (43%)	58 (24%)	2.4 (1.1-5.3)	**0.02**
History of smoking				
Current	3 (9%)	6 (2%)	2.1 (0.3-8.3)	0.39
Ever	3 (9%)	12 (5%)	1.0 (0.2-3.4)	1.00
History of alcohol intake	6 (17%)	58 (24%)	0.7 (0.2-1.8)	0.51

*Significance testing was performed using Fisher's exact or Kruskal-Wallis

4.1.5.2 Histological staging of gastric premalignant lesions

The Operative Link for Gastritis Assessment (OLGA) and Operative Link on Gastritis/Intestinal-Metaplasia Assessment (OLGIM) staging systems were used to stratify GP patients for potential gastric cancer risk. OLGA staging reports were available for 33 patients with GP lesions: 12 (36%) had stage 1, 11 (33%) had stage 2, 9 (27%) had stage 3, and 1 (3%) stage 4 gastric atrophy. OLGIM staging for GIM in 33 patients revealed 11 (33%) in stage 0, 13 (39%) in stage 1, 8 (24%) in stage 2 and 1 (3%) in stage 3.

4.2 Risk factors for gastric adenocarcinoma and its premalignant lesions (objective two)

4.2.1 Gastric adenocarcinoma

4.2.1.1 Socio-economic status

An interviewer-administered questionnaire was used to collect data on the socio-economic status of patients with GA cases and controls. Figure 4.8 shows the socio-economic indicators that were evaluated with the odds ratios and confidence intervals. GA patients were less likely to have good housing, water supply or a kitchen (all *p*-values less than 0.05; Figure 4.8). Overall, GA patient were more likely to be of low socio-economic status [OR 4.2; 95% CI 1.9-9.1, *p*=0.0002].

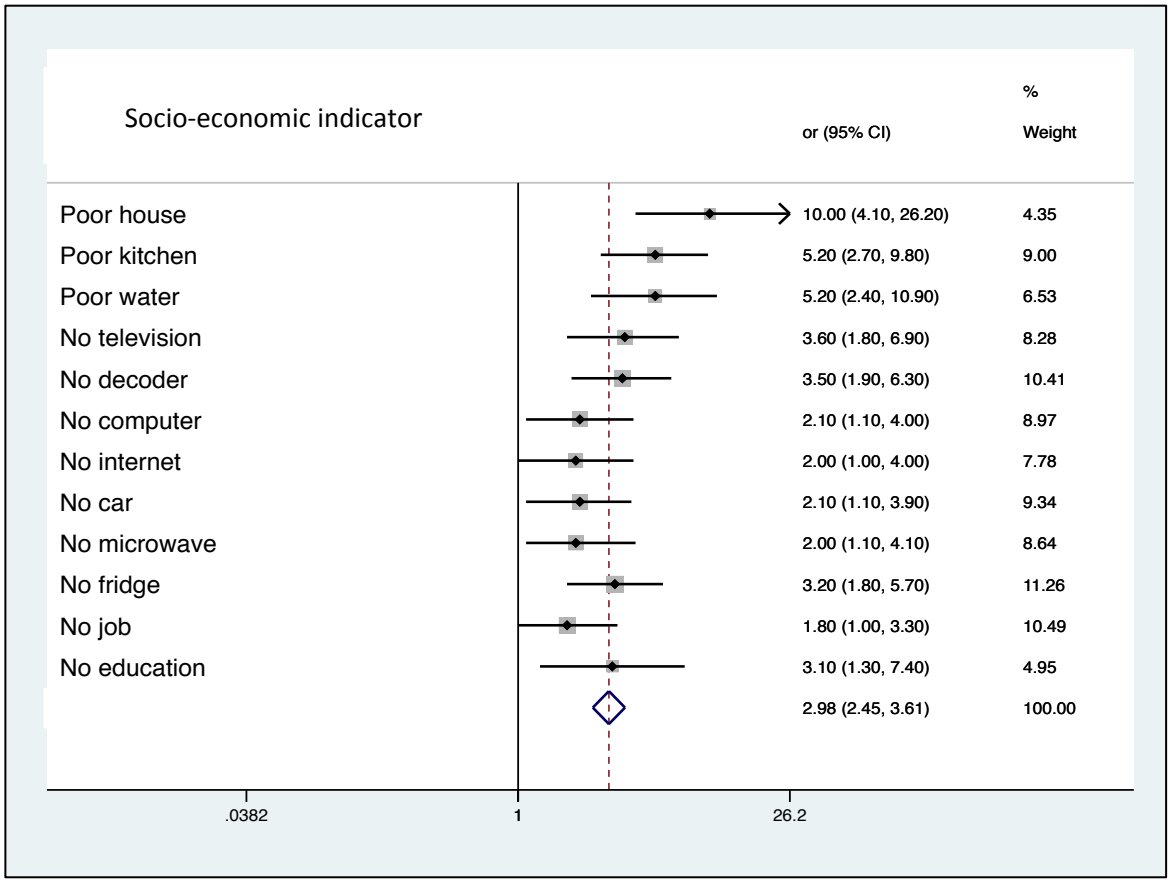

Figure 4.8: Socio-economic indicators of gastric adenocarcinoma patients compared to controls

*The solid vertical line is a null effect line, while the dotted one is showing the summary effect.

**Significance testing was performed using Fisher's exact test.

Applying an unconditional logistic regression adjusted for age, sex and residence, GA patients were less likely to have good housing or a kitchen [OR 5.3; 95% CI 2.1-13.5, $p<0.0001$] and [OR 3.4; 95% CI 1.5-7.4, $p=0.003$] respectively.

4.2.1.2 Biomass smoke exposure and oxidative stress to DNA

Patients were asked about fuel used for cooking in their homes. Overall, 110/316 (35%) of the patients reported that they were completely reliant on biomass fuel for cooking while another 107/316 (34%) used it occasionally as they had access to electric stoves. Thirty-one percent (99/316) did not use biomass fuels in their homes at all. A comparison of the GA cases and controls showed that the cases were more likely to be reliant on biomass fuel than controls ($p=0.001$; Figure 4.9). Adjusting for age, sex, rural residence and socio-economic status in an unconditional logistic regression, GA patients were more exposed to biomass smoke than those without GA [OR 2.3; 95% CI 1.1-4.5, $p=0.02$]. On analysis by histological type of GA, those with intestinal type were more likely to be exposed to biomass smoke [OR 3.6; 95% CI 1.5-9.1, $p=0.003$] but not those with diffuse type [OR 0.9; 95% CI 0.2-3.1, $p=1.00$]. None of the GA patients not using biomass fuels in their homes had microsatellite unstable tumours. On the other hand, 13/26 (50%) of those using biomass fuels all the time had microsatellite unstable tumours, and this difference was statistically significant ($p=0.003$).

Urinary levels of 1-hydroxypyrene (1-OHP) were compared between patients with GA and controls. For this analysis, patients who gave a history of current smoking were excluded (n=8). The median level in the GA group was 0.2 µg/g creatinine (IQR 0.1-0.3 µg/g creatinine), while in the control group it was 0.2 µg/g creatinine (IQR 0.1-0.4 µg/g creatinine; $p=0.18$). The median levels of 1-OHP were similar between patients exposed to biomass smoke and those not regularly exposed ($p=0.07$).

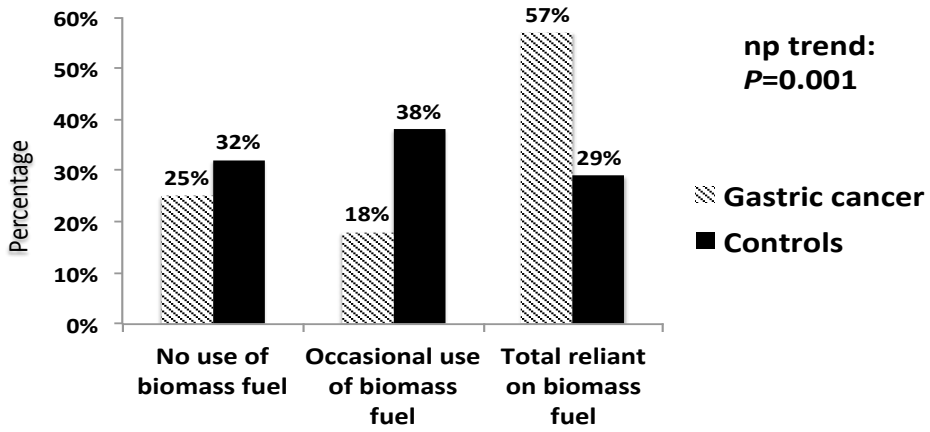

Figure 4.9: Association between gastric adenocarcinoma and reported biomass smoke exposure

*Significance tested using a non-parametric test across ordered groups.

Urinary concentrations of 8-Hydroxydeoxyguanosine (8-OHdG) were higher in GA patients (median 7.1 ng/mg creatinine, IQR 3.2-20.8), than controls (median 4.0 ng/mg creatinine, IQR 2.0-10; *p*=0.01). To explore a probable mechanism of biomass smoke on gastric carcinogenesis, levels of 8-OHdG were compared between patients with minimal, occasional and frequent exposure to biomass smoke.

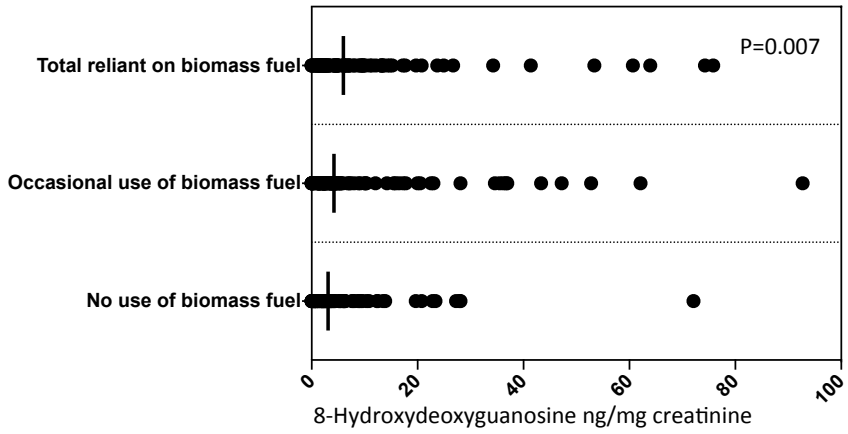

Figure 4.10: Urine levels of 8-Hydroxydeoxyguanosine ng/mg creatinine compared between patients with regular, occasional and infrequent exposure to biomass smoke

*Significance tested using a non-parametric test across ordered groups

The median 8-OHdG was highest in those regularly exposed to biomass smoke (median 6.3 ng/mg creatinine, IQR 2.6-14.8), and least in those with minimal exposure to biomass smoke, (median 3.6 ng/mg creatinine, IQR 1.3-10). Those with

occasional exposure had a median 8-OHdG of 4.3 ng/mg creatinine (IQR 2.3-15.8 ng/mg creatinine; Figure 4.10).

4.2.1.3 Dietary intake

When analysing data from the food frequency questionnaire, consumption of any food type more than once a week was considered regular. Table 4.4 shows a comparison of daily and regular consumption of various food types and groups for GA patients and controls.

Table 4.4: Dietary intake for patients with gastric adenocarcinoma and controls

	Regular consumption (at least 2-4 times a week)				Daily consumption			
Food type	**GA n(%)**	**Controls n(%)**	**Univariate OR (95% CI)**	**P value**	**GA n(%)**	**Controls n(%)**	**Univariate OR (95% CI)**	**P value**
Chicken								
Chicken	42 (60)	175 (71)	0.6 (0.3-1.1)	0.08	2 (3)	8 (3)	11 (0.1-4.6)	1.00
Unprocessed Meat								
Beef	27 (39)	128 (52)	0.6 (0.3-1.0)	0.04	4 (6)	6 (2)	2.5 (0.5-10)	0.23
Pork	13 (18)	39 (16)	1.2 (0.5-2.4)	0.72	1 (1)	0 (0)	-	0.22
Goat	4 (6)	25 (10)	0.5 (0.1-1.6)	0.35	1 (1)	1 (0.4)	3.6 (0.1-284)	0.39
Game	2 (3)	3 (1)	2.4 (0.2-20)	0.31	0 (0)	0 (0)	-	-
Processed red meat								
Polony	9 (13)	29 (12)	1.1 (0.4-1.6)	0.84	0 (0)	2 (0.1)	-	1.00
Hungarian sausage	20 (29)	78 (32)	0.9 (0.4-1.6)	0.66	3 (4)	1 (0.1)	11 (0.9-581)	**0.03**
Bacon	3 (4)	9 (4)	1.2 (0.2-4.9)	0.73	2 (3)	0 (0)	-	0.05
Ham	2 (3)	8 (3)	0.9 (0.1-4.5)	1.00	1 (1)	0 (0)	-	0.22
Canned meat	0 (0)	2 (0.8)	-	1.00	0 (0)	0 (0)	-	-
Other sausage	23 (33)	76 (31)	1.1 (0.6-2.0)	0.77	3 (4)	0 (0)	**-**	**0.01**
Salami	1 (1)	3 (1)	1.2 (0.02-15)	1.00	0 (0)	0 (0)	-	-
Fish								
Bream	34 (49)	152 (62)	0.6 (0.3-1.0)	0.05	2 (3)	3 (1)	2.4 (0.2-21)	0.31
Kapenta	33 (47)	122 (50)	0.9 (0.5-1.6)	0.69	2 (3)	5 (2)	1.4 (0.1-9.0)	0.65
Buka buka	11 (16)	43 (18)	0.9 (0.4-1.9)	0.86	0 (0)	0 (0)	-	-
Tiger fish	3 (4)	11 (5)	0.9 (0.2-3.7)	1.00	0 (0)	0 (0)	-	-
Vegetables								
Green leafy	63 (91)	239 (98)	0.2 (0.1-1.9)	**0.02**	52 (75)	227 (93)	0.2 (0.1-0.5)	**0.0001**
Egg plants	20 (29)	118 (48)	0.4 (0.2-0.8)	**0.004**	3 (4)	21 (9)	0.5 (0.1-1.7)	0.31
Tomatoes	66 (96)	236 (97)	0.7 (0.2-4.5)	0.71	62 (90)	232 (95)	0.5 (0.2-1.4)	0.15
Onions	62 (90)	232 (95)	0.5 (0.2-1.4)	0.15	60 (88)	225 (94)	0.5 (0.2-1.4)	0.11
Fruits (available throughout the year)]								
Bananas	30 (43)	132 (54)	0.6 (04-1.1)	0.15	10 (14)	28 (11)	1.3 (0.5-2.9)	0.54
Oranges	27 (39)	105 (43)	0.8 (0.5-1.5)	0.58	4 (6)	22 (10)	0.6 (0.1-1.9)	0.47
Lemons	18 (26)	73 (30)	0.8 (0.4-1.5)	0.55	4 (6)	14 (6)	1.0 (0.2-3.3)	1.00
Apples	23 (33)	113 (46)	0.6 (0.3-1.0)	0.06	4 (6)	14 (6)	1.0 (0.2-3.3)	1.00
Pineapples	8 (11)	36 (15)	0.7 (0.3-1.7)	0.56	4 (6)	14 (6)	1.0 (0.2-3.3)	1.00
Strawberries	2 (3)	12 (5)	0.8 (0.1-2.7)	0.74	0 (0)	0 (0)	-	-
Fruit combined	38 (54)	167 (68)	0.5 (0.3-0.98)	**0.03**	14 (20)	41 (17)	1.2 (0.6-2.5)	0.59
Seasonal fruit**	58 (81)	182 (75)	1.4 (0.7-2.9)	0.35	-	-	-	-

*Significance testing was performed using Fisher's exact test or Chi square for proportions.

**Seasonal fruit included mangos, papayas, baobabs, watermelons and mulberries.

Also shown in the table are results for the Univariate analysis. Patients who regularly consumed green vegetables, eggplants or fruit were less likely to have GA (Table 4.4). Overall, 279/314 (89%) of the patient admitted to consuming green vegetables on a daily basis. Only 42/314 (18%) were taking at least one type of fruit daily. 30/314 (10%) of all the patients ate meat (red or white) on a daily basis. Using unconditional logistic regression adjusted for age, sex and residence, GA patients were less likely to consume green vegetables daily [OR 0.2; 95% CI 0.1-0.5, *p*=0.0001].

4.2.1.4 Consumption and excretion of sodium

Using an interviewer administered questionnaire, data were collected on salt intake. Only 47/310 (15%) of the patients reported not adding extra salt to their food. Sixty-two percent (191/310) added salt all the time, 28/310 (9%) added it very often while 44/310 (14%) added it infrequently. GA patients did not report adding any more salt than those without cancer (*p*=0.65).

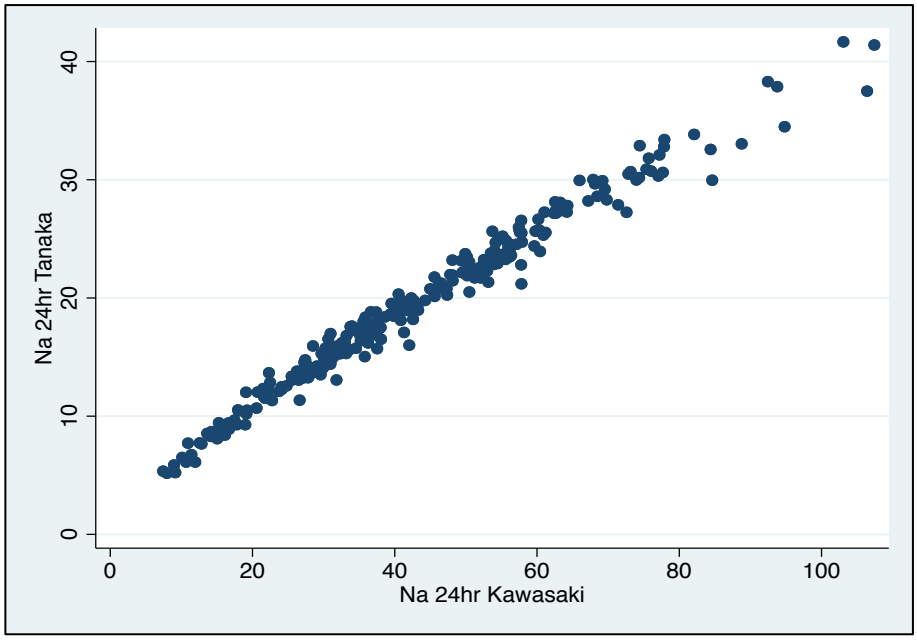

Figure 4.11: Correlation between the Tanaka and Kawasaki methods for estimation of 24-hour sodium excretion

*Significance determined using the Spearman's rank correlation coefficient, as the data were non-parametric

When asked about salt preference, 57/310 (19%) admitted to liking very salty food, 49/310 (16%) salty foods, 171/310 (56%) slightly salty and 30/310 (10%) reported liking their food not salty. Salt preference was not different between the two groups (p=0.43).

Measured spot urine sodium and creatinine levels were available for 262 patients. The Spearman correlation co-efficient between the two methods was 0.99 (Figure 4.11). Estimating the 24-hour sodium intake showed that 99.2% of these patients had high sodium using the Tanaka method and 99.6% using the Kawasaki method. The sodium was considered high if more than the WHO recommended daily intake of 2 g or 5 g for total salt.

The median sodium excretion was 19 g (IQR 14-24 g) with the Tanaka method and 41 g (IQR 30- 56 g) with the Kawasaki method. There was no correlation between estimated sodium excretion and reported salt preference or addition of extra salt to food, (Figures 4.12 (a) and (b)).

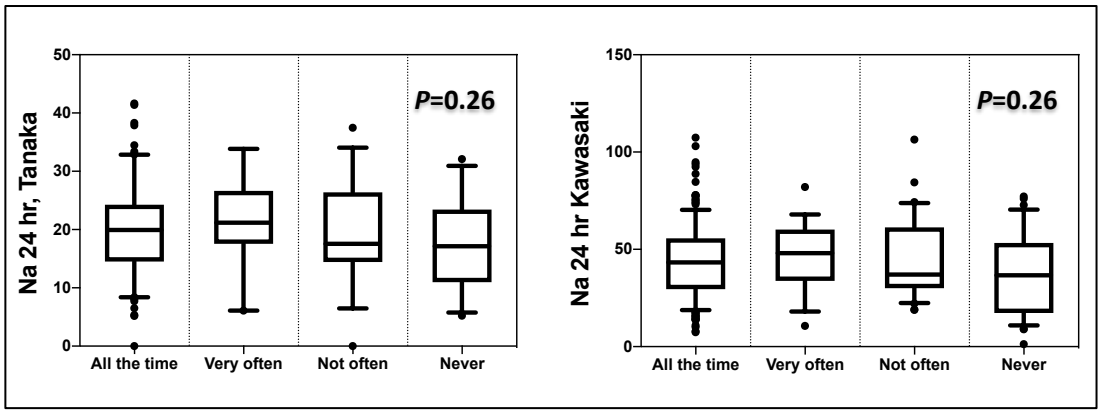

Figure 4.12 (a): Estimated 24 hour urine sodium stratified by reported addition of extra salt to already prepared food

*Significance tested using a non-parametric test across ordered groups. The error bars are showing the 5-95 percentile.

A comparison of sodium excretion between GA cases and controls showed that the cases had significantly less sodium excretion determined by either the Tanaka or the Kawasaki methods.

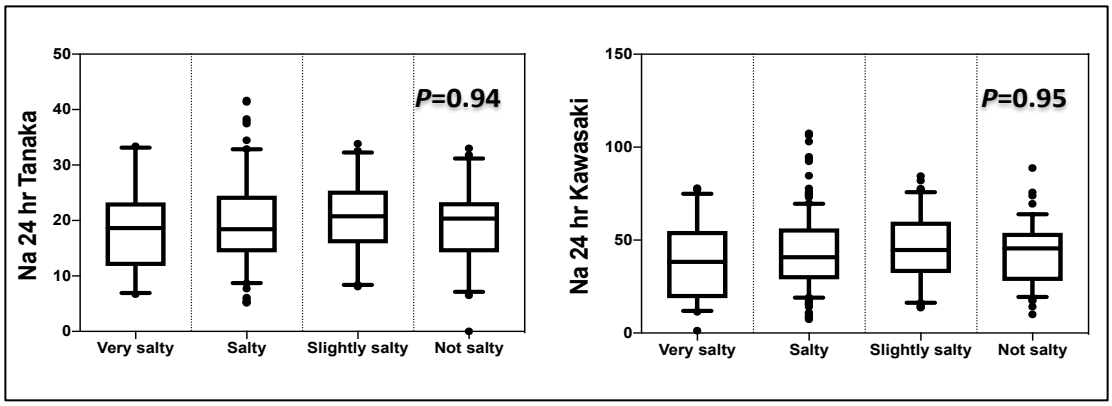

Figure 4.12 (b): Estimated 24 hour urine sodium stratified by salt preference

*Significance tested using a non-parametric test across ordered groups. The error bars are showing the 5-95 percentile.

The median sodium excretion with the Tanaka method was 21 g (IQR 16-25 g) for controls and 12 g (IQR 9-16 g) for GA cases (p=0.0001). Using results for the Kawasaki method, the median for controls was 46 g (IQR 34-58 g), while it was 23 g (IQR 16-34 g) for GA cases (p=0.0001).

4.2.1.5 Mycotoxins

4.2.1.5.1 Urinary aflatoxin M1

Of 313 patients with aflatoxin M1 results, 192 (61%) had detectable toxin in their urine. The median urinary aflatoxin M1 level was 33 (IQR 2-53) ng/ml and after correcting for urine creatinine, the median was 18 (IQR 1.7- 40) ng/mg creatinine. This was not dependent on age (p=0.42) or sex (p=0.46), but was significantly higher in patients living in urban areas (Figure 4.13). Having aflatoxin in urine was not higher in patients without basic household goods (p=0.13), education (p=0.17) or good housing (p=0.11). Patients in the control group had higher levels of corrected urinary aflatoxin M1 than those with GA, median 20; IQR 2-40 ng/mg creatinine and 2; IQR 1-13 ng/mg creatinine respectively (Figure 4.13).

Measured levels of aflatoxin M1 were then compared by month of enrolment. The lowest levels were for months between February and May (Figure 4.14).

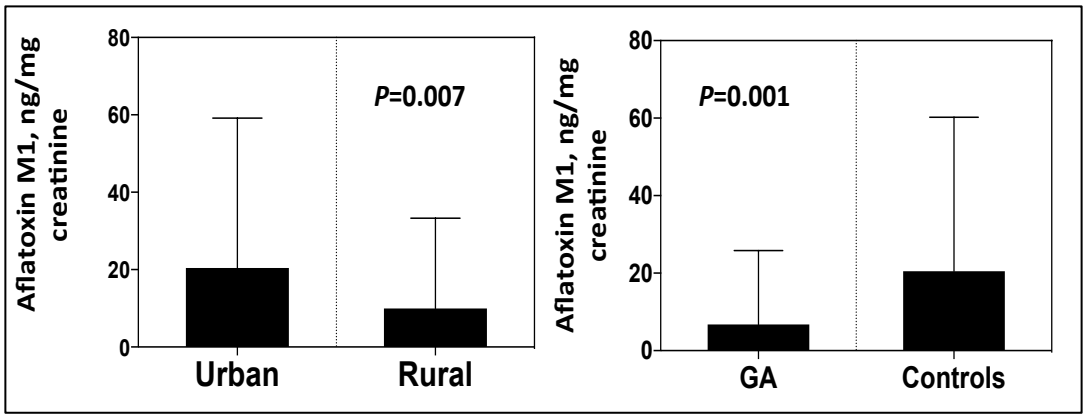

Figure 4.13: Urinary aflatoxin M1 stratified by residence and the presence of gastric adenocarcinoma

*Significance tested using the Mann Whitney test. The error bars are showing the standard deviation.

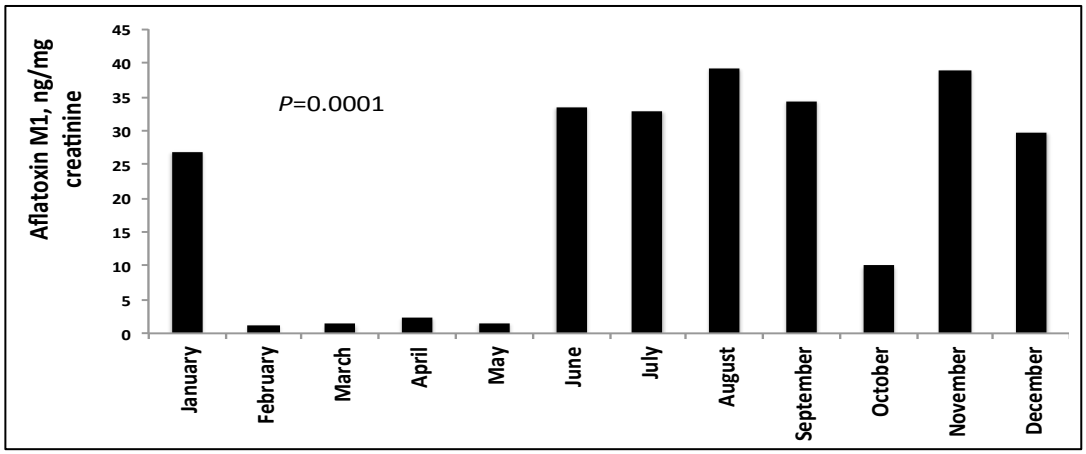

Figure 4.14: Median levels of aflatoxin M1 by month of sample collection

*Significance tested using the Kruskal-Wallis test.

4.2.1.5.2 Serum Ochratoxin A

Of the patients with ochratoxin results, 335/349 (96%) had evidence of ochratoxin A in their blood. The median level was 0.1 ng/ml (IQR 0.2-0.6 ng/ml). There was no significant difference in ochratoxin levels for patients living in rural or urban areas (Figure 4.15). Similarly, ochratoxin levels were not different between GA cases and controls (Figure 4.15). Age, sex and socio-economic class had no influence on ochratoxin levels (p= 0.21, 0.69 and 0.12 respectively).

Levels of ochratoxin A were compared by month of enrolment. The lowest levels were observed in October (Figure 4.16).

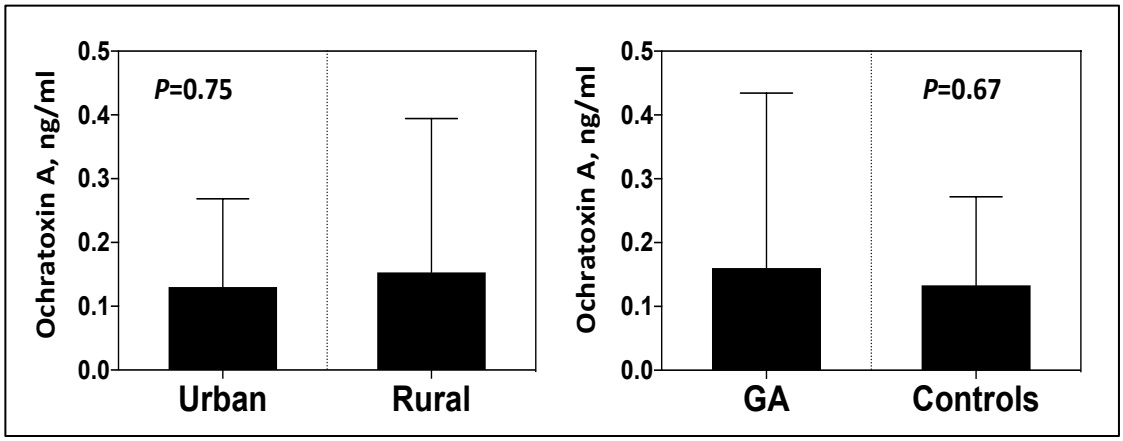

Figure 4.15: Serum ochratoxin a stratified by residence and the presence of gastric adenocarcinoma

*Significance tested using the Mann Whitney test. The error bars are showing the standard deviation.

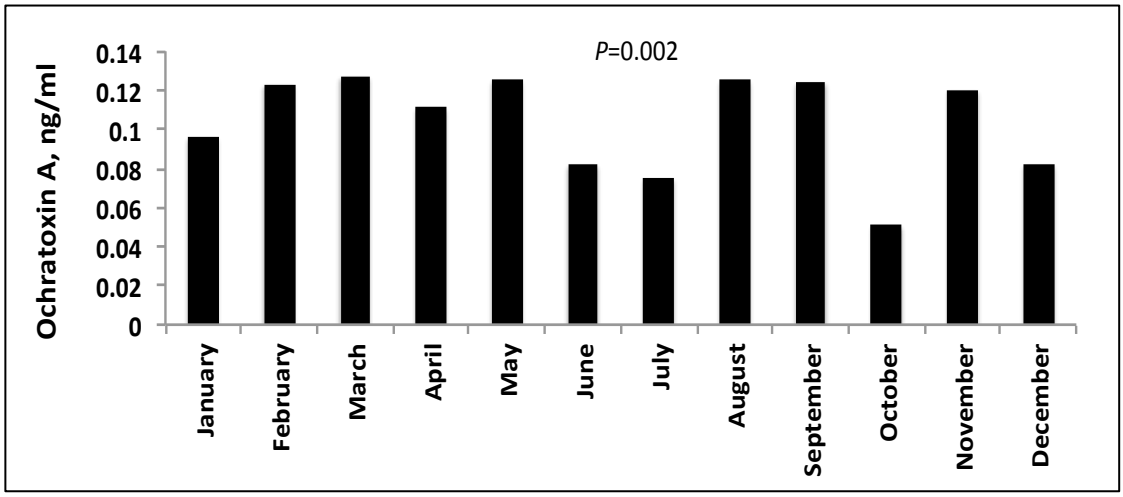

Figure 4.16: Median levels of ochratoxin A by month of sample collection

*Significance tested using the Kruskal-Wallis test.

4.2.1.6 HIV infection

Overall, 61/311 (21%) of the patients included in the risk factor analysis were HIV positive. There was no significant difference in HIV status between GA and controls [OR 1.1; 95% CI 0.5-2.3, *p*=0.86]. Having HIV infection did not increase the odds of EBV associated GA [OR 1.5; 95% CI 0.02-22, *p*=1.0] or microsatellite unstable GA [OR 1.7; 95% CI 0.3-10, *p*=0.69]. HIV infected patients did not have

significantly different levels of aflatoxins (p=0.09) or ochratoxins (p=0.59) than HIV negative patients. Evidence of oxidative stress to DNA was not different by HIV infection (p=0.31).

4.2.2 Risk factors for gastric premalignant lesions

In Figure 4.17, socio-economic indicators were compared between patients with GP and controls. Some of the measured parameters including lack of water, a television, decoder, fridge or job were more likely in patients with GP.

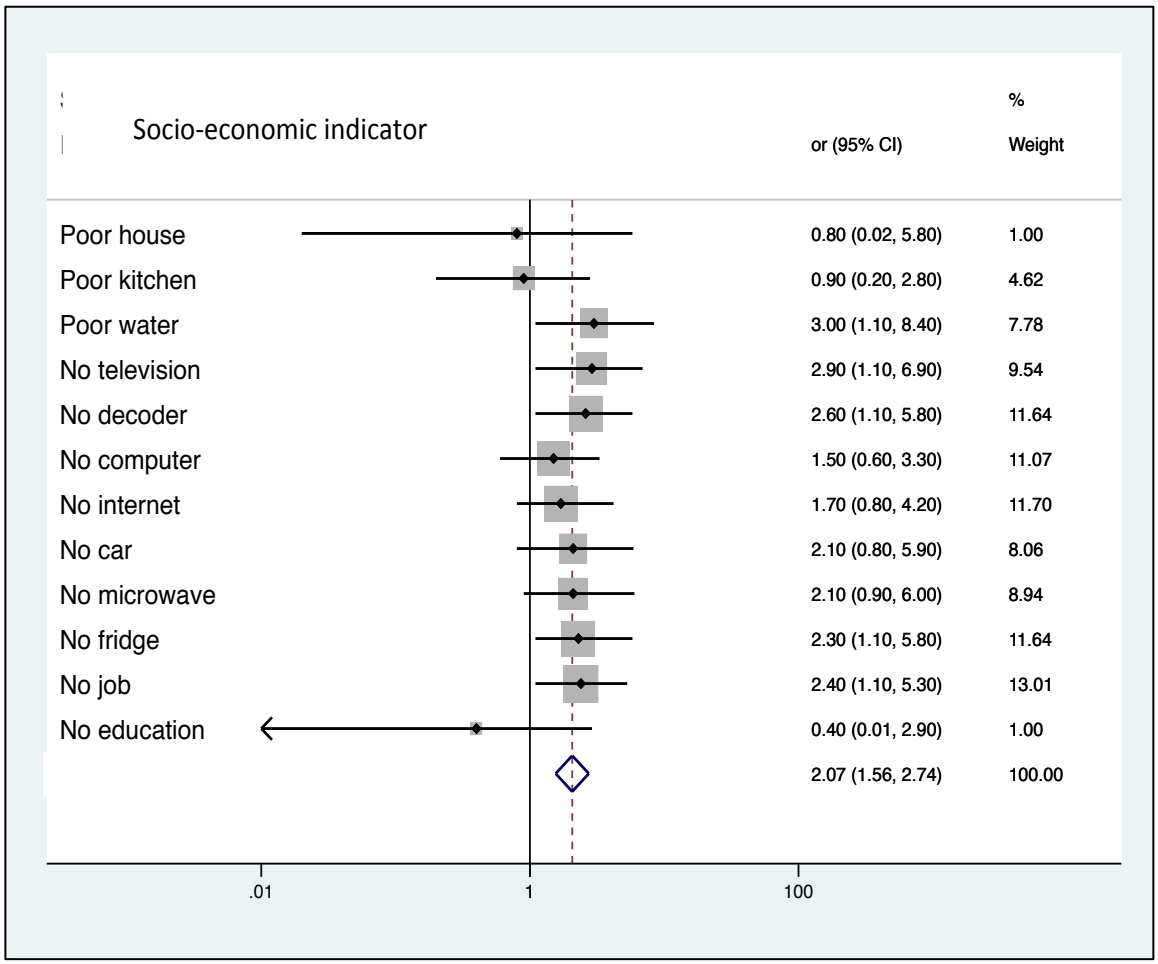

Figure 4.17: Socio-economic indicators of patients with gastric premalignant lesions compared to controls

*The solid vertical line is a null effect line, while the dotted one is showing the summary effect.
**Significance testing was performed using Fisher's exact test.

The rest of the parameters had horizontal lines depicting confidence intervals, which were crossing the solid vertical line and therefore were not statistically significant (Figure 4.17). Unconditional logistic regression adjusted for age, sex and residence did not yield any statistically significant socio-economic indicator. Thirty-seven (13/35) of the patients with GP lesions were reliant on biomass fuel in their homes. Unlike with gastric cancer, there was no association between having GP lesions and biomass smoke exposure [OR 1.5; 95% CI 0.7-3.3, *p*=0.32]. Due to the small numbers of patients with GP, only data for regular food consumption were reported. Regular consumption of eggplants and onions were more likely in patients with GP lesions than those without, *p*=0.03 and *p*=0.001 respectively (Table 4.5). Green vegetables and tomatoes showed a similar trend but were not statistically significant (*p*=0.07 and *p*=0.05 respectively, Table 4.5). Unconditional logistic regression adjusted for age, sex and residence regular consumption of bream [OR 0.4; 95% CI 0.2-0.9, *p*=0.02] and onions remained protective [OR 0.2; 95% CI 0.1-0.5, p=0.002].

Estimated 24 hour urine sodium excretion done by the Tanaka formula did not differ between GP cases, median 21 g (IQR 16-25) and controls 21 g (IQR 16-28, *p*=0.61). With the Kawasaki formula, the levels in the two groups were also similar, 46 g (IQR 34-58) for GP cases and 43 g (IQR 29-70) for controls. Similarly, levels of the two measured mycotoxins were similar between the two groups, aflatoxin M1 (*p*=0.22) and ochratoxin A (*p*=0.06). The proportion of HIV infection was similar between GP patients and those without [OR 0.6; 95% CI 0.1-1.9, *p*=0.47].

Table 4.5: Dietary intake for patients with gastric premalignant lesions and controls

	Regular consumption (at least 2-4 times a week)			
Food type (daily consumption of)	**GP n(%)**	**Controls n(%)**	**Univariate OR (95% CI)**	**P value**
Chicken	15 (43)	128 (52)	0.7 (0.3-1.5)	0.37
Unprocessed meat				
Beef	22 (63)	175 (72)	0.7 (0.3-1.5)	0.32
Pork	7 (20)	39 (16)	1.3 (0.5-3.4)	0.63
Goat	4 (11)	25 (10)	1.1 (0.3-3.6)	0.77
Game	2 (6)	3 (1)	4.9 (0.4-44)	0.12
Combined unprocessed meat	20 (57)	143 (59)	0.9 (0.4-2.1)	0.86
Processed meat				
Polony	1 (3)	29 (12)	0.2 (0.01-1.4)	0.15
Hungarian sausage	9 (26)	78 (32)	0.7 (0.3-1.7)	0.56
Bacon	2 (6)	9 (4)	1.6 (0.2-8.1)	0.63
Ham	1 (3)	8 (3)	0.9 (0.02-6.8)	1.00
Canned meat	0 (0)	2 (0)	-	1.00
Other sausage	8 (23)	76 (31)	0.7 (0.2-1.6)	0.43
Salami	0 (0)	3 (1)	-	1.00
Combined processed meat	12 (34)	121 (50)	0.5 (0.2-1.2)	0.10
Fish				
Bream	14 (40)	152 (62)	0.4 (0.2-0.9)	**0.02**
Kapenta	17 (49)	122 (50)	0.9 (0.4-2.0)	1.00
Buka buka	3 (9)	43 (18)	0.4 (0.1-1.5)	0.23
Tiger fish	2 (6)	11 (5)	1.3 (0.1-6.3)	0.67
Combined fish	26 (74)	194 (80)	0.7 (0.3-1.2)	0.51
Vegetables				
Green leafy	32 (91)	239 (98)	0.2 (0.04-1.5)	0.07
Egg plants	10 (29)	118 (48)	0.4 (0.2-1.0)	**0.03**
Tomatoes	31 (89)	236 (97)	0.3 (0.1-1.3)	0.05
Onions	27 (77)	232 (95)	0.2 (0.1-0.5)	**0.001**
Fruits				
Bananas	22 (63)	132 (54)	1.4 (0.7-3.3)	0.37
Oranges	14 (40)	105 (43)	0.9 (0.4-1.9)	0.86
Lemons	9 (26)	73 (30)	0.8 (0.3-1.9)	0.69
Apples	16 (46)	113 (46)	1.0 (0.4-2.1)	1.00
Pineapples	5 (14)	36 (15)	1.0 (0.3-2.7)	1.00
Strawberries	2 (6)	12 (5)	1.2 (0.1-5.6)	0.69
Fruit combined	25 (71)	181 (74)	0.9 (0.4-2.1)	0.69
Seasonal fruit	22 (63)	182 (75)	0.6 (0.3-1.3)	0.14

*Significance testing was performed using Fisher's exact test or Chi square for proportions. Seasonal fruit included mangos, papayas, baobabs, watermelons and mulberries.

4.2.3 Infection with *Helicobacter* species in relation to gastric disease

4.2.3.1 Antibody seropositivity to thirteen *Helicobacter pylori* antigens

The first *Helicobacter* species analysed was *Helicobacter pylori* (*H. pylori*). Levels of antibodies to thirteen *H. pylori* antigens were measured in 325 patients. Overall, 278/325 (86%) were positive for these antibodies (defined as positivity to four or more antibodies). Results showed that age had no influence on overall *H. pylori* seropositivity with prevalence ranging between 73% and 100% when stratified in five-year age bands (Figure 4.18).

Antibody concentrations obtained with *H. pylori* whole protein ELISA were correlated with antibody responses to each of the thirteen antigens applied in multiplex serology assay.

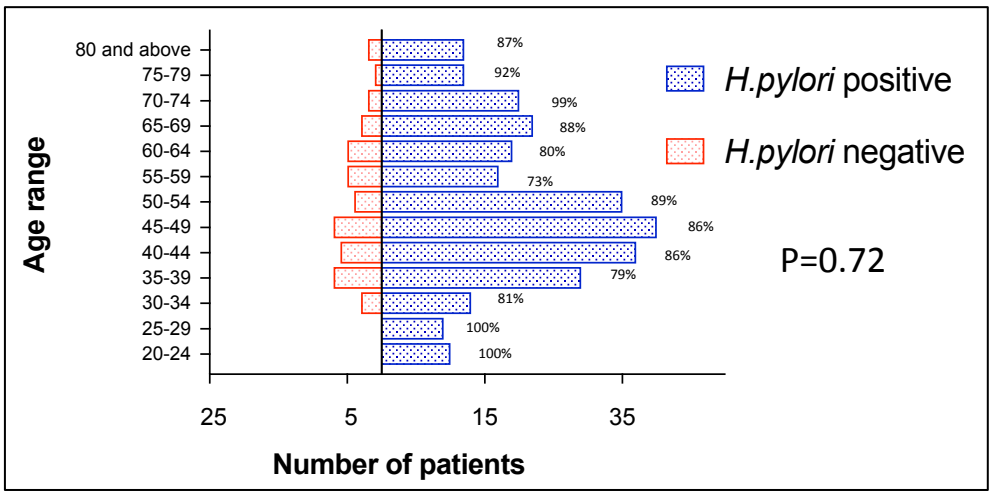

Figure 4.18: *Helicobacter pylori* seropositivity stratified by five-year age bands
*Significance tested using the Kruskal-Wallis test.

Levels of serum IgG antibodies against whole-cell *H. pylori* were well correlated with all of the tested antibodies but catalase, p=0.12. There was a good correlation between individual antibodies. There was an unexpected negative correlation between CagA and NapA (Figure 4.19).

	Anti-bodies	groel	urea	hp0231	napa	hp0350	hpaa	caga	hyua	catal	vaca	hcpc	cad	omp
Antibodies														
Groel	0.0001													
urea	0.003	0.1												
hp0231	0.0001	0.007	0.0001											
napa	0.0002	0.005	0.01	0.12										
hp0305	0.0001	0.0001	0.42	0.0001	0.0009									
hpaa	0.0001	0.0001	0.02	0.0001	0.62	0.0001								
caga	0.0001	0.0001	0.92	0.03	0.0006	0.06	0.0001							
hyua	0.0001	0.0002	0.006	0.0001	0.06	0.0002	0.0001	0.21						
catalase	0.12	0.8	0.0001	0.0001	0.05	0.0009	0.001	0.17	0.0001					
vaca	0.0001	0.0001	0.1	0.0003	0.99	0.0001	0.0001	0.0001	0.0001	0.06				
hcpc	0.0001	0.0001	0.52	0.0001	0.36	0.0001	0.0001	0.0001	0.0004	0.02	0.0001			
cad	0.0001	0.0001	0.0003	0.001	0.05	0.0001	0.0001	0.01	0.0001	0.0001	0.0001	0.0001		
omp	0.0001	0.0001	0.59	0.0001	0.16	0.0001	0.0001	0.0001	0.003	0.004	0.0001	0.0001	0.0003	

Key: Spearman's rho

1.0 | >0.5 | >0.4 | >0.3 | >0.2 | >0.1 | <0.0

*The value in each cell represents the P value

Figure 4.19: Correlation matrix for *H. pylori* antibodies

**Significance determined using the Spearman's rank correlation coefficient, as the data were non-parametric.

4.2.3.2 Comparison of *Helicobacter pylori* antibody levels between patients with gastric adenocarcinoma, premalignant lesions, active and chronic gastric inflammation

Overall, *H. pylori* seropositivity to four or more antibodies was not higher in patients with either GA [OR 1.0; 95% CI 0.4-2.4, *p*= 0.80] or GP [OR 2.2; 95% CI 0.4-19, p=0.39]. The presence of catalase antibodies was significantly higher in patients with GP lesions, while seropositivity to Cad was significantly lower in the GA group. None of the other antibodies were significantly different across the three groups (Table 4.6). *H. pylori* seropositivity was not different when stratified by either BMI (*p*=0.28), gastric pH (*p*=0.35), urinary aflatoxin M1 (*p*=0.11), serum ochratoxin A (*p*=0.89), 1-OHP (*p*=0.36), 8-OHdG (*p*=0.54) or salt excretion determined by Tanaka (*p*=0.10) or Kawasaki (*p*=0.06) methods.

Table 4.6: Seropositivity to *H. pylori* antibodies and gastric cancer or premalignant lesions

		Controls	Gastric cancer			Premalignant lesions		
	Cut-off for positivity	n=223 n (%)	n=66 n (%)	Odds ratio (95%CI)	P value	n=27 n (%)	Odds ratio (95%CI)	P value
GroEL	68	163 (73)	52 (79)	1.4 (0.7-2.9)	0.42	23 (85)	2.1 (0.7-8.7)	0.24
UreA	95	45 (20)	14 (21)	1.1 (0.5-2.2)	0.86	7 (26)	1.4 (0.5-3.7)	0.46
HP0231	95	69 (31)	13 (20)	0.5 (0.3-1.4)	0.09	6 (22)	0.6 (0.2-1.7)	0.50
NapA	51	63 (28)	14 (21)	0.7 (0.3-1.4)	0.27	9 (33)	1.3 (0.5-3.2)	0.65
HP0305	110	78 (35)	21 (32)	0.9 (0.5-1.6)	0.66	7 (26)	0.7 (0.2-1.7)	0.40
HpaA	98	61 (27)	7 (26)	0.9 (0.3-2.4)	1.00	13 (20)	0.7 (0.3-1.3)	0.26
CagA	654	211 (95)	62 (94)	0.9 (0.3-3.9)	0.77	26 (96)	1.4 (0.2-65.6)	1.00
HyuA	114	39 (17)	14 (21)	1.3 (0.6-2.6)	0.47	7 (26)	1.7 (0.5-4.4)	0.30
Catalase	107	70 (31)	19 (29)	0.9 (0.5-1.7)	0.76	16 (59)	3.2 (1.3-8.0)	**0.005**
VacA	114	169 (76)	45 (68)	0.7 (0.4-1.3)	0.26	21 (78)	1.1 (0.4-2.8)	1.00
HcpC	71	168 (75)	44 (67)	0.7 (0.3-1.3)	0.20	20 (74)	0.9 (0.4-2.8)	1.00
Cad	62	65 (29)	8 (12)	0.3 (0.1-0.8)	**0.006**	13 (48)	2.3 (0.9-5.5)	0.05
Omp	137	194 (87)	53 (80)	0.6 (0.3-1.4)	0.23	22 (81)	0.7 (0.2-2.4)	0.39

*Significance testing was performed using Fisher's exact test for proportions

To investigate the dose dependent effects of *H. pylori* antibodies on GA risk, measured median fluorescence intensity (MFI) values were divided into quartiles. Applying unconditional logistic regression adjusting for age and sex and using negative samples as the reference showed statistically significant trends of reducing odds of gastric cancer with increasing antibody responses to VacA and Cad (Table 4.7). Similarly, there was a trend of reducing odds of gastric premalignant lesions with increasing antibody response to catalase (Table 4.7).

Of the 223 controls with *H. pylori* serology results, 48/223 (22%) had active and 172/223 (77%) had chronic gastric inflammation while three had no inflammation. *H. pylori* was seen on histology in 26/47 (55%) of patients with active gastric inflammation and 4/149 (3%) of those with chronic gastric inflammation [OR 30; 95% CI 10-96, p<0.0001].

Serum levels of *H. pylori* antibodies were then compared in four groups, GA, GP, active and chronic gastric inflammation (Figures 4.20 (a) – (d)).

Table 4.7: Gastric cancer and premalignancy risk stratified by strength of antibody response to *Helicobacter pylori* adjusted for age and sex

	Controls	Gastric cancer	Odds ratio	95% CI	Premalignant lesions	OR	95% CI
	n (%)	n (%)			n (%)		
GroEL							
Negative*	60 (27)	14 (21)	1.00 (ref)		4 (15)	1.00 (ref)	
MFI 1st quartile	33 (15)	18 (27)	2.13	0.90-5.00	6 (22)	1.78	0.44-7.21
MFI 2nd quartile	39 (17)	13 (20)	1.37	0.56-3.35	8 (30)	2.65	0.71-9.89
MFI 3rd quartile	38 (17)	15 (23)	1.36	0.56-3.29	6 (22)	1.82	0.45-7.34
MFI 4th quartile	53 (24)	6 (9)	0.38	0.13-1.11	3 (11)	0.61	0.12-3.04
				p=0.08			p=0.62
UreA							
Negative*	178 (80)	52 (79)	1.00 (ref)		20 (74)	1.00 (ref)	
MFI 1st quartile	11 (5)	3 (5)	1.03	0.26-4.08	0 (0)	1	-
MFI 2nd quartile	9 (4)	6 (9)	2.27	0.72-7.12	3 (11)	3.63	0.84-15.71
MFI 3rd quartile	12 (5)	2 (3)	0.57	0.12-2.72	2 (7)	1.21	0.23-6.41
MFI 4th quartile	13 (6)	3 (5)	0.58	0.15-2.24	2 (7)	0.62	0.12-3.38
				p=0.61			p=0.95
HP0231							
Negative*	154 (69)	53 (80)	1.00 (ref)		21 (78)	1.00 (ref)	
MFI 1st quartile	19 (9)	2 (3)	0.20	0.04-1.00	2 (7)	0.42	0.08-2.13
MFI 2nd quartile	15 (7)	6 (9)	1.07	0.37-3.09	1 (4)	0.39	0.04-3.36
MFI 3rd quartile	18 (8)	3 (5)	0.51	0.14-1.90	1 (4)	0.49	0.06-3.95
MFI 4th quartile	17 (8)	2 (3)	0.31	0.06-1.48	2 (7)	0.91	0.18-4.51
				p=0.08			p=0.44
NapA							
Negative*	160 (72)	52 (79)	1.00 (ref)		18 (67)	1.00 (ref)	
MFI 1st quartile	13 (6)	6 (9)	1.70	0.58-5.00	1 (4)	0.77	0.09-6.83
MFI 2nd quartile	14 (6)	3 (6)	0.60	0.16-2.26	5 (19)	3.14	0.96-10.30
MFI 3rd quartile	16 (7)	4 (6)	0.94	0.29-3.07	2 (7)	1.75	0.34-8.98
MFI 4th quartile	20 (9)	1 (2)	0.14	0.02-1.12	1 (4)	0.46	0.05-3.85
				p=0.09			p=0.71
HP0305							
Negative*	114 (59)	34 (62)	1.00 (ref)		17 (71)	1.00 (ref)	
MFI 1st quartile	17 (9)	6 (11)	1.21	0.43-3.44	2 (8)	0.68	0.13-3.60
MFI 2nd quartile	20 (10)	7 (13)	1.25	0.47-3.31	0 (0)	-	-
MFI 3rd quartile	24 (13)	1 (2)	0.14	0.02-1.11	2 (8)	0.62	0.13-3.02
MFI 4th quartile	17 (9)	7 (13)	1.15	0.42-3.15	3 (13)	1.08	0.27-4.36
				p=0.52			p=0.57
HpaA							
HpaA negative*	162 (73)	53 (80)	1.00 (ref)		20 (74)	1.00 (ref)	
MFI 1st quartile	11 (5)	7 (11)	2.02	0.71-5.81	3 (11)	2.29	0.55-9.58
MFI 2nd quartile	14 (6)	2 (3)	0.51	0.11-2.40	3 (11)	1.79	0.43-7.54
MFI 3rd quartile	18 (8)	2 (3)	0.26	0.06-1.23	1 (4)	0.40	0.05-3.28
MFI 4th quartile	18 (8)	2 (3)	0.41	0.09-1.89	0 (0)	-	-
				p=0.52			p=0.28
CagA							
Negative*	12 (5)	4 (6)	1.00 (ref)		1 (4)	1.00 (ref)	
MFI 1st quartile	49 (22)	19 (29)	1.10	0.29-4.24	6 (22)	1.04	0.10-10.73
MFI 2nd quartile	51 (23)	15 (23)	0.77	0.20-3.02	9 (33)	1.47	0.15-14.38
MFI 3rd quartile	50 (22)	18 (27)	1.29	0.33-4.98	7 (26)	1.65	0.16-16.55
MFI 4th quartile	61 (27)	10 (15)	0.60	0.15-2.45	4 (15)	0.76	0.07-8.22
				p=0.40			p=0.83
HyuA							
Negative*	184 (83)	52 (79)	1.00 (ref)		20 (74)	1.00 (ref)	
MFI 1st quartile	11 (5)	3 (5)	1.02	0.26-3.91	0 (0)	-	-
MFI 2nd quartile	8 (4)	6 (9)	1.56	0.48-5.19	3 (11)	1.72	0.37-7.91
MFI 3rd quartile	9 (4)	5 (8)	1.76	0.54-5.67	0 (0)	-	-
MFI 4th quartile	11 (5)	0 (0)	-	-	4 (14)	2.26	0.60-8.45
				p=0.61			p=0.45
Catalase							
Negative*	153 (69)	47 (71)	1.00 (ref)		6 (22)	1.00 (ref)	
MFI 1st quartile	16 (7)	5 (8)	1.29	0.43-3.87	4 (15)	3.80	1.10-13.16
MFI 2nd quartile	18 (8)	5 (8)	0.79	0.26-2.40	5 (19)	1.71	0.40-7.35
MFI 3rd quartile	18 (8)	7 (11)	1.18	0.44-3.13	6 (22)	1.37	0.27-6.97
MFI 4th quartile	18 (8)	2 (3)	0.28	0.06-1.35	6 (22)	4.07	1.25-13.26
				p=0.30			**p=0.04**
VacA							
Negative*	54 (24)	21 (32)	1.00 (ref)		11 (41)	1.00 (ref)	
MFI 1st quartile	40 (18)	14 (21)	0.79	0.35-1.82	5 (19)	0.88	0.22-3.48

MFI 2nd quartile	41 (18)	13 (20)	0.79	0.34-1.82	3 (11)	1.18	0.32-4.36
MFI 3rd quartile	40 (18)	12 (18)	0.78	0.33-1.82	2 (7)	1.29	0.37-4.52
MFI 4th quartile	48 (22)	6 (9)	0.29	0.11-0.82	6 (22)	1.02	0.29-3.55
				p=0.04			p=0.80
HcpC							
Negative*	77 (35)	29 (44)	1.00 (ref)		10 (37)	1.00 (ref)	
MFI 1st quartile	16 (7)	10 (15)	1.94	0.73-5.10	1 (4)	0.58	0.06-5.28
MFI 2nd quartile	43 (19)	9 (14)	0.68	0.28-1.63	5 (19)	1.22	0.36-4.09
MFI 3rd quartile	42 (19)	10 (15)	0.69	0.29-1.60	6 (22)	1.34	0.43-4.15
MFI 4th quartile	45 (20)	8 (12)	0.47	0.19-1.17	5 (19)	0.98	0.30-3.25
				p=0.05			p=0.79
Cad							
Negative*	158 (71)	58 (88)	1.00 (ref)		14 (52)	1.00 (ref)	
MFI 1st quartile	19 (9)	0 (0)	-	-	3 (11)	1.54	0.38-6.17
MFI 2nd quartile	18 (8)	3(5)	0.43	0.12-1.58	1 (4)	0.64	0.08-5.31
MFI 3rd quartile	11 (5)	4 (6)	0.94	0.28-3.23	6 (22)	4.80	1.43-16.11
MFI 4th quartile	17 (8)	1 (2)	0.11	0.01-0.96	3 (11)	1.54	0.37-6.37
				p=0.02			p=0.11
Omp							
Negative*	29 (13)	13 (20)	1.00 (ref)		5 (19)	1.00 (ref)	
MFI 1st quartile	40 (18)	10 (15)	0.46	0.17-1.26	2 (7)	0.23	0.04-1.38
MFI 2nd quartile	53 (24)	15(23)	0.57	0.23-1.42	6 (22)	0.59	0.16-2.24
MFI 3rd quartile	50 (22)	16 (24)	0.72	0.29-1.80	4 (15)	0.59	0.14-2.57
MFI 4th quartile	51 (23)	12 (18)	0.43	0.17-1.13	10 (37)	1.12	0.33-3.83
				p=0.29			p=0.34

*MFL is median fluorescence intensity determined by the multiplex assay

The findings were as follows:

GroEL: Patients with active gastric inflammation had significantly higher levels of GroEL antibodies than those with chronic gastric inflammation (*p*=0.04).

HP0305: Similar to GroEL, patients with active gastric inflammation had significantly higher HP0305 antibodies than those with chronic gastric inflammation (*p*=0.04).

CagA: Antibodies to CagA were significantly higher in patients with active gastric inflammation than those with chronic gastric inflammation (*p*=0.0004), GA (*p*=0.0007) or GP (*p*=0.02).

VacA: Patients with active gastric inflammation had significantly higher levels of VacA antibodies than those with chronic gastric inflammation (*p*=0.002) or GA (*p*=0.0006).

HcpC: Antibodies to HcpC were significantly higher in patients with active gastric inflammation than those with chronic gastric inflammation (*p*=0.007) or GA (*p*=0.0006).

Cad: GP patients had significantly higher levels of Cad compared to those with active gastric inflammation (*p*=0.01) or GA (*p*=0.0009).

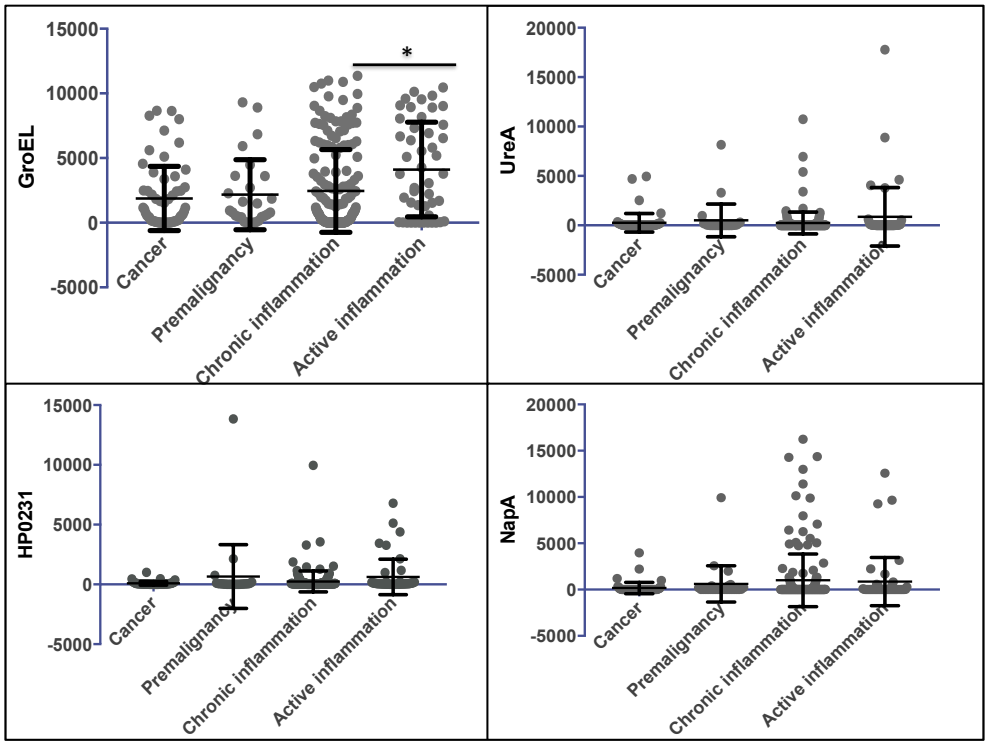

Figure 4.20(a): GroEL, UreA, HP0231 and NapA in patients with GA, GP gastric Inflammation

Significance tested using the Kruskal-Wallis test, *p-value<0.05, **p-value<0.01, ***p-value<0.001.
The error bars are showing the standard deviation.

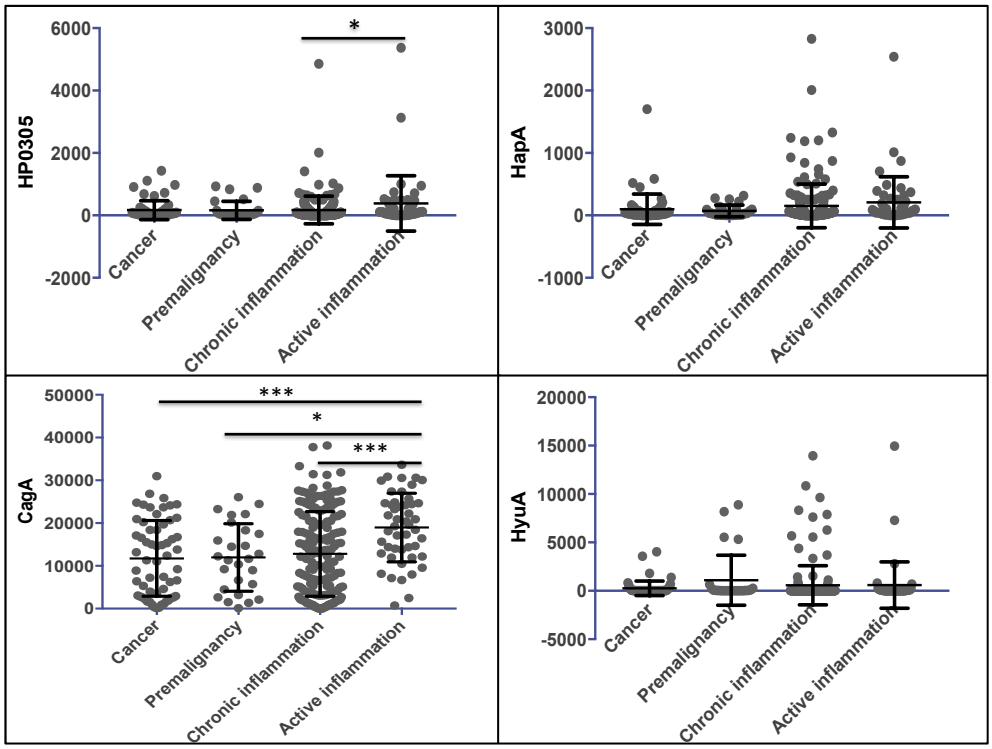

Figure 4.20(b): HP0305, HapA, CagA and HyuA in patients with GA, GP gastric Inflammation

Significance tested using the Kruskal-Wallis test, *p-value<0.05, **p-value<0.01, ***p-value<0.001.
The error bars are showing the standard deviation.

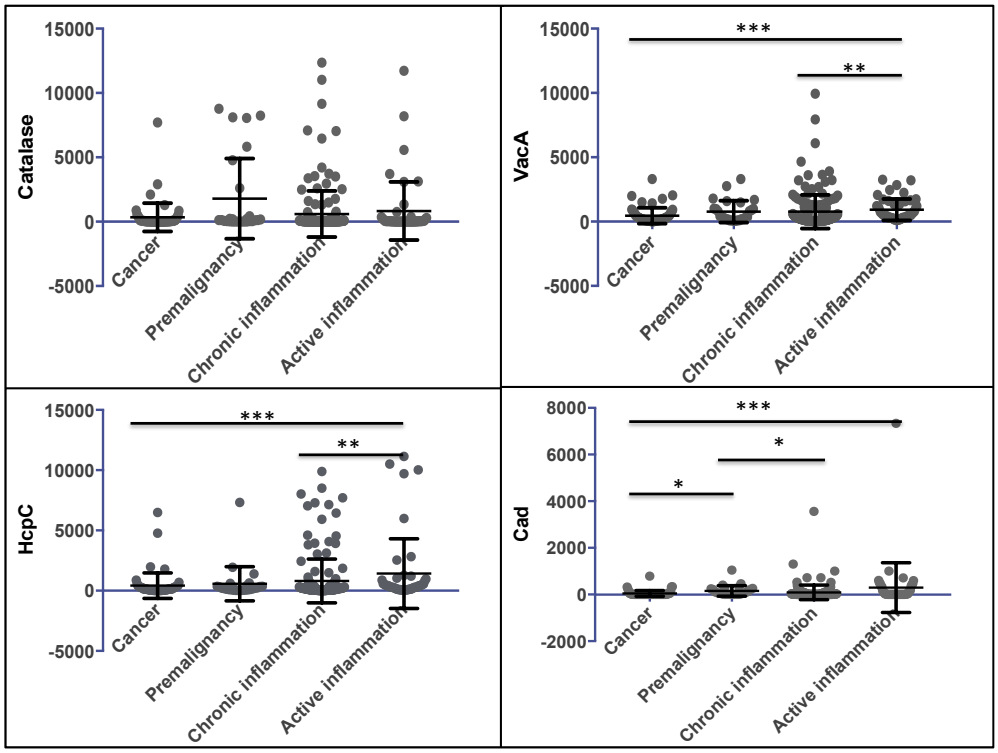

Figure 4.20(c): Catalase, VacA, HcpC and Cad in patients with GA, GP gastric Inflammation

Significance tested using the Kruskal-Wallis test, *p-value<0.05, **p-value<0.01, ***p-value<0.001.
The error bars are showing the standard deviation.

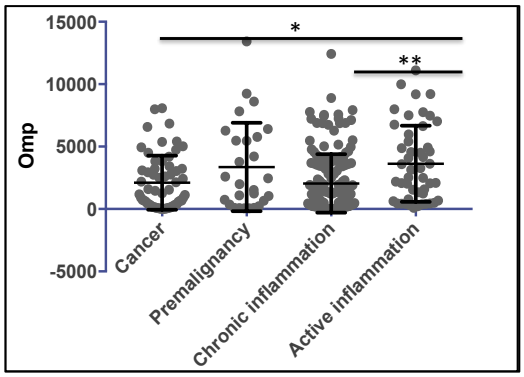

Figure 4.20(d): Omp in patients with GA, GP gastric inflammation

Significance tested using the Kruskal-Wallis test, *p-value<0.05, **p-value<0.01, ***p-value<0.001.
The error bars are showing the standard deviation.

Omp: Antibodies to Omp were significantly higher in patients with active gastric inflammation than those with chronic gastric inflammation (p=0.002) or GA (p=0.03). Serum levels of the antibodies UreA, HP0231, NapA, HapA, HyuA, and Catalase were not significantly different between any of the groups evaluated.

Analysing for the utility of CagA and VacA to identify *H. pylori* positive patients with active gastric inflammation, antibody responses to these two proteins had a sensitivity of 100% but a very poor specificity of 6%. When analysed separately, CagA had a sensitivity of 100% and a specificity of 7%, while the sensitivity and specificity for VacA were 98% and 28% respectively.

Testing for the presence of active *H. pylori* infection using the urease test showed that 32/86 (37%) was urease positive. Urease positivity was not any higher in patients with GA [OR 1.3; 95% CI 0.3-5.0, p=0.76] or GP [OR 1.5; 95% CI 0.2-12, p=0.68]. However, patients with active gastric inflammation were more likely to have positive urease tests than those with chronic inflammation [OR 4.2; 95% CI 1.0-19, p=0.03].

4.2.3.3 Socio-economic status and *Helicobacter pylori* infection

Using the socio-economic indicators described in section 4.2.1.1 above, an association with *H. pylori* infection was sought. Figure 4.21 showed that the horizontal lines representing confidence intervals were crossing the solid vertical line, and therefore having a poor house, kitchen or water source, and not owning a television, decoder, computer, car, microwave or fridge was not significantly linked to the presence of *H. pylori* infection (Figure 4.21). In addition, the education level of patients with *H.pylori* infection was not different from those without.

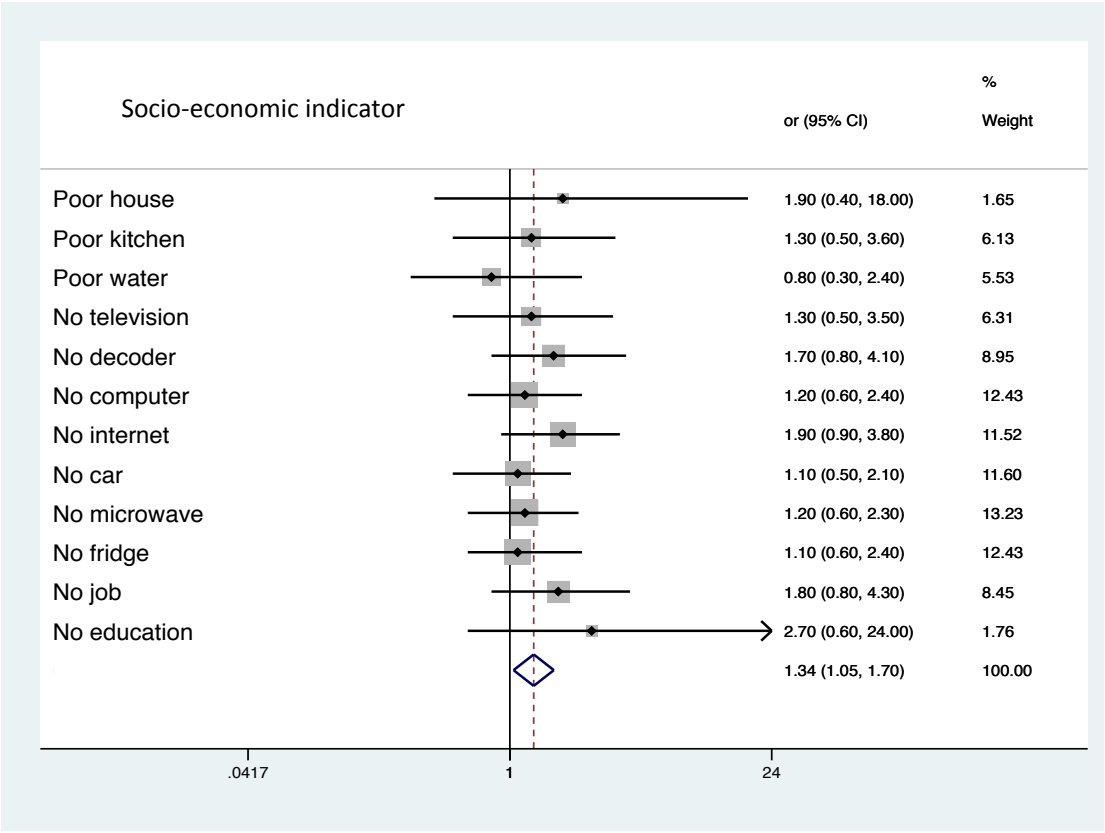

Figure 4.21: Socio-economic indicators of patients with and without *H. pylori* infection
*The solid vertical line is a null effect line, while the dotted one is showing the summary effect. Significance testing was performed using Fisher's exact test.

4.2.3.4 *Helicobacter bilis* and *Helicobacter Hepaticus* in relation to gastric disease

The quantities of antibodies to specific *H. bilis* and *H. hepaticus* were measured. For the former antibodies measured were P167c, P167d and Hrag_01504 while HH0435, HH0713, HH1446 and HH0243 were measured for the latter. Overall 107/296 (36%) of patients had antibodies to *H. bilis* and 269/296 (91%) had antibodies to *H. hepaticus.* Positivity to *H. bilis* and *H. hepaticus* antibodies was compared between patients with GA, GP and in controls. The odds of GA patients having either *H. bilis* or *H. hepaticus* were similar [OR 1.1; 95% CI 0.5-2.0, *p*=0.88] and [OR 0.5; 95% CI 0.2-1.5, *p*=0.19] respectively. The results were similar for GP [OR 2.1; 95% CI 0.8-5.0, *p*=0.09] and [OR 0.7; 95% CI 0.2-3.8, *p*=0.46], respectively. GA patients had significantly higher levels of Hrag_01504, while those with GP had

higher levels of p167d, both antibodies against *H. bilis* (Table 4.8). The levels of *H. hepaticus* antibodies were similar in all the three groups of patients evaluated. The presence of *H. bilis* or *H. hepaticus* antibodies did not influence active gastric inflammation ($p > 0.05$ in all cases).

Table 4.8: Comparison of *H. bilis* and *H. hepaticus* levels between patients with gastric adenocarcinoma, premalignant lesions and controls

	Gastric adenocarcinoma n=59	**Controls n=210**	**P value**
H. bilis			
P167c	7(1-46)	5(1-38)	0.78
p167d	15(6-89)	23(6-93)	0.64
Hrag_01504	29(11-66)	14(6-40)	**0.02**
H. hepaticus			
HH0435	22(11-54)	30(18-70)	0.06
HH0713	31(15-63)	23(14-44)	0.14
HH1446	11(1-29)	8(1-33)	0.39
HH0243	666(161-2220)	921(190-2608)	0.46
	Gastric premalignant lesions n=27	**Controls n=210**	**P value**
H. bilis			
P167c	15(1-200)	5(1-38)	0.06
p167d	77(16-326)	23(6-93)	**0.006**
Hrag_01504	15(9-49)	14(6-40)	0.61
H. hepaticus			
HH0435	17(10-49)	30(18-70)	0.09
HH0713	30(12-166)	23(14-44)	0.93
HH1446	11(2-22)	8(1-33)	0.81
HH0243	1188(185-2512)	921(190-2608)	0.91

*Significance tested using the Kruskal-Wallis test.

4.2.4 Epstein-Barr virus infection in relation to gastric disease

Epstein-Barr virus **(**EBV) serology antibody results were available for GA (n=59), GP (n=27) and controls (n=210).

Table 4.9: Epstein-Barr virus antibodies (presence and titres) compared between patients with GA, GP and controls, and between HIV positive and HIV negative patients

EBV antibodies (median fluorescence intensity cut-off used)	**GA n=59**	**Controls n=210**	**OR (95% CI)**	**P value**
Early antigen (300)	39 (66%)	111 (53%)	1.7 (0.9-3.4)	0.08
Viral capsid antigen (2500)	55 (93%)	188 (90%)	1.6 (0.5-6.7)	0.47
Epstein-Barr nuclear antigen (1800)	59 (100%)	205 (98%)	-	0.59
BZLF1-encoded replication activator protein (200)	47 (80%)	169 (80%)	1.0 (0.4-2.1)	0.86
	GP n=27	**Controls n=210**		
Early antigen (300)	20 (74%)	111 (53%)	2.5 (1.0-7.4)	**0.04**
Viral capsid antigen (2500)	26 (96%)	188 (90%)	3.0 (0.4-130)	0.49
Epstein-Barr nuclear antigen (1800)	26 (96%)	205 (98%)	0.6 (0.1-31)	0.52
BZLF1-encoded replication activator protein (200)	26 (96%)	169 (80%)	6.3 (1.1-265)	0.06
	HIV positive n=64	**HIV negative n=250**		
Early antigen (300)	53 (83%)	131 (52%)	4.4 (2.1-9.7)	**<0.0001**
Viral capsid antigen (2500)	64 (100%)	221 (88%)	-	**0.001**
Epstein-Barr nuclear antigen (1800)	60 (94%)	247 (99%)	0.2 (0.02-1.1)	**0.04**
BZLF1-encoded replication activator protein (200)	61 (95%)	198 (79%)	5.3 (1.6-28)	**0.002**
EBV antibodies determined by the median fluorescence intensity	**HIV positive n=64**	**HIV negative n=250**		
Early antigen	4515 (880-9830)	352 (45-1912)	-	**0.0001**
Viral capsid antigen	12001 (10470-13179)	9178 (5411-11566)	-	**0.0001**
Epstein-Barr nuclear antigen	10220 (7102-11905)	10903 (9274-12383)	-	**0.02**
BZLF1-encoded replication activator protein	3135 (1194-6207)	895 (269-2405)	-	**0.0001**

*Significance testing was performed using Fisher's exact and Kruskal-Wallis tests.

EBV antibodies against Early Antigen (EA), Viral Capsid Antigen (VCA), Epstein-Barr Nuclear Antigen (EBNA) and BZLF1-encoded replication activator protein (ZEBRA) were measured quantitatively. Overall, 98% of the patients had EBV

antibodies in their serum. The presence of EBV antibodies was similar between GA patients and controls. However, EA levels were higher in patients with GP than those without (p=0.04; Table 4.9). Patients with EBER positive gastric cancer had higher median levels of EA (MFI 7290; IQR 1437-13559), VCA (MFI 12903; IQR 5774-15527), EBNA (MFI 11000; IQR 10021-13520) and ZEBRA (MFI 6353; IQR 295-10908), than those with EBER negative tumours EA (MFI 895; IQR 218-3737), VCA (MFI 10090; IQR 7217-12167), EBNA (MFI 10360; IQR 8334-12221) and ZEBRA (MFI 1646; IQR 285-3470). However, these differences did not reach statistical significance (all p values were greater than 0.05).

EBV antibodies were then compared between HIV positive (n=64) and HIV negative (n=250) patients regardless of their clinical or histopathological or diagnosis. HIV positive patients were more likely to have antibodies to EA, VCA and ZEBRA but not EBNA (Table 4.9). A comparison of quantified EBV antibodies between HIV infected and uninfected patients showed that those with the infection had significantly higher levels of antibodies to EA, VCA and ZEBRA. Conversely, the levels of EBNA were significantly higher in the HIV uninfected group (Table 4.9). There was a good correlation between EBV antibodies, apart from VCA and EBNA (Figure 4.22).

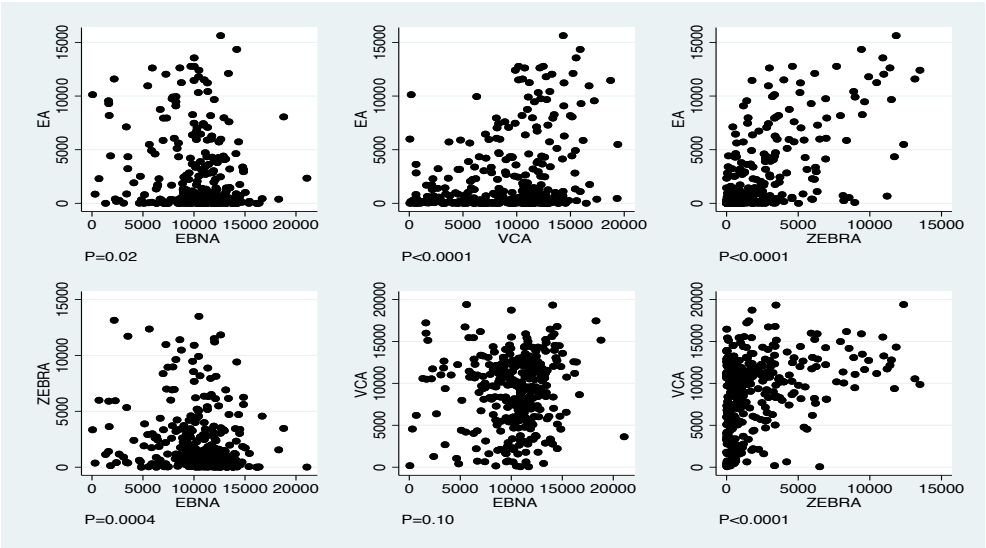

Figure 4.22: Correlation between EBV antibodies, EA, VCA, EBNA and ZEBRA
*Significance determined using the Spearman's rank correlation coefficient, as the data were non-parametric.

4.3 Early detection of gastric mucosal lesions (objective three)

The first part of this chapter (4.3.1) focuses on the time taken for gastric cancer diagnosis while the second part (4.3.2) reports the utility of blood in gastric juice as an indicator of gastric mucosal lesions.

4.3.1 Time in weeks from the first consultation to gastric cancer diagnosis

Patients with gastric tumours were asked about the exact duration of symptoms and when they first presented to a health care centre for assistance. Figure 5.3.1 shows the time in weeks for each GA patient, with the green bars representing time to first consultation and the orange bars being the time to OGD.

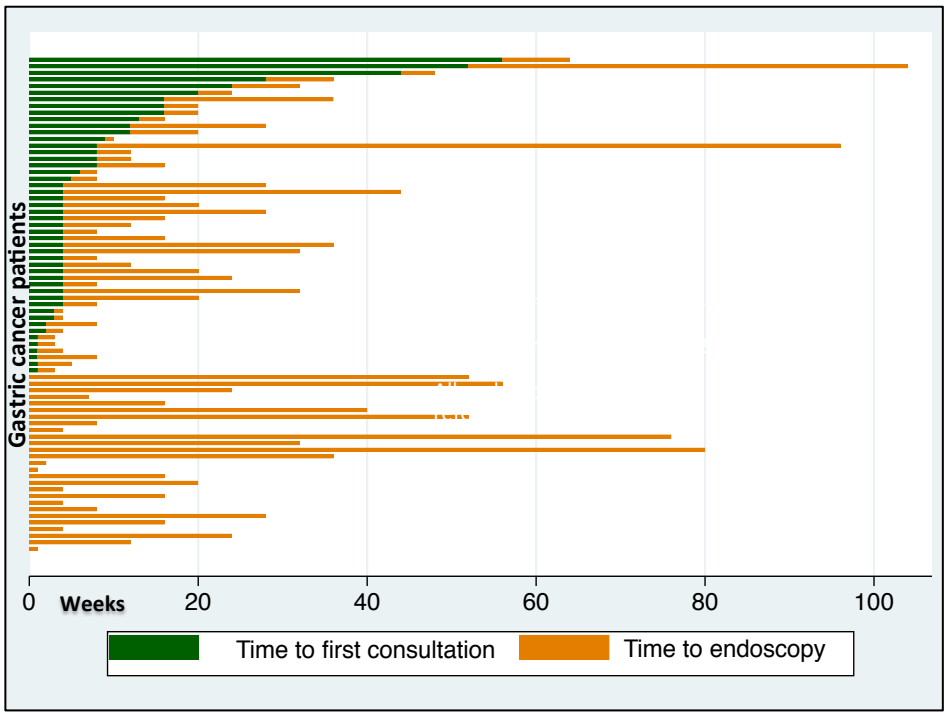

Figure 4.23: Time in weeks from onset of symptoms to clinical diagnosis
*Each horizontal line represents a gastric cancer patient. The x-axis shows time in weeks

The median time to first health care consultation was 2 weeks, IQR 0-4 weeks. However, it took another median of 12 weeks, IQR 4-34 weeks before the diagnosis of a gastric tumour (Figure 4.23). Once requested, all OGDs were done within 2 weeks.

Figure 4.24 shows the median times to clinical diagnosis in weeks and the relative number of gastric cancer patients with their presenting symptoms. The

median time to diagnosis for those with abdominal pain, vomiting, blood loss or dysphagia was 16, 12, 16 and 4 weeks respectively (Figure 4.24).

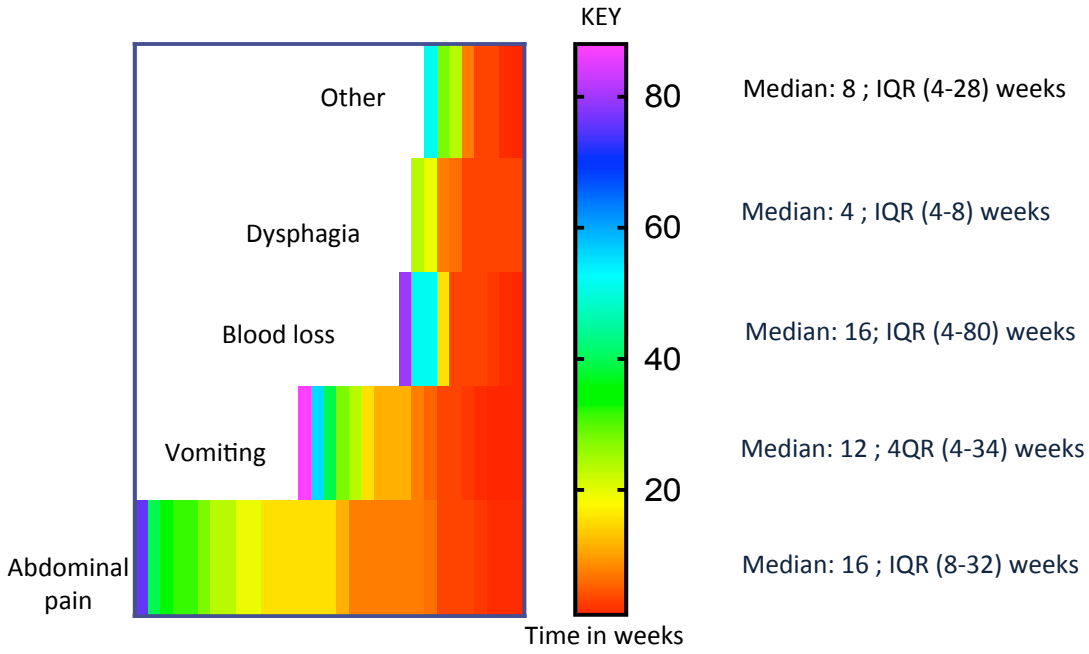

Figure 4.24: Time in weeks from first consultation to clinical diagnosis
*The coloured coding signifies duration in weeks as indicated in the key. Each of the vertical bars represents an individual gastric cancer patient. IQR is the interquartile range

4.3.2 Presence of blood in gastric juice as an indicator of gastric mucosal lesions

The utility of gastric juice to predict presence of mucosal lesions was explored.

4.3.2.1. Basic Characteristics of patients included to analyse the utility of blood in gastric juice

Gastric juice samples were available from 276 patients. Of these, 147 (53%) were female and the median age was 49 years (IQR 40-64 years). 116 (42%) had mucosal abnormalities with 40 (34%) benign gastric ulcers, 34 (29%) duodenal ulcers and 33 (28%) gastric tumours. The remaining 9 (8%) had oesophageal abnormalities, polyps or non-specific inflammation. Of the 33 patients with gastric

tumours, 27 (82%) had adenocarcinoma. Patients with mucosal abnormalities were significantly older than those without (p<0.001) (Table 4.10).

Table 4.10: Comparison of the basic characteristics of patients with normal and abnormal oesophagogastroduodenoscopy findings

	Abnormal OGD n=116: n (%)	Normal OGD n=160: n (%)	OR (95% CI)	*P*
Female	59 (51)	88 (55)	0.8 (0.5-1.4)	0.54
Age in years, n (IQR)	57 (45-69)	45 (39-55)	-	**<0.001**
Residence in capital city	70 (60)	116 (73)	0.6 (0.3-1.0)	**0.03**
No employment	41 (36)	38 (24)	1.7 (1-3.1.0)	**0.04**
No secondary education	55 (47)	44 (28)	2.3 (1.4-4.1)	**0.001**
History of blood loss or anaemia	34 (29)	20 (13)	2.9 (1.5-5.7)	**0.001**
History of abdominal pain	75 (65)	122 (76)	0.6 (0.3-1.0)	**0.04**
History of vomiting	12 (10)	12 (8)	1.4 (0.6-3.6)	0.52
History of acid suppressing drugs	71 (65)	108 (75)	0.6 (0.3-1.0)	0.07
Current smoker	6 (6)	10 (10)	0.8 (0.2-2.5)	0.80
Current intake of alcohol	30 (27)	32 (21)	1.4 (0.7-2.6)	0.30
	Gastric tumour n=33: n (%)	No gastric tumour n=243: n (%)	OR (95% CI)	P
Female	18 (55)	129 (53)	1.1 (0.5-2.4)	1.00
Age in years, n (IQR)	63 (53-71)	48 (39-60)	-	**<0.001**
Residence in capital city	16 (48)	170 (70)	0.4 (0.2-0.9)	**0.02**
No employment	16 (48)	63 (26)	2.6 (1.2-5.9)	**0.01**
No secondary education	18 (55)	81 (33)	2.4 (1.1-5.4)	**0.02**
History of blood loss or anaemia	9 (27)	45 (19)	1.7 (0.6-4.0)	0.25
History of abdominal pain	20 (61)	177 (73)	0.6 (0.3-1.3)	0.15
History of vomiting	5 (15)	19 (9)	2.1 (0.6-6.4)	0.18

*Significance testing was performed using Fisher's exact, Chi square or Kruskal-Wallis tests

4.3.2.2 Blood in gastric juice as a marker of gastric pathology

Overall, 95/276 (34%) of the patients had hypochlorhydria with gastric pH greater than 4. 179/276 (65%) had history of having taken acid suppressing medication within two weeks of enrolment. The median pH for the patients with

normal OGD was 6 while it was 5.5 in those with abnormalities, *p*=0.15. 57/276 (21%) of the patients had pH less than 3 including 7 with pH 1, 21 pH 1.5, 20 pH 2 and 9 pH 2.5. All these were diluted as outlined in the methods above. Excluding these samples from the analysis did not alter the results (data not shown). The occurrence of blood in gastric juice was higher in patients with abnormal endoscopic findings than those without, even at 1:10 and 1:100 dilutions (Table 4.11).

Table 4.11: The presence of blood in gastric juice is associated with abnormal oesophagogastroduodenoscopy

Gastric juice	Presence of blood	OGD findings		OR(95% CI); P	Area under ROC curve	Sensitivity	Specificity
		Abnormalities seen n=116	Normal endoscopy n=160				
Undiluted	Blood present	85 (73%)	90 (56%)	2.1(1.2-3.7); 0.004	0.58	73%	44%
1:10 dilution	Blood present	61 (52%)	45 (28%)	2.7(1.6-4.7); <0.001	0.62	51%	73%
1:100 dilution	Blood present	22 (19)	10 (6%)	3.4(1.5-8.5); 0.001	0.56	18%	94%

*Significance testing done with Fisher's exact test

The odds of having blood in gastric juice for patients with a history of blood loss or anaemia was 0.9; 95% CI 0.5-1.7, *p*=0.75.

Patients were then stratified by endoscopic diagnosis including normal mucosa, gastric or duodenal ulceration or tumours. Figure 4.25 showed that increasing the dilution factor reduced the proportion of blood detection in patients with normal gastric mucosa. However, dilution also further increased the number of patients having mucosal abnormalities but without blood in gastric juice.

4.3.2.3 Blood in gastric juice and gastric cancer

The proportion of patients gastric cancer patients with detectable blood in gastric juice was 30/33 (91%), while it was 145/243 (60%) in those without tumours, *p*=0.0005. The results were similar at 1:10 and 1:100 dilutions (Table 4.12).

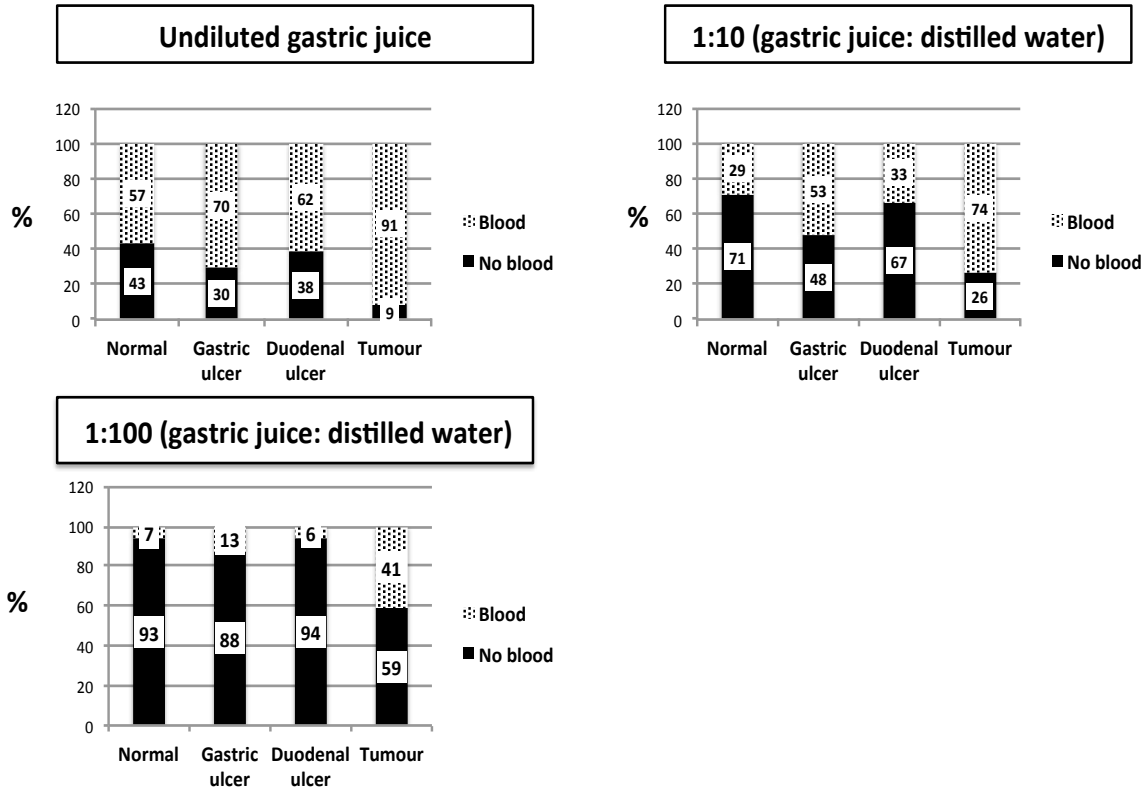

Figure 4.25: Presence of blood in diluted and undiluted gastric juice, stratified by oesophagogastroduodenoscopy diagnosis

A high intensity of blood in gastric juice defined by colour change signifying 2+ or 3+ was higher in patients with gastric cancer 26 (79%) than in those without 55 (23%) [OR 12.7; 95% CI 5-36, *p*<0.0001]. The sensitivity for cancer detection using blood in neat gastric juice was 91% with a specificity of 41%.

The area under the receiver operating characteristic (ROC) curve for gastric cancer detection was 0.66 (95 % CI of 0.6-0.72). Considering the intensity of blood (as defined above) in gastric juice for detection of gastric cancer, the area under the

ROC curve was 0.78 (95% CI of 0.71-0.86). The sensitivity of this approach was 79% with a specificity of 77%.

Table 4.12: The presence of blood in gastric juice was associated with gastric tumours

Gastric juice	Presence of blood	Gastric tumour n=33	No tumour n=243	OR; 95% CI	P
Undiluted	Blood present	30 (91%)	145 (60%)	6.7; 2-35.3	0.0005
1:10 dilution	Blood present	24 (72%)	79 (33%)	5.4; 2.3-13.8	<0.0001
1:100 dilution	Blood present	13 (39%)	16 (7%)	9.1; 3.5-23.3	<0.0001

*Significance testing done with Fisher's exact test

CHAPTER 5: DISCUSSION

In this study, gastric cancer risk factors for adenocarcinoma were investigated. In addition, a strategy that could contribute towards early case detection was explored. The study demonstrated that gastric cancer was widespread throughout Zambia with similar occurrence in men and women; but as these data represented a convenience sample based on referral for OGD and not population-based, they do not permit determination of national prevalence. The majority of gastric adenocarcinoma (GA) was of the intestinal type using the Lauren classification, and of the microsatellite unstable subtype (MLH1 negative). GA was associated with low socio-economic status, frequent exposure to biomass smoke and regular consumption of processed meat. However, regular consumption of fruits and vegetables were protective against GA. *Helicobacter pylori* antibodies did not show any usefulness for GA risk stratification. The study demonstrated a high consumption of salt in Zambia (although an inverse association with GA) and that testing for blood in gastric juice could be used as a simple, cheap and readily available strategy for identification of patients with gastric mucosal lesions. The information presented serve as a basis for future gastric cancer research in Zambia.

Staging for gastric premalignant (GP) lesions showed that most of the patients did not need subsequent endoscopic follow-up. Overall, risk factors evaluated for GP showed similar trends to those of GA but many of the differences did not reach statistical significance.

5.1 An overview of gastric cancer and premalignant lesions in Zambia

This was a hospital-based study and patients referred for oesophagogastroduodenoscopy (OGD) were enrolled. Despite the known limitations of a hospital based study, mapping the town of permanent residence showed a fairly good representation of patients across the country correlating well with the

population distribution in Zambia. Most of the patients enrolled were from highly populated areas including Lusaka, the Copperbelt, Central and Southern provinces.

As previously published, the median age for patients with gastric tumours was at least a decade less than that reported from developed countries with 20% of them being under the age of 45 years (early onset cancers). The prevalence was not significantly different between males and females. Gastric cancer patients had a lower median BMI than controls and coupled with poor outcomes, is suggestive of advanced disease. In addition, gastric cancer patients were more likely to present with persistent vomiting or dysphagia, symptoms most prominent in late gastric cancer.

GA was the predominant type of cancer and studies from developed countries have linked it to low socio-economic status (Barker et al., 1990; Balakrishnan et al., 2017). A similar effect was therefore sought within a predominantly poor population. GA patients were less likely to possess basic household items and they were living in poorer quality houses without ready access to piped or treated water. This effect remained significant after adjusting for rural residence, which is where many of the GA patients were resident.

A high proportion of GA observed was of the intestinal type. None of the proximal tumours were of the diffuse type, conforming to the understanding that diffuse GA has a predilection for the distal stomach and the body-antral transitional zone (Charlton et al., 2004). It has been estimated that globally, 20% of GA is of the diffuse type (Cancer.Net, 2017) but proportions as high as 48% have been reported from New Zealand (Ellison-Loschmann et al., 2017). The proportion of diffuse GA found in this study (which was 33%) is consistent with global estimates and therefore, cannot fully explain the high number of early-onset cancers. In addition, only a fifth of the diffuse cancers were early-onset.

Evidence of loss of MLH1 expression in GA was investigated. Loss of MLH1 expression is generally thought to occur in 13 to 44% of GA (Halling et al., 1999).

The higher proportion of MLH1 loss found in this study and its link to biomass smoke exposure therefore, warrants further investigation. In addition, loss of MLH1 was less likely in patients below the age of 45 years. Carvalho et al. (2004) reported that patients with early onset GA all had MLH1 positive tumours without germline mutations of CDH1, TP53 or RUNX3.

The OLGIM and OLGA staging systems were used to determine GA risk in patients with gastric premalignant (GP) lesions. Many of the patients with GP did not have stages of lesions high enough to warrant further endoscopic follow-up, using current guidelines (Capelle et al. 2010). Compounded with the patchy nature of these premalignant lesions, screening for GA using endoscopy is not only unaffordable but would not be practical to implement in Zambia.

5.2 The role of infectious agents on gastric carcinogenesis in Zambia

5.2.1 *Helicobacter* species

Helicobacter pylori (*H. pylori*) infection is the single most important risk factor for gastric cancer and it is a class 1 carcinogen (IARC, 1994). The prevalence of *H. pylori* in Zambia is high even among healthy volunteers but previously published data did not show any association with gastric cancer (Kayamba et al., 2013). In this study, antibody responses to different immunogenic *H. pylori* proteins were analysed. With the help of collaborators at the Germany Cancer Research Centre in Heidelberg, antibodies to thirteen proteins, which had previously been identified by them as important for *H. pylori* pathogenicity, were tested and compared them between patients with GA, GP and controls.

There was no evidence of an age cohort effect (increasing prevalence with age) for *H. pylori* seropositivity as reported from many western populations (Murray et al., 1997; Kokkola et al., 2003; Michel et al., 2014). *H. pylori* antibodies were generally well correlated with each other. Only levels of Cad (for GA) and Catalase (for GP) were significantly different between cases and controls. To test for a

probable dose effect, the median fluorescent intensity values for each antibody were categorised into quartiles. Overall, this analysis did not change the conclusions showing no significant association with GA or GP. With an understanding that *H. pylori* is the main cause of gastric inflammation, the controls were then divided into those with active or chronic inflammation. Separate comparison of each of these groups with GA and GP showed that active inflammation had significantly higher quantities of CagA, VacA, HcpC and Omp than GA. These data suggest that *H. pylori* alone might not be sufficient for gastric carcinogenesis. It could be an initial trigger, causing acute gastritis, but then other factors are needed to complete the process (Correa et al., 2007). Patients with active inflammation also had higher quantities of GroEL, HP0305, CagA, VacA, HcpC and Omp, than those with chronic inflammation. Therefore, these antibodies could be helpful in discriminating *H. pylori* positive patients with active inflammation without necessarily getting endoscopic biopsies. It could be a helpful biomarker to guide *H. pylori* eradication therapy.

Data on other *Helicobacter* species with gastric disease are scant, and associations have not previously been investigated in Africa. Serological measurements of *H. bilis* and *H. hepaticus* antibodies showed that less than half the patients had positive readings for the former but more patients had evidence of exposure to the latter infection. Higher levels of *H. bilis* antibodies were found in GP patients while only one antibody was significantly associated with GA. Whether or not these findings are of any biological significance is yet to be established. The lack of association of these antibodies with inflammation is against the possibility of a similar pathway to that induced by *H. pylori*.

5.2.2 Epstein Barr Virus and Human Immunodeficiency Virus

Using *in situ* hybridisation, a similar proportion of Epstein-Barr virus (EBV) positive GA to that seen in populations with a lower burden of HIV infection was observed. A previous study reported that HIV infected patients were more likely to

have active EBV infection (Kayamba et al., 2016). This viral activity does not seem to influence EBV associated gastric carcinogenesis. Similarly, neither GA nor GP showed an association with serological measurements of EBV antibodies. However, there was an association between HIV and antibodies against EA, VCA and ZEBRA. EBNA, which mainly signifies past exposure, was higher in the HIV negative group. EBV is a very common viral infection acquired by almost everyone in childhood, and these results suggest that later acquisition of HIV infection could drive the re-activation of EBV. Similar to the assessment of *H. pylori* antibodies, the correlation of these EBV antibodies with each other was evaluated. The results showed that one antibody does not necessarily predict the response of another.

Over half of persons living with HIV infection are in east and southern Africa resulting into a very high disease burden (Avert, 2018). It was previously shown that gastric cancer was not associated with HIV infection (Kayamba et al., 2013), and that antiretroviral therapy did not significantly alter measures of gastric physiology (Kayamba et al., 2018). This study has similarly shown no association between gastric cancer and HIV infection, and that HIV infection itself does not influence oxidative stress to DNA, or increase the risk of EBER positive GA.

5.3 The influence of diet and mycotoxin exposure on gastric cancer and premalignant lesions

Green vegetables, fruits and meat

The traditional Zambian diet, particularly in rural areas is predominantly vegetarian. This study was however still able to demonstrate that daily consumption of green vegetables reduces GA. Many Zambians do not eat fruit on a daily basis and the widely available or affordable fruits are predominantly seasonal. There is considerable evidence from industrialised countries, and these data add more evidence, in support of the protective properties of regular fruit intake. Some investigators reported a benefit of allium vegetables such as onions for protection

against GA (You et al., 1989; Zhou et al., 2011). These data did not show any significant protection against GA, but there was a statistically significant inverse relationship with GP. Therefore, the protective effect of onions could be most prominent during the early stages of gastric carcinogenesis. Regular consumption of eggplants was found to be protective against GA. Eggplants are not part of the traditional Zambian diet, but their consumption is increasing particularly in urban areas. They are solanaceous plants containing glycoalkaloids that are believed to have some anti-carcinogenic properties (Friedman, 2015). This could explain their negative association with GA.

Daily consumption of processed meat was low but still showed a significant association with GA. Neither fish nor chicken consumption had any association with GA.

Consumption of salt

To estimate the 24-hour urinary sodium excretion, the Tanaka and Kawaski methods were used. There was a near perfect correlation of the estimates by these two methods, but the Kawasaki method generated higher values. High salt intake has been linked to several diseases (D'Elia L et al., 2014) and data from this study showed that Zambians consume significantly higher than the WHO recommended levels (median 19 g with Tanaka and 41 g Kawasaki methods). These levels are higher than the 13.5 g average reported from Korea, a country with the highest incidence of gastric cancer in the world (Shin et al., 2011). More than half of the patients enrolled admitted to adding extra salt to their food all the time, a practice strongly suggestive of excessive salt intake. GA patients, however, had less sodium excretion values than the controls. One reason for this observation could be that anorexia and abdominal pain associated with gastric cancer modify appetite and food intake dramatically. Also, hyponatraemia (a common reason for low urine sodium) occurs frequently in very ill patients. Patients who admitted to adding extra salt or preferring very salty food did not necessarily have the highest sodium excretion. This

could have been because individuals who took less salt the day before enrolment had less urinary salt regardless of their past intake or preferences. Therefore, measuring the salt content of food taken over a period of time would be a more accurate way of determining salt intake. Comparing the controls and GP patients showed no difference in estimated 24-hour urine sodium. With evidence of the influence of salt on gastric cancer, (which has not been shown here), it is possible that this effect occurs much later in the carcinogenic pathway after the development of GP.

Aflatoxins and Ochratoxins

The proportion of patients with detectable aflatoxin M1 in their urine was high particularly for those living in urban areas. It could be an indication of poor grain storage especially maize, which is the staple food in Zambia. Many rural households grow their own grain, with domestic storage and are less reliant on commercial maize or groundnuts. Socio-economic status did not show any influence on exposure to aflatoxins suggesting that the source of the toxin could also be from well-packaged commercially available maize and groundnut products consumed by urban dwellers, rich or poor alike. Gastric cancer patients had significantly lower aflatoxin levels than the controls. This could be because of changes in food intake as a result of the illness itself. Also, the metabolism of aflatoxins is quite rapid and the assay used was only validated to determine exposure of aflatoxin ingestion in the prior 2-3 days (Smith et al., 2017). Aflatoxins are well-established hepatic carcinogens and this study has shown that exposure to these toxins is high in Zambia. Similar to a study done in Zimbabwe involving over 1500 pregnant women (Smith et al, 2017), seasonal variations in the levels of aflatoxins measured were observed. Aflatoxin M1 levels were lowest in the months around harvest time when most of the produce is fresh and consumption of stored grain is lowest. In Zambia, the major food crops

such as maize and groundnuts are typically planted in November to December and harvested in April and May.

Exposure to ochratoxins was very high in this patient group. The proportions found were much higher than those reported among Koreans (42%), a population with the highest gastric cancer rates in the world (Jung et al., 2015). But unlike the aflatoxins, there was no significant difference between urban and rural residents or between GA/GP cases and controls. The quantities of ochratoxin excreted in urine were also much lower than those of aflatoxins.

5.4 Biomass smoke and gastric cancer

Zambia has one of the highest deforestation rates in the world with an estimated 250,000 hectares lost per year (NASEM, 2018), most of which is due to over-dependence on charcoal. Data from this study have shown that use of firewood and charcoal and ultimately exposure to biomass smoke contributes to gastric carcinogenesis. Patients exposed to biomass smoke had significantly higher levels of 8-Hydroxydeoxyguanosine (8-OHdG) than those not frequently exposed, suggesting a link between biomass smoke and oxidative stress. This corroborates well with a Norwegian study in which they found that oxidative damage to DNA and repair was induced by wood smoke particles in human A549 and THP-1 cell lines (Danielsen et al., 2009). In addition, long-term inhalation of biomass smoke was reported to induce DNA damage in airway cells (Mukherjee et al., 2013). Differences in measured 8-OHdG between cases and controls could be a result of carcinogenesis and not an aetiological factor. However, these observations suggest a causal pathway, in that DNA damage was increased in patients without GA who were exposed to biomass smoke. The association of GP with biomass smoke was not statistically significant, but this could have been due to the small number of cases evaluated. In addition, it could have been a manifestation of the variable gastric carcinogenic pathways, some of which are known not to follow the Correa pathway.

Measured urinary levels of 1-Hydroxypyrene (1-OHP), a metabolite of polycyclic aromatic hydrocarbons (PAH) was not associated with GA, GP or exposure to biomass smoke. However, 1-OHP might not be the best biomarker for assessment of long-term biomass smoke exposure as metabolism of PAH is very rapid, and therefore checking for metabolites in urine might not necessarily be an accurate reflection of such exposure. As with salt and mycotoxins, changes in behaviour when gastric cancer developed could have led to reduced exposure to biomass smoke resulting in lower 1-OHP levels. In addition, measured PAH metabolites could have also come from other sources such as fumes from diesel engines, resulting in higher readings in patients not reliant on biomass fuels. A study cohort including 256,357 men in Sweden showed that there was an increased risk of gastric cancer in workers exposed to diesel fumes (Sjödahl et al., 2007). Additionally, a 15-year UK cohort study involving over 34 000 employees in eight UK oil refineries found that gastric cancer risk was increased in labourers with long service compared to the UK general population (Rushton et al., 1981). All these data illustrate the need for further research on environmental pollution and gastric cancer.

5.5 Need for, and feasibility of, new approaches to early detection

Poor gastric cancer patient outcomes have usually been attributed to advanced disease stage on presentation. This study showed that many of patients did seek medical attention but were not promptly identified as requiring OGD to facilitate definitive diagnosis. Delays reported by the patients stretched even up to one year after first presentation. The lack of specific alarm symptoms for early gastric cancer makes it difficult for health care providers to suspect early cancer. This study, therefore, explored the feasibility of using a simple method for identifying individuals likely to have gastric mucosal lesions and in need of endoscopic evaluation. The results showed that testing for blood in gastric juice was sensitive for suspected gastric cancer. This strategy could assist health care providers in low-resource

settings. I collected gastric juice during OGD but the juice could also be obtained using a thin nasogastric tube as a simple bedside sample collection tool. Alternatively, the patient could swallow a tethered capsule for detection of haemoglobin.

Endoscopy with biopsy is the gold standard for gastric cancer diagnosis, but it is expensive, invasive and requires trained personnel, making it difficult to implement on a population level in most sub-Saharan countries. In Korea, a high gastric cancer incidence country, its national screening programme using endoscopy was shown to significantly reduce the likelihood of dying from gastric cancer (Jun et al., 2017). Such a programme however cannot be implemented in regions with scarce endoscopic facilities. A more effective strategy, therefore, would be to direct the limited resources to individuals most likely to have early gastric lesions. In Zambia for example, a 38-year audit showed that close to 70% of the endoscopies done were non-revealing (Kayamba et al. 2015). With the correct screening tool, it could have been possible to identify patients who were more likely to have pathology and in need of endoscopy.

Recently, there has been a surge of publications on non-invasive ways of diagnosing gastric cancer and premalignant lesions using easily obtained specimens such as urine or blood but these use molecular technologies, which are difficult to set up in rural Africa. A simpler bedside test, which can deliver the results instantly, would be more useful. The use of urinary reagent strips is one such strategy as they are fairly cheap and readily available even in the most basic centres, using health workers with very basic training.

5.6 Limitations of the case-control approach in understanding cancer risk

There were some limitations that were considered for this study.

5.6.1 Case-control design

With this design, the study was evaluating risk factors that patients had been exposed to. Recall bias could have impacted some data, particularly from the food frequency questionnaire. There could have ultimately been some mis-classified dietary consumption patterns. Secondly, being hospital based, all cases and controls alike had some sort of symptoms.

5.6.2 Biomarkers measured

The 1-OHP, aflatoxin and ochratoxin assays used could not determine long-term exposure. This reduced the power of the study to detect associations, as behaviour or dietary modifications driven by disease could have affected biomarker concentrations in body fluids. Similarly, the 24-hour urine sodium did not account for long-term salt intake.

5.6.3 Inadequate tissue for EBER CISH and immunofluorescence

It was not possible to run EBER CISH on all the GA biopsies as some of the tissue had been depleted when evaluating for histological diagnosis. Endoscopic biopsies are very small about 2-3 mm (Turk et al., 1991) and even after taking a minimum of six biopsies, processing for histopathological diagnosis used most of the tissue in some cases. There was not access to any resected specimens as very few of the patients were offered operative therapy. However, the data obtained, were still very helpful as they correlated well with serology.

5.6.4 Blood in gastric juice

Endoscopic intubation could have caused some mucosal abrasions before collecting the gastric juice. The urinary reagent strips used in this study were sensitive even to small amounts of blood. This could have lead to over estimation of patients with blood in gastric juice and therefore lowering the specificity of the test. It

is therefore imperative that more studies be carried out to confirm the utility of this strategy.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Although these data do not address the true prevalence of gastric cancer in Zambia, they provide evidence that it is widespread geographically around the country, equally affecting males and females, and disproportionately affecting persons from rural areas and of low socio-economic status. The most common type was gastric adenocarcinoma (GA) of the intestinal type, located in the distal part of the stomach. Despite its association with Epstein-Barr virus (EBV) activity, HIV infection had no influence on the prevalence of EBV-associated GA. Biomass smoke exposure was a risk factor for GA possibly mediated through oxidative stress to DNA. Regular consumption of green vegetables, eggplants and fruits reduced, while processed meat increased the odds of developing GA. The median consumption of sodium in Zambia was higher than the WHO recommended maximum but it was not associated with gastric cancer. Mycotoxin contamination of food was common but there is no evidence of it driving gastric carcinogenesis. The multiplex serology assay for *H. pylori* showed limited usefulness for gastric cancer risk stratification in Zambia. There is therefore need to identify other proteins that can be used in populations with the African *H. pylori* strain. The presence of blood in gastric juice was associated with gastric mucosal lesions including GA and showed potential for use as a cheap screening tool.

6.2 Recommendations

In this study, risk factors for a cancer that is poorly investigated in Zambia were investigated. With the above findings, the following are recommendations to the government of Zambia and policy markers, clinicians and the other researchers.

6.2.1 To the government of Zambia and policy markers

1. To include gastric cancer among the national health research priorities to facilitate programmes that will be able to collect population based data
2. To advocate for and facilitate the availability of cleaner fuels such as electricity or solar, in order to reduce exposure to biomass smoke
3. To formulate programmes aimed at sensitizing Zambians on the health benefits of consuming green vegetables and fruits
4. To increase regular food checks for mycotoxin contamination

6.2.2 To Clinicians

1. To take a thorough and comprehensive history from all patients with symptoms suggestive of gastrointestinal disease
2. To be on the lookout for gastric cancer 'alarm' symptoms such as persistent vomiting and dysphagia even among younger adults below the age of 45 years
3. To promptly refer patients with 'alarm' symptoms for OGD
4. To promptly consult available gastroenterologists or trained physicians when in doubt

6.2.3 To the scientific community

1. To conduct clinical and molecular genetic studies on gastric pathology including cancer
2. To design studies that will describe pathophysiological mechanisms of factors that I have identified as influencing gastric cancer development
3. To identify molecular signatures necessary for the formulation of precision treatment for gastric cancer in Zambia.

6.3 Future work

In this study, biomass smoke exposure has been identified as one of the factors contributing to higher occurrence of gastric adenocarcinoma in poorer communities. As part of future work, metabolic pathways of the constituents of biomass smoke that lead to gastric carcinogenesis will be investigated. Also to be explored are associated genetic and host response mechanisms involved. With the consistent finding of a high proportion of early onset gastric cancer in Zambia, a longitudinal follow-up study to run over eight to ten years will be designed. This study will provide conclusive evidence of gastric cancer risk factors predominantly affecting young adults. It will also provide insights into pathogenesis and progression of premalignant lesions.

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APPENDICES

APPENDIX 1: PROTOCOL FOR I*N SITU* HYBRIDISATION FOR EPSTEIN-BARR VIRUS

Commercially available EBV CISH Detection Kits (Master Diagnostica, Granada, Spain) were used to determine the presence of EBV in gastric tumours. With strict adherence to the manufacturer's instructions, the following procedure was followed:

1. Dewaxing and hydrating

To begin the dewaxing process, biopsies mounted on electrically (charged (polylysine-coated) slides were placed in a 60^{o}C oven overnight. They were then immersed in xylene twice for ten minutes each. Rehydration started with absolute ethanol for five minutes twice, followed by 80% ethanol for 5 minutes, then 70% ethanol, and lastly the slides were rinsed off with distilled water for ten minutes.

2. Enzymatic digestion

For enzymatic digestion, 5 µl of concentrated proteinase K solution was diluted in 2ml of Tris-buffered saline (TBS) and applied onto the tissue with an eight-minute incubation at room temperature (RT). The slides were then washed three times with TBS.

3. Hybridisation

For hybridisation, EBER CISH an artificially synthesized Peptide Nucleic Acid (PNA) Probe was applied and a cover slip placed on top. The slides were then incubated for one hour at 37 ^{o}C.

4. Detection and visualization

After the incubation, the slides were washed with TBS three times. In a careful series, the slides were treated as follows:

i. 200 µl of peroxide blocking reagent for ten minutes at RT, then washed with TBS three times

ii. 200 µl of digoxigenin antibody for ten minutes at RT, then washed with TBS three times

iii. 200 µl of primary antibodies amplifier master for ten minutes at RT, then washed with TBS three times

iv. 200 µl of master polymer plus HRP for ten minutes at RT, then washed with distilled water three times

v. A drop of chromogen concentrate was then mixed with DAB substrate buffer and applied onto the tissue for five minutes at RT and followed by a wash with distilled water three times.

5. Contrast staining and mounting

For enhanced tissue visualization, the slides were then stained with contrast heamatoxylin, blueing in tap water. Dehydration was then performed with increasing concentrations of alcohols and xylene and finally slides were mounted under coverslips and visualized under a microscope at X100, X200 and X400 magnifications. The kits from Master Diagnostica Granada, Spain, included positive control slides.

APPENDIX 2: DATA COLLECTION SHEET (QUESTIONNAIRE)

The investigator administered this questionnaire

Basic demographic characteristics

DATE:_____ ID number _____

Cell number: _____

Relative or caregiver's number: _____

Cost of transport to UTH (using public bus) _____

Town/city of permanent residence: _____

Duration of stay in this town/city: _____

Sex: 0- male
1- female

Age: _____ years

Weight: _____ Height:_____

Marital status:

0- Single 1- Married 2- Widowed
3- Divorced 4- Separated 5- Co-habiting

Occupation: _____

Level of education attained:

0-none 1-primary 2-secondary 3-tertiary

Household characteristics

Type of house:

0- brick 1- mud 2- grass 3- metal sheets 4- other (specify)_____

Location of the cooking area:

0- inside the house, separate room 1- inside the house, in the room for sleeping
2- outside the house, separate structure 3- outside the house, no structure

How frequently respondent cooks:

0- more than once a day 1- once a day 2- more than once a week

3- once a week 4- more than once a month 5- once a month
6- very rarely 7- never

Fuel used for cooking:

0- electricity 1-charcoal 3-firewood 4-gas 5- other (specify)_____

Duration of use for the fuel mentioned above: _____ years

If applicable, fuel used in the past:

0- electricity 1-charcoal 3-firewood 4-gas 5- other (specify)_____

Household items:

	Yes	No
Television		
Decoder		
Fridge		
Computer		
Internet		
Microwave		
Car		

Source of water for the household:

0- piped into the house 1- piped to outside tap 2- drawn from neighbours
3- borehole 4- covered well 5- open well
6- stream/ River/ Lake 7- other (specify)

Medical history

Main symptoms:

_____

_____

Duration of symptoms:
_____(days, months or years)

Time since the first contact with a health care worker
_____(days, months or years)

History of acid suppressive drugs PPI or other 0-No 1-Yes

History of gastrointestinal cancer in the family:

0- None 1- Oesophageal 2- Gastric 3- Duodenal 4- Colon
5- Rectum 6- Liver 7- Pancreas 8- Biliary

Relation to the cancer patient: _____

FOOD FREQUENCY QUESTIONNAIRE

Date: …….. Participant's ID number……

Foods	Average use in the past year Please put a mark (√) on every line as appropriate Never or less than once a month	1-3 times a month	Once a week	2-4 times a week	5-6 times a week	Once a day	2-3 times a day	More than 3 times a day	Write down the typical serving size
Animal Protein									
Beef									
Goat									
Pork									
Game									
Chicken									
Hungarian									
Polony									
Ham									
Bacon									
Salami									
Sausage									
Canned meat									
Fish									
Kapenta									
Bream									
Buka Buka									
Tiger									
Other fish									
Vegetables									
Green leafy vegetables									
Egg plant									
Tomatoes									
Green beans and peas									
Onions									
Fruits									
Banana									
Mangoes									
Papaya									
Guava									
Orange									
Lemon									
Baobab									
Apple									

Foods	Never or less than once a month	1-3 times a month	Once a week	2-4 times a week	5-6 times a week	Once a day	2-3 times a day	More than 3 times a day	Write down the typical serving size
Pineapple									
Strawberries									
Mulberry									
Avocado									
Watermelon									
Fruit juices									
Zambian fruits									
Beverages									
Chibuku beer									
Kachasu									
Packaged spirits									
Bottled beer									
Ordinary spirits									
Wines									
Ciders									
Traditional brews									
Current smoking									
Former Smoking									

QUESTIONNAIRE ON SALT INTAKE

	All the time	Very often	Not often	Never
Added salt				
Salt preference				
Not salty				
Slightly salty				
Salty				
Very salty				

APPENDIX 3: INFORMATION SHEET

You are invited to take part in a study looking at the factors that leading to the development of stomach cancer. Stomach cancer is a disease characterized by a mass or growth in the stomach. As it grows, the affected person may start vomiting, have stomach pain and the blood levels can go very low. The person will also start losing weight. However, many people with this disease do not show any obvious signs until the stomach growth has become really big or even spread to other parts of the body. Many people who develop this disease get very sick and die quickly, because they come to the hospital when it is too late. In order to reduce such deaths, there is need to find out which factors are associated with the development of this disease. In this study, we are looking at these factors and trying to find ways of preventing stomach cancer and having it treated before it spreads to other parts of the body.

Who is doing the study?

Dr Violet Jolezya Kayamba is the main investigator on this study. The study is being conducted at the endoscopy unit of the University Teaching Hospital.

What is the purpose of the study?

The purpose of this study is to find out the factors around us that could be leading to the development of stomach cancer.

How is the selection being done?

We are including any individuals sent to this unit to have the test you are about to have called endoscopy, as long as they are willing to participate. We will however, not include those who are not able to swallow food properly.

What procedure is going to be done?

You have already been asked to come for a procedure known as endoscopy to check your food pipe, stomach and the first part of your small intestines. Before we do the endoscopy, we will ask you to swallow a capsule, which will then be pulled out of your stomach using a small string attached to it. This will take about two minutes. During the endoscopy, we will carefully examine the stomach to see if there are any abnormalities that could lead to cancer of the stomach. We will take some tissue, called a biopsy from the stomach that will be taken to the laboratory for analysis. Taking biopsies from the stomach is completely painless and there is just minimal bleeding which stops almost immediately. We will also get some fluid to check how much acid is in the stomach. After the procedure, 10ml blood will be taken and sent to the laboratory for testing. In addition, we are going to ask you some questions regarding your health do a physical examination.

What are we going to do with the samples we take?

Tissue samples collected from the stomach will be sent to the laboratory within the UTH for evaluation. At the laboratory, they will check for the presence of any changes that can lead to the development of cancer. The blood samples will be sent to the laboratory where chemicals that could lead to cancer will be checked. In addition the blood will be checked for the presence of HIV infection. We will offer you counseling before checking for HIV. After getting the results, counseling will again be offered. You are free to tell us if you are not willing to have an HIV test.

What are the possible benefits to you?

As a participant in this study, you will be have the chance of being thoroughly checked for the presence of stomach changes that could lead to development of cancer in future. If you already have cancer, we will have you referred to the Cancer Diseases Hospital and delays will be minimized. Otherwise, there is no financial or

material gain for participation but you will assist doctors to understand how stomach cancer develops and find ways of preventing it. Participation in this study will not cost you any money. We will give you a transport refund for coming to the hospital to collect relevant results for tests done in the study.

What are the possible disadvantages to you?

There are no major risks that will result from study participation. However, as we draw blood from your arm you will feel some discomfort at the prick site and there is a small chance of you bleeding excessively or getting an infection at the site. This is very rare as we draw blood from many patients without any problems. We assure you that only well-trained and experienced personnel will draw the blood. The Hemopill is the size of a standard capsule and is easily swallowed with water. Just like swallowing a capsule, you will fill it as you swallow it and also as it is being taken out. It is however, completely painless.

Study related injuries

Should there be any study related injuries, the principal investigator, who is a trained physician, will be readily available to treat them. Appropriate referrals will be made should that be deemed necessary. However, there are no major risks to the patients that will be merely due to study participation.

Confidentiality

Your details will be recorded in a form, which will be locked away in an office here in the UTH. Your details will be entered on a computer but only in coded form and your name will not be included. Any information and results obtained will remain absolutely confidential, and other family members or work colleagues will not be granted access to this information.

The study is voluntary.

You do not have to participate in the study if you do not want to, and even if you refuse to participate in the study, you will be provided with the best care available and there will be no discrimination of any sort. If you do agree, you are also free to change your mind at any time. You are also free to decline giving answers to any questions that you do not want to respond to. The Research Ethics Committee of the University of Zambia has approved this research study and their contact details are given below.

Contact details of the Principle Investigator: Dr Violet Jolezya Kayamba, Department of Internal medicine, University of Zambia, PO Box 50398, Lusaka, Phone 0977 254 854, e-mail viojole@yahoo.com.

Contact details of Research Ethics Committee: The chairman, UNZABREC office, Department of Anatomy, Ridgeway Campus, Nationalist Road, PO Box 50110, Lusaka (phone 0211 256067) unzarec@unza.zm

Nyanja translation

MAU OLONGOSOLA

Mwaitanidwa kutengako mbali kumaphunzilo kuona za magulu amene aleta kuchuluka kwazilonda zamumala ndi unyinji makalidwe kapena kukula mumala. Pamene zikula munthu amene ali ndi vuto iyi nthawi zina amaluka, kubaba kwamumala ndipo magzi agate ochepa. Munthu anga yambe kuonda ngakale nditelo saonesa zizindikilo mphanka mimba kukula kapena kupeleka kuziwalo zina zatupi. Anthu ambili amene ankala ndi matenda aya akala odwala kwambili ndikufa musanga, chifukwa amabwela kuchipatala muchedwa. Pofuna kuchepesa infa

zachoncho, chofunikila nikuziwa mbali ziti zimene zili pamozi kuti zikulise nthenda iyi, pamhunzilo awa tifuna kuona zofunikila ndiku chilisa kalibe kupita mbali zina zathipi.

Ndani achita mamphuzilo?

Dotolo Violet Jolezya Kayamba ndi akulu amene akufufuza mamphuzilo awa, mamphuzilo awa achitika kukiliniki ya endoscopy ya University Teaching Hospital.

Chilnga cha mamphunzilo nichabwanji?

Chilinga cha mamphuzilo awa ndi ku peza magulu mwaife zimene zingalete kukula kwazilonda zamumala.

Kusanka kuzankala bwanji?

Kizatenga munthu aliyense amene azathumidwa kukiliniki akapimindwe yochedwa endoscopy. Mulinga alikufuna kutengako mbali. Sitizaikamo aja amene alepela kumena bwino chakudya.

Ndi njilayambwanji azachitilamo?

Munafunsidwa kale kubwela kukapimindwa kuchedwa endoscopy. Kuona pipe yanu yachakundya mumala ndi njila yoyamba mbali ya makumbo yanyono. Yikalibe kukupimani ku endoscopy, tizakupempani kumwako kamankwala paneme pambuyo pache kachoka mumala mwanu. Kuzebezensa kanthu kamene ali pamozi, ichi izachitika panthawi inyono yak u pima endoscopy. Kizapima bwino mumala kuona agti muli zinthu zimine zingalese zilonda zamumala, tizachosa tuntu tochedwa (biopsy) kuchosa mumala mwanu kuti akapime kupimila kutenda tuntu (biopsy). Kuchosa mumala sichiwawa ndipo kumachoka tumagazi kunyono kumene tumaleka thawi inyono, kizathengako timadzi kuti ti pime kuona acidi yamumala. Pambuyo pazopima, tizathengako madzhi ndikupeleka kuti akapime. Tizakufunsani mafunso pa zaumoyo wanu ndi kupima kwina.

Zimenekizatenga tizachitanazo chani?

Zonze zimene tizatenga kuchokela kizathuma kopimina kulabu muchipatala cha Univeristy Teaching Hospital muno kuit tione zochitika kuja. Azaona zones zamene zipezeka angati chachinja zamene zingaletele chichulka kwazilonda mumala, magazi onse amene azathengedwa azpita kopimila kumene mankwala amene aleta zilonda zamumala azaona, pakupima zine, mazazi azapimamo a doyo ka HIV. Tikapeza zotulukamo tizakuyuzani zopezekamo. Muliomasuka kutiuza kuti fumufuna kupima HIV.

Ndipindu ya bwanji yamene muzapeza?

Ngati muthengako mbali mumamphuzilo awa, muzankala ndi mupata kuona bwino pa kusintha kwamumala pazimene zileta kuchuka kwazilonda kusogolo, ngati mulindizilonda kale, tizakutumani kuchipatala chachikulu chazilonda cha cancer disease hospital. Sikuzankala kuchendwa angankale ndi choncho ndipi sikuzanthala malipilo kapena zina zopezamo akutengako mbali koma muzathandiza a dotolo kuziba kuti zilonda zipaka bwanji ndikupeza ngila yo chingiliza kukenga ko mbali mumamphuzilo, simukapeleka ndalama ilionse. Tizakupasani, ndalama yukweleleku kupita kuchipatala ku kengako zithulukamo mamamphuzilo.

Zovuta nizabwanji zizapezekamo?

Kulibe zikulu ziyofya zingayofwe kutengako mbali mumamphuzilo ngankale choncho pamene titenga magadzi kukwanja kwanu. Muzamva muwawa panyono. Kumbali thuwi zina munga mve kuwawa pangono nthawi zina mungachose magandhi kapenna kuchosako zina, chamene chimachitika kuodwala pakuchosamagadzi ayo opanda mavuto. Tikulonjeza kuti ameneyo amene anamphuzila kufikapo ndiyamene azachosa magadzi. Hemopill siyizi yamapilusi ndipo sivuta kumela ndimdzi pakumela pilusi ya capsule muzanvela ndi kuvulula ndipo siwawakonse.

Ngati kukala kuzichita mumamphuzilo

Asinyanga ochuka azakalako kupasa tandizo. Ngati kuzichita kuzankala kwakukulu, asinyanga azauthumizani zipatala zoyenela, koma muyenela kuziwa kuti kulibe kozichita kunga chitike mumamphuzilo aya.

Zachnsinsi

Zonse zimene tizalemba zizasungidwa pamondzi, zimene zizakomedwa muofesi kunoku UTH. Zonse zimene tizakambilana, kizalowesa mucompta koma, muchokambila chapmuta. Zinayano sizankalapo zokambilana ndi zi tulukamo. Zonzs zizankala zachinsinsi. Abale anu kapena akunchito sazanthala ndi mpata kuzokambilana.

Kumamphuzilo nikuzipeleka

Shimunga tengeko mbali ku mamphuzilo ngati simufuna kutengako mbali. Muzathandiziwa bwino. Sitizayikapo kapatulula mulimonse, ngati mwavomela. Munga chinje nzelu zanu panthwi ilionse. Muliomasuka kusanyanka mafunso alionse amene simufuna kuyanka. Bungwe ayikulu yoyanganila pazamamphuzilo ya UNZA avomela kuti mamphuzilo awa angakalepo ndi mungawa beze pa ma numbala olemba pansikwa ofufuza a dotolo.

Yaba dotolo ni: Dr Violet Jolezya Kayamba, Department of Internal medicine, University of Zambia, PO Box 50398, Lusaka, Phone 0977 254 854, e-mail viojole@yahoo.com.

Yabo pasa mbali ni: The chairman, UNZABREC office, Department of Anatomy, Ridgeway Campus, Nationalist Road, PO Box 50110, Lusaka (phone 0211 256067) unzarec@unza.zm

APPENDIX 4: CONSENT RECORD SHEET

I confirm that I understand the information I have been given about the study. I agree to participate in the study. I confirm that I am joining the study of my free will and that I can withdraw at any time without affecting the care available to me. I understand what will be required of me. I also understand that I can refuse to answer any questions that I do not want to respond to.

(Nyanja translation)

CHIBVOMERESEDWE

Ndi ku tanthauza kuti ndamvensesa maphunzilo aya. Ndipo nabvomera kunkalamo. Ninga chokemo panthawi iliyonse gati nafuna. Ndipo ndabvera kuti nigakane kuyanka mufunso yamene sinilikufuna kuyanka.

Name of Participant: …….

Signed: ……..or thumb print……....

Date: ……..

Name of Witness: ……..

Signed: ……or thumb print……...

Date: ……

I confirm that I have explained the information fully and answered any questions

Signed by Investigator: ……

Name: ……....

Date: …….

APPENDIX 5: UNIVERSITY OF ZAMBIA BIOMEDICAL RESEARCH ETHICS COMMITTEE APPROVAL

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
E-mail: unzarec@unza.zm

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

Assurance No. FWA00000338
IRB00001131 of IORG0000774

16th June, 2016.

Our Ref: 005-03-16.

Dr. Violet J. Kayamba,
University of Zambia,
School of Medicine,
Department of Medicine,
P.O Box 50398,
Lusaka.

Dear Dr. Kayamba,

RE: RESUBMITTED RESEARCH PROPOSAL: **"GASTRIC CANCER AT THE UNIVERSITY TEACHING HOSPITAL IN LUSAKA; AN ASSESSMENT OF RISK FACTORS AND USE OF THE HEMOPILL" (REF. No. 005-03-16)**

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 14th June, 2016. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr. S.H Nzala
VICE-CHAIRPERSON

Date of approval: 16th June, 2016. **Date of expiry:** 15th June, 2017.

APPENDIX 6: NATIONAL HEALTH RESEARCH AUTHORITY APPROVAL

THE NATIONAL HEALTH RESEARCH AUTHORITY
C/O Ministry of Health
Haile Selaisse Avenue,
Ndeke House
P.O. Box 30205
LUSAKA

MH/101/23/10/1

7th July, 2016

Dr. Violet Kayamba
Department of Medicine
P. O. Box 50398
Lusaka

Dear Dr. Kayamba

Re: Request for Authority to Conduct Research

The National Health Research Authority is in receipt of your request for authority to conduct research titled **"Gastric cancer at the University Teaching Hospital in Lusaka: an assessment of risk factors and use of hemopill."**

I wish to inform you that following submission of your request to the Authority, our review of the same and in view of the ethical clearance, this study has been approved to carry out the above mentioned exercise on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, and all key respondents.

Yours sincerely,

Dr. P. Chanda-Kapata
For/Director
National Health Research Authority