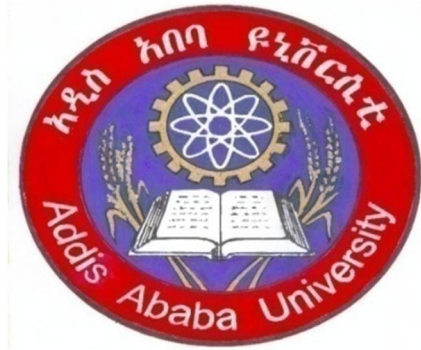
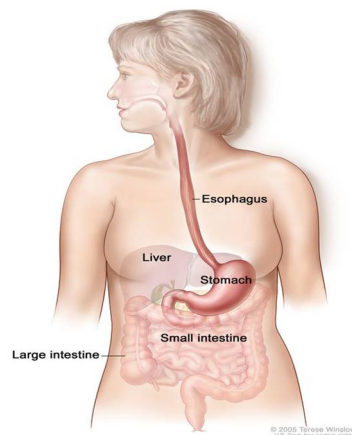


**Addis Ababa University, College of Health Sciences,
School of Medicine, Department of Medical Microbiology
Immunology and Parasitology**



**The Epidemiology, Oral Microbiome Signature, and Mycotoxins
Exposure of Esophageal Cancer Patient in Ethiopia**



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March, 2025

Addis Ababa, Ethiopia

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Dissertation Declaration

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I, Girma Mulisa Misgana, declare that this dissertation is my original work. I have written and submitted to Addis Ababa University College of Health Sciences, Department of Microbiology, Immunology and Parasitology never to any other institution in any form for evaluation. All the information here is dully acknowledged and I have never used any other source except those cited ones.

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College of Health Sciences
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**The Epidemiology, Oral Microbiome Signature, and Mycotoxins Exposure of
Esophageal Cancer Patient in Ethiopia**

By
Girma Mulisa Misgana

A thesis submitted to the Department of Microbiology, Immunology and Parasitology; College of
Health Sciences; Addis Ababa University in the fulfilment of the requirements for the Degree of
Doctor of Philosophy in Medical Microbiology

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June 19, 2025

Statement of author

Here I justify that the general research question and its general scientific and social perspective were proposed by me and my advisors. Also I have not used any sources other than those listed in the bibliography and identified as references. I further declare that I have not submitted this thesis at any other institution in order to obtain a degree.

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.

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Abbreviations

AFB2	Aflatoxin B2
ASVs	Amplicon sequence variants
CIT	Citrinin
CPA	Cyclopiazonic acid
DNA	Deoxy ribonucleic acid (DNA)
DON	Deoxynivalenol
EAC	Esophageal adenocarcinoma
EC	Esophageal cancer
EFSA	European Food Safety Authority
ENN B	Enniatin B
ESCC	Esophageal Squamous Cell Carcinoma
IARC	Agency for Research on Cancer
NIV	Neosolaniol, nivalenol
OTA	Ochratoxin A
SSA	Sub-Saharan Africa
TA	Tenuazonic acid
TME	Tumor microenvironment
ZAN	Zearalanone
α -ZEL	Alpha-zearalenol

Abstract

Background: Ethiopia has a disproportionately high prevalence of esophageal cancer (EC), with 80% patients diagnosed at advanced stages and poor five-years survival rates. The disease exhibit notable geographic variation, particularly in the Arsi-Bale district, where traditional risk factors like alcohol and tobacco use are uncommon. This study aimed to characterize the epidemiology and histology of EC in this high-incidence region, assess the etiological roles of the oral microbiota and multi- mycotoxin exposure, and evaluate the oral microbiome as a potential none-invasive diagnostic biomarker.

Methods: The study was conducted in the Arsi-Bale district of Ethiopia's Oromia region. A retrospective cross-sectional study design was used to analyze the epidemiology and histology of 630 EC cases, while a residence-matched case-control study assessed association between oral microbiota, mycotoxins exposure, and EC. The oral microbiome of 103 EC cases and 108 healthy controls was characterized using V4 16S rRNA sequencing, with data processed via QIIME 2 and R 4.4.1. Comparative analysis included datasets from Tanzania, Venezuela, Uganda, and the American Gut Project. Biomarker discovery models were trained and validated on Chinese cohort. Plasma samples from 166 EC patients and 166 controls were analyzed for 39 mycotoxins using liquid chromatography-tandem mass spectrometry, and multivariate binary logistic regression analysis was applied to assess risk factors.

Results: A retrospective cross-sectional study of 630 EC cases revealed unique epidemiological features, including higher prevalence among younger females and predominance of esophageal squamous cell carcinoma in the distal esophagus. Oral microbiome profiling using V4 16S rRNA sequencing in 103 EC cases and 108 controls identified low microbial diversity and enrichment of Cluster 2 communities positively associated with EC (AOR=3.4[95% CI: 1.8-6.0]). Forty-nine differentially abundant species were identified, with distinct patterns between cases and controls. The oral microbiome demonstrated high diagnostic accuracy for distinguishing EC cases from healthy controls in Ethiopia (AUROC=0.91) and predicts disease status in an external cohort from China (AUROC=0.74), highlighting its potential as a non-invasive biomarker. Additionally, plasma analysis of 166 EC patients and 166 control revealed significant associations between EC and exposure to multiple mycotoxins (AOR= 2.54 [95% CI: 2.10-3.07]), particularly tenuazonic acid (AOR= 1.88 [95% CI: 1.68-2.11]), suggesting their etiological role in the region.

In conclusion, EC in Ethiopia is characterized by unique epidemiological patterns, with younger females disproportionately affected. The oral microbiome and mycotoxins exposure may contribute to its etiology, offering insights into localized risk factors for prevention and control. The oral microbiome also show promise as non-invasive diagnostic tool for EC, under scoring the need for targeted interventions addressing socio-cultural, dietary, and environmental factors specific to Ethiopia.

Key words: Esophageal cancer, Esophageal cancer Epidemiology, Mycotoxins, Oral Microbiome, and Biomarkers

Chapter 1: General Introduction

1.1. Background

Cancer is a disease in which transformed cells proliferate uncontrollably and acquire epigenetic and genetic changes in the surrounding environment as a result of interactions with the host and other causes (1). The biological characteristics that distinguish cancer cells from healthy cells during their development are known as cancer hallmarks, which include maintaining proliferative signaling, avoiding growth suppressors, preventing cell death, permitting replicative immortality, triggering angiogenesis, initiating invasion, and metastasis (2). The foundation of these cancer cell capacities is the accumulation of genetic and epigenetic changes triggered by environmental exposure (3). Cancer may begin in any part of the body and progress to other organs in advanced stages (4).

The formation of cancer is a complex process that unfolds over an extended period and involves both host-related factors and environmental exposure variables (**Figure 1**). During this process, normal cells undergo changes to become preneoplastic and then invasive carcinoma (5,6). Depending on the type and vulnerability of the tissue, these exposures can cause inflammation. Chronic inflammation predisposes individuals to gastrointestinal cancer, the most common and fatal type of cancer (carcinoma of the esophagus, stomach, duodenum, colon, rectum, liver, gall bladder, and bile ducts) (7). Furthermore, altered cells encourage the inflow of cells expressing inflammatory mediators that boost pro-tumoral activities (8).

Cancer is becoming an increasingly global health concern. According to the International Agency for Research on Cancer (IARC), there were approximately 10 million cancer-related deaths and nearly 20 million new cancer cases globally in 2022. According to projections that consider population growth and age, over 35 million additional cancer cases are expected to occur by 2050, which is a 77% increase from 2022 (9). Global projections showed a large increase in cancer incidence and a decline in mortality rate; however, in sub-Saharan Africa (SSA), both cancer incidence and mortality were anticipated to increase considerably(10). Lung, female breast, prostate, colorectal, and stomach cancers are the top five cancer types worldwide (9,11) while esophageal, liver, and pancreatic cancers are the most prevalent aggressive cancer types with a very poor five-year prognosis (12). The control and prevention of cancer are made more difficult by the low awareness of cancer and the limited understanding of its causation in SSA. Significant mortality in SSA is also a result of the lack of adequate diagnostic resources and available cancer treatment options in the region.

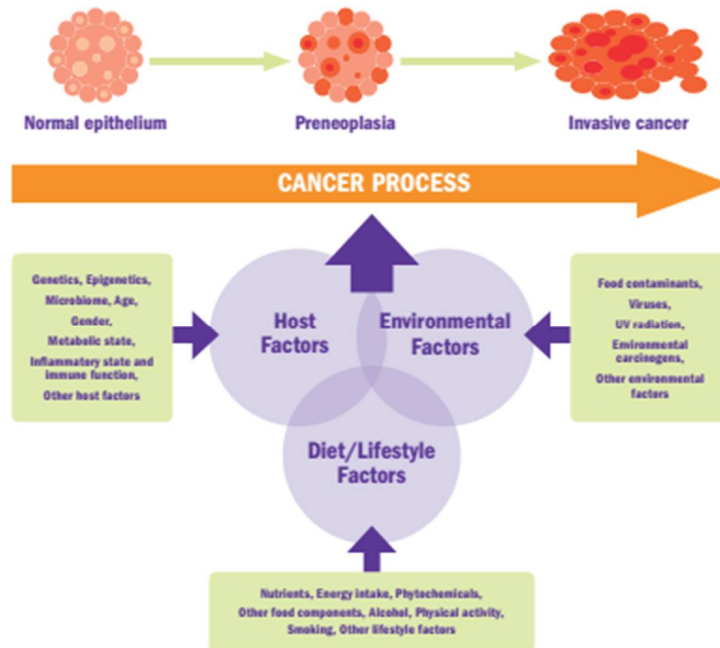


Figure 1: Cancer process, adapted from the World Cancer Research Fund Network (13).

Esophageal cancer (EC) ranks as the ninth most prevalent cancer globally and is the sixth foremost cause of cancer-related mortality, distinguished by a poor prognosis and a five-year survival rate below 20% (14). The two common histological subtypes of EC are Esophageal Squamous Cell Carcinoma (ESCC) and esophageal adenocarcinoma (EAC), of which ESCC accounts for more than 85% of its histological types (11,15). These two subtypes are biologically and epidemiologically distinct (16). ESCC originates from the inner layer of the esophagus, which is the mucosa that is naturally covered with squamous cells (**Figure 2**). ESCC results from a multistep process in which the accumulation of genetic mutations leads to the generation of precancerous lesions that progress into invasive carcinomas (17).

The esophagus is a muscular tube organ that carries solid food and liquid from the mouth to the stomach for further digestion, as shown in **Figure 3** (18). It shares the complex architecture of the gastrointestinal tract anatomy with a special difference in having submucosal glands that have a specific protective function, squamous lining that exists nowhere else in the gut except the anus, and submucosal nerve plexus, all of which have specific responses to injury (19).

Prominent geographic variations in the incidence and prevalence of EC have been observed. Globally, the incidence and mortality associated with EC are high in regions of Eastern and

Southern Africa and Eastern and South Central Asia(20). Geographically based marked histological type variation was also observed. EAC is most common in developed countries (21,22) with increasing trend has been reported worldwide (14) while ESCC is the major histological type in developing countries mainly in African and Asian EC belt (14,23,24). Different risk factors were also identified for both histologies, with alcohol consumption and tobacco use being strongly associated with an increased risk of ESCC, while gastroesophageal reflux disease, obesity, and cigarette smoking were established risk factors for EAC (21,22). In developing countries, deficiency of essential micronutrients, exposure to polycyclic aromatic hydrocarbons, thermal injury due to hot food and beverage consumption, oral health, and exposure to biomass fuels (25–27) have been reported to be associated risk factors for EC. The use of chemical containers and exposure to X-rays have also been reported to be risk factors for EC (28), showing a combination of multi-factorial involvement in the occurrence of EC.

Globally, the incidence of EC is higher in males (70%) than in females (29,30). Similarly, in Africa, where EC incidence is high (Eastern and Southern Africa), a significant male sex excess with EC has been reported (31). This sex-based difference in the incidence of EC has been attributed to the fact that males are more likely to engage in risk factors associated with the development of EC, such as smoking and heavy alcohol consumption (32,33). Furthermore, hormonal differences between males and females may contribute to the development of ESCC. For example, estrogen has been shown to have a protective effect against the development of ESCC, which could explain the lower incidence rates in females in most populations worldwide (32,34).

In Africa, EC is a major public health problem, with high incidence and mortality rates reported in several countries (35). The lack of awareness and screening test for EC contributes to late diagnosis, leading to poor outcomes (36,37). Moreover, therapeutic alternatives are sometimes constrained by the high cost of therapy and the scarcity of cancer care facilities in numerous African nations. In Ethiopia, EC is among the top ten cancer types, with an age-standardized incidence rate of 2.7(38–40), with a higher prevalence in the Arsi-Bale district of the Oromia regional state (41,42). Similar to other African countries, there is no screening program for EC in Ethiopia, leading to late diagnosis, poor survival, and limited treatment options. This warrants prioritizing primary prevention by identifying the specific risk factors of EC in Ethiopia.

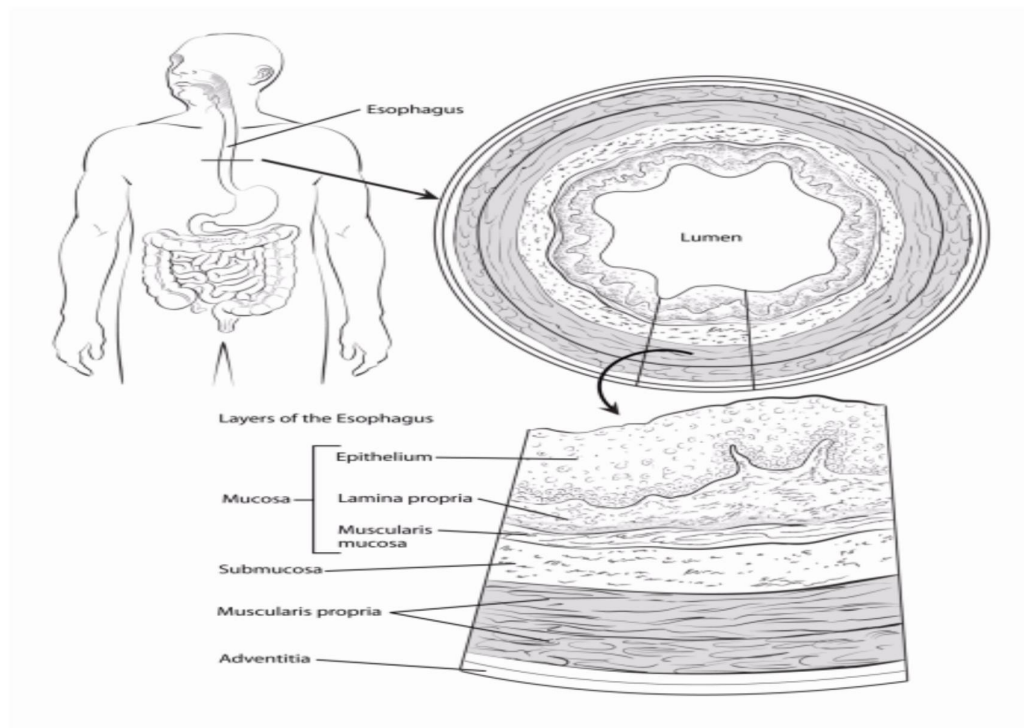


Figure 2: The structure of esophagus wall (Adapted from American Cancer Society, cancer.org | 1.800.227.2345)

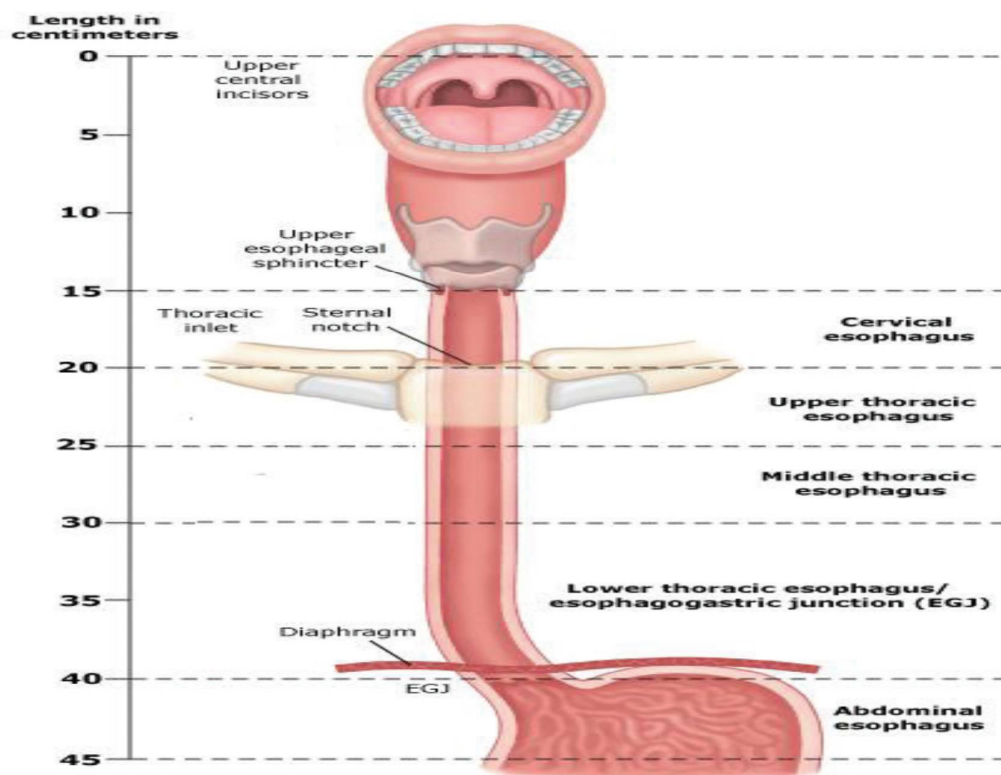


Figure 3: Anatomy and different parts of esophagus (18)

1.2. Literature Review

1.2.1. Esophageal Cancer

EC is an aggressive form of cancer that affects the esophagus, which is the muscular tube that transports meals from the oral cavity to the stomach (43). ESCC and EAC are the two primary histological subtypes of EC (44). With its molecular subgroups of ESCC 1, ESCC 2, and ESCC 3, ESCC makes up over 90% of EC worldwide and is more prevalent in nations with limited resources. Esophageal EAC is the most prevalent histologic type in industrialized nations and is on increase worldwide (16,45). EAC occurs at the distal and middle esophagus, whereas ESCC typically occurs along the proximal and middle esophagus. In addition to these clear distinctions, each has unique molecular characteristics. For example, ESCC frequently has genomic amplifications of CCND1, SOX2, and/or TP63, while EAC has more frequent amplifications of ERBB2, VEGFA, and GATA4 and GATA6(45). Additionally, this histological classification is important for both therapeutic and prognosis purposes(16,22).

1.2.1.1. *Epidemiology of Esophageal Cancer*

EC ranks sixth globally in terms of cancer-related deaths and is the ninth most frequent type of cancer, with a poor five-year survival rate of 20% (14). According to estimates, there were 544,100 EC deaths and 604,100 new cases worldwide in 2020. These figures translate into age-standardized incidence and mortality rates of 6.3 and 5.6 per 100,000, respectively (11). 880,000 EC deaths are projected in 2040, based on data from 2020(15). The incidence of EC has shown clear geographic diversity. Incidence of EC and related mortality are high mainly in Eastern and Southern Africa and Eastern and South Central Asia (20). Geographical variation was also observed in histology. The most prevalent histological type in industrialized nations is EAC, whereas ESCC is the most prevalent in developing nations, primarily in the African and Asian esophageal cancer belt(14,23,24). EAC is more common in countries like the United States, the United Kingdom, France, Switzerland, Denmark, Italy, Slovakia, the Netherlands, Australia, and New Zealand. However, ESCC is prevalent in developing countries(46) and its frequency has been rising over time in East, Central, and Southern Africa(47) as well as in a north-south corridor that runs from Ethiopia and Sudan to South Africa(48). There were notable increases in the number of EC cases in South Africa's rural and Eastern Cape districts(49), Western Kenya (50,51), Darfurian tribes in Sudan(52), and Tanzanian communities residing in Kilimanjaro regions(53). Given its rising prevalence

and related mortality, it was proposed as early as 2010 to classify EC as the top health problem in SSA (54).

The African Esophageal Cancer Corridor includes Ethiopia(**Figure 4**)(55). EC is one of the top ten cancer types in Ethiopia(38) and is on the rise (39), with a notably elevated incidence in the Arsi-Bale district of Oromia regional state(56–58). In Ethiopia, the majority of patients were from rural areas and work as farmers, and the five-year survival rate was low. Patients are diagnosed at an advanced stage of the disease (59).

The incidence and death rate of EC varied by gender. Worldwide, the prevalence of EC is two to three times higher in men than in women, with males having a higher incidence rate (70%) than females (11,60). Males are more likely than females to have EAC(22). Male gender is more markedly affected by geographic heterogeneity in EC incidence and histological type (61).

In Africa, gender-based differences in EC prevalence have also been observed (31). In most regions of Africa, EC is more common in men than in women, mostly because men are more likely to engage in risk factors such greater rates of alcohol and tobacco use(62). This is in contrast to Northern and Western Africa, where EC incidence is similar for both sexes. EC is more prevalent in men in South Africa(63) but more common in women, according to a 2015 study conducted in Sudan by Gasmelseed et al. Similarly, a somewhat greater frequency among female cases was identified in previous Ethiopian studies (57,64).

Age is a measure of the significance of time as a moderator of exposure-response in chronic diseases, including cancer risk factors (65,66). EC driver-mutated clones start to appear several times in early childhood, grow in size and number as people age, and eventually replace nearly the whole oesophageal epithelium in the very old (67). EC peaked between the ages of 70 and 80 worldwide (22) while in Africa, it rapidly rose starting at age 40 and peaked at age 75 (62). In contrast to other parts of the world, African Esophageal Cancer Corridor was more likely to impact younger age groups, and the number of asymptomatic cases was also higher in this region (68–70). Ethiopian EC patients frequently arrive with the disease in advanced stages (III and IV), which is made worse by an increased incidence that rises with age up to 60(59). A poor prognosis for treatment is predicted by the increased incidence of ESCC in older males, since poor treatment results are predicted by advanced age (71).

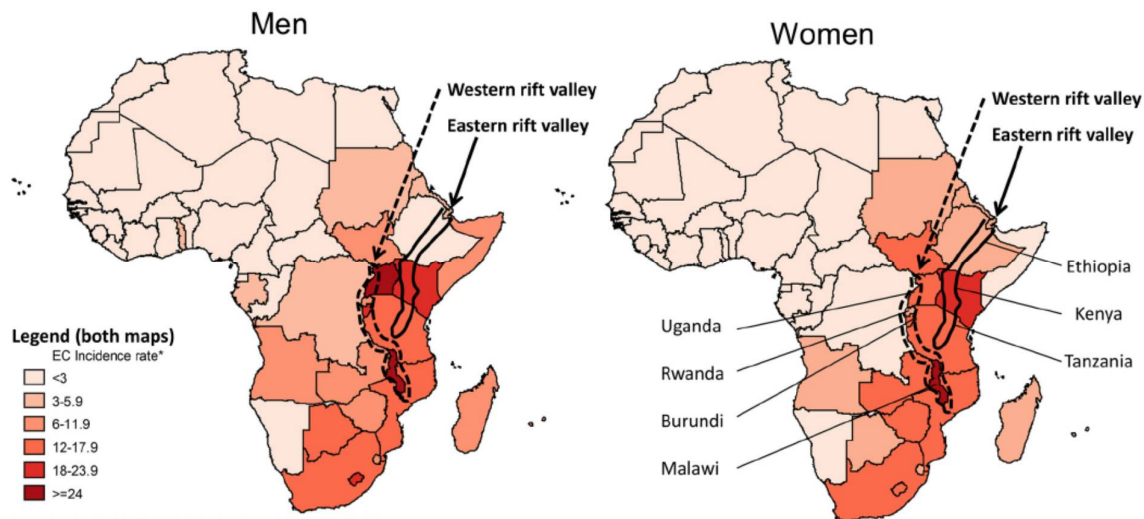


Figure 4: Eastern and western rift valley overlaid on a map of esophageal cancer incidence rates in Africa

1.2.1.2. *Risk Factors of Esophageal Cancer*

For the two main histologies of EC, ESCC, and EAC, distinct risk factors have been identified (72). Numerous ESCC risks were found, with significant regional variation. The two primary known risk factors for ESCC in wealthy countries were heavy alcohol consumption and cigarette smoking (23). The use of hot foods and beverages, betel liquid, pickled vegetables, exposure to polycyclic hydrocarbons, lack of essential micronutrients, low use of fruits and vegetables, low socioeconomic status, and poor oral health were all identified as risk factors for ESCC in countries with limited resources, in addition to alcohol use and tobacco exposure(14,23). Obesity, cigarette smoking, and gastroesophageal reflux illness are known risk factors for EAC (21,22). Here, we go discuss a few EC risk factors.

A. Alcohol consumption, tobacco Exposures and indoor air pollution

According to the IARC, alcohol consumption increases the risk of developing cancer (73). Long-term alcohol exposure raises the risk of EC in individuals who use it (74), but it has been shown that the effect of alcohol is more dosage dependent than exposure duration (75). One of the main risk factors for ESCC is heavy alcohol consumption (76). Furthermore, patients with EC have a lower survival rate when they drink alcohol(77). In China, consuming large amounts of alcohol (>53.3 g ethanol per day) over brief periods of time in men was found to be a major risk factor for EC (78). Alcohol intake, whether heavy or light, has been identified as a significant factor in the development of EC in the United States of

America (79). Additionally, in Kenya, alcohol consumption was found to be independently linked to an elevated risk of EC (80). Similarly it has been identified as a substantial risk factor for ESCC in three Eastern African countries including Kenya, Tanzania, and Malawi primarily among male individuals(81). Several mechanisms of alcohol carcinogenesis have been found, including direct deoxyribonucleic acid damage from its metabolites, direct tissue damage and stimulation of inflammatory processes, as well as oxidative stress resulting from alcohol metabolic products (82).

Significant risk factors for a number of human cancers, including upper gastrointestinal cancer, have been linked to tobacco use(83). The IARC publication from 2004 claimed that smoking and involuntary smoking are risk factors for both ESCC and EAC(84), and that the risk varies with dose. According to *Asombang et al. 2016*, smoking increases the risk of EC by 11 times when compared to those who do not smoke in the study area(85). Additional supporting data showed that smoking duration, frequency, and male smoker status were all correlated with the development of EC (86). Despite these findings, a matched case control research conducted in China showed that smoking did not significantly increase the risk of ESCC(78).

The risk of developing EC is increased more by co-exposure to alcohol and tobacco than by their additive. In the Chinese population, exposure to alcohol and tobacco elevated the chance of developing EC to 1.50 and 1.57, respectively, whereas co-exposure raised the risk to 7.32 for males(87). Similarly, concurrent exposure to alcohol and tobacco elevated the risk for both men and women in South America by 12 to 19 times, respectively(88). However, in many developing nations with high EC prevalence, such as Ethiopia, Sudan, and China, alcohol and tobacco exposure do not significantly contribute to the development of EC (57,87,89,90), supporting other factors probably playing a role in the disease's occurrence.

Throughout the African EC corridor, exposure to indoor air pollution (91,92), firewood smoke during cooking, nitrosamines, and both inhaled and swallowed polycyclic aromatic hydrocarbons(93), were all linked to varied degrees of elevated risk (94). According to reports, one of the main risk factors for EC in SSA is biomass fuel(27). A strong correlation was found between cooking in the living room and an elevated risk of EC in Ethiopia, an area with a high incidence of EC (28).

B. Nutrition

Using an ecological study methodology, the relationship between EC and important micronutrient deficiencies has been identified from African countries. Particularly, a higher risk of EC incidence was associated with deficiencies in zinc (Zn) and/or selenium (Se)(95). The fact that around 2 billion individuals globally suffer from vitamin deficiencies and that the epidemiology of EC overlaps strongly in East and Southeast Asia but weakly in South Asia and SSA is another discovery that corroborates the concern(96). Critical micronutrient deficiencies were found in both East and South African countries, according to evidence from a big study conducted in Africa. Some countries are particularly vulnerable to deficiencies in a number of micronutrients. The estimated risks for Ca, I, Se, and Zn deficiencies in Malawi are 61, 27, 64, and 33%, while in Ethiopia they are 100, 64, 36, and 81%, respectively. Low consumption of dairy products, together with low consumption of the vitamin and calcium, has been associated with a significantly increased risk of EC in the Arsi-Bale district of Ethiopia(28).

Food processing type was identified as an EC risk factor. For example, a pro-inflammatory diet that includes fried or processed foods high in trans fatty acids or saturated fat has been linked to an increased risk of ESCC in Iran (97) but eating fruits, raw vegetables, and fish has been linked to a lower risk of ESCC. A lower risk of developing Barrett's esophagus has been linked to dietary intake of omega-3-fatty acids, polyunsaturated fat, total fiber, fiber from fruits and vegetables, dietary vitamin C, beta-carotene, and vitamin E, whereas red meat and fatty meals may be risk factors(98).

According to *Bravi et al. 2012* findings, eating a diet high in animal products and low in vitamin and fiber-rich foods raises the risk of EC (99). Micronutrient deficiencies in zinc and selenium were substantially positively correlated with EC in the African Esophageal Cancer Corridor(100). Additionally, it was noted that the normal microbial flora of the gastrointestinal system is greatly impacted by the type of food and feeding habits(101) and that the microbiota also influences the feeding habits of their host (102).

C. Hot food and beverage (Thermal injury)

Hot foods and drinks were found to be the primary cause of EC in Sub-Saharan Africa(27), as they cause thermal damage (91,103). According to the IARC working group, "very hot (>65 Co) beverages" are likely carcinogenic for humans (104). Since hot substances like coffee, mate, and other beverages are not chemically carcinogenic, the working group reasoned that

even though there is little mechanistic and other pertinent evidence for very hot beverages, there is biological plausibility for an association between very hot beverages and cell injury and the sequel that could lead to cancer. Supporting data indicates that recurring thermal lesions cause hyper proliferative premalignant lesions, as demonstrated by an animal model that produced a significant proliferation stimulation of esophageal epithelial basal cells(105). By maximizing the necessary factor, the thermal lesion improves the effect of recognized carcinogenic substances. Acute esophageal thermal damage and the infiltration of inflammatory cells can result from tradition and culture of providing highly hot food and beverages(106).

The average pain threshold for coffee was measured in a pilot study with 87 people. The results were 67°C , which is higher than the threshold danger(107). Similar studies also revealed that the ideal temperature for serving coffee in the food service sector was 75°C , whereas the suggested temperature for dispensing 110 coffees in private homes was based on cooling mechanisms(108).

There have been many reports linking the incidence of ESCC in high incident areas to the temperature of the food consumed. In Tanzania, where CE is prevalent, male participants' rapid consumption of hot milky tea before it cools is a predictor of the disease's prevalence(109). In North West China, a retrospective study revealed a favorable correlation between the occurrence of ESCC and the consumption of hot food and beverages (110). Contrary to what was previously said, a meta-analysis of coffee intake and the incidence of EC revealed that regular hot coffee consumption protects against EC in Europe and East Asia(111).

A higher risk of ESCC has been linked to the quantity and temperature of hot foods and beverages(112). Consumption of hot milky tea is substantially linked to ESCC in Tanzania (113). This ESCC is also prevalent in the Arsi-Bale areas of the Oromia regional state in Ethiopia. Milky tea was thought to cause damage to the tongue and esophagus since it cools more slowly than black tea. Either by triggering persistent inflammation or by impairing the mucosa's ability to heal itself, heat damage to the esophageal mucosa may be directly linked to carcinogenesis. Other carcinogens have the potential to behave further when they are present.

D. Oral hygiene

According to some observational studies, poor oral hygiene is a predictor of EC (114–117). Inadequate dental care and the number of decaying or lost teeth are correlated with the incidence of EC. According to *Abnet et al. 2008*, after adjusting for other common risk factors, those who have lost a lot of teeth and do not clean their teeth are at a five-fold higher risk than those who have lost thirteen teeth and those who brush their teeth every day(114). Because eating becomes difficult when teeth are lost, people may swallow food that irritates their esophagus.

E. Bacterial Oral Microbiome

A population of microorganisms, such as bacteria, fungi, viruses, protists, and archaea, that inhabit a specific human body environment is known as a microbiota. Bacteria are the most dominating part of this human microbiota, which is much more numerous than human cells(118). With a human cell population of 3.72×10^{13} (119), the human body is home to 100 trillion bacteria and other microbiota members. There are several microbiomes on the body at various locations(118). The gastrointestinal (GI) tract is by far the most extensively inhabited part of the human body (120) with more than 700 different species of bacteria that live on the hard surfaces of teeth and the soft tissues of the oral mucosa, the mouth is home to the second most diverse microbial community in the body(121).

Microbes in the oral cavity are linked to the hard surfaces of teeth and braces, as well as the mucosal surfaces of the tongue and cheeks. With over 10^{12} bacterial of various species from the phyla Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, Spirochaetes, Synergistetes, Tenericutes, and additional possible phylums, the oral microbial environment is incredibly diverse. Actinomyces, Streptococcus, Neisseria, Veillonella, Porphyromonas, and Selenomonas are among the most common genera (120). Healthy subjects have varying levels of human microbiome diversity and abundance, with significant niche specialization within and across individuals (122).

In many ways, colonizing bacteria are advantageous to their host. The most widely recognized advantages of a healthy microbiota include host mucosal differentiation, nutrition and digestion, metabolism regulation, environmental chemical processing and detoxification, immune system development and maintenance, and defense against pathogen invasion and growth (123). Numerous human illnesses, including inflammatory bowel disease, antibiotic-associated diarrhea, and chronic periodontitis, are linked to changes or disruptions in the

human microbiome. Furthermore, microbiota are linked to cancer by either increasing or lowering the host's risk of developing cancer. By directly harming the host DNA with genotoxins and bacterially generated inflammatory mediators, they can contribute to the development of cancer cells (124).

The normal bacterial flora affects cancer treatment. The effectiveness of anti-immune checkpoints T-lymphocyte-associated protein 4 (CTLA-4) mAb therapy is considerably impacted by the absence of bacterioids and Burkholderiales(125). Similar results were reported by Vétizou *et al.* 2015, who demonstrated that CTLA blocking therapy had no effect on tumors in mice treated with antibiotics or germ-free mice(126). Anti-PD-1 immunotherapy for melanoma patients is significantly impacted by the presence or absence of Ruminococcaceae/Faecalibacterium(127). Research shows that altering the gut microbiome's makeup in an animal model has a major impact on adoptive T cell therapy's effectiveness(128).

Research indicated that the occurrence of extraoral organ malignancies was associated with specific oral bacterial communities (129–132). It was shown in a prospective case control research that carrying *P. gingivalis* and *A. actinomycetemcomitans* were associated with a higher risk of pancreatic cancer, along with a lower relative abundance of the phylum Fusobacteria and its genus Leptotrichia (131). Similarly, the abundance of Peptostreptococcus, Parvimonas, and Fusobacterium was found to be protective against colorectal cancer in health control groups, whereas the relative higher abundance of Streptococcus and Prevotellas species in the oral cavity was identified as the etiologic agent of colorectal cancer (132). Additionally, Bar *et al.* 2017, found that oral bacterial flora is linked to esophagus, head and neck, lung, and breast cancers (133).

Studies have shown that the oral microbiome of patients with EC differs significantly from that of healthy controls, with notable changes in the abundance of specific bacterial phyla and genera (129,134–136). ESCC patients often have increased levels of certain bacteria such as *Streptococcus*, *Prevotella*, and *Planctomycetes*, and decreased levels of *Neisseria* and *Proteobacteria*. Some genera, such as *Actinomyces* and *Atopobium*, are also associated with a higher risk of ESCC, while others like *Fusobacterium* and *Porphyromonas* are more prevalent in healthy individuals(137). In the United States of America Peters *et al.* 2017 reported, *Tannerella forsythia* and *Porphyromonas gingivalis* in the oral cavity were linked to increased risk of EAC and ESCC,

respectively (129) while from China *Chen et al. 2015* reported, ESCC patients have lower carriage of the genera *Lautropia*, *Bulleidia*, *Catonella*, *Corynebacterium*, *Moryella*, *Peptococcus*, and *Cardiobacterium* than healthy controls (138).

In other study periodontal pathogen *Porphyromonas gingivalis* has been detected at significantly higher rates in ESCC tissues compared to adjacent or normal esophageal tissue, suggesting it may be a risk factor and potential biomarker for ESCC (139). Depletion of commensal bacteria such as *Neisseria* and *Streptococcus pneumoniae* was associated with a lower risk of EAC (140). Recent Mendelian randomization study suggests a bidirectional causal relationship between the oral microbiome and esophageal cancer, with certain oral bacteria acting as either risk or protective factors. For example, species within the phyla *Firmicutes*, *Patescibacteria*, and *Actinobacteria* have demonstrated significant associations with EC risk, with some species being protective and others increasing risk (141).

Proposed mechanisms by which microbiota contribute to carcinogenesis include the direct adhesion and invasion of esophageal tissue by pathogenic oral bacteria, resulting in chronic inflammation and altered host immunity(142); the induction of a pro-inflammatory microenvironment and the depletion of protective commensal bacteria (143); and the interaction among oral bacteria, dietary factors, and esophageal mucosa, which may further influence cancer risk (144).

Given the available data regarding microbiota, it is reasonable to infer that the oral microbiome may function as a non-invasive biomarker for the early detection and risk stratification of esophageal cancer. Additionally, enhancing oral hygiene and targeting specific pathogenic bacteria could be viable strategies for mitigating ESCC risk, particularly in high-risk populations. The available results indicate the necessity for additional study to elucidate the mechanistic connections and to create microbiome-based therapies for prevention and treatment.

Different variables are linked to variations in the oral bacterial flora. The transition in ESCC to EAC from 1990 was thought to be caused by the disruption of the human normal flora community caused by the introduction and widespread use of antibiotics in the western world in the 1950s, which preceded the rise of EAC(145). In patients with gastroesophageal reflux

diseases, it has been postulated that antibiotics change the microbiome to which the esophagus is exposed and that long-term exposure to this aberrant microbiome.

Depending on their host age, sex, nutrition, time, harsh environment, breastfeeding as a baby, and educational attainment, healthy individuals may have different oral microbiota(146). The microbial composition of the oral cavity at the species level has also been found to be influenced by dental hygiene and health(120). A brief cessation in oral hygiene causes a shift in the diversity of oral microbes, which does not reverse compositional changes 14 days following the break (147). Regular oral hygiene is essential for maintaining oral homeostasis because stopping it was linked to a considerable rise in the relative abundance of potentially cariogenic *Leptotrichia* species and a decrease in *Streptococcus* species.

According to a study done in the USA, the most prevalent genera among children under five are *Streptococci*, *Neisseria*, and *Prevotella*(148). Proteins, dietary fibers, and fats are examples of macronutrients that have distinct effects on the microbiota of the gastrointestinal tract(149). Saturated fatty acid intake was favorably associated with the relative abundance of *Fusobacteria* and *Beta proteobacteria*, whereas vitamins were associated with the *Clostridium* family and *Leptotrichiaceae*(150).

Personal habits that affect the composition of the oral microbiota include drinking alcohol(151), smoking (152,153), and drinking coffee. Compared to non-drinkers, higher alcohol consumers were more likely to have the genera *Actinomyces*, *Leptotrichia*, *Cardiobacterium*, and *Neisseria*, whereas drinkers were observed to have a lower abundance of the order *Lactobacillales*. Smoking has been linked to oral microbiome dysbiosis, namely a decrease in the relative abundance of the phylum *Proteobacteria* and other taxa such as *Capnocytophaga*, *Peptostreptococcus*, and *Leptotrichia*. *Streptococcus* and *Atopobium*, on the other hand, were enriched when compared to non-smokers. Green tea consumption affects the oral cavity's microbial makeup by increasing the relative number of *Staphylococcus* and *Streptococcus*(154).

Dietary association between oral microbiomes of traditional farmers and hunter-gatherers in the Philippines was observed. This implies that diets within or closely related to ecological or socioeconomic conditions are important salivary differentiation drivers(155). Hunter-gatherer groups were shown to have a greater diversity of oral-cavity microbiomes and a higher incidence of *Haemophilus* than agricultural groups(156). A similar study that included people

from three continents—Africa, North America, and Europe—found that there was a unique and common bacterial population with a wide range of abundances(157). Additional supporting data about the diversity of gut microbiota across altitudes and genetic backgrounds revealed a decrease in the number of Bacteroidetes intern and an increase in Firmicutes at high altitudes(158). The oral bacterial community is more influenced by shared environments than by community genetics, according to a study that used genetic relatedness based on genome-wide single nucleotide polymorphisms to examine the extended families of Ashkenazi Jews who lived in multiple cities(159).

F. Mycotoxins

Mycotoxins, which are secondary metabolites produced by fungi that contaminate agricultural crops and commodities, including *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* toxigenic species, have been linked to an increased risk of developing a number of cancer types(160,161) with a proven mechanism of carcinogenesis(162). IARC classified fumonisins (including fumonisin B1 and B2) (163)and ochratoxin A (164) as possibly carcinogenic to humans (Group 2B), and aflatoxins (including aflatoxin B1, B2, G1, G2, and M1) as carcinogenic to humans (Group 1)(165). Other mycotoxins were also assessed and categorized as not classifiable as to their carcinogenicity to humans (Group 3) based on the evidence that was available(166).

There have been reports of mycotoxin contamination in common food cereal crops across the African and Asian EC belt (27,167–170). The epidemiology of EC and the regional overlap of mycotoxin prevalence in food may indicate that mycotoxin exposure could be a risk factor for EC. In China and Iran, for instance, the amount of FB1 in rice and corn samples taken from regions with a high prevalence of EC was linked to a higher risk of EC (167,168,170).

As major agricultural food commodities were contaminated by carcinogenic mycotoxins (171), mycotoxin contamination has been reported to be a health concern in Ethiopia. In areas of high EC incidence in Ethiopia, common foodstuffs include maize, wheat, barley, and raw milk, all of which are known to expose humans to mycotoxins(28). Coffee, which is also reported to be contaminated with mycotoxins (172), is consumed at least three times a day in that local community.

Due to inherent limitations, food occurrence data and population data on food consumption have limited application in assessing mycotoxin exposure (173). Instead, a human

biomonitoring approach that analyzes biomarker compounds in biological fluids and tissues is the most effective way to determine internal exposure to mycotoxins in humans. Evidence from epidemiological (27,170), biomonitoring by Xue et al., 2019(174), and mechanistic experimental (175) investigations points to the significant contribution of mycotoxins to the high incidence of EC in regions with no established or unenforced mycotoxin-related food safety legislation.

The majority of studies in the literature described evaluating mycotoxin exposure by combining population data on food consumption with food occurrence data. Nevertheless, this method is recognized for its inherent drawbacks because of the uneven distributions of mycotoxins in food, individual differences in toxicokinetics and bioavailability, and imprecise food consumption estimations(173). The most effective method for determining a person's internal exposure to mycotoxins is human biomonitoring, which involves examining biomarker compounds in biological fluids and tissues.

1.2.1.3. Gene and Epigenes associated with Esophageal cancer

In the Chinese population, a close family history of EC was linked to a higher incidence of the disease (odds ratio = 1.85, 95% (CI=1.42–2.41)) after adjusting for other possible its risk factors(176). Similar familial associations demonstrating a genetic link for the development of EC were also identified in African countries (177–180) with high EC incidence. Exposure of genetically predisposed individuals to environmental stimuli results in modifications of essential growth-regulatory genes and triggers a progression of epithelial changes from dysplasia to cancer in esophageal carcinogenesis(181). The accumulation of genetic changes in normal cells at their driver are caused by aging, carcinogenic chemicals, UV light, and others, while aging and chronic inflammation are the main causes of epigenetic changes (182). All of these factors have an effect on somatic mutations, which are increased throughout life and linked to many types of cancer including EC (183).

The exposure to EC risk factors, the stage of EC, and its prognosis are all substantially correlated with the TP53 gene mutation on germline or somatic cells (184). It has been established that TP53 mutations in ESCC have carcinogenic properties (185), and that TP53 biallelic loss is a necessary condition for the formation of copy number changes in genes involved in the cell cycle, DNA repair, and apoptosis pathways in ESCC (186). Additionally, somatic TP53 mutations are common in both EAC and ESCC histological types(187).

There are other genes exclusive to the two main histological groups. Key genetic anomalies in ESCC include changes in genes that control cell cycle, cell differentiation (particularly the NOTCH pathway), and EGFR (HER1) signaling(188). Farther more NFE2L2, MLL2, ZNF750, NOTCH1, TGFBR2 (187,189), JAK3, BRCA2, FGF2, FBXW7, MSH3, PTCH, NF1, ERBB2, CHEK2 (190), CCND1, CDKN2A, FBXW7, MLL2, EP300, CREBBP, TET2; NOTCH1, NOTCH3, FAT1, YAP1, AJUBA, PIK3CA, EGFR, and ERBB2 (191) were found to be significantly mutated genes in ESCC, whereas chromatin-modifying factors ARID1A, ERBB2, CDKN2A, SMAD4, PIK3CA, SPG20, TLR4, ELMO1, and DOCK2 were significantly mutated in EAC (189,192). Furthermore, whilst PIK3CA, PTEN, SOX2, FGFR1, and RB1 genes are more commonly altered in ESCC and KRAS, EGFR, FGF3/4/19, VEGFA, CCNE1, and GATA4/6 are altered more frequently in EAC (189). Additionally, it was noted that EAC has comparatively little resemblance to ESCC and is related to gastric cancer, but ESCC shares similarities with other body squamous cell carcinomas, such as lung and head and neck cancer (193,194)..

Additionally, EC was linked to epigenetic modifications (86). EC is linked to changes in the epigenetics, including DNA methylation and histone modification(195). Epigene is susceptible to environmental factors, which can change how genes are expressed (196). This changes are important in tumorigenesis because they contribute to the dysregulation of vital cellular pathways through chromosomal instability, oncogene activation, and tumor suppressor gene inactivation (197). As epigenetic states are more dynamic and susceptible to environmental impacts at particular phases than the genome, this made it relevant to find new biomarkers for risk assessment and prevention of cancer(198). Numerous environmental, dietary, and lifestyle factors linked to the development of a variety of human cancers by causing epigenetic alterations(199).

Change in DNA methylation is commonly associated with occurrence of cancer (198). It is a major epigenetic mechanism that mostly involve in control of both normal cellular processes and abnormal events associated with tumor development and progression(200). DNA methylation regulates gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA(201). Throughout cancer start and progression, alterations in genome-wide and gene-specific DNA methylation arise due to mutant or dysregulated chromatin regulators. Initial anomalous DNA methylation patterns observed after transformation seem to persist throughout tumor progression. Similarly, DNA

methylation differences among different regions of a tumor reflect the history of cancer cells and their response to the tumor microenvironment(202). As it is frequently linked to tumor growth from early in carcinogenesis (203) change in DNA methylation can serve as markers for cancer diagnosis and prognosis.

The association between environmental and epigenetic factors in the development of esophageal cancer was identified in the Indian population in a high-incidence area.(204). The observation of concomitant p16 promoter hypermethylation and p53codon 72 polymorphisms result from dietary, lifestyle, and environmental variables were specific to that high ESCC incidence. Similarly, there was a substantial correlation between ESCC and tobacco-induced promoter hypermethylations of tumor suppressor genes (205). Tobacco(206) and alcohol(207) use, two recognized risk factors for ESCC, promotes carcinogenesis by interfering with retinoid metabolism and blocking DNA methylation. In other study a total of 37 differentially methylated CpG site were observed between ESCC and healthy tissue of esophagus(208). These CpG sites were associated with several genes involved in the pathway including IL-10 anti-inflammatory signaling pathway showing the role inflammation in carcinogenesis.

1.2.1.4. Biomarkers of EC

Cancer (tumor) biomarkers are classified in to six types based on their purpose of use and classified as biomarkers of early detection, diagnosis, prognosis, prediction, therapeutic target, and surrogate end point. Various challenge were associated with the use of biomarker which are the influence of biologic factors on characteristics and concentration of biomarkers, limited technology for discovery of accurate biomarker of preneoplastic neoplasia, the low analytical sensitivity and specificity to detect the biomarkers and the high risk of false positivity arise from bulk of molecules during identifying biomarkers(209).

A. DNA methylation a Biomarkers of EC

Molecular alterations may precede histopathological alterations, making molecular biomarkers of early mucosal transformation a potential tool for use in screening procedures(181,210). The clinical status including survival periods for EC patients and individual susceptibility to EC were linked to those gene alterations (211). DNA methylation is a molecular bias for esophageal cancer, making it a promising choice for EC biomarkers. It was suggested for a number of biomarkers, such as prognostic or predictive biomarkers and diagnostic biomarkers (212). To evaluate the use of circulating free DNA as a potential

cancer biomarker, one can look for tumor-specific changes in the DNA or changes in the total amount of circulating free DNA(213).

This method is especially crucial in low- and medium-resource populations around the world, where the incidence of esophageal cancer is high and treatment and prognosis monitoring resources are minimal (214). Abnormal methylation of a collection of marker genes found in oral rinse samples was utilized to detect oral squamous cell carcinoma with >90% sensitivity and specificity for early diagnosis of this cancer (215). With 64.3% sensitivity and 100% specificity, the plasma aberrant DNA methylation of three distinct epigenes—EPB41L3, GPX3, and COL14A1—in ESCC demonstrated a promising biomarker from a non-invasive sample (216). It was suggested to the researchers that more research be done on the clinical value of their findings (plasma biomarkers) in a wider population using more precise techniques like bisulfite pyrosequencing.

Machine was trained to discriminate ESCC from healthy tissue of esophagus using Methylated DNA markers and showed excellent ability in discriminating the disease from healthy showing areas under the receiver operating curve 0.90 and 0.87(217). DNA Methylation from samples obtained using less invasive techniques exfoliative cytology show significant association (($P < 0.004$) with EC and its progress showing its candidacy for effective method for large-scale screening of at-risk populations. Other study identified differentially methylated points and regions that able to differentiate ESCC from healthy tissue from samples collected from EC high-incidence countries of Africa, Asia, and South America which also show the potential applicability of this biomarker(218). In other work PAX9, SIM2, and THSD4 genes were the top three prioritized gene which were hypermethylated in ESCC and they were known gene involved in cancer. Though DNA methylation markers are able to diagnosis the EC using sample collected via less invasive techniques and used to identify the progression of the disease it has impacted with limitation such as extensive heterogeneity and inter observer variability(219).

B. Other Serum Tumor Markers

Carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) , cancer antigen 125(CA 125) and Squamous Cell Carcinoma Antigen (SCC) as the biomarkers of EC was long studied(220). The subsequent study revealed low sensitivity of CEA and CA 19-9 in detecting EC which was 34.3% and 28.6% respectively(221). Other study which aimed to evaluate the sensitivity of tumor markers for diagnosing EC using serum CEA, CA19-9,

alpha-fetoprotein and beta-human chorionic gonadotropin observed the low sensitivity of these markers(222).

1.2.1.5. Esophageal cancer patient presentation, diagnosis, staining, and management

According to the UK cancer research dysphagia, weight loss, indigestion, and pain in throat are main prominent presentations of patient with EC. Other symptoms like food coming back up after swallowing, persistent cough, hoarseness, cough up blood, dark stool and tiredness are also associated with EC(223,224). Reports from Ethiopia indicated that the primary complaints of esophageal cancer patients include dysphagia, weight loss, vomiting, epigastric pain, and anemia (39,42,225). Due to overlapping of this symptom with other disease, upper intestinal endoscopy is required for identifying the presence of lesion or mass and sampling for pathological examination (226).

According to the ESMO Clinical Practice Guideline all patients with new dysphagia, gastrointestinal bleeding, recurrent aspiration or emesis and weight loss and/or loss of appetite should undergo an upper intestinal endoscopy (226) to obtain tissue for diagnosis, histological classification and molecular biomarkers. A thorough approach necessitates a complete blood count to assess iron deficiency anemia, renal and hepatic function tests are required to determine appropriate therapeutic strategies.

Imaging plays a central role in the diagnosis, staging, and restaging of EC. Endoscopic ultrasound for accurate tumor and lymph node staging in potentially resectable tumors, bronchoscopy with endobronchial ultrasonography to evaluate tumor encroachment on central airways, and computed tomography of the thorax, abdomen, and pelvis for staging metastatic disease are required (226). Acknowledging to the limitation of these imaging techniques, magnetic resonance imaging is an emerging technique for evaluating esophageal cancer and offers improved local staging, especially for advanced tumors (227).

Information on cancer stage provides how big the tumor and whether it has spread that help the physician to decide on the patients' treatment. Tumor, node and metastasis staging is the most common way to stage EC. Tumor describes the size of the tumor (area of cancer), node describes whether the cancer has spread to the lymph nodes and metastasis describes whether the cancer has spread to a different part of the body (226,228). The tumor grades inform provide information on how abnormal the cells look under the microscope. For instance high

grade dysplasia means that there are severely abnormal cells in the inner lining of the esophagus(226).

The treatment of EC depends on various factors including the cancer histological type, stage, grade, and the general health of the patient. Before starting treatment the consideration dietary verity, physical activity and psychological preparation are listed as worth important and this are termed prehabilitation. The treatment and management of both EAC and ESCC depend on the tumor stage (229). Esophagectomy is frequently advised upon the diagnosis of high-grade dysplasia (230). The development of minimally invasive esophageal surgery, including the use of robotic esophageal surgery, has been enhanced throughout time (231). However, esophageal resection can cause postoperative problems such anastomotic leaks and long-term symptoms like reflux and dysphagia, which can significantly affect quality of life (232,233). Consequently, in patients with a low risk of lymph node metastases, other techniques such as endoscopic mucosal resection and endoscopic submucosal dissection were employed(234,235). In addition to surgery, the primary treatment modalities for esophageal cancer encompass radiotherapy, chemotherapy, chemoradiotherapy, targeted therapy, and immunotherapy (223,226).

The biological characteristics of ESCC which are high frequency of neoantigens, radio-sensitive tumor and multiple immunogenic cancer antigens made it suitable for immunotherapy(236). An optimal and rational approach to ESCC treatment may involve the combination of anti-PD-1/anti-PD-L1 mAb with CTL-inducing therapies such as anti-CTLA-4 mAb, cancer vaccination, chemoradiation, and cytotoxic and/or molecular target medications. It was also suggested immunotherapy showed superior efficacy than chemoradiotherapy. Its efficacy in pre-operative treatment and the growing clinical trials made it promising for feature development on its applicability globally(237). Toripalimab and Camrelizumab plus chemotherapy were advised in the first-line context for the treatment of advanced ESCC, whereas Sintilimab and Camrelizumab were probably the preferred treatments in the second-line scenario(238).

1.2.1.6. *Screening and prevention of EC*

The purpose of early screening is to minimize the risk associated with advanced state EC and to reduce its associated mortality. Barrett's Esophagus follows a progression from intestinal metaplasia, low-grade dysplasia, high-grade dysplasia, intramucosal carcinoma, and finally invasive EAC (239). Barrett's Esophagus is a metaplastic alteration of the esophageal lining

in which specialized columnar epithelium replaces the typical squamous epithelium(240). The motivation for Barrett's Esophagus screening and surveillance comes from the step-by-step advancement of Barrett's Esophagus patients and the stage-dependent survival of EAC. The only histology that can predict the onset of ESCC is esophageal squamous dysplasia (241).

Thus the purpose of EC screening is to identify Barrett's esophagus and squamous epithelium dysplasia, which are precursor lesions to EAC and ESCC, respectively. There are several methods that differ in accuracy, suitability for primary care, cost-effectiveness, and scientific proof, such as circulating or stool markers, trans-nasal endoscopy, capsule endoscopy, cytosponge, and conventional endoscopy(242). Conventional endoscopy is widely utilized as the gold standard for interim accuracy. Also the prominent established risk factors were in consideration for screening to optimize resource associated with screening. For instance risk factors include male sex, age older than 50 years, Caucasian ethnicity, tobacco use, obesity, and a family history of BE or EAC in a first-degree relative are used with conventional endoscopy for screening for EAC. Geographical based variation in screening strategy was also observed which depends on countries resources and risk factors associated(243)

Primary prevention by identification and avoidance of modifiable risk factors can decrease the occurrence(244). This primary prevention strategy basically differs for both common histological types with respective of their risk factors. Secondary prevention by implementation of effective screening programs and application of novel diagnostic tools is lifesaving, through early detection of asymptomatic neoplasia and treat them effectively(245). Tertiary prevention by the development of effective treatment modalities in symptomatic tumors is the most common clinical scenario and may prolong survival and improve quality of life(181).

1.2.1.7. Conclusion

The epidemiology and histological type of EC varies based on geography. Its prevalence shown to be slightly decreased in some countries while death associated remain no change. ESCC more common in low income countries and its etiologies were not well known. EAC which is common in high income countries is shown to be increased in low income countries with increasing with its global incidence. This is associated with change in life style that increase the risk of EAC, decrease in the infection of *H. pylori* and increase of obesity among populations. Globally EC is prevalent among male gender showing more male exposure its

risk factors and hormone may also partly play the role. However, in Eastern African nations such as Sudan, Eritrea, and Somalia, EC is prevalent among women. The early younger age onset of EC is increasingly reported from Kenya showing the population exposure to EC risk factors early in their life. In Ethiopia variable results of epidemiology of EC were reported based on the study area within the country. This warrants the basic the need of basic epidemiological study to reveal the basic epidemiology of EC that provide clue for research on possible risk factors and etiology.

Heavy alcohol drinking and tobacco use were the risk factors of ESCC in high income countries while in low income countries varies risk factors including dietary factors, exposure to environmental factors, oral hygiene, cultural and life style were reported. The risk factors reported in low income countries were local specific and they have methodological limitation. For instance the oral microbiome beyond the oral hygiene and mycotoxin exposure assessment using biomonitoring approach were not assessed in African high EC incidence including Ethiopia. However in global western countries and China Oral microbiome and exposure to mycotoxins were reported as the causal link with EC. More over so far no study conducted for the assessment of noninvasive biomarker in African context using oral microbiome.

1.3. Statement of the Problem

In terms of prognosis and mortality, EC is an aggressive malignancy. The global incidence and mortality rates for esophageal cancer (EC) among all cancer types were 604,100 (3.1%) and 544,076 (5.5%), respectively (29). A notable health concern, its prevalence is anticipated to increase (246) and mortality rates are elevated in underdeveloped nations with limited resources (247). Due to the diagnosis of EC occurring at an advanced stage based on observable clinical symptoms and the absence of a validated screening method, the five-year survival rate for EC patients is below 20%. In resource-limited environments, such as Sub-Saharan Africa, there is an inadequate availability of diagnostic and treatment facilities for EC patients.

One of the top ten cancer types in Ethiopia is EC (38) with a very low five-year survival rate (64) and a markedly rising incidence trend (39). Ethiopia also showed notable differences in EC frequency by gender, histological type, and geography; the majority of cases were from Oromia regional state, accounting for over 50% of Ethiopian cases (39,248,249). This observed variation serves as an indicator of the part played by regionally specific EC risk

factors in Ethiopia. Alcohol and tobacco use, two known risk factors, were not substantially linked to the incidence of ESCC, the most common histological type in Ethiopia (28,57). Few descriptive studies were conducted in Ethiopia to assess the epidemiological, behavioral, nutritional, and environmental factors of EC (28,57,59,250). However, because of the descriptive nature of the research and referral bias, we are unable to draw firm conclusions from them.

The disparity in gender in EC incidence is one of the difficulties in identifying the cause and understanding the disease. Globally, men are 70% more likely than women to have EC (29,30). Similarly, sex-based differences in EC incidence have also been documented in the high-risk region of Africa (33), with South Africa having a higher prevalence of EC in men (63) than Sudan (89) and Ethiopia (41,42). It was also observed a slightly higher prevalence of female cases (57,58) while in some other studies slightly more male cases were reported (59,64).

There is increasing evidence that gastrointestinal microbiota influence the occurrence, pathogenesis, and treatment outcomes of various cancer types (251), as 99% of healthy human microbiota resides in the gastrointestinal tract, exerting both local and long-distance effects(252). Dysbiosis enables pathogens to effectively establish themselves, leading to inflammation and the production of genotoxins and other carcinogenic microbial metabolites (253). The absence of Clostridiales, Faecalibacterium, Blautia, and Bifidobacterium in the colon of a patient with colorectal cancer contributed to its consideration additionally (254). Certain bacterial normal flora and their byproducts can promote wound healing and reduce the growth of tumors(255). A healthy microbiome also enhances the effect of other prominent cancer risk factors, such as obesity, alcohol consumption, and smoking (256–260). By regulating the human immune system, microbiota also influence the development, pathophysiology, and management of cancer (261).The host immune response is activated, trained, and modulated by the local microbiota (262), which impacts the use of immune checkpoint medication for cancer treatment.

In developing countries where heavy drinking and smoking were uncommon, the results of cross-sectional and prospective follow-up studies provide evidence that poor oral hygiene and health were the major risk factors for EC (263–267). There have also been reports of tooth decay and loss as EC predictors (268,269). Given that the oral microbiota determines oral health, including dental caries, tooth decay, and tooth loss, this emphasizes how the oral microbiota contributes to the development of EC.

Tannerella forsythia, *Streptococcus pneumoniae*, and commensal *Neisseria* species in the oral cavity were linked to EAC in the United States, while *Porphyromonas gingivalis*, a periodontal infection, was linked to a higher risk of ESCC (270). Similar findings indicated that ESCC participants had a generally lower microbial diversity than control and dysplasia subjects in China, where EC cancer incidence is high (271). Additionally, it was revealed that the oral bacterial flora, *Fusobacterium nucleatum*, was linked to the survival and pathogenesis of EC patients (272). Furthermore, because it is strongly linked to esophageal squamous dysplasia, the precursor lesion of EC, the oral microbiota was found to be a predictor of EC (273). Various factors including dietary, altitude, genetic, behavioral, age and gender influence the diversity of oral microbiome that necessitate the similar study in Ethiopia to evaluate the role of oral microbiome for EC occurrence.

Numerous cancer types have been linked to mycotoxin exposure (160,161), and their carcinogenic process has been established(162). Aflatoxin, fumonisin, and ochratoxin A were designated by the IARC as carcinogenic in humans based on information gathered from mechanistic, epidemiological, and animal research(166). Mycotoxin exposure was common even in nations with mycotoxin regulations in place and people aware of the negative health effects of mycotoxin (274).

There have been reports of mycotoxin contamination in common food crops, such as cereal grains, in regions with a high incidence of EC. The prevalence of EC was shown to be positively correlated with aflatoxin levels in wheat flour in the Golestan area of northeastern Iran(169). Likewise, fumonisin B1 (FB1) levels in rice and corn samples taken from EC-high risk regions in China and Iran were linked to an increased risk of developing EC (167,168,170).

Numerous studies indicate that mycotoxin exposure may be a contributing factor to EC in the African EC belt (275–277). Given the geographic overlap between the epidemiology of EC and the incidence of mycotoxins in food, exposure to mycotoxins may be a risk factor for EC. Since key agricultural food items have been contaminated, mycotoxin exposure has also been recognized to be a health risk in Ethiopia(171). In Ethiopian regions with an elevated prevalence of EC, common food items include maize, wheat, barley, and row milk all of which are known to expose humans to mycotoxins(278). The majority of people living in Ethiopia's EC high occurrence area drink coffee at least three times a day, despite the fact that it has been found to be contaminated with mycotoxins(172). Even though there is evidence

linking mycotoxins exposure to EC, and mycotoxins contamination of food and coffee has been identified in Ethiopia, its relationship to EC has not been evaluated.

In Ethiopia, we assessed the profile of EC by gender and age group in order to better determine the risk factors associated with it. We additionally addressed its histology and location along the esophagus to gain a better estimate and stratify the risk factors for future study. In order to characterize the oral microbiota of healthy rural Ethiopians and identify potential biomarkers for EC, we characterized the salivary microbiota of EC cases and matched controls. This was done in light of the oral microbiome's role in the development of EC and the lack of evidence regarding its relationship to EC in African countries, including Ethiopia. Since ecological, biomonitoring, and mechanistic experimental investigations have demonstrated a strong correlation between mycotoxin exposure and EC in regions with a high frequency of this disease, we have also evaluated the role of mycotoxin in the occurrence of EC in Ethiopia where alcohol and tobacco are not linked to EC. Thus, in the Arsi-Bale district of the Oromia Region of Ethiopia, we evaluated the contribution of oral microbiota, multi-mycotoxin exposure, and basic demographic variables such as gender, age, and place of residence to the prevalence of EC.

1.4. Research hypothesis

We hypothesize that;

- Patients with EC from Arsi-Bale district have distinct epidemiological and histological features compared to earlier reports from other parts of Ethiopia.
- The composition and relative abundance of oral microbiota in EC patients is different from health controls.
- Oral microbiome of EC patients is functionally distinct and different from healthy controls.
- Oral microbiome correctly identifies EC patients from health controls.
- Western population healthy oral microbiome composition and abundance are different from developing countries.
- The plasma level and number of mycotoxins are different in EC patients and health controls.

1.5. Objectives:

General objective:

To determine the distribution and determinants of esophageal cancer, characterize oral microbiome signature, and assess mycotoxins exposure of esophageal cancer patient in the Arsi Bale district of Ethiopia.

Specific objectives:

- To assess the epidemiology and histological distribution of esophageal cancer in the Arsi-Bale Districts of Ethiopia.
- To identify the composition and diversity of oral microbiome associated with esophageal cancer in the Arsi-Bale districts of Ethiopia.
- To determine factors influencing the composition and diversity of oral microbiome in the Ari-Bale district of Ethiopia.
- To compare the composition and diversity of Ethiopian oral microbiome with global data of oral microbiome
- To assess oral microbiome functional pathways associated with Esophageal cancer
- To identify the potential diagnostic biomarkers of Esophageal cancer using oral microbiome
- To determine the plasma level mycotoxin exposure associated with esophageal cancer in the Arsi-Bale Districts of Ethiopia.

1.6. Significance of the study

In Oromia, esophageal cancer is the second most prevalent upper gastrointestinal condition, behind gastroesophageal varices, with a significant rise in prevalence nationwide from 2.7% during 1979-1994 to 11.2% from 2016-2024 (279). It ranks sixth in cancer-related mortality (280), and its incidence has markedly risen in Ethiopia (38). The disease is diagnosed at its advanced state and resulted in poor prognosis with low five year survival rate of patients. Geographical based variation in its epidemiology, etiology and histological types were noticed. In areas of Ethiopia with a high incidence of EC, there is limited knowledge regarding the etiology, epidemiology, and histology of this disease. These with inaccessibility of diagnostic and treatment service in resource limited setting made difficult for early treatment of EC. Prior research in Ethiopia, specifically within the Arsi-Bale district, indicated that alcohol use and tobacco use, previously recognized as risk factors for EC, were not significantly associated with the condition (41,42), underscoring the necessity for investigation into local risk factors.

The oral microbiota and mycotoxin exposure, identified as potential etiological and diagnostic markers of EC in the global West and China (174,281,282), have not yet been assessed in Africa, including Ethiopia. Additionally, differences in the proportions of incident gender, high risk age group, and histological type were noted in Ethiopia. Noninvasive feasible diagnostic method is also the priority area in EC research.

By avoiding the referral bias that obscure the effects of spatial variation, we uncovered essential epidemiological and histological data that facilitates the prediction and comprehension of the etiology and associated risk factors of EC in this high-incidence region of Ethiopia. At a time when there is a great demand for non-invasive diagnostic and screening biomarkers of this cancer, we also found a strong correlation between the oral microbiota and its possible diagnostic biomarker of EC using a case control study design. Additionally, we discovered through a human biomonitoring study, for the first time in Africa, that individuals with EC exhibited significantly higher levels of mycotoxin exposure compared to healthy controls, suggesting that mycotoxin exposure may be a contributing factor to EC in regions with elevated EC incidence in Ethiopia.

Our study's gender disparity findings suggest that social and cultural risk factors that contribute to EC prevention in the study area need to be evaluated. This study also emphasizes the significance of prospective study in determining the dimensionality of oral microbiome associated with EC, their impact on patient treatment response and disease prognosis, and the role mutimycotoxin exposure plays. Additionally, the significance of evaluating multimycotoxin exposure using a matrix that can indicate long-term exposure by involving non-endemic healthy controls is highlighted when longitudinal prospective investigations are impractical.

1.7. Scope of the study

Study area and setting

The study was carried out in Ethiopia's Oromia regional state's Arsi-Bale district, where a high prevalence of EC has been observed regionally. The district, which has four zones—Arsi, West Arsi, Bale, and East Bale—is located in South East Ethiopia. Adama Hospital Medical College (AHMC), Adama General Hospital and Medical College (AGHMC), Muse Genera Hospital (MGH), Asella Rehoboth General Hospital (ARGH), Medda Wolabu Hospital (MWH), Asella Hospital (AH), Yanet Internal Medicine Hospital (YIMH), Yoya Hospital (YH), and Negelle Arsi General Hospital and Medical College (NAGHMC) were

the nine hospitals from which the study participants were recruited. **Figure 5** displays these hospitals' approximate geographic locations.

Since these hospitals are situated in the catchments of Ethiopia's Arsi-Bale districts, which have a high prevalence of EC, purposely they were selected. Suspected patients were sent to these institutions from other hospitals in the catchment areas because they offer endoscopic and pathological examination services. Cancer patients can receive chemotherapy and palliative care at Asella Hospital and AHMC. The analysis of mutimycotoxin exposure was performed in one of the Center of Excellence in Mycotoxicology at Faculty of Pharmaceutical Sciences, Ghent University, Belgium. The other most important analysis performed was the oral microbiome and this was conducted at Department of Biochemistry and Molecular Biology, The Pennsylvania State University, USA.

Aim: The aim of this study is to describe the epidemiology and histology of esophageal cancer in the Arsi-Bale district of Ethiopia, investigate the potential link between mycotoxin exposure and the oral microbiome in relation to esophageal cancer, and to evaluate the role of these noninvasive biomarker for detection of the disease. Overall, this research seeks to contribute valuable insights into the factors influencing esophageal cancer in this region, addressing a significant public health concern.

Study design

A cross-sectional study design based in a healthcare facility was employed to characterize and evaluate the epidemiology of esophageal cancer from data of patients diagnosed August 2019 to August 2022 in the selected hospitals. Data was gathered using interviewer-administered questionnaires and patient chart reviews. A case-control study design was employed from January 2022 to June 2024 to evaluate the relationship between the oral microbiota and several mycotoxin exposer and esophageal cancer. Ethiopian oral microbiome data was used to train global oral microbiome data in order to find a possible diagnostic biomarker for esophageal cancer.

Source population

The source population of this study was esophageal cancer cases residing in the catchment of selected hospital.

Study populations

The study population comprised all cases of esophageal cancer and their corresponding healthy relatives who attended the hospital during the study period.

The Study Methods

To elucidate the epidemiology and histology of esophageal cancer, data from 630 cases were collected using data abstraction chart and analyzed using descriptive statistics. Saliva samples from 211 participants (108 cohabiting healthy controls and 103 treatment-naïve esophageal cancer patients) were collected for oral microbiota characterization and analyzed via V4 16S rRNA sequencing. Additionally, liquid chromatography-tandem mass spectrometry assessed internal exposure to 39 mycotoxins and metabolites in plasma samples from 166 esophageal cancer patients and 166 location-matched healthy controls.

Ethical Consideration

This study was reviewed and approved by Research Ethics Committee of the Department of Microbiology, Immunology, and Parasitology and the Institutional Review Board of the College of Health Science at Addis Ababa University. Before beginning the data collection, a formal letter requesting for cooperation was obtained from the Department of Microbiology, Immunology, and Parasitology to the selected hospitals to acquire their full consent. A copy of this letter was sent to each hospital's oncology department or unit by the local institutional review board to request assistance in the study. Additionally, the ethical approval was obtained from the Oromiya regional health Bureau. A specimen was collected after informed consent was obtained from each participant.

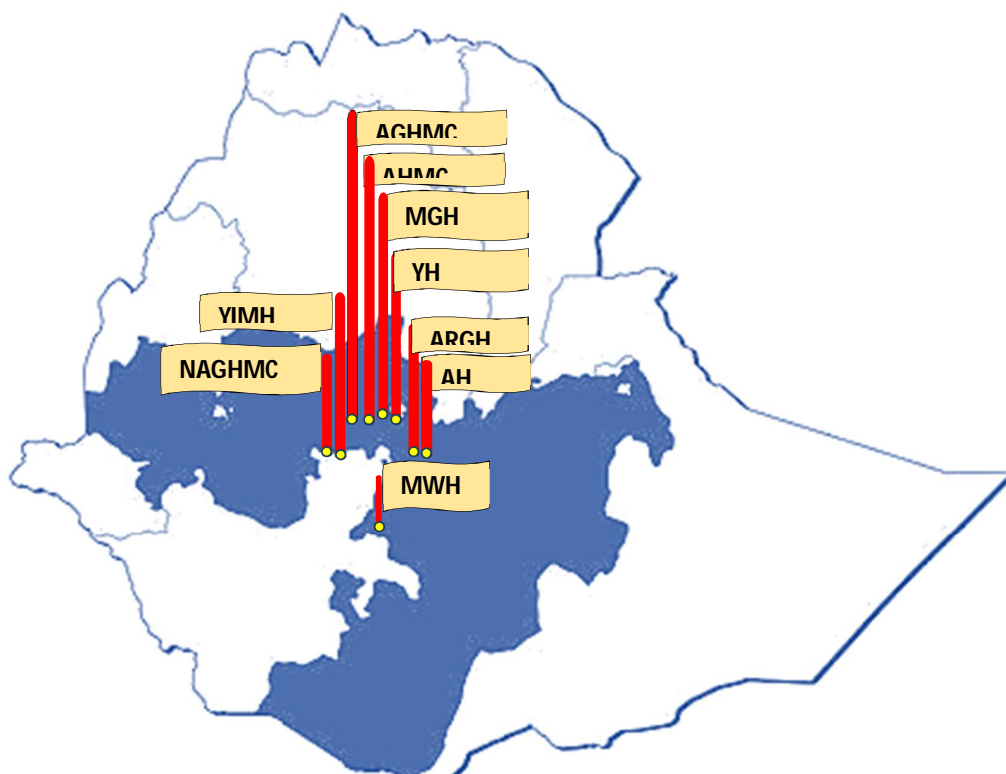


Figure 5: Map of hospital locations included in this study from the Oromia region (colored in blue) of Ethiopia.

AGHMC- Adama General Hospital and Medical College, AHMC- Adama Hospital Medical College, AH- Asella Hospital, ARGH- Asella Rehoboth General Hospital, MWH- Medda Wolebu Hospital, NAGHMC- Negele Arsi General Hospital and Medical College, YH-Yoya Hospital, YIMH-Yanet Internal Medicine Hospital

1.8. Limitations of the study

Disease condition may alter the oral microbiome's composition, which could add bias for establishing a cause-effect link. Additionally, compared to healthy controls, the dietary habits of EC patients were altered by their disease status, which modifies the degree of mycotoxin exposure through diet. Mycotoxins have a short half-life in plasma, and some polar mycotoxins are difficult to detect in plasma samples that need another matrix. This calls for establishing of a prospective cohort research that incorporates various matrices and a potential marker of long-term exposure.

1.9. Structure of the study

This dissertation consists of five chapters. In the **chapter 1**, the epidemiology, risk factors, and etiology of EC were introduced. A brief literature review on EC that mostly outlines its

epidemiology, etiology, diagnosis, and treatment were also included. In this chapter the goals, problem statement, and significance of the study, methods, and study scope were also included.

Chapter 2 examined and provided the epidemiological risk factors and histological types of esophageal cancer which was published in Cancer Reports. In **Chapter 3**, the association of oral microbiome with esophageal cancer and their potential biomarker for EC was presented. The manuscript of this work was submitted to peer-reviewed journal and it's under review. The **chapter 4** provides detail of work on mycotoxin exposure assessment for a possible risk factor for EC in Ethiopia and this work was published in Internal Journal of Hygiene and Environmental Health. The **chapter 5** summarizes the findings of the previous chapters, provides a conclusion, and offers suggestions for further study. The structure of this dissertation is shown schematically in **Figure 6**.

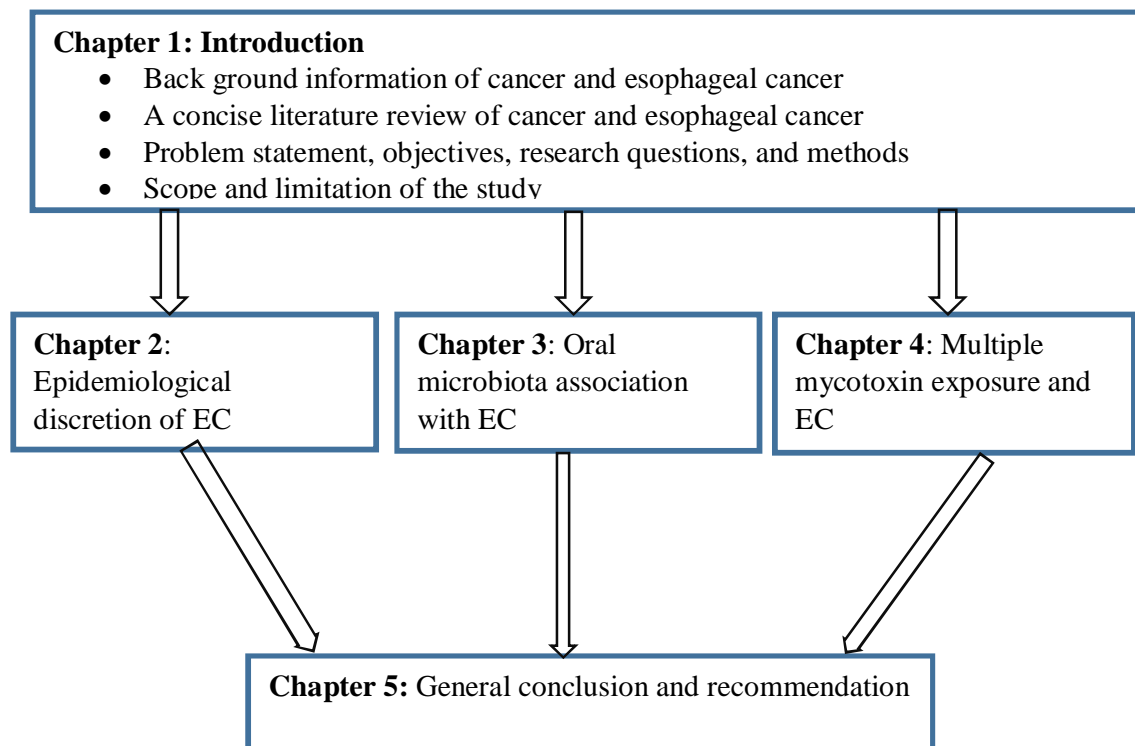


Figure 6: Schematic presentation of the fellow the thesis

Chapter 2: Exploring Esophageal Cancer in Ethiopia: Elevated Incidence in Females and Younger Cases

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Authors contributions:

Girma Mulisa: conceptualization, investigation, writing – original draft, methodology, validation, visualization, writing – review and editing, formal analysis, project administration, software, data curation, supervision, resources, funding acquisition. Tamrat Abebe: conceptualization, writing – original draft, methodology, visualization. Bekele Gutema: project administration, writing – review and editing, writing – original draft, formal analysis, data curation. Jannatul Mahmuda: methodology, visualization, writing – review and editing, formal analysis. Md. Al Amin Khan: methodology, visualization, writing – review and editing, software. Tarik Gheit: methodology, visualization, writing – review and editing, formal analysis. Zdenko Herceg: writing – review and editing, methodology, formal analysis, conceptualization. Fazlur Rahman Talukdar: conceptualization, investigation, writing – original draft, methodology, visualization, writing – review and editing, formal analysis.

2.1. Abstract

Background: Esophageal cancer is a public health concern in Ethiopia. The first steps in determining the burden of the disease and possible etiopathological connections are determining the incidence and demographic characteristics of the two histological subtypes, ESCC and EAC.

Aim: To identify the age and gender-specific incidence patterns of the most common subtype of esophageal cancer in a high-incidence area of Ethiopia.

Methods: Using retrospective cross-sectional study conducted from August 2019 to August 2022, nine hospitals' pathology registries yielded 630 cases from a high-incidence esophageal cancer area in Ethiopia. The patient records were carefully reviewed and data on age, gender, tumor location, and histological types was systematically compiled. The patient data were retrieved and descriptive statistics were used to generate results.

Results: ESCC subtype, accounted for constituting 500 (79.437%) of the cases. A gender disparity was observed, with 62.80% of cases occurring in females and 37.20% in males. This distribution of higher female ESCC incidences aligns with previous findings indicating a regional consistency and probable aetiological factor. Furthermore, ESCC incidence peaked at 40-50 years in females, highlighting an age-related incidence trend. EAC was observed in 67(51.5%) female and 63(48.5%) male showing similar prevalence. Spatial analysis revealed that the majority of ESCC cases were located in the lower esophagus, followed by the middle part, with fewer instances in the upper esophagus.

Conclusion: This study from Ethiopia identified ESCC as the predominant subtype, with a marked female predominance and age-related gender disparities. EAC with a lesser proportion identified with consistent spatial distribution patterns in both gender genders provide valuable insights into the epidemiological landscape of this disease. These findings emphasize the urgency of targeted research to uncover the underlying factors.

Keywords: Esophageal cancer, Esophageal squamous cell carcinoma, Ethiopia, Gender and Age

2.2. Introduction

ESCC in Africa has been linked to a number of risk factors, including as poor nutrition, alcohol use, and smoking. The development of ESCC in Africa has also been linked to environmental variables, including exposure to aflatoxins from contaminated food and high amounts of nitrosamines in traditional food preservation methods (43,283) (284). Traditional alcoholic beverages such kachasu in Malawi, Mozambique, and mahewu in Zimbabwe have been linked to ESCC in various parts of Africa (285).

Africa has a high incidence of EC, which is concerning because this histological subtype is frequently identified at an advanced stage, which restricts treatment options and results in poor outcomes (36,37). Many EC cases remain undetected until it is too late for effective treatment because of a lack of awareness and screening programs in many African countries. Additionally, there are sometimes few options for cancer therapy due to the high expense of care and the scarcity of facilities in many African countries. Furthermore, epidemiological data from African countries are essential since any distinctions from those in Europe or other Western nations can help to provide light on the etiology of esophageal cancer.

Research has indicated that men are more prone to smoke and drink excessively, two risk factors linked to the development of EC, which may help to explain why incidence rates are higher in men.(32,33). Furthermore, EC may arise as a result of hormonal variations between males and females. For instance, it has been demonstrated that estrogen protects against the development of EC, which may account for the lower incidence rates in females in the majority of countries worldwide(32,34). Prevention measures that specifically target the groups at the highest risk can be devised by recognizing the underlying risk factors and the sex-specific incidence patterns of ESCC. For instance, preventative efforts have to concentrate on lowering alcohol and tobacco use among men and raising knowledge of the protective benefits of estrogen for women. Furthermore, research and conclusions about risk groups might be used to design innovative preventative strategies.

Additionally, programs for screening and early detection might be created to target the highest-risk groups. Nonetheless, the frequency of ESCC varies by age and sex based on local risk factors in East Africa and other countries (286). Reviewing medical records from tertiary hospitals makes it challenging to establish a temporal relationship and evaluate the male-to-female ratio in place and time for EC patients because of referral biases. In order to better understand the aetiology of the disease, we examined sex-specific incidence patterns

from referral hospitals located within the catchment of Ethiopia's EC high-incidence district. We also looked at other clinical and demographic characteristics.

2.3. Materials and Methods

Study design and setting

Nine general hospitals including Adama Hospital Medical College (AHMC), Adama General Hospital and Medical College (AGHMC), Muse Genera Hospital (MGH), Asella Rehoboth General Hospital (ARGH), Medda Wolabu Hospital (MWH), Asella Hospital (AH), Yanet Internal Medicine Hospital (YIMH), Yoya Hospital (YH), and Negelle Arsi General Hospital and Medical College (NAGHMC)—were included in the cross-sectional study design using healthcare facilities. These hospitals' approximate geographic locations are displayed in **Figure 7A**. These medical facilities were chosen because they are situated in the catchments of Ethiopia's Arsi-Bale provinces, which have a high prevalence of EC. Suspected patients are sent to these institutions from other hospitals in the catchment areas because they offer endoscopic and pathological examination services. Cancer patients can receive chemotherapy and palliative care at Asella Hospital and AHMC.

Data sources and study population

Retrospective data was gathered from August 2019 to August 2022 on 464 confirmed cases of EC patients from AGHMC, AHMC, and AH. After receiving consent from the cancer department unit and hospital administration, the data was taken from the pathology laboratory registration logbook and patient records. Included were all pathologically verified EC patients diagnosed throughout the study period, complete with age, sex, histological type, and cancer site. The remaining patient information was gathered prospectively from the medical facilities listed in the study setting. The data collection numbers for each hospital are shown in **Figure 7B**.

Data collection tools and procedures

Demographic information (age, sex, and place of residence) and clinical information (EC histological type, anatomical location of EC on the oesophagus, and clinical presentation) were gathered from the patient card and registration logbook using a standardized data extraction chart. To collect data, oncologic nurses with bachelor's degrees and data collection experience were sought out. They received a half-day training that covered the checklist's contents and the study's goal. To get acquainted with the data extraction checklist, pre-tests were carried out. We acknowledge the possibility of incomplete data collection in some

cases. We used many sources whenever possible and put in place a strict data verification procedure to mitigate this. Where appropriate, the results indicate that cases with substantial missing data for the parameters under investigation were not included in the study.

Data management and analysis

Precautionary steps were made to guarantee the data's quality and prevent duplicate data from being collected from oncology departments' patients cards and pathology laboratory registration logbook. To characterize EC cases by age, sex, and cancer location, descriptive statistics were calculated, including percentages and measures of central tendency and dispersion. Figures and tables were used to display all of the EC patients' demographic information.

2.4. Results

Patients' characteristics

The male to female ratio was 1:1.53, with 381 (60.5%) of the 630 EC patients being female. The majority of patients, 398 (63.17%), were below the age of 60 years with 72% of total female patients and 50% male patients within this category. The mean age of the patients was 53.58 with an SD of ± 12.9 with the overall age range of 18–105 years. The mean ages of men and females were 57.33 (SD ± 13.2) and 51.12 (SD ± 12.108), respectively.

Distribution of ESCC and EAC Incidence Patterns

Among the two histological types of EC, ESCC was the dominant type, accounting for 79.37% ($n = 500$) of the total cases (**Figure 8A**). In contrast, EAC constituted a smaller, yet noteworthy proportion of 20.63% (130 cases). A higher proportion of individuals with ESCC were observed among younger age groups when compared to those with EAC (**Figure 8B**).

Sex Distribution among ESCC Patients

The present study also examined the sex distribution among patients diagnosed with ESCC. The sex distribution among the 500 ESCC patients is shown in (**Figure 8C**), providing insights into the demographic composition of our study participants. This distribution revealed that approximately two-thirds (314 patients) were females, whereas the remaining third (186 patients) were males. This sex-specific distribution contributes to an essential component in the larger context of our findings. Unlike the usual ESCC incidence pattern, our study population demonstrated a higher incidence rate among females, accounting for ~62.80% of all cases, whereas males constituted approximately 37.20% of the total cases. The presence of sex-related differences with increased female incidence not only adds

convolution to our findings but also prompts investigations into the potential vulnerabilities associated with females in Ethiopia that may contribute to these divergent phenomena. However, in EAC, the male–female ratio is almost equal with a slightly higher number of females (51.5%) than males (48.5%) (**Figure 8D**).

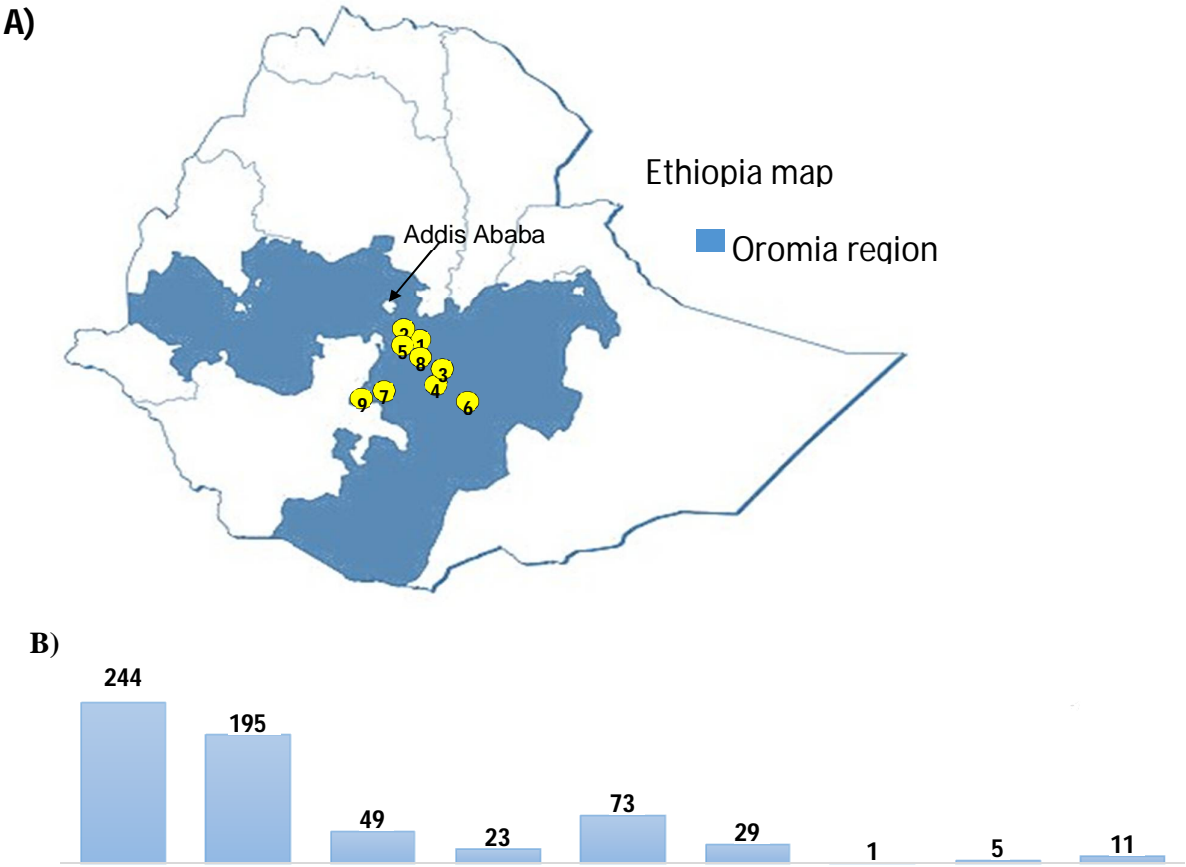


Figure 7: Map of hospital locations included in this study from the Oromia region (colored in blue) of Ethiopia.

A) Map of selected hospitals from the Oromia state in Ethiopia. **B)** Names of hospitals and the number of cases enrolled from each hospital.

1) Adama General Hospital and Medical College	2) Adama Hospital Mediacal College	3) Asella Hospital	4) Asella Rehoboth General Hospital	5) Muse General Hospital	6) Medda Wolabu Hospital	7) Negelle Arsi General Hospital and Medical College	8) Yoya Hospital	9) Yanet Internal Medicine Hospital
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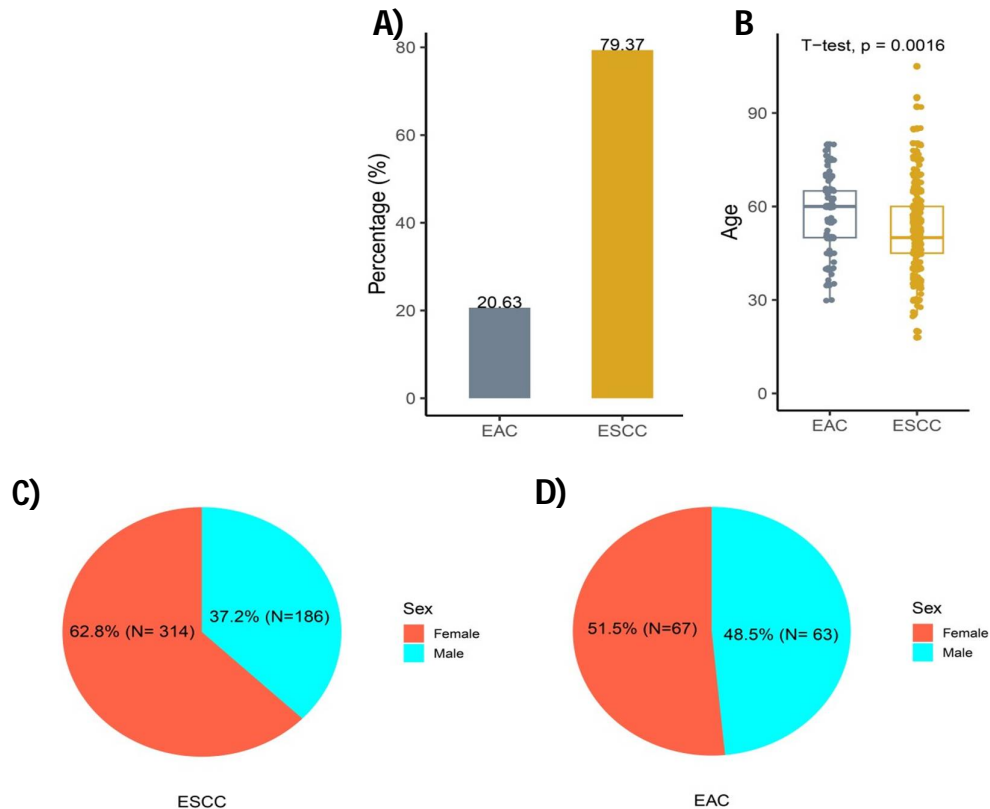


Figure 8: Distribution of the subtypes of esophageal cancer by histological type and sex

A) The proportion of ESCC and EAC in this study, **B)** The pattern of ESCC and EAC with the age of the age patients, **C)** The proportion of ESCC with respective patients' sex, **D)** The proportion of EAC with respective of patient's sex.

ESCC- Esophageal Squamous Cell Carcinoma, EAC- Esophageal Adenocarcinoma.

Age-specific Distribution of ESCC

The analysis of the distribution of ESCC in relation to age revealed that its prevalence varies across different age groups. One notable observation pertains to the substantial disparity in age distribution between males and females within the age range of 30–59 years (**Figure 9A**). Significantly, there was a notable disparity in the incidence of female ESCC cases within this particular age group, with prevalence nearly twice as high as that observed among their male counterparts. The average age of individuals with ESCC is notably significantly greater in males than in females (**Figure 9B**). Moreover, the number of female cases who were ≤ 45 years old was twice as high as the number of male cases in the same age group (as shown in **Figure 9C**). On the other hand, both sexes had nearly equal proportions of incidence among cases aged > 45 as shown in (**Figure 9D**).

Anatomical Distribution

We also investigated spatial distribution patterns of ESCC tumors within the esophageal anatomy (**Figure 10**). These findings offer critical insights into the tumor's preference for specific anatomical segments and contribute significantly to our understanding of preferential locations of ESCC tumors within the esophagus in our study population. The lower third of the esophagus has emerged as the epicenter of tumor prevalence, exhibiting a substantial share of ~50.20% of cases. Subsequently, approximately 37.80% of ESCC cases occur in the middle third of the esophagus. While displaying a slightly reduced prevalence compared to the lower third, this segment's involvement remains a significant component of the ESCC distribution. Interestingly, the upper third of the esophagus exhibited the lowest incidence, accounting for ~12.0% of all cases. This relatively low prevalence in the upper esophagus underpins the complex interplay between anatomical factors and tumor initiation. The spatial distribution of ESCC for male and female along the esophageal anatomy is depicted in (**Figure 10B, C**).

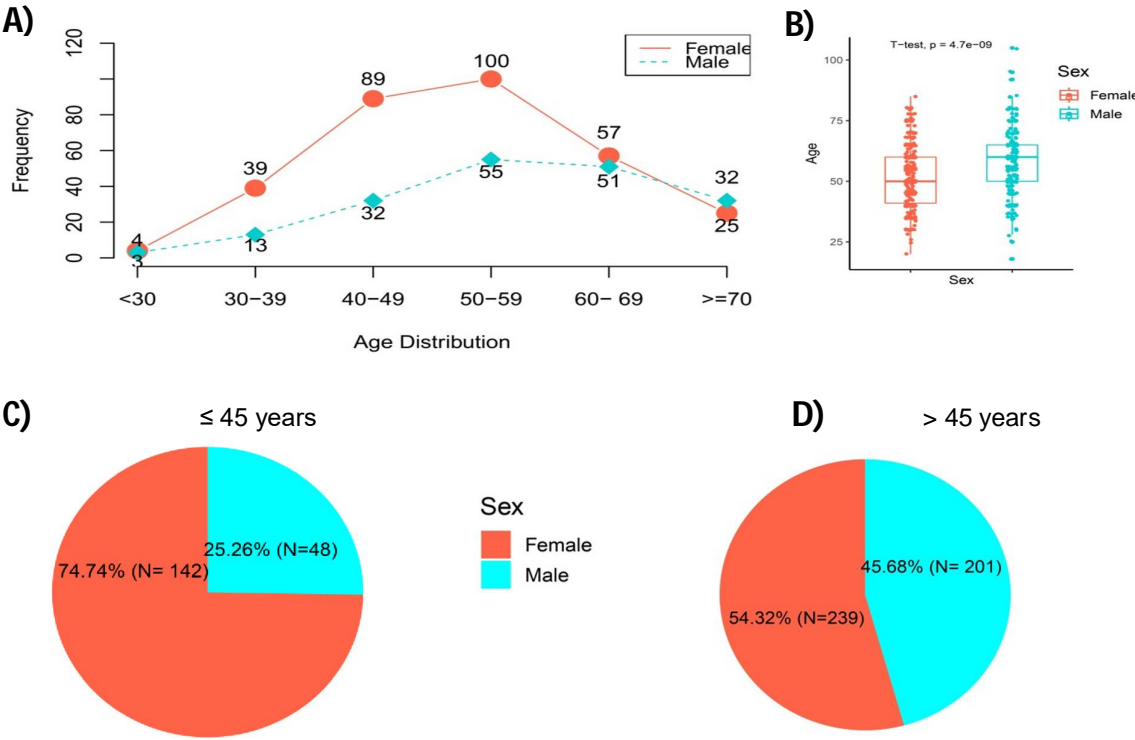


Figure 9: Age distribution between males and females.

A) Comparison of age distribution across various age groups between males and females **B)** Mean age distribution between males and females, **C)** Percentage of individuals with an age of ≤45 who have been diagnosed with esophageal cancer, categorized by gender, **D)** Percentage of individuals with an age of >45 who have been diagnosed with esophageal cancer, categorized by gender.

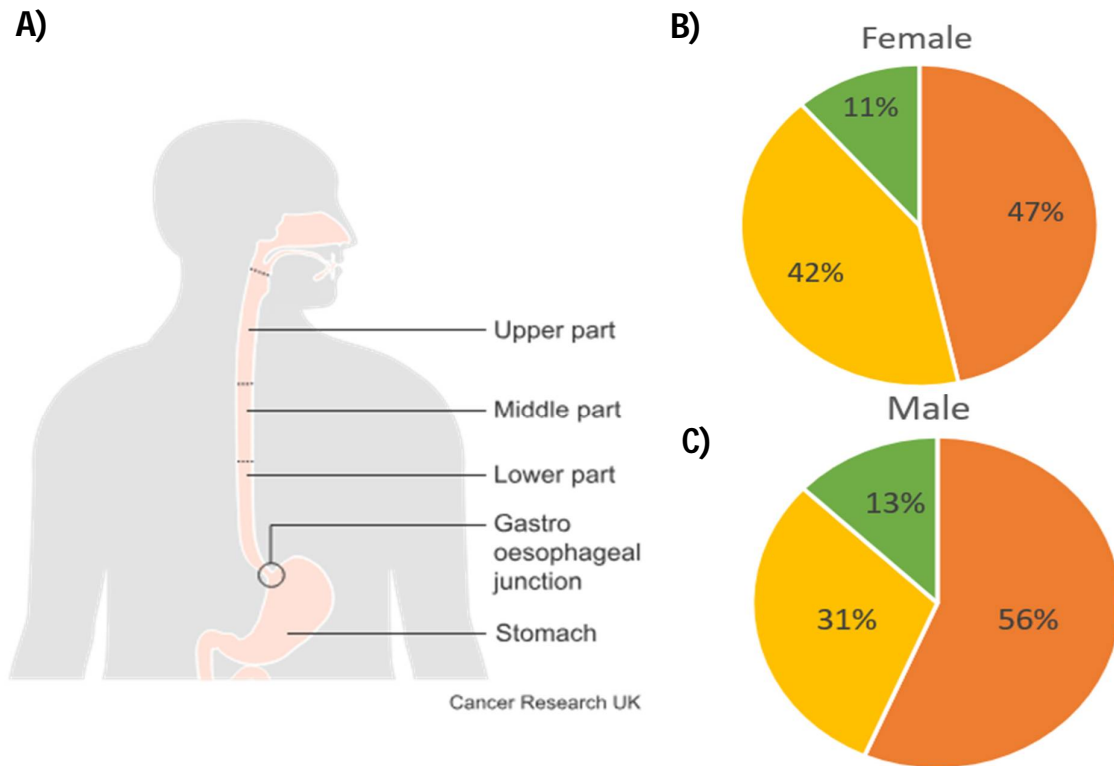


Figure 10: Anatomic location of ESCC tumors across the esophagus.

A) Anatomic division of the esophageal lumen, **B)** ESCC cases in females based on the anatomic location of the esophagus and **C)** ESCC cases in males based on the anatomic location of the esophagus.

2.5. Discussion

The current cross-sectional study on esophageal cancer, carried out in Ethiopia, makes a substantial addition to the body of knowledge on the subject. Our results show that compared to EAC, the incidence rate of ESCC is four times higher. More emphasis should be paid to this significant difference between the incidence patterns of ESCC and EAC. According to a recent systematic review and meta-analysis, a number of environmental and lifestyle factors combine to influence the development of ESCC, increasing the disease's overall risk (58). Our results align with the larger esophageal cancer epidemiological context. The high prevalence of ESCC highlights the importance of investigating Ethiopian-specific environmental, genetic, and lifestyle factors that may be involved in this frequency.

Both alcohol use and cigarette smoking were found to have no significant correlation with the incidence of EC in earlier Ethiopian studies (28,57). On the other hand, it was found that EC risk factors were more closely linked to the consumption of hot porridge, dairy products, food preparation techniques, exposure to x-rays, and chewing khat (28,57,250). These findings

emphasize the necessity for further investigation into the unique associations of these risk factors in association with the diversity of Ethiopia.

According to comparable data from Sudan, the risk factors in Ethiopian and Sudanese contexts are same (89,287). On the other hand, Ethiopian data regarding the prevalence of ESCC were inconsistent. ESCC was found to be present in almost all (98.3%) of the identified cases in one study by Deybasso et al. (58) whereas Wondimagegnehu et al. found in another investigation that ESCC accounted for just over half (56.70%) of the cases studied (58). The reason for the discrepancy in numbers is because Wondimagegnehu et al.'s data covers multiple regions of Ethiopia, whereas Deybasso et al.'s data was collected from a single district. Tumor histological type variations are influenced by geographic variation, which includes variations in lifestyle and exposure to the environment.

Geographical heterogeneity in the location and histological type of tumors was also observed in Pakistan (288). Obesity and smoking are two risk factors for this histological type of gastro-intestinal reflux disease that were not previously noted in the catchment population (28), which may be the reason for the low incidence of EAC in this study. Even though our results indicate a smaller percentage of EAC, the fact that EAC is becoming more common worldwide makes future research crucial for possible etiological studies (14). This highlights the significance of closely observing changes in lifestyle and other possible causes.

The gender difference in ESCC incidence complicates our knowledge of the condition. The increased incidence of ESCC in women raises major research on the interaction of behavioral, genetic, and hormonal variables. This insight has led to additional research on the distinct risk profiles of men and women. Interestingly, the incidence of ESCC is significantly skewed by gender, with a greater proportion of female patients in our cohort. Males have a significantly higher incidence of EC (70%) than females worldwide (29,30).

Furthermore, **Figure 11A** shows that Ethiopia has a greater incidence of EAC than other countries in the region, which is consistent with studies of an increase in EAC worldwide, even in economically disadvantaged countries (14). A notable male preponderance in ESCC cases has been documented in Africa, especially in the Eastern and Southern regions where EC rates are elevated (33). For example, ESCC is more common in men in South Africa (63). Similar trends have been found in other countries with high male incidence rates, including Tanzania (289), Kenya (290,291), and Uganda (292,293). On the other hand, other research

found that women in Somalia (294) and Eritrea (295) and Sudan (89,287) had greater rates of ESCC. A slightly higher prevalence of female patients has also been reported by previous Ethiopian research (41,296).. The findings of this study were compared with those of other earlier research projects conducted in African EC Corridor nations, such as Sudan, Kenya, Somalia, Uganda, Tanzania, Malawi, and Eritrea (297). Our findings were compared with those of earlier research from both Eastern and Southern Africa. **Table 1** summarizes these findings and presents the incidence rates by gender from research done in Ethiopia and its neighboring countries.

This comparison shows that the prevalence of female cases is higher than that of male cases in Ethiopia's northwest (Sudan), northeast (Eritrea), and east (Somalia) (89,287,294,295). On the other hand, the prevalence of male cases is two to three times greater in southwestern countries like Kenya, Uganda, and Tanzania (289,290,292). **Figure 11B** shows that the incidence of EC in males increases steadily from East to South Africa. This geographical-gender-based incidence association is also evident in ESCC which is the predominant cancer in this region (**Figure 11C**). These discrepancies could be explained by regional and culturally particular behaviors influencing risk variables in different countries.

Our analysis revealed a lower incidence of EAC than ESCC, which is in contrast to patterns seen in many wealthy countries. This discrepancy might result from the African population's lower prevalence of obesity and gastric reflux disease, both of which are recognized to be risk factors for EAC. This disparity is also influenced by the tobacco, alcohol, and socioeconomic trends that are different in our area compared to certain industrialized countries. In order to fully understand these variations and their consequences for preventative and therapeutic approaches, further research is required. Additional research is required to gain a better understanding of the risk factors linked to ESCC. Unfortunately, we were unable to collect thorough clinical and demographic data, which made it challenging for us to remark on the etiological variables influencing this incidence pattern. In particular, research needs to focus on pinpointing regional risk factors that are unique to particular age groups and gender-specific. This knowledge will improve our overall knowledge of the illness and be crucial in creating focused preventative and treatment plans.

Women under 45 years of age have a much higher incidence rate of ESCC (75% of cases in this age group), indicating that they are more susceptible to ESCC than their younger counterparts or that the exposure risk is higher for younger women than for men. In contrast,

women over 45 years of age had a relatively lower frequency of ESCC (57.18% of cases). Tanzania has previously reported on the occurrence of ESCC in young people (less than 45 years old) (298) and identified specific risk factors such as infrequent tooth brushing, secondhand tobacco smoke exposure, and pest infestation of grain and/or nuts. Significantly more young people (less than 30 years old) in Kenya were reported to have ESCC, while the risk factors for this disease had not been identified(299).However, according to another study from a cases series report on cases of young age EC risk factors in Kenya, the majority (79%) of the participants reported having a family history of cancer, while only 15% reported having exposure to alcohol or tobacco. These findings suggest that alcohol and tobacco were not important predictors of EC (300). Our results, along with the earlier report from Eastern Africa, call for further research to determine the possible risk factors for ESCC's early development in this area.

These results generate several valuable insights that can be acquired. The observation that ESCC exhibits a higher likelihood of occurrence among women aged 30 to 59 underscores the necessity of scrutinizing sex-specific risk factors during this specific age group. Variations in hormonal levels and specific lifestyle factors have been identified as potential contributors to the increased susceptibility of women to develop various forms of cancer. Furthermore, our observed findings demonstrate the intricate nature of the aetiology of ESCC in Ethiopia by elucidating the interplay between two major factors such as early age and higher incidence in women which collectively contribute to its divergent patterns of distribution compared to other high-incidence populations(33).

An increased distribution of ESCC frequency in males with increasing age was observed. Men aged greater than 45 years exhibit a higher incidence rate of ESCC (42,82%), while men aged less than 45 years demonstrate a lower frequency of ESCC, constituting only 25% of the total cases. These findings highlight a direct age-related trend in men, indicating that older men are more susceptible to developing ESCC compared to younger men. Patients in Ethiopia often present with advanced stages (III and IV) of the disease, which is compounded by an increased incidence with age until 60(59). The high incidence of ESCC among older men predicts a poor treatment prognosis, as advanced age is a predictor of poor treatment outcomes(71). It is noteworthy that the incidence rates of ESCC in both sexes begin to converge within the age bracket of 60 to 69 years. This interesting observation implies that, in the context of ESCC, age may exert a greater influence than sex during this specific stage

of life, potentially diminishing the significance of sex-specific factors that were more prominent in earlier stages of life.

The observed sex-specific variations in the age-related distribution of ESCC frequency emphasize the complex interplay between age, sex, and the development of the disease. The underlying mechanisms driving these differences warrant further investigation in consider. Potential factors contributing to the increased susceptibility of younger women to ESCC may include hormonal factors and early exposure to risk factors. Conversely, the higher ESCC frequency in older males could be influenced by distinct risk factors prevalent in this age group, such as lifestyle choices, dietary habits, or long-term occupational exposures.

The importance of time as a modifier of exposure-response in chronic disease including cancer risk factors is measured by the age of patients(65,66). Understanding the age and sex dynamics in ESCC incidence is crucial for designing targeted prevention strategies, early detection measures, and personalized treatment approaches. Tailoring interventions based on age and sex-specific risk profiles can improve the effectiveness of prevention and management efforts. Furthermore, these findings underscore the importance of age and sex considerations in clinical decision-making, patient counseling, and public health initiatives aimed at reducing the burden of ESCC. Future research endeavors should focus on elucidating the underlying mechanisms behind these age and sex disparities, paving the way for improved risk stratification and more precise interventions in ESCC management.

The predominant histological type in Africa is ESCC, which is consistent with findings from our and other developing regions(301,302). The incidence of EC is higher in males than females across most of Africa, except in North Africa where the rates are similar(31,62). This male predominance is particularly evident in high-risk regions such as Eastern and Southern Africa(31).

In contrast, European countries, especially in the West, have seen a rise in esophageal adenocarcinoma, largely attributed to lifestyle factors like obesity and gastroesophageal reflux disease. This shift in histological type is less pronounced in Africa, where lifestyle and environmental factors such as tobacco use and low socioeconomic status remain significant risk factors for ESCC(62).

The differences in EC incidence and histological types between Africa and Europe highlight the need for region-specific prevention and treatment strategies. In Africa, the high mortality-to-incidence ratio underscores the challenges in healthcare access and the need for improved diagnostic and treatment facilities(62,170). In Europe, addressing lifestyle risk factors could help mitigate the rising incidence of adenocarcinoma. These comparative insights are crucial for tailoring public health interventions to effectively manage and reduce the burden of EC in diverse populations, especially in Africa.

The anatomical distribution of ESCC tumors along the esophagus provides valuable clinical insights. Our findings align with previous global results (303,304) and those from Ethiopia (42) indicating that the lower esophagus is the most affected anatomical structure. The concentration of tumors in the lower third of the esophagus necessitates focused strategies for early detection, particularly in this region. The findings also highlight the need for further research to elucidate the underlying factors contributing to the differential tumor distribution within the esophagus. Factors such as local anatomy, exposure to carcinogens, genetic predisposition, and cellular and molecular characteristics may play a role in determining the site-specific susceptibility to ESCC. Moreover, obtaining valuable information regarding anatomical distribution is crucial for planning potential palliative care, including esophagostomy, while considering associated complications and patient benefits(305).

Overall, the results emphasize the importance of considering the anatomical distribution of ESCC tumors within the esophagus in clinical practice and research. Understanding the patterns of tumor occurrence can guide targeted approaches for prevention, early detection, and treatment strategies. Moreover, this knowledge may contribute to the development of personalized medicine approaches tailored to the specific needs and risks with tumor location in ESCC patients as tumor location of esophageal cancer is the predictors of patient survival,(71) guide the treatment approach (306) and also the proxy marker of patients' severity of disease (predictors of metastasis)(307).

The results presented here hold implications for clinical practice and public health strategies. Integrating sex and age considerations into screening, diagnosis, and treatment protocols can enhance patient care. The observed disparities also serve as a call for future research aimed at elucidating the underlying mechanisms responsible for the observed trends. Such insights are crucial for the development of targeted interventions, personalized treatments, and improved outcomes in the management of esophageal squamous cell carcinoma.

Table 1: Male-to-female ratio of incident Esophageal cancer cases in Ethiopia in comparison to previous research conducted in the neighboring regions.

Studies	Country	Male-to-Female Ratio		
		Overall esophageal cancer	ESCC	EAC
Our study	Ethiopia	1: 1.53*	1: 1.7*	1: 1.06*
Wondimagegnehu, <i>et al.</i> 2020 (Ref-3)	Ethiopia	1:0.80	NA	NA
Deybso et al.2021 (Ref-8)	Ethiopia	1:1.08*	NA	NA
Shewaye et al. 2016 (Ref 7)	Ethiopia	1:1.24*	NA	NA
Gasmelseed <i>et al.</i> 2015 (Ref-20)	Sudan	1: 1.76*	1:2	2.1
Hamad, <i>et al.</i> 2017 (Ref-21)	Sudan	1: 1.47*	NA	NA
Mengistu et al. 2024 (Ref-33)	Eritrea	1:2.78*	1:3.48*	1:1.06*
Kadle and Dufle. 2017 (Ref-34)	Somalia	1:1.19*	1:1.26*	1.85:1
Degu et al.2022 (Ref -29)	Kenya	1:0.68	NA	NA
Abdihamid et al. 2024 (Ref-28)	Kenya	1:0.94	NA	NA
Gabel et al. 2016 (Ref- 30)	Tanzania	1:0.49	1:0.49	1:0.54
Obayo et al. 2023 (Ref-32)	Uganda	1:0.42	NA	NA
Alema and Iva. 2014 (Ref-31)	Uganda	1:0.34	NA	NA

**Ratios where female incidence> male incidences; NA=Not available, Ref=reference*

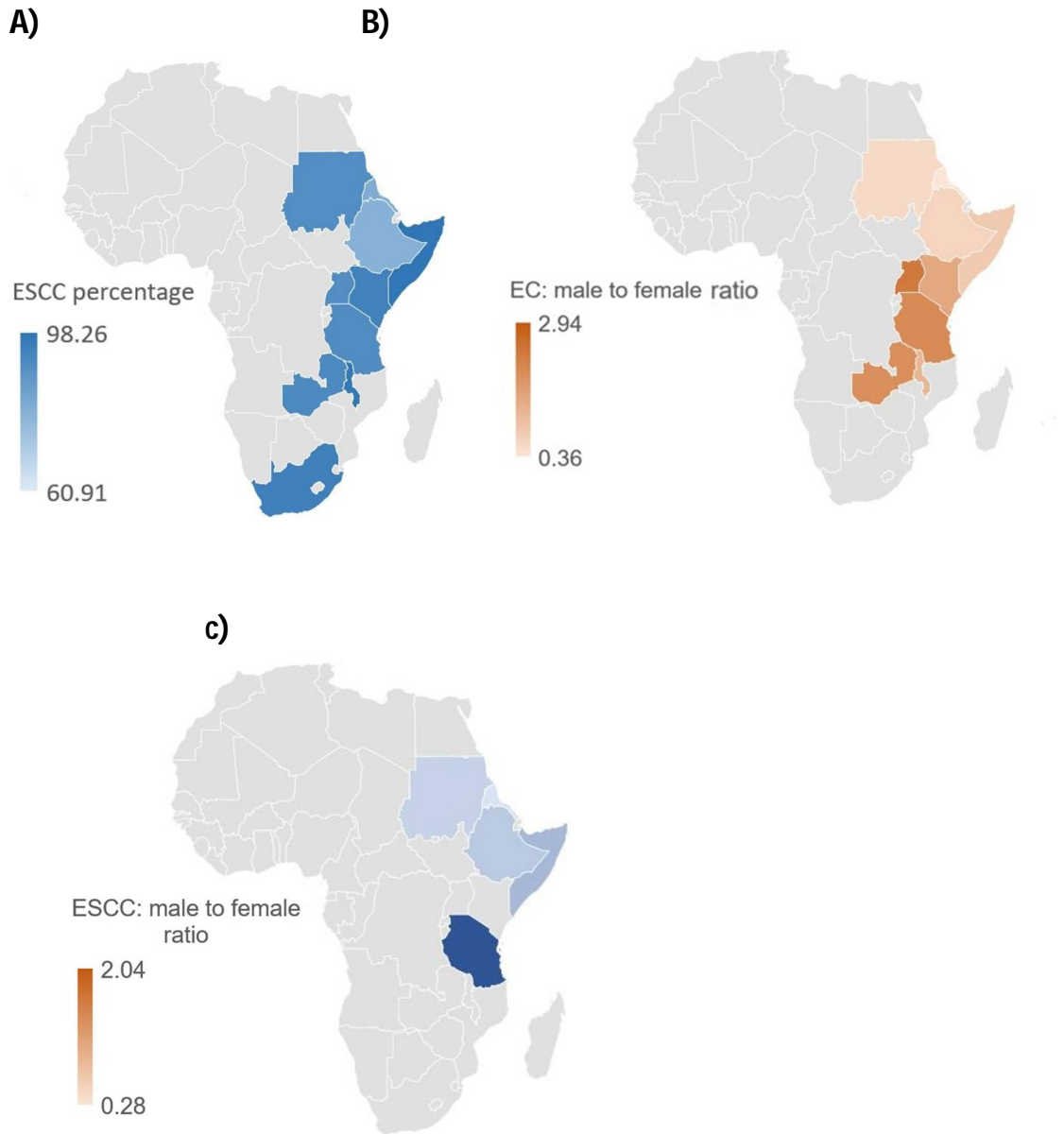


Figure 11: EC distribution across Eastern and Southern Africa

A) ESCC percentage across Eastern and Southern Africa. **B)** Male-to-female ratio of EC across Eastern and Southern Africa **C)** Male-to-female ratio of ESCC across Eastern and Southern Africa

2.6. Strength and limitation of the study

This is the largest dataset collected from several hospitals in a region of Ethiopia where EC is common. While our study provides valuable epidemiological data on EC in Ethiopia, we acknowledge that we were unable to thoroughly investigate the causes of observed differences compared to developed countries. This limitation stems from incomplete demographic and clinical data for many patients in our cohort. Future studies with more

comprehensive data collection are needed to elucidate the specific risk factors contributing to the unique epidemiological profile of EC in Ethiopia and other resource-limited settings.

2.7. Conclusion

The results of our study differ somewhat from prior findings, highlighting the necessity for further investigation into the intricate mechanisms governing age- and sex-related changes in ESCC among the Ethiopian population. Understanding the possible causes behind the increased incidence ESCC among younger age female patients could lead to help in prevention strategy by increasing the awareness among this population in Ethiopia.

This study is one of the largest cross-sectional studies conducted in Ethiopia. We identified that ESCC is the most common subtype of esophageal cancer, accounting for a significant number of cases. Its widespread occurrence has a significant impact on the health landscape of Ethiopia, making it as one of the major health concerns. The marked sex disparity, with a higher incidence among females, not only highlights the need for sex-specific investigations but also aligns with previous similar findings in Ethiopia and Sudan, hinting at shared underlying factors. Particularly noteworthy is the age-related trend observed among females, with ESCC peaking at early age <50 emphasizing the importance of considering age-specific risk factors in future research and intervention strategies. Spatial analysis revealing most cases in the lower esophagus further contributes to our understanding of the disease distribution within the Ethiopian context. These findings collectively emphasize the need for a comprehensive, multi-faceted approach to unravel the complex interplay of factors contributing to ESCC in Ethiopia, providing a foundation for targeted public health initiatives and further research endeavours in this unique population.

Chapter 3: Salivary microbiota predicts esophageal cancer in a rural Ethiopian cohort with low behavioral risk factors

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Authors contributions:

G.M., A.M., G.L., and T.A. designed the Ethiopian study. G.M. and A.M. oversaw sample collection. J.Z., A.D., and J.E.B. were responsible for extraction, sequencing, and analysis of Ethiopian microbiome data. J.Z. was responsible for qPCR analysis. I.J.A-P. and L.S.W. were responsible for data collection and processing of international oral microbiota cohorts. I.J.A-P., L.S.W. and J.E.B. were responsible for comparative analysis of international oral microbiota cohorts. R.P-G., M..D.B. and S.D.S. were responsible for mycotoxin analysis. J.Z. and J.E.B. were responsible for cross-cohort machine learning analyses. S.D.S., T.A., and J.E.B. were responsible for obtaining funding. G.M., J.Z., I.J.A-P., L.S.W., R.P-G., T.A. and J.E.B. were responsible for writing the manuscript; all authors reviewed and approved of final submission. J.E.B. has primary responsibility for the final manuscript.

1.1. Abstract

Esophageal cancer (EC) is a malignant disease with high mortality rates due to difficulty in early diagnosis. Ethiopia is among the countries of the African EC belt typified by high EC prevalence, early onset age, and poor disease prognosis. While the precise etiological factors remain unknown, common behavioral risk factors, including alcohol and tobacco use, are relatively low in many areas where EC prevalence is high including regions of Ethiopia. Previous reports in western populations correlated oral microbiota composition to EC risk; however, little is known about the oral microbiota in African populations, especially in the EC belt, and its role as a potential risk factor for EC. In this study, we characterized the salivary microbiota of 211 individuals using V4 16S rRNA sequencing (103 treatment naive EC and 108 cohabitating healthy controls) residing in rural agricultural regions of Oromia, Ethiopia. We find that the salivary microbiota in the healthy population is highly diverse, forming two functionally distinct community clusters differing in diversity, composition, and absolute abundance. Salivary microbiota composition was associated with sex and alcohol consumption, but not age. Comparison against reference groups from geographically distinct populations showed that cluster 2 resembled other east African populations while cluster 1 was unique to the Ethiopian cohort. Both EC subtypes, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma, were associated with a loss of microbial diversity and an increased probability of having a cluster 2 microbiota (OR=3.3). Models trained on the Ethiopian cohort could accurately predict disease status in an external cohort of mid and late stage ESCC from China, a second region of high EC prevalence (AUROC=0.74). Taken together, these results support a role for the salivary microbiota as a non-invasive biomarker of EC and a potential for detection and prevention.

1.2. Introduction

There is growing evidence that human-associated microbes, the microbiota, play a role in the occurrence, pathogenesis and treatment outcomes of many cancer types. While much research has focused on the gut microbiota(308), which constitutes 99% of the healthy human microbiota that exerts both local and long distance effects(252), the oral microbiota is associated with increased risk of pancreatic(309), colorectal(310), and lung cancer(311). Poor oral hygiene (114–117) and teeth loss/decayed were associated with increased risk of EC (114) which is the indicative of oral microbiome involvement in this cancer type. Bacteria causes of chronic inflammation that induce genome instability one of cancer cause. It was observed that EC is caused by multiple factors that synergize each other in a dose-response manner(312). Injury to Esophagus due environmental exposure is possibly fueled by bacteria which are accessible from oral cavity and dysbiosis is associated with esophagitis. This implies the possibility of oral microbiome in the development of EC.

Connections between EC and the oral microbiota have been previously explored in the USA and China. A prospective study in the USA reported higher richness and abundances of *Tannerella forsythia*, *Streptococcus pneumoniae*, *Neisseria* spp., and *Porphyromonas gingivalis* as predictors of EC(313). Alternatively, a Chinese study showed that ESCC cases have an overall decreased microbial diversity compared to healthy control and dysplasia subjects(314). The lower abundance of oral microbiome was significantly associated with low Pepsinogen I to pepsinogen II ratio which is the validated predictors of gastric cancer and esophageal squamous dysplasia which the precursor lesion of EC (273). Combined epidemiological risk factors and oral microbiome biomarkers have been proposed to be used as non-invasive early screening markers of ESCC in China, although similar studies in other countries are needed to increase the global validity of the method(315).

Oral microbiota research, has been mainly conducted industrialized societies in the global west(316), although oral microbiota are distinct across different human populations(317), and shaped by one's evolutionary history, diet, environment, genetics, hygiene, and lifestyle(318–320). It is plausible that unique oral microbes in understudied populations play roles in disease or can aid in identifying population-specific disease biomarkers(321–323). These findings suggest the need for replicating studies in other population of the world including Africa(324). Understanding and accurately classifying similarities and differences between industrialized and non-industrialized populations' oral microbiome allow for more accurate

biomarker identification and provide a more comprehensive global picture of oral microbiome diversity. Further, comparative oral microbiota studies across global populations will help uncover variation in disease susceptibility, novel adaptive mechanisms, and potential prophylactic strategies.

For these, we characterized the salivary microbiota of EC cases and matched controls to characterize the oral microbiota of healthy rural Ethiopians, and to uncover potential biomarkers for EC. We further perform cross-cohort analyses from other east African, Asian, South American, and westernized populations cohorts and demonstrate that patterns of EC-associated dysbiosis can be translated to Chinese cohorts.

1.3. Materials and Methods

Participant recruitment and sample collection

Participants were recruited from the Oromia catchment area of the Adama Hospital Medical College, Adama General Hospital and Medical College, Muse General Hospital, Asella Reboth Hospital, and Meda Wolabu Hospital. The enrollment criteria were either being newly diagnosed with biopsy proven EC and being treatment-naïve, or being a healthy cohabitating relative. Individuals receiving antibiotics in the past two weeks and those with active oral disease were excluded from the study. After obtaining written informed consent, participants were interviewed using a semi-structured questionnaire to obtain socio-demographic and behavioral data relevant to EC risk factors. Information on tobacco usage (both smoking and chewing), alcohol use and khat use were collected as a binary outcome based on current self-reported usage. Tumor histological types were identified by hematoxylin and eosin stained microscopic histological examination.

The study protocol was approved by Institutional Review Boards of Addis Ababa University, College of Health Sciences with protocol number 024/21/DMIP and by the Federal Ministry of Education National Ethics Committee with protocol number 03/246/221/22.

For microbiota analysis, participants were instructed to deposit approximately 5 mL of saliva into a sterile 25 mL conical tube. The saliva was then mixed with an equal volume of 96% ethanol before transfer for long-term storage. For mycotoxin exposure, a whole blood sample of 5 mL was collected using an EDTA coated tube from each participant. Plasma was separated by centrifugation at 5000 rpm for 5 minutes, transferred to a sterile cryotube using a sterile pipet. Both saliva and plasma were stored at -80°C in a registered biobank until processing.

Microbiota characterization

200 µL of each saliva sample was extracted using the ZymoBiomics 96 MagBead kit (Zymo D4308) following the manufacturer's protocol. Mechanical disruption was performed using a FastPrep96 (MPBio) with a total disruption time of 5 minutes. Positive controls consisting of a defined community containing 8 bacteria were included and a reported purity of <0.01% contaminant DNA (Zymo D6300). Negative controls consisting of only extraction reagents were included on each plate. gDNA subsequently underwent primary amplification of the V4 16S rRNA region using the following primers V4_515Fmod_Nextera: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*GGTGYCAGCMGCCGCGGTAA* and V4_806Rmod_Nextera:

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTAC*NVGGGTWTCTAAT*

wherein the italicized portion represents the amplification primer and the remainder is used for subsequent indexing and sequencing. Samples were amplified using a high fidelity enzyme (KAPA HiFi Hot Start PCR KK2502) in the presence of SYBR green (Sigma S9430) with 5 minutes at 95°C, and 32 cycles of 20 sec @ 98°C, 15 sec @ 55°C, and 60 sec @ 72°C. Reactions were monitored in real time using a QIAquant 384 (Qiagen). Lack of amplification of negative controls was further confirmed through gel electrophoresis to ensure the absence of a true amplicon. All samples, including negative controls, were subsequently diluted 100x and indexed using 10 cycles of the same reaction conditions with 1 µM unique dual index (UDI) sequences available at github.com/BisanzLab/OHMC_Colaboratory. Amplicons were subsequently quantified using Quant-it Picogreen (Life Technologies P7589) and pooled at equimolar concentrations. Negative controls (failed reactions) were pooled at full available volume (9 µL). All liquid handling steps were performed using QIAcube HT, Integra mini96 or Opentrons OT-2. The pooled library was subsequently size selected using both gel (Qiagen MinElute 28604) and magnetic capture beads (Ampure XP Beckman A63881). The final library was inspected by TapeStation 4200 (D1000) and quantified using the NebNext library quantification kit (NEB E7630S). The library was loaded on a P1 600 cycle kit with 40%PhiX using an Illumina NextSeq2000. Raw sequencing data is available in the NCBI Sequence Read Archive (SRA) under accession PRJNA1137386.

Resulting data was processed using v2.1 of the following script: github.com/BisanzLab/OHMC_Colaboratory/blob/main/analysis_scripts/AmpliconSeq_q2.R Briefly, reads were processed using QIIME2 version 2023.5. Primer sequences were removed allowing for an error rate of 0.15 discarding any read without a valid primer

sequence on both ends. Next reads were denoised and overlapped using Dada2. Taxonomy was assigned using the Dada2 taxonomic classifier against the SILVA version 138.1 database. Metagenomic inference was performed from subsampled ASV data using PICRUSt v2.4.1 using the MetaCyc database.

Microbiota analysis

All data was processed using R 4.4.1. QIIME 2 artifacts were imported using qiime2R. Samples with less than 10,000 reads were discarded prior to downstream processing which included all negative controls. Alpha diversity metrics were generated after subsampling to the lowest available read depth for the sample set of interest using vegan::diversity (v2.6-6.1) for Shannon's diversity index and picante::pd (v1.8.2) for Faith's phylogenetic diversity and ASV (species) richness. CLR euclidean (Aitchison) distances were calculated on genus-summarized abundances by first using qiime2R::make_clr to conduct a centered-log2-ratio transformation followed by stats::dist to calculate the euclidean distance. Clustering was performed with the partitioning around medoids method using cluster::pam (v2.1.6) with the gap statistic calculated using cluster::clusGap.

Absolute quantification of saliva microbial load

Total salivary bacteria were quantified by qPCR analysis targeting the 16S rRNA gene. The microbial DNA was extracted with ZymoBiomix 96 MagBead kit (Zymo D4308) as mentioned above. The assay was 891F forward primer: TGGAGCATGTGGTTTAATTCGA, 1003R reverse primer: TGCGGGACTTAACCCAACA, and 1002P probe: [6FAM]CACGAGCTGACGACARCCATGCA[BHQ1], each at 200 nM. The qPCR reactions were amplified using iTaq™ Universal Probes Supermix (BioRad 1725132) on a BioRad CFX384 thermocycler following the procedure: Initial denaturation at 95°C for 5 minutes, and 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 15 seconds. Absolute quantification was performed using a standard curve of purified 8F/1542R primer amplified *Escherichia coli* MG1655 DNA(325). Each assay was performed in triplicate in a 10uL reaction mixture.

Statistical analysis

Univariate analysis was performed using appropriate base R functions for Welch's t-test (stats::t.test), Mann-Whitney U test (stats::wilcox.test), or Fisher's exact test (stats::fisher.test) as indicated in figure legends. Statistical analysis of distances were performed by PERMANOVA (vegan::adonis2). Statistical analysis of differentially abundant

taxon features were performed using ALDEx2 (v1.36.0) using either Welch's t-test for cross sectional analysis of community type (ALDEx2::aldex) or generalized linear model (ALDEx2::glm), both using Benjamini Hochberg false discovery rate correction. GLMs for alpha diversity metrics were performed using stats::glm. Model terms were subsequently reduced to minimize the Akaike information criterion (AIC). Differential pathway abundances were performed by normalizing to relative abundance and conducting a log2-transformation after addition of a pseudocount corresponding to 2/3s of the lowest per-feature abundance. Analysis was subsequently performed by Welch's t-test with Benjamini Hochberg false discovery rate correction. All plots were generated using ggplot2 and relevant extensions. Error bars represent standard error (SE) unless otherwise noted.

Comparison to global oral microbiota data

Ethiopian datasets generated in this study were compared to 16S rRNA V4 raw datasets, including those from Tanzania, Venezuela, Uganda, and westernized populations represented in the American Gut Project, as described elsewhere(326). Quality filtering of the data was performed with the `q2-quality-filter` plugin, and denoising was carried out using Deblur(327) via the `q2-deblur` plugin with a trim length of 150 bases, resulting in amplicon sequence variants (ASVs). The representative sequences were aligned using MAFFT(328) with the `q2-alignment` plugin, and a phylogenetic tree was constructed using FastTree2(329) via the `q2-phylogeny` plugin. The dataset was then filtered to include only adults, oral samples, single-timestamp samples, and those without antibiotic use or other treatments that could alter oral microbiome composition using the `qiime feature-table filter-samples` plugin. To ensure data robustness, features present in only one sample and with <5 occurrences across all samples were removed. Decontamination was applied using all available project controls (i.e., extraction reagent controls and no-template amplification controls) using decontam, and sequences associated with chloroplasts and mitochondria were removed. Finally, samples with fewer than five taxa and high levels (>40%) of identified contaminants were excluded, including individual "11492.A19489.TW99" (46.592% contamination from *Synechococcus*) and individual "11492.A19451.TW51" (45.833% contamination from JG30-KF-CM45). Additionally, duplicate samples were also removed (i.e., "11492.A19451.KG59e"). Alpha and beta diversity analysis were performed as described above. Statistical analysis of Shannon's diversity was performed by ANOVA with Tukey HSD post hoc test. All the code for obtaining and processing data from these studies is available at: github.com/microARCHlab/UGanadaGlobalMicrobiome_2025.

Model training and validation on external cohort

A literature search was conducted to identify additional oral microbiota studies of health and EC individuals which had used Illumina-based short-read amplicon sequencing covering the V4 region for characterization. Two Chinese studies were identified: Chen et al. (Bioproject accession PRJNA964904), and Jiang et al. (PRJNA853196) which had available sequencing data and unambiguous metadata. Both datasets were V3-V4 sequencing performed on an Illumina NovaSeq with 150x150 reads. Due to issues with denoising and overlapping V3-V4 amplicons on this platform, only the reverse read covering the V4 region was carried forward for denoising, taxonomic assignment and diversity characterization as above. For cross-cohort comparisons, 10 iterations of the following training/testing strategy were applied. First the Ethiopian cohort was randomly divided into a training set (139 participants), and a test set (72 participants). A random forest classifier (randomForest v4.7-1.1) model predicting case vs control was trained on genus-summarized proportional abundances from the training set and then used to predict both the test sets, and then the external cohorts. ROCs, and AUROCs were derived using the ROCR (v1.0-11) and pROC (v1.18.5) packages. Feature importances were derived by extracting the per-feature mean decrease in GINI coefficients from each iteration of the classifier.

Multiple mycotoxin biomonitoring

Mycotoxin determination in plasma samples is described in Mulisa *et al.*(330). Briefly, 300 μ L of acetonitrile was added to 300 μ L of each plasma sample for protein precipitation. Samples were vortexed for 2 minutes and centrifuged at 3300 g for 15 minutes at 4°C using a Multifuge 3 S-R centrifuge from Heraeus (Hanau, Germany). The supernatants were transferred to Eppendorf glass tubes in a Turbo Vap LV evaporator from Biotage (Dusseldorf, Germany) and evaporated at 40°C under gentle nitrogen gas flow until completely dry. The residues were re-dissolved in 150 μ L of injection solvent (60:40 v/v mobile phase A/mobile phase B) by vortexing for 2 minutes, then centrifuged at 2100 g for 1 min. The dissolved residue was transferred to a tube with a PVDF centrifuge filter of 0.22 μ m from Millipore (Cork, Ireland) and centrifuged at 9000g for 5 minutes at 4°C. The filtrate was transferred into a HPLC vial and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Water Acquity UPLC I-class system coupled to triple quadrupole XEVO TQ-XS mass spectrometer equipped with an electrospray ionization source. Chromatographic separation was done in an Acquity UPLC HSS T3 column (1.8 μ m particle size, 2.1 mm id x 100.0mm) with a matching VanGuard precolumn (5 mm). Analytes

were quantified against authentic analytical standards of 39 mycotoxins (3-acetyldeoxynivalenol, aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1, aflatoxin G2, aflatoxin M1, alternariol methylether, alternariol, beauvericin, citrinin (CIT), cyclopiazonic acid (CPA), diacetoxyscirpenol, deepoxy-deoxynivalenol, deoxynivalenol (DON), enniatin A, enniatin A1, enniatin B (ENN B), enniatin B1, fumonisin B1 (FB1), fumonisin B2, fumonisin B3, fusarenone-X, hydrolysed fumonisin B1, HT-2 toxin (HT2), neosolaniol, nivalenol (NIV), ochratoxin A (OTA), ochratoxin alpha, roquefortine-C, sterigmatocystin, tenuazonic acid (TA), T-2-toxin (T2), T-2 tetraol, zearalanone (ZAN), alpha-zearalanol, beta-zearalanol, zearalenone, alpha-zearalenol (α -ZEL) and beta-zearalenol) using matrix-matched calibration curves and normalized using an appropriate isotopically labeled internal standard (^{13}C -AFB1, ^{13}C -CIT, ^{13}C -DON, ^{13}C -FB1, ^{13}C -HT2, ^{13}C -T2 and ^{13}C -TA).

1.4. Results

Demographic characteristics of the study cohort

A total of 108 non-cancerous control individuals and 103 cases were successfully sequenced. Relevant participant demographics are displayed in **Table 2**. All the cases and controls were rural residents of Arsi, Bale, East Shoa, and West Harage districts of the Oromia region in Ethiopia. ESCC was the most common histological type accounting for 85% of cases (87/103 individuals). Cases were on average 12.5 years older than controls ($P=2.3\text{e-}14$, Mann-Whitney U test). Compared to controls, there were significantly more men in the case group representing 26% and 40% of the cases and controls respectively ($P=0.028$, Fisher's exact test). Usage of cigarettes, chewing tobacco, and khat were reported by less than 5 individuals per group and found to not be significantly different (**Table 2**). Alcohol usage was reported by 12% of participants and was significantly lower in the cases ($P=0.006$, Fisher's exact test). All participants reported occupations associated with farming/agriculture. Coffee consumption was reported by 95.7% of participants, which was marginally higher in cases ($P=0.003$, Fisher's exact test).

Table 2: Participant Demographics

Variable (Units)	Controls (N=108)	Cases (N=103)	P-value ¹
Age (mean±sd)	39.0 ± 7.1	51.5 ± 13.8	2.3e-14
Sex (M/F)	28/80	42/61	0.028
Cigarette Use (N)	0	3	0.11
Chewing Tobacco Use (N)	0	3	0.11
Alcohol Use (N)	20	6	0.0060
Khat Use (N)	4	5	0.74
Coffee Use (N)	99	103	0.0033
1- Mann Whitney U test for age and Fisher's exact test for categorical variables			

Microbiome sequencing

A total of 211 oral samples were successfully sequenced resulting in a mean post-processing read depth of 129,651±92,473 reads (mean ± sd) with a median depth of 115,188 [10,992-556,826] (median [range]). There was no significant difference between cases (N=103) and controls (N=108) in terms of read depth (P=0.94, Welch's t-test, 130,137±83,934 controls vs 129,141±101,068 cases). Samples were randomized across 4x96-well plates for DNA extraction with library preparation performed in a single 384-well plate to minimize potential batch effects in processing. No significant effects on alpha or beta diversity were observed across extraction batches. A total of 10 negative controls (DNA extraction with reagents only) were included across the 4 plates. While successful amplification by qPCR or gel electrophoresis was not observed in the negative controls, they were still pooled into the sequencing run at the maximum available reaction volume (*see Methods*). Negative controls resulted in <1,592 reads per sample (771±393) which were primarily assigned to Burkholderiales and *Escherichia coli*, known reagent contaminants(331), but which are also present in the oral microbiota. A total of 4 positive controls of a defined community were included (134,136±73,325 reads; *see Methods*). Less than 43 reads could be assigned to organisms not reported found in the reference community standard (0.0097±0.0087%, range 0-0.02%; 17.3 ± 18.3 reads, range 0-43 reads), an approximation of the manufacturer's reported purity of <0.01% foreign microbial DNA. Conservative filtering of contaminant taxa was completed to ensure major findings were not driven by potential contaminant taxa (see below); however, given that the origin of low abundance features is difficult to determine and

may be driven by both biological and technical sources, all taxa were retained for downstream analysis.

Rural Ethiopian oral microbiotas are highly diverse and form two distinct clusters

A total of 3,533 denoised amplicon sequence variants (ASVs) were detected across the healthy control cohort (N=108). The median number of ASVs on a per-individual basis was 302 ranging from 79 to 546 (**Figure 12A**). Richness of ASVs was high and also evenly distributed within individuals (**Figure 12B**). The most abundant oral microbes belonged to the Prevotellaceae, Streptococcaceae, and Neisseriaceae families (**Figure 12C**). In both analysis of taxonomic composition, and through beta-diversity analysis, a qualitative pattern emerged suggesting the presence of discrete compositional clusters. An unsupervised partitioning around medoids (PAM) clustering approach was applied which provided evidence of two compositional clusters as determined through maximizing the gap statistic in the minimal number of clusters (**Figure 12D**). Visualization of these community clusters provided visual confirmation that these clusters mapped to an apparent bifurcation in community composition(**Figure 12E**) which was consistent with our previous results identifying two distinct oral communities in pre-industrialized European populations(332). As above, this clustering was robust to the strict removal of any putative contaminant ASV observed in either negative or positive controls. Notably, community clusters had no correspondence with sample processing batches, physical locations in multiwell plates, sex, age, or other demographic variables. Cluster 2 microbiotas exhibited lower microbial diversity(**Figure 12F**).

Differential abundance analysis between clusters revealed 73 significantly different genera, 43 associated with cluster 1, and 30 with cluster 2 (FDR<0.1, ALDEx2;(**Figure 12G**)). Cluster 1 was associated with a broad spectrum of bacterial genera previously described in the oral cavity, but notably included the archaeal genus *Methanobrevibacter*. Alternatively, cluster 2 was associated with a number of Proteobacteria genera including *Bordetella*, *Brevundimonas*, and *Escherichia*, as well as the common gut genus *Akkermansia*. Rationalizing that some of the features most strongly associated with cluster 2 may, in a non-mutually exclusive manner, also be potential background organisms, we quantified the absolute abundance of microbes and discovered a reduction in the microbial load (difference in log₁₀ means=0.8 [0.5-1.1 95% CI]; (**Figure 12H**)). Through metagenomic inference Cluster 1 was associated with archaeal pathways involved in methanogenesis, while Cluster 2 was

associated with a number of pathways including those involved in amino acid metabolism. Taken together these findings indicated a potential population structure within the healthy cohort defined both by the composition and absolute abundance of salivary microbes.

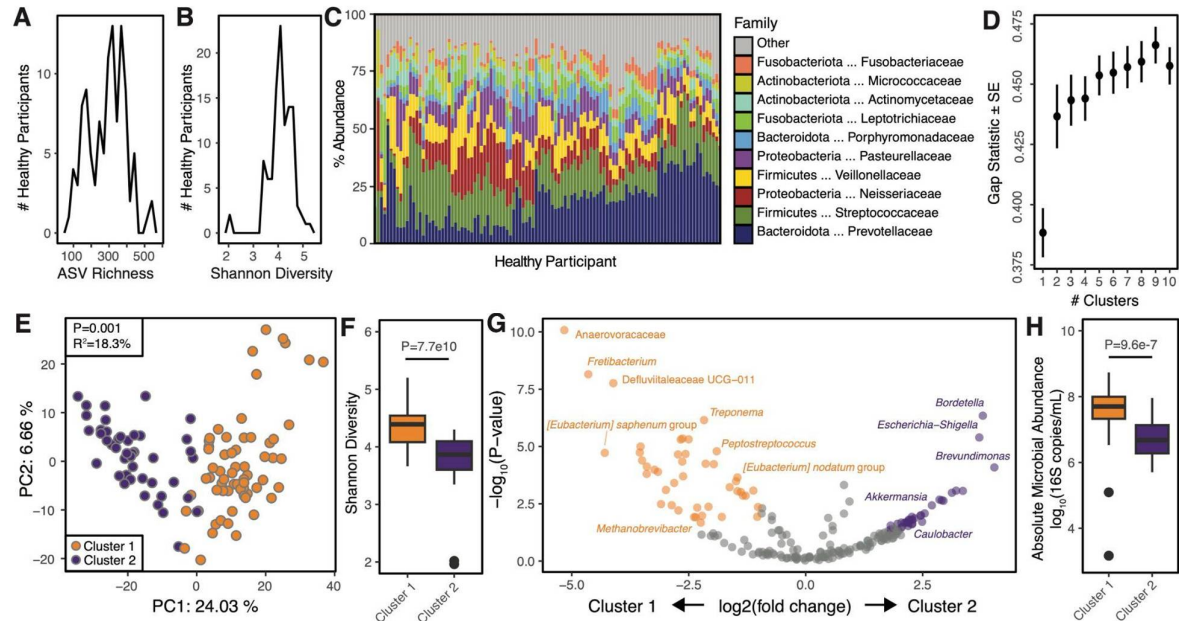


Figure 12: Two diverse and compositionally distinct oral microbiota types are observed among healthy rural Ethiopians

Diversity of oral microbiotas in healthy controls measured by **(A)** richness of amplicon sequence variants (ASVs) and **(B)** Shannon's diversity index. **(C)** A barplot of family-level abundances illustrates the most commonly observed oral microbes belong to the Prevotellaceae and Streptococcaceae. **(D)** Partitioning around medoids (PAM) clustering with analysis of the gap statistic demonstrates that two clusters best describe the number of community types. **(E)** PCoA analysis of Aitchison distance based on genus-level abundances between healthy participants demonstrates two coherent clusters with community state type describing 18.3% of variation ($P=0.001$, PERMANOVA). **(F)** Microbial diversity is significantly lower in Cluster 2 (Welch's t-test). **(G)** Genera which are significantly different between community clusters (ALDEx2 FDR-corrected Welch t-test <0.1). **(H)** The microbial load is significantly lower in cluster 2 as determined through qPCR (Welch's t-test). $N=108$ healthy participants in all panels, $N_{\text{Cluster1}}=64$, $N_{\text{Cluster2}}=44$.

To contextualize these findings, the composition of the healthy Ethiopian controls was contrasted against oral microbiota profiles previously collected from other east African

cohorts from noncancerous individuals in Uganda(326), and those we previously characterized in Tanzania(333). These were further contrasted against a non-westernized population from Venezuela(334), and samples from the American Gut Project(335). Samples from the AGP are primarily from individuals in the United States; however, it also contains samples from other westernized populations (i.e. mostly the United Kingdom and Australia). Oral microbiota diversity was significantly higher in all non-westernized populations compared to the AGP cohort ($P < 2.9 \times 10^{-6}$ ANOVA with TukeyHSD; **Figure 13A**). The Ethiopian and Ugandan cohorts displayed significantly higher diversity than the Tanzanian and Venezuelan cohorts, but were not significantly different from each other. Analysis of taxonomic abundances revealed notable differences among the Ethiopian cohort and all other reference cohorts (**Figure S2**). Among the notable differences contrasting the healthy Ethiopian cohort against the AGP were higher levels of Prevotellaceae and lower levels of Enterobacteriaceae, Micrococcaceae and Pseudomonadaceae ($FDR < 0.1$, ALDEx2). When beta-diversity analysis was performed (**Figure 13B**), the samples formed a gradient along PC1 drawing a clear delineation between western and non-westernized populations (effect of cohort: $R^2 = 0.33$, $P = 0.001$ PERMANOVA). Interestingly the previous population structure of the two Ethiopian clusters was retained when ordinated with the reference cohorts demonstrating that Ethiopian Cluster 2 was compositionally similar to other east African cohorts, but Ethiopian Cluster 1 was distinct.

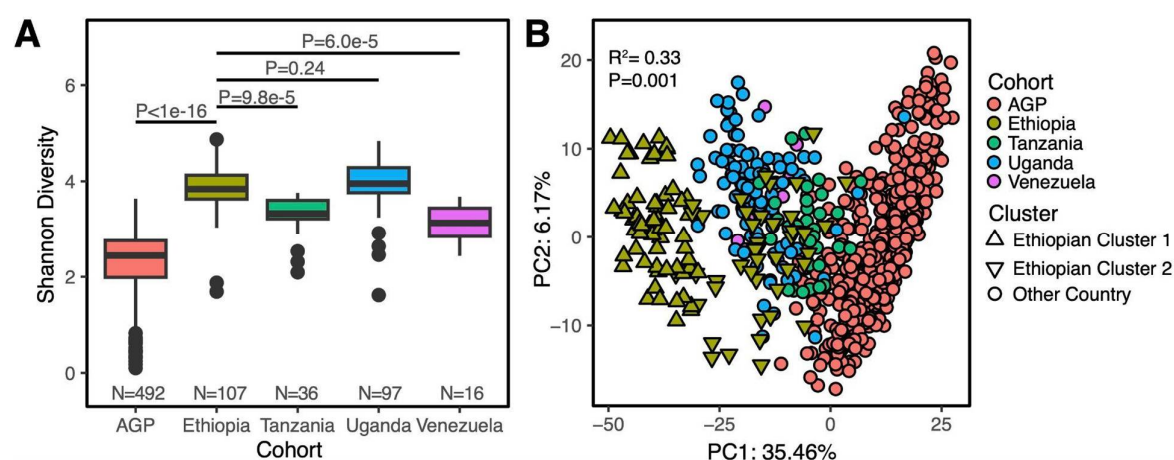


Figure 13: Cluster 1 healthy rural Ethiopian oral microbiotas are distinct from other non-western populations

(A) Rural Ethiopian oral microbiotas display higher diversity than westernized (AGP American Gut Project) and Tanzanian communities, but are not significantly more diverse than those from Uganda (ANOVA with TukeyHSD). **(B)** PCoA analysis of Aitchison

distances displays distinct compositional differences between westernized (AGP) and non-westernized communities (inset statistical analysis by PERMANOVA). While Cluster 2 Ethiopian microbiota are not distinct from other east African populations, Cluster 1 communities are distinct.

To better understand the factors that may shape oral microbiota composition in the healthy Ethiopian cohort, we examined relevant covariates that determined community diversity and composition. We included additional data on plasma mycotoxin levels which we have previously described(330). Notably, mycotoxin burden differs significantly between cases and controls, although both groups have significant exposure(**Table S1**). The final generalized linear model which best fit the data found significant effects of sex and alcohol use, but not age on alpha diversity (**Figure 14A**). Coffee consumption and khat usage, as well as the number of mycotoxins detected in plasma, and the plasma concentration of ochratoxin were found to be nearly significant. Reporting as male was associated with a higher Shannon diversity index (0.227 ± 0.11 , $P=0.0495$), while alcohol consumption was associated with a lower alpha diversity (-0.330 ± 0.129 , $P=0.0117$ (**Figure 14B**). In a similar analysis of community composition/beta diversity, significant effects of sex ($R^2=0.018$, $P=0.024$, PERMANOVA) and plasma ochratoxin A ($R^2=0.015$, $P=0.047$) were observed (**Figure 14C**). None of these variables appear to be predictive of the gradient along PC1 which defined the community clusters (**Figure 12C**), although sex appeared to be represented on the second axis of the PCoA plot (**Figure 14D**).

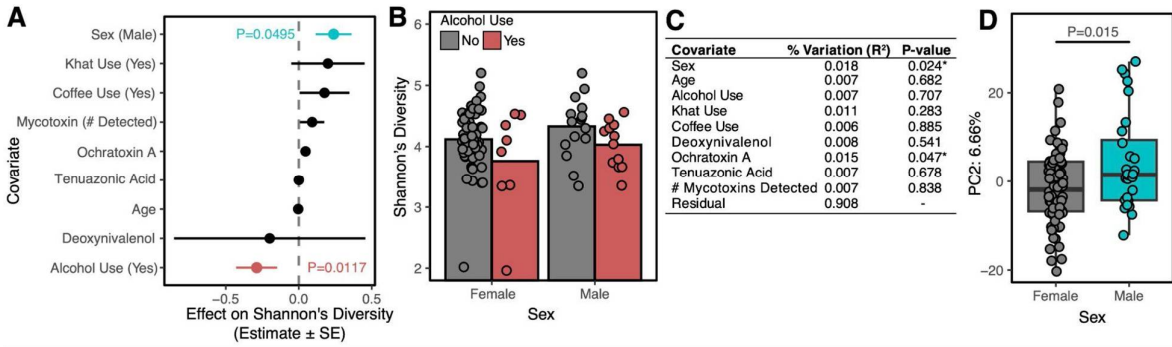


Figure 14: Oral microbiota composition is impacted by sex and alcohol consumption

(A) Oral microbiota diversity is significantly associated with sex and alcohol consumption ($P<0.05$, generalized linear model, $N=108$, $AIC=153.84$). (B) Identifying as male is associated with higher microbial diversity while both sexes show a decrease in diversity associated with reported alcohol consumption. (C) Analysis of microbial community

composition demonstrates that only sex is associated with altered community composition ($P < 0.05$, PERMANOVA of CLR Euclidean distances). **(D)** Sex is associated with the second axis of the principle coordinates plot shown in **Figure 1D** rather than the community clusters associated with PC1 (Welch's t-test).

The oral microbiota is disrupted in cases compared to controls

Cross-sectional analysis of microbial diversity measured through multiple alpha diversity metrics and accounting for relevant covariates revealed significant reductions in microbial diversity in cases (**Figure 15A-C**). Analysis of community composition by ordination demonstrated clustering by disease status which was found to be statistically significant ($R^2 = 0.046$, $P = 0.001$ PERMANOVA (**Figure 15D**). Subgroup analysis with the cases contrasting EAC and ESCC subtypes did not find significant differences in alpha or beta diversity (**Figure S3**). Noting a trend of separation between cases and controls along the PC1 axis, the full set of samples ($N = 211$) were re-clustered as before using unsupervised methods which further supported the notion of the two community composition clusters previously observed with a high correspondence to the previous assignments observed in the healthy cohort (**Figure 12D,E**). Analysis of the distribution between community clusters revealed that community cluster 2 was enriched in the cases (odds ratio = 3.4, 95% CI = 1.8-6.0, Fisher's exact test). While cluster 2 was more common in cases, it was still prevalent in the control group (**Figure 15E**) which may suggest potential value as a predictive tool in accessing disease risk.

Cross-sectional analysis between cases and controls reported 49 differentially abundant species, 24 elevated in cases, and 25 elevated in controls (ALDEx2 GLM FDR < 0.1 (**Table S1**). These organisms represented a broad phylogenetic range of common host-associated microbes with closely related organisms differing in their association (**Figure 16A**). As representative, *Streptococcus sanguis* and *S. intermedius* were negatively associated with cases, while *S. anginosus* was positively associated. Similarly, *Prevotella shahii* was associated with controls, while 4 other clades of *Prevotella/Alloprevotella* were associated with cases. Interestingly the most enriched taxa in cases belonged to the Actinobacteria representing *Bifidobacterium* spp., *Actinomyces* and *Alloscardovia* (**Table S1**). Functional inference revealed the most strongly enriched pathway in cases was related to glycerol degradation to 1,3-propanediol (**Table S2**). There is an apparent bifurcation in quinone biosynthesis wherein controls indicate higher abundance of pathways involved in ubiquinone biosynthesis while cases indicate higher abundance of those involved in biosynthesis of

menaquinone (**Figure 16B**). Cases were further associated with decreased pathway abundances involved in biosynthesis of amino acids and nucleotides, and multiple pathways involved in fermentation, respiration, and degradation (**Figure 16B, Table S2**). Some of these metabolites, for example arginine, are associated with modulating anti-tumor activity of host immune cells(336).

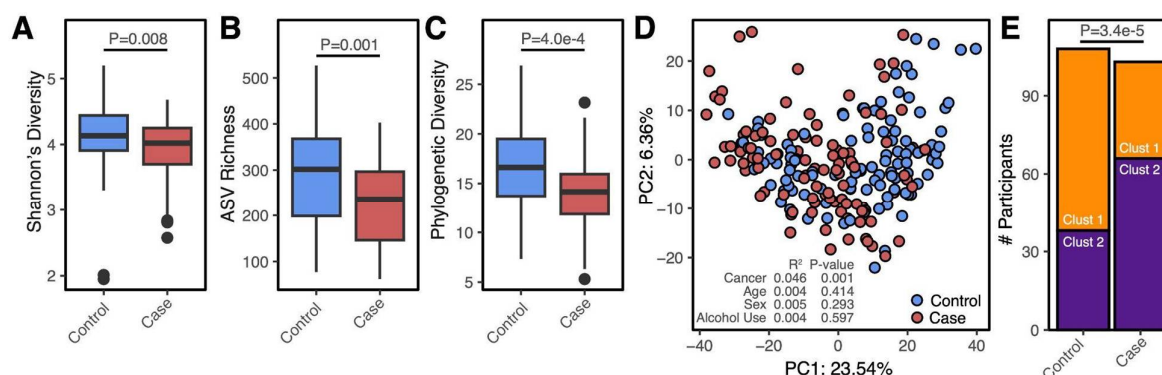


Figure 15: Oral cancer is associated with reduced diversity and community cluster 2

Microbial diversity is significantly reduced irrespective of choice of alpha diversity metric including (A) Shannon's diversity index, (B) ASV richness, and (C) phylogenetic diversity. (D) Visualization by PCoA demonstrates subtle variation in microbiome composition related to cancer status which is supported by statistical analysis (inset, PERMANOVA). (E) Clustering analysis, as in **Figure 1** demonstrates cases are enriched in community cluster 2 microbiotas (OR=3.4 95%CI=1.8-6.0; Fisher's exact test). Statistical analysis for planes A and C by GLM with covariates of sex, age, alcohol use. Statistical analysis for panel B same using negative binomial distribution. $N_{\text{cases}}=103$, $N_{\text{controls}}=108$ for all panels.

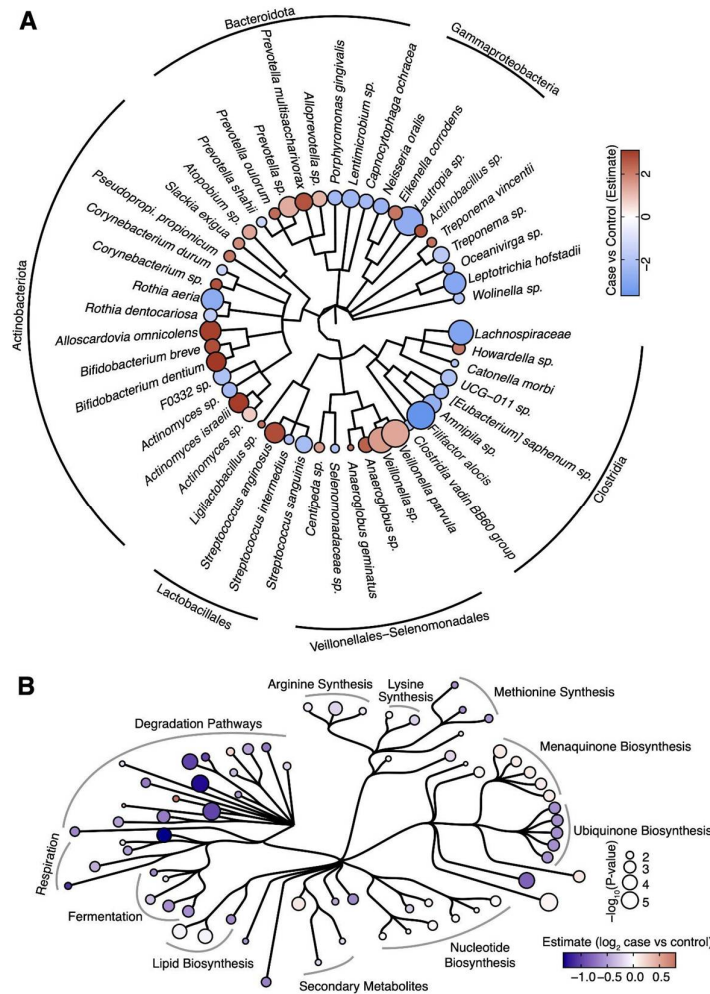


Figure 16: Esophageal cancer is associated with a functionally distinct microbiota

(A) Prokaryotic species differentially abundant between case and controls displayed in a cladogram based on taxonomy. (B) Inferred genetic pathways differentially abundant between case and controls drawn in a functional hierarchy. Statistical analysis for panels A and B by GLM with covariates of sex, age, alcohol use. Significance was determined as an FDR-corrected P-value <0.1 . No significant effects were observed for covariates. $N_{\text{cases}}=103$, $N_{\text{controls}}=108$ for all panels.

Esophageal cancer-associated microbiota signatures generalize across geographical cohorts

To better understand the reproducibility of the EC-associated shifts in the oral microbiota and if these may generalize to other populations, we sought to use a meta-analysis framework to conduct cross-population analysis. Of previous studies characterizing the oral microbiota using comparable sequencing methodologies, we were only able to obtain publicly available

data from two (337,338), both from Chinese populations which also exhibit increased rates of ESCC. The cohort reported by Chen *et al.* consisted of individuals with early-stage ESCC (N=31) and controls (N=21) located around Nanjing. Alternatively, the Jiang *et al.* cohort consisted of 109 individuals recruited from Huaian with either biopsy-diagnosed ESCC (N=56) and healthy controls (N=53). These locations are approximately 140 km apart and found in eastern China. Taxonomic comparison across cohorts revealed similar compositions at the family level; however, the Chen *et al.* cohort exhibited lower levels of oral Prevotellaceae while the Jiang *et al.* cohort exhibited lower levels of Streptococcaceae (**Figure S4A**). Ordination of beta diversity metrics revealed that the Chinese cohorts most closely resembled each other as compared to the Ethiopian cohort(**Figure S4B**). Cohort explained the major source of variation in the combined dataset ($R^2=0.161$, $P=0.001$); while the effect of disease state was still statistically significant ($R^2=0.019$, $P=0.001$, PERMANOVA). Notably, differences in microbial alpha diversity were not reproduced in the Chinese cohorts(**Figure S4C**).

To better understand how finer-scale changes in microbiota generalize across cohorts, and to identify the most important predictors of cancer status, we used a robust machine learning approach consisting of multiple iterations of randomly sampling the Ethiopian cohort into a training and test set, and then using random forest classifiers generated on the training set to predict both the test set and external Chinese cohorts (*see Methods*). Models trained within the Ethiopian cohort had excellent accuracy in predicting the withheld Ethiopian samples reporting areas under the receiver operator curve (AUROCs) of 0.91 ± 0.05 (mean \pm SD (**Figure 17A**)). These models also gave good performance on the Jiang *et al.* cohort (AUROC= 0.74 ± 0.03), but not the Chen *et al.* cohort (AUROC= 0.55 ± 0.03). These observations may reflect a biological difference between early stage (Chen) and later stage EC (Jiang); however, they may also result from technical difficulties in direct comparison of studies employing differing methodologies for sample collection and processing(339). Examination of the predictive power of individual taxa revealed an inflection point wherein approximately 20 genera most effectively differentiated cases and controls of which *Lautropia* spp. were the strongest predictor (**Figure 17B, C**). Taken together, these results indicate that while the Ethiopian and Chinese cohorts have distinct oral microbiotas, there is a common signature of disease observed across populations which suffer from high EC/ESCC burden.

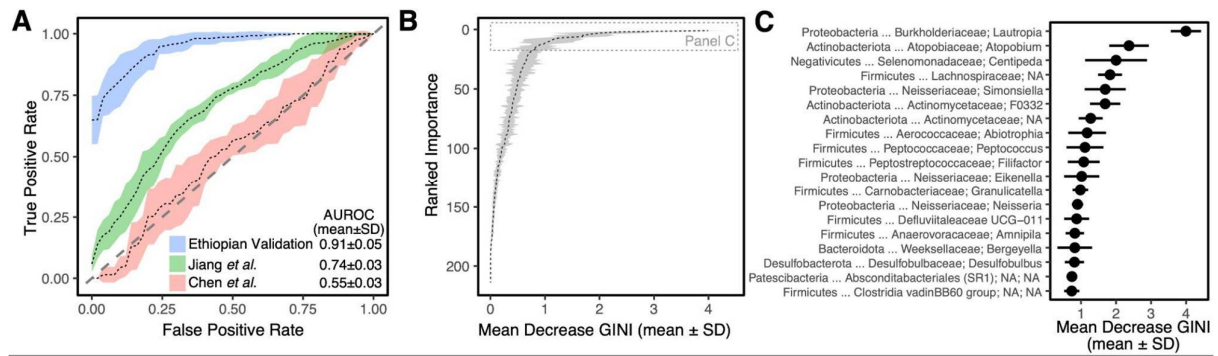


Figure 17: Shifts in the Ethiopian cohort predict disease status in a Chinese ESCC cohort, but not an early stage ESCC cohort

(A) Random forest classifier models were trained on multiple iterations (N=10) of random sampling of the Ethiopian cohort into a training (N=139) and validation set (N=72, *see Methods*). Models were then used to predict cancer status in Chinese cohorts with good predictive power in the Jiang *et al.* cohort, but not the Chen *et al.* cohort (see inset table of AUROCs). Ribbons represent mean±SD of 10 replications. (B) A limited set of bacterial genera predict cancer status (N=20) which are displayed in panel (C).

1.5. Discussion

In this report, we characterized the salivary microbiota of healthy individuals residing in the rural region of Oromia, Ethiopia and contrasted these profiles against other east African, Asian, South American, and westernized populations uncovering a unique population structure found only within Ethiopians. Contrasting healthy controls against individuals with esophageal cancer, we uncovered a loss of microbial diversity and an altered microbial composition associated with cancer: both the ESCC variant common to Africa, and the EAC variant more common in the global west. Through machine learning approaches, we demonstrated that taxonomic shifts in microbial community composition could predict cancer status in an external Chinese cohort highlighting a minimal number of strong predictors of disease status. Collectively, our study demonstrates the importance of studying non-westernized populations and reinforces a role for the oral microbiota as a predictor and/or mediator of esophageal cancer.

In this work 3,533 amplicon sequence variants (ASV) were detected in healthy controls with median value of 302 ASV and ranging from 79 to 546 showing high diversity. *Prevotellaceae*, *Streptococcaceae*, *Nisseriaceae*, *Veillonellaceae*, *Pasteurellaceae*,

Leptotrichiaceae, *Actinomycetaceae*, *Micrococcaceae*, and *Fusobacteriaceae* were the most abundant families. These families are among the core oral microbiome of healthy human which are least variable microbiota of a niche (340). In population of Southern Africa similar results were reported where *Streptococcus* and *Prevotella* were among the top five most frequent genera in saliva samples of healthy individuals (341). The genera of *Prevotella* that belongs to *Prevotellaceae* is the most second abundant microbial flora of oral cavity of healthy human next to *Streptococcus*(342). Similarly *Prevotella spp* were enriched the oral microbiome of non-Westernized populations including Ethiopian populations which follow a more traditional diet and lifestyle (343). The frequent occurrence of *Prevotella* in human microbiome reflects its functional importance in health and disease. The inverse association of *Prevotella* from the oral cavity and *H. pylori* in the stomach reflecting the protective role of *Prevotella* from the virulent pathogen (344). However, *P. intermedia* from the genera of *Prevotella* involved in promoting growth, invasion, angiogenesis, and metastasis of ESCC.

Our finding were also in agreement with the study results of oral microbiome study from the Atlantic Canadian samples of healthy cohort where the family of *Veillonellaceae*, *Neisseriaceae*, *Streptococcaceae*, and *Prevotellaceae* were the dominant oral microbiome (345). Similarly, these observed dominant families in our study were similar with those listed in human oral microbiome data base V3.1(<http://www.homd.org/>) of oral cavity. Also in Chinese healthy population the genera of *Prevotella*, *Streptococcus*, and *Veillonella* were the dominant family in saliva samples(346,347). This observation showed the specified community of microbiome colonize healthy oral cavity and maintain the health of host. From this and previous studies we observed core oral microbiome were identical in unrelated healthy individuals showing the universality of these resilient and adapted oral microbiome (348).

While evidence has been provided of discrete community configurations in the gut and urogenital tract, commonly termed enterotypes (349) and community state types (350) respectively, this conceptual framework has been less frequently applied to the oral cavity. One study of adolescents in Spain reported two clusters, which they termed stomatotypes, although the taxa which define these clusters differ substantially from those we report (351). While these clustering approaches have drawn criticism for considering gradients as discrete classes, and potentially having issues in underlying statistical considerations (352), they remain useful tools for dimensional reduction and interpretation of highly complex data

(353). Mimicking what has been described with gut enterotypes (354), we report that the community clusters observed in the healthy Ethiopian population differ in absolute abundance by nearly an order of magnitude. The observation that the lower abundance/diversity Cluster 2 was associated with EC lends itself to a hypothesis that a loss of protective microbes in the saliva may predispose an individual to EC. This is further supported by an enrichment in Proteobacteria in Cluster 2 whose inflammatory LPS may drive states of chronic inflammation and disrupt the mucosal barrier which may aid in carcinogenesis (355).

Also the observed two communities were functionally distinct. Community cluster 1 associated with methanogenesis while community cluster 2 associated with various metabolism path ways including amino acid metabolism. The oral microbiome composed Saccharolytic bacteria which include mainly the genera of *Streptococcus*, *Actinomyces*, and *Lactobacillus species*, Proteolytic or amino acid-degrading bacteria, including mainly the *Prevotella* and *Porphyromonas species* (356). The Saccharolytic bacteria degrade carbohydrates into acids and triggers dental caries, while acid neutralization via the peptide or amino acid metabolism counteract acidification but responsible for periodontal disease and periodontal mal-odor. In this study the predominance of the *Prevotella*, *Streptococci*, *Poryphromonas* and *Fusobacterium* in agreement with the functional pathway of proteolytic nature of this organisms. The metabolites produced from amino acid by oral microbiome in the oral cavity reported to be associated with host taste perception (357). This in oral metabolism of protein and carbohydrate by oral microbiome reported be associated with change test receptors on tongue and result in behavioral change on feeding preference which associated with obesity and metabolic disease. Using metabolomics-based assay it was also identified that gut microbiota efficiently utilize host amino acid and deplete it with affecting host glucose tolerance via peripheral serotonin (358).

Comparison to international cohorts from east Africa (Tanzania and Uganda), South America (Venezuela), and a predominantly American cohort (American Gut Project), revealed the non-westernized cohorts to exhibit significantly higher microbial diversity and higher levels of the family representing *Prevotella* spp., organisms which are also known to be significantly higher in the fecal microbiota of African individuals (359). The composition and diversity of microbiome is significantly affected by the human life style shown by the oral microbiome diversity of non-westernized population was more diverse compared to

westernized population. It was supported by that urbanization is associated with a loss of microbial organisms and genes (360). In supporting this the oral microbiomes from hunter-gatherers and traditional farmers in Filipins was highly diverse which linked to diet and other life style (155). It was also reported higher oral microbiome diversity was observed in hunter-gatherer group than agricultural group (156).

The observation that Ethiopian Cluster 2 individuals from rural environments are more compositionally similar to westernized populations may be indicative of signatures of an westernization process taking place within these individuals. Taken together these may support that loss of microbial diversity and colonization may predispose individuals to the development of cancer, although caution is needed without prospective human studies or understanding of underlying mechanisms.

Irrespective of unsupervised clustering, we observed the diversity of salivary microbes to be reduced in EC individuals which has been variably reported across previous studies (314,361–363). Microbial diversity within the tumors themselves has also been reported to decreased (361); however, the study of tumor-associated microbes has come under recent scrutiny and must be interpreted with care (364,365). Reduced microbial diversity may be associated with latent infection and inflammation (366). While it is difficult to establish cause and effect, the esophagus is prone to disruption of barrier function due to both external factors, for example hot food/beverages and dietary carcinogens, and due to its proximity to the low pH content of the stomach. These factors may interact with both resident and transitory microbes from the oral cavity to exacerbate inflammatory processes leading to carcinogenesis (367). Supporting these observations, gastroesophageal reflux disease (GERD) a known risk factor for EAC, is also associated with disrupted microbial communities including elevated abundance of Gram negative microbes (368).

Future studies will need to carefully incorporate highly accurate long-term and short-term dietary intake as diet is a major determinant of oral and gut microbiome diversity(369–371). Previous studies in Ethiopia have identified low dairy intake(28), and low intake of fruit and vegetables with increased risk of EC(28,250), both of which may interact with gut microbes. Further, prolonged caloric restriction has been observed to decrease the absolute abundance of gut microbes with a concurrent increase in microbial diversity⁷⁸. While effects of caloric intake on salivary microbes has not been well established, there is a possibility that

discomfort associated with EC and associated altered dietary patterns may contribute to the observed microbial signals.

Some of the species we associated with EC have been previously associated with various malignancies including esophageal cancer. *S. anginosus* has been experimentally demonstrated to promote gastric tumorigenesis via inducing gastritis which has been proposed as a potential biomarker for gastric cancer(372,373). *Prevotella* and related genera including *Alloprevotella* have been previously associated with EC(374–376), and *Alloscardovia* have been proposed as biomarkers of human papillomavirus and cervical cancer(377).

These organisms comprised a microbial fingerprint of EC which was observed to cross-cohorts demonstrating a potential for using the salivary microbiota as a non-invasive biomarker for disease detection without the need for biopsy. While these technologies are not yet widely available in the clinical context, rapid developments in inexpensive and field-deployable sequencers offer promise for diagnostics in the near future(378). Alternatively, PCR-based assays may also provide utility; however, sequencing at scale may be less resource intensive than highly multiplexed PCR³⁴. That *Lautropia* spp. were the strongest predictor of health is an observation of note. A single species exists in standing nomenclature (*L. mirabilis*)(379); however, a second species has also been described differing at the level of whole genome average nucleotide identity(380). *Lautropia* has been isolated from a variety of oral microenvironments including saliva, dental plaque, and gingival biofilms with rate reports considering it an opportunistic pathogen(381). Further studies are needed to understand how microbes in the upper gastrointestinal tract influence carcinogenesis in the mucosa.

1.6. Limitations of Study

There are several limitations to be considered with respect to the interpretation of the findings. Given that our study was not prospective, we are unable to establish causality of shifts in microbial community structure in the development of EC. While this has been previously reported in American populations(313), data were not made available for direct comparison; however, through qualitative analysis, many key indicator organisms overlap between our report and the American cohort including reductions in commensal *Neisseria* spp. Further mechanistic studies are required to understand the extent to which oral microbes are a driver, or a marker, of esophageal carcinogenesis. Comparison of microbiota sequencing data between studies is imperfect owing to many technical variables which may be collectively termed ‘study effects’ and explain the greatest

proportion of variance in such analyses(339). Furthermore, by nature of data availability, populations are not matched on important covariates such as sex or age, and this data is often incomplete in public repositories. As an example, the Tanzanian cohort was derived from postpartum mothers within a month of delivery and some of whom variably received other interventions relating to maternal health(333). This may partially explain why the lower diversity and abundance Ethiopian cluster 2 more closely resembled this Tanzanian cohort. We performed functional characterization of the microbiota by inferring pathway abundances(382) rather than through direct metagenomic sequencing due to low sample biomass and high host DNA content in saliva rendering metagenomic sequencing resource prohibitive. Further, careful interpretation is required when machine learning approaches are applied as overfitting may pick up technical noise rather than biologically meaningful signals. This was mitigated through a careful internal and external validation strategy minimizing the potential for overfitting and data leakage; however, additional cohorts and datasets would be needed for conclusive validation and model refinement.

1.7. Conclusions

In summary, the salivary microbiota of rural Ethiopians is highly diverse with an apparent bifurcation into two subtypes defined by differing diversity, microbial composition, function, and absolute abundance. While sex and alcohol use are significant determinants of oral microbiota composition, the factors which determine the community subtype remain to be determined but cannot be explained by technical variables. The lower diversity and abundance cluster 2 was over-represented in individuals with esophageal cancer potentially indicating a protective role of the oral microbiota against esophageal cancer; however, this signature also demonstrated power as non-invasive diagnostic tool which could also predict disease state in a Chinese cohort. Understanding the mechanisms that drive these associations, and their directionality will be key for early detection and prevention of this disease, in particular esophageal squamous cell carcinoma, which disproportionately impacts individuals in east Africa and central Asia.

Chapter 4: Multiple mycotoxin exposure assessment through human biomonitoring in an esophageal cancer case-control study in the Arsi-Bale districts of Oromia region of Ethiopia

Redrafted from: Mulisa G, Pero-Gascon R, McCormack V, Bisanz JE, Talukdar FR, Abebe T, et al. Multiple mycotoxin exposure assessment through human biomonitoring in an esophageal cancer case-control study in the Arsi-Bale districts of Oromia region of Ethiopia. *International Journal of Hygiene and Environmental Health*. 2025 Jan; 263:114466.

Authors contributions: Girma Mulisa: Writing original draft, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Roger Pero-Gascon: Writing original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. Valerie McCormack: Writing – review & editing, Methodology. Jordan E. Bisanz: Writing review & editing, Visualization, Formal analysis. Fazlur Rahman Talukdar: Writing – review & editing, Formal analysis. Tamrat Abebe: Writing review & editing, Resources, Conceptualization. Marthe De Boevre: Writing review & editing, Resources, Funding acquisition, Conceptualization. Sarah De Saeger: Writing review & editing, Resources, Funding acquisition, Conceptualization.

4.1. Abstract

Background: Esophageal cancer (EC) is a malignancy with a poor prognosis and a five-year survival rate of less than 20%. It is the ninth most frequent cancer globally and the sixth leading cause of cancer-related deaths. The incidence of EC has been found to vary significantly by geography, indicating the importance of environmental and lifestyle factors along with genetic factors in the onset of the disease. In this work, we investigated mycotoxin exposure in a case-control study from the Arsi-Bale districts of Oromia regional state in Ethiopia, where there is a high incidence of EC while alcohol and tobacco use – two established risk factors for EC – are very rare.

Methods: Internal exposure to 39 mycotoxins and metabolites was assessed by liquid chromatography-tandem mass spectrometry in plasma samples of EC cases (n = 166) and location-matched healthy controls (n = 166) who shared similar dietary sources. Demographic and lifestyle data were collected using structured questionnaires. Principal Component Analysis and machine learning models were used to identify the most relevant demographic, lifestyle, and mycotoxin (co-)exposure variables associated with EC. Multivariate binary logistic regression analysis was used to assess EC risk.

Result: Evidence of mycotoxin exposure was observed in all plasma samples, with 10 different mycotoxins being detected in samples from EC cases, while only 6 different mycotoxins were detected in samples from healthy controls. Ochratoxin A was detected in plasma from all cases and controls, while tenuazonic acid was detected in plasma of 145 (87.3%) cases and 71 (42.8%) controls. Using multivariable logistic regression analysis, exposure to tenuazonic acid (AOR = 1.88 [95% CI: 1.68–2.11]) and to multiple mycotoxins (AOR = 2.54 [95% CI: 2.10–3.07]) were positively associated with EC.

Conclusion: All cases and controls were exposed to at least one mycotoxin. Cases were exposed to a statistically significantly higher number of mycotoxins than controls. Exposure to tenuazonic acid and to multiple mycotoxins was associated with increased risk of EC in the study population. Although aflatoxin B1-lysine and the ratio of sphinganine to sphingosine (as a biomarker of effect to fumonisin exposure) were not assessed in this study, our result emphasizes the need to characterize the effect of mycotoxin co-exposure as part of the exposome and include it in risk assessment, since the current mycotoxin safety levels do not consider the additive or synergistic effects of mycotoxin co-exposure. Moreover, a prospective study design with regular sampling should be considered in this high incidence area of EC in Ethiopia to obtain conclusive results on the role of mycotoxin exposure in the onset and development of the disease.

KEYWORDS: Human biomonitoring; Mycotoxins; Risk factors; Esophageal cancer; Case-control study

4.2. Introduction

Cancer is a disease of uncontrolled proliferation of transformed cells which undertake genetic and epigenetics changes in the microenvironment following the interaction with host and other factors (1). Cancer is increasingly a global health issue. The IARC estimated that there were almost 20 million new cases of cancer and nearly 10 million cancer deaths worldwide in 2022. Projections considering population growth and aging predict over 35 million new cases of cancer in 2050, an increase of 77% compared to 2022 (9). EC is the ninth most common cancer worldwide and the sixth leading cause of cancer-related deaths, characterized by a poor prognosis and a five-year survival rate of less than 20% (14). The two common histological subtypes are ESCC and EAC of which ESCC accounts for more than 85% of its histological types (15,23).

EC is a major public health concern worldwide, although its incidence and mortality has been found to vary significantly by region (35). The highest incidence of EC and associated mortality globally is found in the areas referred as the African and Asian EC belts, which extend from eastern to southern Africa (Ethiopia to South Africa) and from western to eastern Asia (eastern Turkey to northern and central China), respectively (23). Marked histology variation was also noticed based on geography. ESCC is the major histological type in economically less developed countries in the African and Asian EC belts while EAC is most common in more developed countries (14,23,24). Different risk factors were established for both histologies: alcohol consumption is strongly associated with increased risk of ESCC, gastro esophageal reflux disease and obesity are risks factors for EAC, while smoking is a risk factor for both ESCC and EAC (21,22).

In most high-risk areas in Asia and Africa, the lack of awareness and screening programs of EC contribute to late diagnosis, leading to poor outcomes (36,37). Furthermore, treatment options are often limited due to the high cost of treatment and the limited availability of cancer care facilities in many African countries. In Ethiopia, EC is among the top ten cancer type (38) with a significant increasing trend in its incidence (39), which is particularly high in Arsi-Bale districts of Oromia regional state (56–58). There is no screening program of EC leading to late diagnosis and poor survival (383,384). The treatment options available to this

cancer are limited. This warrants the need to prioritize the implementation of primary preventive strategies based on the identification of specific etiology as well as associated risk factors of EC in Ethiopia.

In addition to alcohol consumption and smoking, several risk factors have been linked to ESCC in Africa. These include poor nutrition and exposure to environmental contaminants, such as high levels of nitrosamines present in traditional food preservation methods (43,283). Exposure to aflatoxins from contaminated food, has also been associated with the development of ESCC in Africa (284). ESCC has also been associated with the consumption of local alcoholic beverages, particularly kachasu in Malawi and Zambia, chang'aa in Kenya, and gongo in Tanzania (285). In Ethiopia, where a high incidence of EC has been reported, very few etiological studies have been conducted. In recent years, already established important risk factors for ESCC, i.e. alcohol consumption and tobacco use, were observed to be rare in the affected Ethiopian population (57,58). However, in African and Asian population where alcohol and tobacco are not common, exposure to mycotoxins, including fumonisins from contaminated cereal crops and maize, was identified as a significant risk factor for EC (170,385,386). Based on these findings, we hypothesized that exposure to mycotoxins, among other environmental exposures, could play a role in areas of high EC incidence in Ethiopia.

Mycotoxins, i.e. secondary metabolites produced by fungi such as *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* toxigenic species that contaminate agricultural crops and commodities, have been associated with increased risk of various cancer types (160,161) with proven mechanism of carcinogenesis (162). IARC listed aflatoxins (including aflatoxin B1, B2, G1, G2 and M1) as carcinogenic to humans (Group 1) (165) and fumonisins (including fumonisin B1 and B2) (163) and ochratoxin A (164) as possibly carcinogenic to humans (Group 2B), while other some mycotoxins were also evaluated and classified as not classifiable as to its carcinogenicity to human (Group 3) based available evidence (166). In the African and Asian EC belt, common cereal crops used for food have been reported to be contaminated with mycotoxins (27,167–170). This geographical overlap of mycotoxin occurrence in food and the epidemiology of EC may suggest exposure to mycotoxins may be a potential risk factor for EC. For example, the level of FB1 in corn and rice samples collected from areas of high risk of EC in Iran and China were associated with increased risk of EC (167,168,170).

Mycotoxin contamination has been reported to be a health concern in Ethiopia, as major agricultural food commodities were contaminated by carcinogenic mycotoxins (171). Maize, wheat, barley and raw milk, which are known sources of human exposure to mycotoxins, are used as common foodstuff in areas of high EC incidence in Ethiopia (28). Coffee, which is also reported to be contaminated with mycotoxins (172), is consumed at least three times per day in that local community.

Most studies in the literature reported the use of food occurrence data combined with population data on food consumption to assess mycotoxin exposure. However, this approach is known for its intrinsic limitations due to the non-uniform distributions of mycotoxins in food, individual variations in toxicokinetics and bioavailability, and inaccurate estimations of food consumption (173). Assessment of human internal exposure to mycotoxins is best achieved using a human biomonitoring approach by analyzing biomarker compounds in biological fluids and tissues.

The evidence generated by epidemiological (27,170), biomonitoring (Xue et al., 2019) and mechanistic experimental (175) studies suggests the important role of mycotoxins in the high incidence of EC in areas where food safety policy on mycotoxins is not established or not enforced. In this work we determined the concentrations of multiple mycotoxins in plasma samples of EC patients and healthy controls to investigate associations between mycotoxin exposure and the onset of EC in the high incidence area of Arsi-Bale districts of Oromia regional state of Ethiopia.

4.3. Materials and methods

Study design and setting

A health care facility-based case-control study design was used. Cases were pathologically confirmed newly diagnosed and treatment-naïve EC patients. Controls were residence matched endemic healthy relatives of the patients. Because controls were recruited at the health care facility from accompanying relatives of the case, other potential confounders, such as age, sex, and body mass index, were not matched. Female participants were more frequent in the control group because female relatives were more likely to accompany the patients to the hospital. Cases and controls were recruited from purposively selected hospitals located in the catchment area of high EC incidence namely Adama Hospital Medical College (AHMC), Adama General Hospital and Medical College (AGHMC), Muse General Hospital, Asella Rehoboth Hospital and Meda Wolabu Hospital. After signed written informed

consent, socio-demographic data, dietary patterns, and mycotoxin awareness data were collected from 166 cases and 166 controls using interviewer administered semi-structured questionnaires. A whole blood sample of 5 mL was collected using an EDTA coated tube from each participant. EDTA plasma was immediately separated by centrifugation at 5000 rpm for 5 minutes, transferred to a sterile cryotube using a sterile pipet and stored at -80°C until processing. The study protocol was approved by Institutional Review Boards of Addis Ababa University, College of Health Sciences with protocol number of 024/21/DMIP and by the Federal Ministry of Education National Ethics Committee with protocol number of 03/246/221/22.

Chemicals and reagents

ULC–MS grade glacial acetic acid and LC–MS grade absolute methanol (MeOH) from Biosolve (Deuze, France), analysis grade ammonium acetate from Merck (Darmstadt, Germany), and ultrapure water (18 mΩ · cm) from an Arium® Pro water purification system from Sartorius (Goettingen, Germany) were used to prepare the chromatographic mobile phases and the injection solvent. LC–MS grade acetonitrile from Biosolve was used for plasma samples preparation.

Analytical standards of 3-acetyldeoxynivalenol (3-ADON), aflatoxin B1 (AFB1), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), beauvericin (BEA), cyclopiazonic acid (CPA), enniatin A (ENN A), enniatin A1 (ENN A1), enniatin B (ENN B), fumonisin B1 (FB1), fumonisin B2 (FB2), fumonisin B3 (FB3), hydrolysed fumonisin B1 (HFB1), neosolaniol (NEO), ochratoxin alpha (OT α), roquefortine-C (ROQ-C), sterigmatocystin (STC), T-2 tetraol, and zearalanone (ZAN) were purchased from Fermentek (Jerusalem, Israel); aflatoxin B2 (AFB2), alternariol methylether (AME), alternariol (AOH), citrinin (CIT), diacetoxyscirpenol (DAS), deoxynivalenol (DON), enniatin B1 (ENN B1) HT-2 toxin (HT2), nivalenol (NIV), tenuazonic acid (TA), alpha-zearalanol (α -ZAL), beta-zearalanol (β -ZAL), zearalenone (ZEN), and alpha-zearalenol (α -ZEL) were purchased from Sigma-Aldrich (Overijse, Belgium); aflatoxin M1 (AFM1), deepoxy-deoxynivalenol (DOM), fusarenone-X (FUS-X), ochratoxin A (OTA), T-2-toxin (T2), and beta-zearalenol (β -ZEL) were purchased from Food Risk Management B.V. (Oostvoorne, Netherlands). Two multi-mycotoxin standard working solutions were prepared: (i) containing 60 μ g/L of the 5 AFs and OTA standards and 600 μ g/L of the other 36 mycotoxin standards in methanol; and (ii) containing 6 μ g/L of the 5

AFs and OTA standards and 60 µg/L of the other 36 mycotoxin standards in methanol. These solutions were stored at -20°C when not in use.

Isotopically labeled internal standards ^{13}C -AFB1, ^{13}C -CIT, ^{13}C -DON, ^{13}C -FB1, ^{13}C -HT2, ^{13}C -T2 and ^{13}C -TA were purchased from Food Risk Management B.V. An internal standards working solution was prepared containing 19 µg/L of ^{13}C -AFB1 and 300 µg/L of the other 6 ^{13}C -labeled internal standards in methanol. This solution was stored at -20°C when not in use.

Plasma samples for matrix-matched calibration curves and quality controls

EDTA plasma purchased from the Red Cross East Flanders (Ghent, Belgium) was used to prepare matrix-matched calibration curves and quality controls. Upon receipt, the EDTA plasma was pooled and analyzed for the presence of mycotoxins by UHPLC-MS/MS. The result was negative for all mycotoxins, except OTA. The concentration of OTA was 0.13 µg/L, quantified using the standard addition method spiking with an OTA standard in the range of 0.03 to 0.50 µg/L.

Plasma samples pretreatment

EDTA plasma samples were transferred from -80°C to -20°C and stored overnight. Then, they were taken to room temperature, thawed and homogenized by vortex for a few seconds. Three hundred microliter (300 µL) of each sample was transferred to Eppendorf tubes of 2 mL. Twenty µL of the internal standards working solution was added to all samples and appropriate concentrations of mycotoxin standards were added to the EDTA plasma samples from the Red Cross East Flanders to prepare matrix-matched calibration curves for mycotoxin quantification and quality controls. Then, 300 µL of acetonitrile was added to all samples for protein precipitation. Samples were vortexed for 2 minutes and centrifuged at 3300 g for 15 minutes at 4°C using a Multifuge 3 S-R centrifuge from Heraeus (Hanau, Germany). The supernatants were transferred to Eppendorf glass tubes in a Turbo Vap LV evaporator from Biotage (Dusseldorf, Germany) and evaporated at 40°C under gentle nitrogen gas flow until completely dry. The residues were re-dissolved in 150 µL of injection solvent (60:40 v/v mobile phase A/mobile phase B) by vortexing for 2 minutes, then centrifuged at 2100 g for 1 min. The dissolved residue was transferred to a tube with a PVDF centrifuge filter of 0.22 µm from Millipore (Cork, Ireland) and centrifuged at 9000g for 5 minutes at 4°C. The filtrate was transferred into a HPLC vial with insert and closed with a cap. Air bubbles on the bottom of the insert were removed by gently tapping the vial.

UHPLC-MS/MS analysis of multiple mycotoxins, identification criteria and method validation

A Waters Acquity UPLC I-class system coupled to a triple quadrupole XEVO TQ-XS mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) source was used for targeted analysis. Analytes were separated chromatographically using an Acquity UPLC HSS T3 analytical column (1.8 μm particle size, 2.1 mm id \times 100.0 mm) from Waters with an Acquity UPLC HSS T3 VanGuard precolumn (1.8 μm , 2.1 mm id \times 5 mm). The chromatographic separation has already been described by Martins et al. (387,388). The optimized UHPLC-MS/MS parameters for the analysis of mycotoxins are shown in **Table S-3**.

Matrix-matched calibration curves were obtained by analyzing EDTA plasma samples from the Red Cross East Flanders spiked with the mycotoxin standards at 7 concentration levels, while the concentration of internal standards was constant. Response was calculated as the integrated area of the chromatographic peak of each mycotoxin divided by the area of the corresponding internal standard. To obtain accurate calibration models, a weighting factor of $1/x^2$ was applied to the regression modeling of the calibration curves to account for the fact that the variability (standard deviation) of the response increased proportionally with the concentration of the analytes over the entire concentration range, as is typical in bioanalytical LC-MS/MS assays (389). The midpoint of the calibration curve (at the 4th concentration level) was reinjected throughout the analytical run every 11th analysis as a quality control.

Stringent criteria were used for the identification of mycotoxins by UHPLC-MS/MS based on SANTE/12089/2016 guidelines (390): the retention time of the analyte in the sample extract should correspond to that of the average of the calibration standards measured in the same sequence with a tolerance of ± 0.2 min, chromatographic peaks with a similar peak shape should be observed in the extracted ion chromatograms of the two product ions and the ion ratio should be within $\pm 30\%$ (relative) to that obtained from the average of the calibration standards from the same sequence. The chromatographic peaks should have a signal-to-noise ratio of at least 3.

Validation assays were performed constructing three calibration curves during four different working days. The lower limit of quantification (LLOQ) was calculated using the formula $\text{LLOQ} = k \cdot \text{SD}$, where $k=5$ to ensure an absolute coefficient of variation within 20% for acceptable precision and SD represents the standard deviation of the replicate analyses (of the

lowest point of the calibration curve), while the accuracy was within the range 80-120% (391). The linearity was interpreted graphically using a scatter plot. The limit of detection (LOD) was calculated as three times the standard error of the intercept, divided by the slope of the calibration curve (392) (except for BEA, ENN A, ENN A1 and ENN B1 for which the linearity in the calibration range was poor and the LOD was determined experimentally analyzing samples spiked at low concentration and considering the identification criteria of SANTE/12089/2016 guidelines). Accuracy was expressed as: $\text{Accuracy (\%)} = 100 * \text{measured concentration/spiked concentration}$ (391); the measured concentration was obtained by averaging the results of three analyses conducted over four days (12 replicates). Precision was estimated by the coefficient of variation using the following formula: $\text{CV} = \text{SD/measured concentration}$ (391); the SD and the measured concentration were obtained by considering the results of three analyses conducted over four days (12 replicates). Selectivity was evaluated by analyzing (non-spiked) EDTA plasma from the Red Cross East Flanders. Matrix effect was not evaluated during method validation as it was expected to have a negligible impact on quantification due to the use of matrix-matched calibration curves, and no plasma samples indicated hemolysis or special conditions (icterus, lipemia). The absence of carry-over was confirmed by analyzing (non-spiked) EDTA plasma from the Red Cross East Flanders after a calibrant spiked at the highest (7th) concentration level. The results of the method validation are shown in **Table S-4**.

Statistical analysis

For statistical analysis, binary categories were replaced with integers (0, 1) for the following demographic and lifestyle variables: group (case or control), gender, soup drinking, coffee drinking, porridge eating, alcohol drinking, use of separate dwelling house, use of separate kitchen and smoking of utensils. Left-censored data were substituted following the guidelines of the European Food Safety Authority (EFSA) (393) considering the middle-bound scenario: mycotoxin biomonitoring measurements with values between the limit of detection (LOD) and the lower limit of quantification (LLOQ) were assigned a concentration of $\text{LLOQ}/2$, and non-detects (i.e. below the LOD) were replaced by $\text{LOD}/2$.

Data was analyzed using IBM SPSS software version 29 (p-value<0.05 was considered as statistically significant). Normality of continuous data was tested using the Shapiro-Wilk test. Differences between the case and control groups in age, binary categorical variables for demography and lifestyle, and mycotoxin (co)-exposure were tested using independent

samples t-test, chi-square test or Mann–Whitney U test, respectively. Multivariate binary logistic regression analysis was used to identify demographic, lifestyle and mycotoxin (co-)exposure variables that may potentially be risk factors for the development of EC.

JupyterLab (version 3.6.3) software based on Python programming language (version 3.9.7) was used to create violin plots, perform Principal Component Analysis (PCA), and compute classification and regression models based on 13 machine learning algorithms (Logistic Regression, K Neighbors Classifier, Naïve Bayes, Decision Tree Classifier, SVM – Linear Kernel, Ridge Classifier, Random Forest Classifier, Quadratic Discriminant Analysis, Ada Boost Classifier, Gradient Boosting Classifier, Linear Discriminant Analysis, Extra Trees Classifier, Light Gradient Boosting Machine) included in the PyCaret (Classification and Regression Training) library (<https://pycaret.gitbook.io/docs>). When considering only participants under age of 50, Synthetic Minority Over-Sampling Technique (SMOTE) was used to address the imbalance between the number of cases and controls in the data set (394).

4.4. Results

Demographic and lifestyle variables

Table 3 shows the descriptive statistics of demographic and lifestyle variables for the cases and location-matched controls in Ethiopia. The mean age of the case and control groups was statistically significantly different (independent samples t-test, $\alpha = 0.05$), with the case group being older. The minimum and maximum age of the cases was 18 years and 105 years, respectively. Females were more frequent in the control group (chi-square test, $\alpha = 0.05$). All participants were residents of the rural district of Oromia regional state with agricultural occupations. Based on food frequency questionnaires, all participants were reported to use a similar dietary source for lunch and dinner, predominantly wheat, barley and teff (*Eragrostis teff*). However, many cases were unable to eat solid food at the time of sample collection due to severe dysphagia. The majority of study participants were unaware of mycotoxins (90 (54%) cases and 88 (53%) healthy controls). Most study participants reported no history of exposure to alcohol and tobacco. Of the two histological types identified, ESCC accounted for 86%. There was a relationship (chi-square test, $\alpha = 0.05$) between disease status and the variables alcohol drinking, coffee drinking, porridge eating, use of separate dwelling house, use of separate kitchen and smoking of utensils.

Table 3: Descriptive statistics of demographic and lifestyle variables for esophageal cancer cases and location-matched controls in Ethiopia.

Variables		Cases (n=166)	Controls (n=166)	Statistical test		
				T	df ¹	p-value
Continuous		Mean ± standard deviation				
Age (years)*		52±14	39±7	11.4	246	<0.001
Categorical		Frequency (n (%))		χ ²	df ¹	p-value
Gender*	Female	96 (57.8)	126 (75.9)	12.2	1	<0.001
	Male	70 (42.2)	40 (24.1)			
Established risk factors						
Alcohol drinking*	Yes	8 (4.8)	26 (15.7)	10.6	1	0.001
	No	158 (95.2)	140 (84.3)			
Smoking	Yes	3 (1.8)	0 (0)	3.0	1	0.082
	No	163 (98.2)	166 (100)			
Potential risk factors						
Thermal injury						
Soup drinking	Yes	142 (85.5)	130 (78.3)	2.9	1	0.087
	No	24 (14.5)	36 (21.7)			
Coffee drinking*	Yes	166 (100)	149 (89.8)	17.9	1	<0.001
	No	0 (0)	17 (10.2)			
Porridge eating*	Yes	164 (98.8)	145 (87.3)	15.9	1	<0.001
	No	2 (1.2)	20 (12.0)			
Exposure to indoor air pollution						
Use of separate dwelling house*	Yes	62 (37.3)	121 (72.9)	42.4	1	<0.001
	No	104 (62.7)	45 (27.1)			
Use of separate kitchen*	Yes	75 (45.2)	102 (61.4)	8.8	1	0.003
	No	91 (54.8)	64 (38.6)			
Smoking of utensils*	Yes	143 (86.1)	80 (48.2)	54.2	1	<0.001
	No	23 (13.9)	86 (51.8)			

*Variables with a statistically significant difference between the case and control groups.

¹ Degrees of freedom.

Mycotoxin exposure

Evidence of exposure to 10 of the different mycotoxins investigated was observed in plasma samples of the participants as shown in **Figure 18**. Ochratoxin A (OTA) was detected from all participants both in the cases and control groups, while tenuazonic acid (TA) was detected in plasma of 145 (87.3%) cases and 71 (42.8%) controls. Whereas the mycotoxins citrinin (CIT), cyclopiazonic acid (CPA), deoxynivalenol (DON), and zearalanone (ZAN) were detected both from cases and controls. Aflatoxin B2 (AFB2), enniatin B (ENNB), nivalenol (NIV), and α -zearalenol (α -ZEL) were detected only in the plasma of cases. Representative chromatograms of all mycotoxins detected in plasma samples and a comparison with a

plasma sample spiked with the mycotoxin standard at a similar concentration are shown in **Figure S-5**.

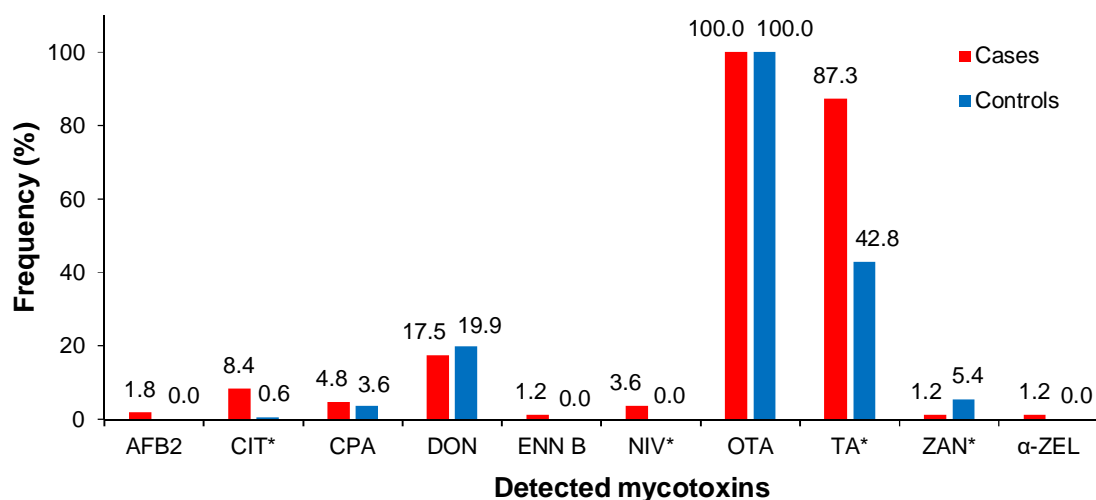


Figure 18: Frequency of detection of mycotoxins in plasma from esophageal cancer cases and location-matched controls in Ethiopia

*Variables with a statistically significant difference between the case and control groups. Results of the chi-square test (χ^2 , p-value): AFB2- aflatoxin B2 (3.0, 0.08), CIT- citrinin (11.8, <0.001), CPA- cyclopiazonic acid (0.3, 0.59), DON- deoxynivalenol (0.3, 0.57), ENNB- enniatin B (2.0, 0.16), NIV- nivalenol (6.1, 0.01), OTA- ochratoxin A (-,-), TA- tenuazonic acid (73, <0.001), ZAN- zearalanone (4.6, 0.03), α -ZEL- α -zearalenol (2.0, 0.16).

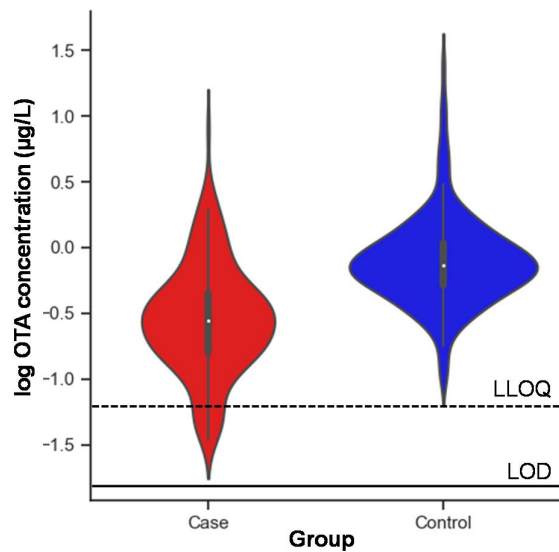
Table 4 shows the LOD, LLOQ, median and the highest concentration for the mycotoxins detected in the plasma samples. Violin plots were prepared for the mycotoxins with a frequency of detection >50% in cases and/or controls, i.e. OTA and TA (**Figure 19**). The violin plots pointed out some differences in exposure for case and control groups. The concentration of OTA in case and control groups was compared using the Mann-Whitney U test and there was a statistically significant difference (p-value<0.001), with OTA plasma concentrations being lower in cases than in controls. Regarding exposure to TA, there was also a statistically significant difference between the groups (p-value<0.001), with TA plasma concentrations being higher in cases than in controls.

Table 4: Limit of detection (LOD), lower limit of quantification (LLOQ), median, 95th percentile (P95) and highest concentration for the mycotoxins detected among esophageal cancer cases and location-matched controls in Ethiopia.

Mycotoxins	LOD (µg/L)	LLOQ (µg/L)	Median (µg/L)		P95 (µg/L)		Highest concentration (µg/L)	
			Cases	Controls	Cases	Controls	Cases	Controls
AFB2	0.018	0.024	<LOD	<LOD	<LOD	<LOD	0.14	<LOD
CIT	0.11	0.46	<LOD	<LOD	>LOD and <LLOQ	<LOD	1.6	0.66
CPA	0.18	0.66	<LOD	<LOD	<LOD	<LOD	>LOD and <LLOQ	>LOD and <LLOQ
DON	0.027	0.082	<LOD	<LOD	0.39	>LOD and <LLOQ	32	320
ENN B	0.011	0.16	<LOD	<LOD	<LOD	<LOD	0.42	<LOD
NIV	0.31	1.36	<LOD	<LOD	<LOD	<LOD	2.4	<LOD
OTA*	0.020	0.070	0.28	0.73	1.7	3.8	8.0	24
TA*	5.0	10	>LOD and <LLOQ	<LOD	133	68	320	115
ZAN	0.27	0.72	<LOD	<LOD	<LOD	>LOD and <LLOQ	>LOD and <LLOQ	>LOD and <LLOQ
α-ZEL	0.11	0.50	<LOD	<LOD	<LOD	<LOD	>LOD and <LLOQ	<LOD

*Variables with a statistically significant difference between the case and control groups (Mann-Whitney U test, p-value<0.05). AFB2- aflatoxin B2, CIT- citrinin, CPA- cyclopiazonic acid, DON- deoxynivalenol, ENN B- enniatin B, NIV- nivalenol, OTA- ochratoxin A, TA- tenuazonic acid, ZAN- zearalanone, α-ZEL- α-zearalenol.

A)



B)

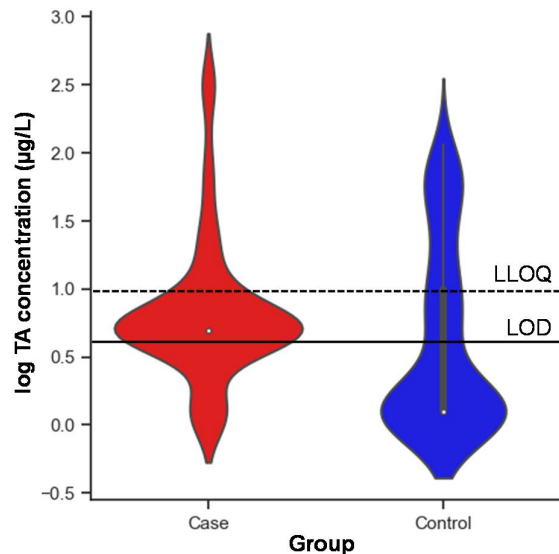


Figure 19: Violin plots for mycotoxin exposure in the esophageal cancer case-control study (A) ochratoxin A (OTA), and (B) tenuazonic acid (TA). Mycotoxin concentrations are indicated in logarithmic scale. The limit of detection (LOD) is indicated as a solid line. The lower limit of quantification (LLOQ) is indicated as a dashed line. Values between LOD and LLOQ were assigned a concentration of LLOQ/2, and non-detects (i.e. below the LOD) were replaced by LOD/2.

Identification of potential risk factors for esophageal cancer

Principal component analysis (PCA) was used to investigate the relationships among demographic and lifestyle variables, and mycotoxin concentrations to identify patterns and potential clusters in the data (Figure S-6). The Scores plots of the PCA show that PC2 was

useful in differentiating cases and controls. Variables positively correlated with the case group were age (which is a risk factor for the development of most cancers) (395), smoking of utensils, coffee drinking, soup drinking, porridge eating, and the exposure to several mycotoxins, e.g. AFB2, CIT, and NIV, consistently with the higher number of positive samples in cases compared to controls. Concentration of OTA and use of separate dwelling house were positively correlated with the control group.

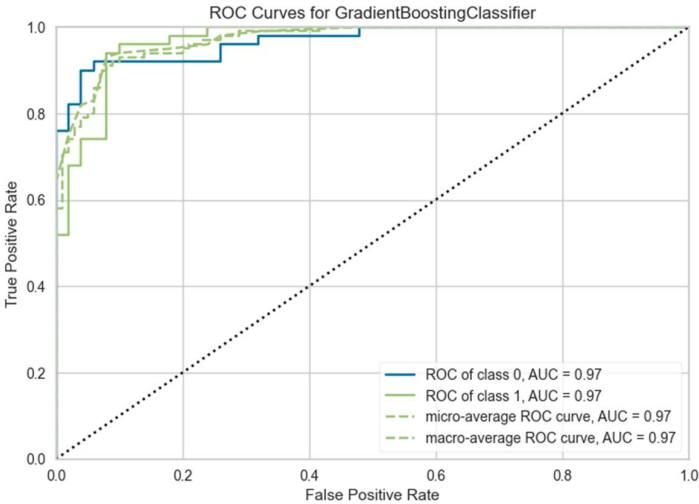
To further investigate the datasets, machine learning algorithms included in PyCaret library were used to compute classification and regression models. The model with the highest accuracy was based on the Gradient Boosting Classifier algorithm. The area under the Receiver Operating Characteristic curve (AUC) of the computed model was 0.97 for both case and control groups and the five most important features for classification of cases and controls were age, concentration of OTA, concentration of TA, smoking of utensils and use of separate dwelling house (**Figure 20**). In view of the importance of mycotoxin exposure in the classifier model, the machine learning models were recomputed to discriminate EC patients from controls based solely on mycotoxin concentrations. The model with the highest accuracy was again based on the Gradient Boosting Classifier algorithm, the AUC was 0.92 for both cases and controls and the most relevant mycotoxins for classification of cases and controls were OTA and TA (**Figure S-7**).

Considering the promising results of the machine learning models to classify EC patients and controls, a multivariate binary logistic regression model was computed for the onset of EC based on demographic and lifestyle variables and mycotoxin exposure quartiles (Q1- low exposure, Q2- medium-low, Q3- medium-high, Q4- high exposure) (**Table S-5**). **Table 5** shows the results of the final multivariate binary logistic regression analysis considering the five most important predictor variables from the Gradient Boosting Classifier model (**Figure 20-B**). Variables statistically significantly associated with an increased probability of developing EC were age, smoking of utensils and mycotoxin exposure quartile for TA. Use of a separate dwelling house and mycotoxin exposure quartile for OTA were positively correlated with the control group.

Sensitivity analysis was performed to evaluate the influence of participant age on mycotoxin exposure. To balance the age of participants in the case and controls groups, only participants younger than 50 years were considered for further analysis (n=61 and 157 for case and control groups, respectively). However, the imbalance in the number of participants in the two groups negatively affected the sensitivity of the multivariate binary logistic regression models. SMOTE was used to address the imbalance between the number of cases and

controls in the data set by over-sampling of cases. **Table 6** shows the results of multivariate binary logistic regression analysis considering age and mycotoxin exposure for participants under age of 50. TA exposure was statistically significantly associated with an increased probability of developing EC, independently of age, while OTA was statistically significantly associated with the control group.

A)



B)

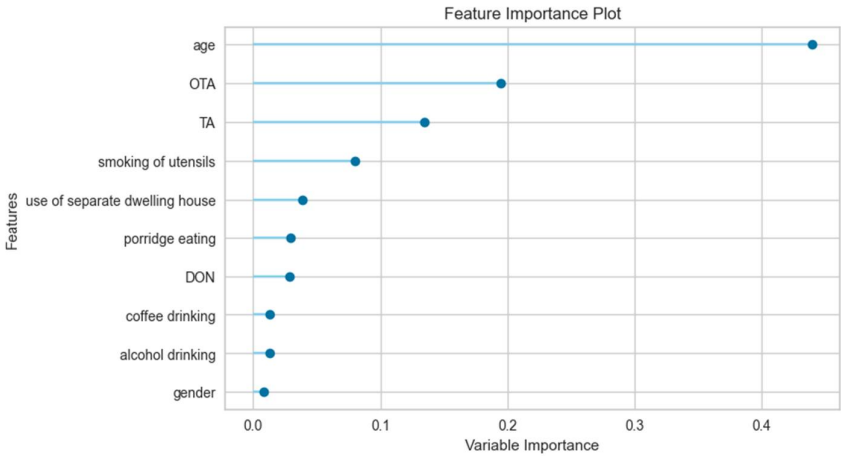


Figure 20: Gradient Boosting Classifier model to classify esophageal cancer patients from controls based on demographic and lifestyle variables, and mycotoxin concentrations

(A) Receiver Operating Characteristic curve, and (B) feature importance plot.

Table 5: Multivariate binary logistic regression analysis for the onset of esophageal cancer based on demographic, lifestyle and mycotoxin exposure variables selected from the Gradient Boosting Classifier model (Figure 3-B) for cases and location-matched controls in Ethiopia.

Variables		Cases (n=166)	Controls (n=166)	Multivariate binary logistic regression analysis ¹	
				AOR (95% CI)	p-value
Continuous		Mean ± standard deviation			
Age (years)*		52±14	39±7	1.17 (1.12-1.23)	<0.001
OTA quartile*		1.90±1.03	3.11±0.85	0.28 (0.19-0.41)	<0.001
TA quartile*		2.67±0.91	2.34±1.28	1.80 (1.26-5.58)	0.001
Categorical		Frequency (n (%))			
Use of separate dwelling house*	Yes	62 (37.3)	121 (72.9)	1	-
	No	104 (62.7)	45 (27.1)	11.9 (4.70-30.0)	<0.001
Smoking of utensils*	Yes	143 (86.1)	80 (48.2)	28.7 (9.72-84.7)	<0.001
	No	23 (13.9)	86 (51.8)	1	-

AOR- adjusted odds ratio; CI- confidence interval.

*All variables are statistically significantly different between the case and control groups.

¹ Percentage of correct classification (cases / controls): 86.1% / 87.3%.

Table 6: Multivariate binary logistic regression analysis for the onset of esophageal cancer based on age and mycotoxin exposure for cases and location-matched controls in Ethiopia under the age of 50 years.

Variables	Cases (n=61)	Controls (n=157)	Multivariate binary logistic regression analysis ¹	
			AOR (95% CI)	p-value
Continuous	Mean ± standard deviation			
Age (years)	39±6	38±5	1.01 (0.99-1.04)	0.301
OTA quartile*	1.93±1.12	3.10±0.85	0.21 (0.18-0.23)	<0.001
TA quartile*	2.75 ±0.92	2.34±1.28	1.88 (1.68-2.11)	<0.001

AOR- adjusted odds ratio; CI- confidence interval.

*Variables with a statistically significant difference between the case and control groups.

¹ Due to the imbalance in the number of cases and controls, cases were over-sampled using SMOTE. Percentage of correct classification (cases / controls): 88.9% / 80.4%.

Figure 21 shows the histogram of the number of mycotoxin exposures per participant in the case and control groups. All participants were positive at least for one type of mycotoxin (i.e. OTA). There was a statistically significant difference in the number of mycotoxin exposures

between case and control groups (Mann-Whitney U test; p-value<0.001), indicating that cases were exposed to more types of mycotoxins than controls. Multivariate binary logistic regression analysis based on number of mycotoxin exposures, age and gender for all participants of the case-control study revealed the number of mycotoxin exposures as a predictor of EC independently of gender (**Table S-6**). **Table 7** shows the results of multivariate binary logistic regression analysis considering age and number of mycotoxin exposures for participants under age of 50. The number of mycotoxin exposures was statistically significantly associated with the probability of developing EC, independently of age.

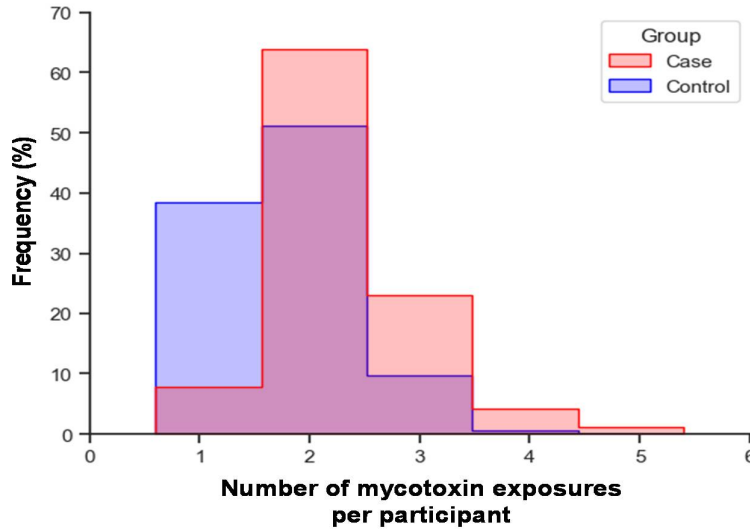


Figure 21: Number of mycotoxin exposures per participant among esophageal cancer cases and location-matched controls in Ethiopia

Table 7: Multivariate binary logistic regression analysis for the onset of esophageal cancer based on age and number of mycotoxin exposures for cases and location-matched controls in Ethiopia under the age of 50 years.

Variables	Cases (n=61)	Controls (n=157)	Multivariate binary logistic regression analysis ¹	
			AOR (95% CI)	p-value
Continuous	Mean ± standard deviation			
Age (years)	39±6	38±5	1.02 (0.99-1.05)	0.073
Number of mycotoxin exposures*	2.30±0.72	1.73±0.65	2.54 (2.10-3.07)	<0.001

AOR- adjusted odds ratio; CI- confidence interval.

*Variables with a statistically significant difference between the case and control groups.

¹ Due to the imbalance in the number of cases and controls, cases were over-sampled using SMOTE. Percentage of correct classification (cases / controls): 86.7% / 55.0%.

4.5. Discussion

In Ethiopia, where there is a high EC incidence and already established important risk factors for EC, i.e. alcohol consumption and tobacco use, are rare (57,58), we assessed the exposure to 39 mycotoxins and metabolites through human biomonitoring and their association with occurrence of EC. The risk associated with individual as well as multiple mycotoxin exposure and their levels was evaluated using classification and regression models. All cases and controls were found to be exposed to at least one mycotoxin, suggesting evidence of (multi-)mycotoxin exposure as a potential risk factor for the onset EC for the first time in Ethiopia. In EC patients, a wider variety of mycotoxins and a statistically significant greater number of co-exposures were observed, including higher frequency and concentration of tenuazonic acid (TA). Multivariate binary logistic regression analysis indicated that TA exposure and the number of mycotoxin exposures were positively associated with EC, independent of age. These findings warrant the importance of considering mycotoxins in the study of the etiology of EC in Ethiopia. We also identified other potential risk factors for EC, i.e. coffee drinking and porridge eating as proxy indicator of thermal injury and use of separate dwelling and smoking utensil as indicators of indoor air pollution levels.

Environmental exposures have been identified as potential risk factors of EC in Africa including exposure to heavy metals (396,397), polycyclic aromatic hydrocarbons metabolites from in-door air pollution (398), N-nitrosamines from traditional brews (399), smoking and daily use of spicy chilies and salted foods (400). Dietary and various environmental exposure determinants of EC were reported from Ethiopia (28). Mycotoxins are a major cause of food intoxication in Sub-Saharan Africa, where the hot and humid tropical climate favors the growth of fungi (401). Recently, dietary exposure to mycotoxins in Nigerian mothers and infants was investigated by analyzing breast milk, complementary food and urine (402,403). Multiple mycotoxin exposure was higher in urine samples from non-exclusively breastfed compared to exclusively breastfed infants, demonstrating dietary exposure to mycotoxins through complementary foods.

Using an epidemiological approach the significance of mycotoxin exposure as a risk factor of EC was reported in high incidence areas in Africa (276,277). However, these epidemiological studies are known for their limitations due to the non-uniform distributions of mycotoxins in food and inaccurate estimations of food consumption and recall bias associated food frequency questionnaire. To bridge this gap we assessed the human internal exposure to multiple mycotoxins using a human biomonitoring approach which better estimates human exposure to mycotoxins (173,404).

A notable gender imbalance in the study participants was observed, in particular a higher representation of female controls. This is because the majority of the patients' relatives who accompanied them were female. Globally, the incidence of EC is higher in males (70%) than in females (11,60). Gender-based variations in the incidence of EC have also been reported in Africa EC high-risk region (31). In South Africa, EC is more common among males (63) whereas, a study in Sudan by Gasmelseed et al. in 2015 found a higher incidence of EC among females. Similarly, other studies conducted in Ethiopia (57,64) found a slightly higher prevalence among female cases. This variation may be due to difference in risk factors based on geographical and gender-associated cultural activity variation across countries.

In this study, OTA was detected in all cases and controls, with OTA concentrations being higher in controls than in cases. A possible explanation for the lower OTA concentrations in cases may be related to the disease status. From the same district, a large proportion (87.6%) of cases presented with dysphagia, which is associated with weight loss due to reduced food intake (58), probably causing a decrease in the level of exposure to OTA. The median OTA concentration in plasma in the present study was 0.28 and 0.73 $\mu\text{g/L}$ for cases and controls, respectively (**Table 5**), which is higher compared to the results of mycotoxin biomonitoring studies from China (median OTA concentration was 0.16 $\mu\text{g/L}$) (406) and Italy (median OTA concentration was 0.17 $\mu\text{g/L}$) (407), and similar to a study in Bangladesh (median OTA concentration was 0.57 $\mu\text{g/L}$) (408). In contrast, a study in Northern Spain reported a median OTA concentration in plasma of 2.60 $\mu\text{g/L}$ (409), which is around 10 times and 2 times higher compared to the results in the present study for cases and controls, respectively.

The high frequency of OTA detection is consistent with previous human biomonitoring studies, e.g. OTA was the mycotoxin most frequently detected in serum of healthy individuals of Tunisian population, with no variation across age group but with the region of residence (410). It was also the most frequently detected mycotoxin among the healthy population of Spain with increasing frequency (409,411) and among Chinese healthy volunteer individuals living in Nanjing (406).

The widespread presence of OTA in cereals and coffee may contribute to the high prevalence of this mycotoxin. OTA is among the major mycotoxin contaminants of cereal grain and its products in different parts of the world, e.g. high levels of OTA contamination in food and feeds were reported in South and Central America, Asia and Africa (412). These factors likely play a role in the high frequency of OTA detection in our study, as the cereals in which OTA contamination was frequently reported are a source of bread and injera, which are staple food in our study setting (28). Furthermore, coffee bean and its derivative products were

reported to be contaminated with OTA (413) and are also frequently used by cases and controls of this study. The properties of OTA, including its high thermal resistance to food processing (stable up to 180°C) (414) and its long half-life in human plasma and serum (35 days) (415) may contributed to its high frequency of detection in plasma from both cases and controls. Moreover, the absence of mycotoxin regulatory policy in Ethiopia and low awareness of the study participants on mycotoxins, may also contribute to chronic exposure to this mycotoxin.

OTA is classified by the IARC as possibly carcinogenic to humans (Group 2B) since 1993 (164), and more recent data show evidence of the carcinogenic activity and toxicity of OTA (166). Since OTA is widely prevalent in food and feed and also commonly reported in human biomonitoring studies, the EFSA recommended further research on OTA mechanistic toxicity and genotoxicity (416). An experimental study showed that exposure to OTA in a dose of 0.125 µM–0.5 mM over two time periods within 48 hours was able to induce biomarkers of hypoxia and transformation in human kidney cell (417). Some mechanisms including inhibition of protein synthesis, induction of oxidative stress, and DNA adduct formation were mentioned for the toxic action of OTA (418). In addition, the ability of OTA in damaging esophageal epithelial cell by reactive oxygen species generation and oxidative DNA damage was demonstrated *in vitro* (419). This oxidative process was well mentioned in cancer development previously (2,182). OTA is also linked to liver and kidney damage. A benchmark dose lower confidence limit (BMDL₁₀) of 4.73 µg/kg body weight (bw) per day for the non-neoplastic endpoint was calculated from kidney lesions observed in pigs (416). This BMDL₁₀ corresponds to an OTA concentration in plasma of 1046 µg/L using the Klaasen equation and considering an OTA bioavailability of 0.5, a body weight of 70 kg, and a daily renal clearance of 0.1099 mL/min, (409). One hundred and sixty-five cases (99.4%) and 161 controls (97.0%) resulted in a margin of exposure (MOE) of more than 200, indicating a low health concern. For neoplastic effects, a BMDL₁₀ of 14.5 µg/kg bw per day (i.e., 3208 µg/L in plasma) was calculated from kidney tumors seen in rats (416). Only 69 cases (58.4%) and 15 controls (9.0%) resulted in a MOE of more than 10000, indicating that human exposure to OTA in the Arsi-Bale districts of Oromia regional state in Ethiopia could be a potential health concern.

On the other hand, TA is the second most frequently detected mycotoxin in this study in samples from both cases and controls and is positively associated with EC after adjusting for demographic variables. TA is among the main *Alternaria* mycotoxins that contaminate food commodities (420). The fungi genus of *Alternaria* grows under a wide range of conditions

and global climate change is increasing the prevalence of this fungi and its toxins in tomatoes (421,422) and cereals, mainly wheat and its products(423,424), and barley (425).

A few toxicity studies showed that TA inhibits protein synthesis (426) and induces cytotoxicity to human intestinal epithelial cells and hepatocytes (427). In animal trials it was observed that TA causes emesis and hemorrhagic gastro-enteropathy among other effects (428). It was also described that animal exposure to TA for a ten-month period resulted in the development of precancerous lesion on esophageal tissue from which authors concluded that in cases where exposure to TA occurs for longer periods of time, progression to EC might occur (429). Since the dietary habits of the population in the current study did not change over a long period of time, the higher frequency of TA detection and the statistically significantly higher concentration among cases indicate that TA exposure may play an etiological role of EC.

Furthermore, a wider variety of mycotoxins were detected in cases than in healthy controls, i.e. 10 and 6 different mycotoxins were detected in the case and control group, respectively. The number of mycotoxin exposures was statistically significantly higher among cases, including two subjects that were positive for five different mycotoxins, while co-exposure was less frequent in controls and for up to three different mycotoxins. AFB2, ENN B, NIV and α -ZEL were detected only among cases. In addition, CIT was detected in 14 cases but only in one participant of the control group. This exposure difference may be attributed to differences in food storage, particularly post-harvest grains, which may be prone to fungal contamination at house hold level. Of those mycotoxins, AFB2 was classified by IARC under group 1, which is carcinogenic to human, while NIV and CIT were classified under group 3 (166). Although there is no safe dose of AFs in human, the exposure dose-dependent association between aflatoxins and liver cancer risk was explained by a comprehensive review on mycotoxins and cancer risk in humans (161), while an EC case-control study in China with a number of 570 participants reported that internal exposure to aflatoxin B1 and fumonisin B1 was associated with the risk of EC and the synergic effect due to co-exposure may contribute to increased risk (430). Our result is in line with another study in which CIT was detected in 90% of plasma samples (LOD 0.07 μ g/L) analyzed from 104 participants in Bangladesh (431). Moreover, CIT was reported to be associated with nephropathy and promote the renal cell carcinogenesis (432).

The results of multivariate binary logistic regression analysis after adjusting for gender and age indicate that TA concentration in plasma and the number of mycotoxin exposures (of the 10 mycotoxins detected in plasma in this study, **Figure 19**) per participant are positively

associated with EC. This suggests that mycotoxin exposure may play a role in the occurrence of EC, although other potential confounders of the exposome such as, micronutrients deficiency, thermal injury and infectious agents were not controlled. It is known that co-exposure to OTA with other mycotoxins enhances their toxicity and carcinogenicity in human and other animals cell lines, as observed with *Alternaria* toxins, FB1, AFs and CIT (433–435). For example, OTA co-exposure with CIT increased OTA-DNA adduct formation and its associated oxidative stress damage. The results in this study emphasize the need to characterize the effect of mycotoxin co-exposure and include it in risk assessment, as currently mycotoxin safety levels do not consider the additive or synergistic effects of co-exposure to mycotoxins.

Human exposure to mycotoxins occurs primarily through the consumption of contaminated food (436). Since patients with upper gastrointestinal tract cancer, including EC, are unable swallow solid food due to the early obstruction of the gastrointestinal passage (437) and often transition to a cereal porridge-based diet, it is worth noting the possibility of reverse causality in the mycotoxin biomonitoring results presented in this case-control study. A prospective study design with regular sampling should be considered in this high incidence area of EC in Ethiopia to obtain conclusive results on the role of mycotoxin exposure in the onset and development of the disease.

4.6. Strengths and limitations of the study

To the best of our knowledge, this is the first study to evaluate the risk of multiple mycotoxin exposure as an etiological factor of EC in a high incidence region in Ethiopia using a human biomonitoring approach. The main strengths of this study are the case-control design, in which location-matched healthy controls shared similar dietary sources with the cases, the optimum sample size (overall sample size = 332) and the broad panel of mycotoxins analyzed. However, we are unable to determine the levels of aflatoxin B1-albumin or aflatoxin B1-lysine, which are biomarkers for aflatoxin exposure, due to unavailability of standards. Additionally, the analysis of plasma samples provides evidence of chronic exposure to several mycotoxins (438), but polar mycotoxins, which are typically cleared from the body within a few hours, can be better assessed by analysis of 24h-urine samples, and fumonisins, which have frequently been associated with an increased risk of EC, have low absorption and are mainly excreted via the fecal route. Although it is possible to assess human exposure to FB1 in plasma by determining the ratio of sphinganine to sphingosine (439), unfortunately sphinganine and sphingosine standards and internal standards were not available in our laboratory at the time of analysis and we did not have a validated method. Also we are unable to assess other potential confounders of the exposome such as

micronutrients deficiency, thermal injury and infectious agents. Due to the limited detail of the food frequency questionnaire, we are unable to relate mycotoxin concentrations in plasma to the source of exposure. Furthermore, it was not possible to match the age of the case and control groups, although we overcame the dissimilarity by conducting an analysis restricted to participants under 50 years of age and over-sampling cases to balance the number of participants in both groups.

4.7. Conclusion

Mycotoxin exposure was frequent in both cases of EC and healthy controls from the Arsi-Bale district of Oromia region of Ethiopia, while a wider variety of mycotoxins and greater number of mycotoxin exposures per participant were observed in the plasma samples of the cases. In particular, the frequency of detection and concentration of TA and exposure to multiple mycotoxins were found to be positively associated with EC. The low awareness of participants on mycotoxins and the absence of mycotoxin regulatory policies adds to the potential overall health risk of mycotoxin exposure in the district. The findings on the high prevalence of OTA and TA observed in this study highlight the need for further research on exposure to these two dietary contaminants. In the future, a conclusive result on the role of mycotoxin exposure in the occurrence of EC may be obtained using a prospective cohort study in this high incidence area in Ethiopia.

Chapter 5: General Discussion, Conclusion and Recommendation

5.1. General discussion

Geographical differences in EC demographic epidemiology and histology were noted, indicating that risk factors vary according on where a person lives. This demonstrates the significance of exposure to the environment, communal culture, and individual behaviors that influence or complement one another. According to earlier research, there are differences in Ethiopia's EC prevalence and histology based on area of residence. Some studies from Ethiopia which involves cases from various districts of Ethiopia showed slightly higher prevalence of EC among males and an increased proportion of EAC(59,64,250), while the study results of patients primarily from the Arsi-Bale district showed a higher prevalence among females and a larger proportion of ESCC histological type(56–58). We used data from a medical institution in the Arsi-Bale district catchment area to examine the demographic profile and descriptive epidemiology of EC, taking into consideration the role that local factors play in this variability. 630 EC patients' data was collected and analyzed. ESCC is the most prevalent histological subtype, accounting for 79.43% of cases. The disease was most prevalent in people aged 40 to 50, and women comprised the majority of these cases (62.80%). Male and female exposure risk would be comparable, as evidenced by the almost identical distribution of EAC incidence in both genders. The lower esophagus is where the majority of cancers begin, followed by the middle esophagus.

The demographic epidemiology of EC demonstrates regional similarities. We found that the prevalence of EC was higher among women in Ethiopia's East African neighbors, such as Sudan(89,287), Eritria(295), Somalia(440) and Djibouti(441), while the predominance of male cases increased from Eastern Africa to South Africa, beginning in Kenya (**Figure 11B**). It has been widely recognized that there is a disparity between men and women in cancer, especially with respect to cancers such as thyroid, liver, colon, bladder, and EC (442). The differences have been attributed to biological factors such as sex chromosomes, hormone levels, and sex-biased molecular changes, as well as external exposures such as social behaviors, smoking, and alcohol use (442,443).

Although more research is needed to determine the precise risk factors for this gender-based variation, the high frequency of EC among females in our sample and the findings of other studies in the area demonstrated the role played by shared environmental and cultural factors. Women living in the study district were more at risk than men, as evidenced by the decreased oral microbial diversity that was substantially linked to EC (**Figure 15 A–D**) and female gender (**Figure 14 D**). Dietary diversity and preference may have contributed to the gender-

based variance in EC incidence in the area where patients were recruited, as evidenced by the correlation between low oral and gut microbiome diversity and reduced host dietary diversity(444,445). This is supported by reports of poor dietary habits (78.4%) among female residents of the study's district, which were linked to low perceived severity of poor dietary practices, low nutritional knowledge, food insecurity, and a negative attitude toward dietary diversity (446–448). Furthermore, fruits (3.48%) and meat (2.8%) were the least consumed foods (447). The higher frequency of EC in women may also be related to their exposure to polycyclic aromatic hydrocarbons (PAH), which is linked to cooking. There have already been reports linking PAH to a higher risk of EC in underdeveloped nations (398,449). In Kenyan women, PAH metabolite concentrations were positively and substantially correlated with indoor cooking, age <50, and no post-primary education, but not with tobacco use (398). Although definitive results on its risk factors were not obtained from earlier studies, geophagia, which is more common among females living in the African Esophageal Cancer Belt, may also contribute to this difference(450).

Additionally, the region's reported higher ESCC proportion implies that comparable risk factors play a role in the occurrence of the disease. Compared to men in the same age range (40–50 years), we observed that younger girls had a higher prevalence of ESCC. Similar results were reported from Eretria (295), Somalia(294), and Tanzania(298). Compared to males, young adult females may have been exposed to the causative agent earlier or in comparatively higher amounts. For instance, poor dental hygiene were identified as early-life risk factors for EC (451). Proper study is required in the region to uncover factors and etiology associated with EC among thinner age female which intern help for primary prevention. Increased awareness of the risk variables linked to early EC onset aids in preventive and early screening to lower the disease's morbidity and mortality.

One of the primary concerns is that Ethiopia has a significantly greater prevalence of EAC than other countries in the region. With a poor five-year survival rate, this histological type is becoming increasingly prevalent globally, particularly in young adults (14,452). Our identification of oral microbiomes linked to cancer cases that characterize a western lifestyle where EAC is common raised the possibility that Ethiopia has more risk factors for EAC than other nations in the region.

From the lesson that microbiome involvement in the development of many cancer type (309–311) and no similar work in Africa and Ethiopia where other risk factors were play minimal role, we identified oral microbiome of EC patients were compositionally and functional distinct and able to differentiated EC cases from healthy individuals residing in the same

district. EC oral microbiome was lower in their absolute diversity and compositionally similar to western microbiome reflecting westernization process takes place. This could be attributed to over processing of food during in house preparation as reported earlier(453) or due to nutrition switching from local traditionally processed solid food to industrially processed liquid food upon disease onset. Additionally, the presence of disease-associated taxa in healthy individuals indicates that the residing healthy population is at risk and suggests common risk factors that drive difference in that oral microbiome. Farther more the low diversity oral microbiome among female's in general and higher incidence of EC among this gender (**Figure 10 A, and figure 14 D**) highlight the oral microbiome or other factors leads to this difference is/are the potential etiology of EC. Farther more the result of global oral microbiome comparison showed Tanzanian and Ugandan results were close diversity with Ethiopian cohort where shared nearly the same Epidemiology of EC high lighting shared regional risk factors. In support of this oral hygiene is significantly associated with early age onset of EC in Tanzania (298), showing the possible etiologic role oral microbiome.

The taxa including *S. anginosus*, *Alloprevotella* and *Alloscardovia* which were previously associated with various cancer were differentially abundant in oral microbiome of EC patients showing their potential etiological role and diagnostic markers. Experimentally it has been demonstrated *S. anginosus* promote gastric tumorigenesis via inducing gastritis (372,373). In Chinese cohort it was also reported *S. anginosus* was more abundant in stool and tissue samples of gastric cancer patients compared to samples from patients with chronic gastritis(372). Similarly, from the Japanese cohort salivary *S. anginosus* was significantly associated with EC(454). *Prevotella* and related genera including *Alloprevotella* have been previously associated with EC(374–376). Similarly *Alloscardovia* was reported to be biomarkers of human papillomavirus and cervical cancer showing its involvement in cancer occurrence (377). The genera of *Prevotella*, *Streptococcus* and *Veillonella* which are abundant in the Ethiopian population were also significantly associated with ESCC in the African Esophagus corridor (361).

With these similar findings and the result of our finding with the ability of correct classification diseased from non-disease of Ethiopians and Chinese cases (**Figure 17 A**) salivary microbiome are the potential non-invasive diagnostic biomarker of EC and possibly play etiological role or proxy indicators of EC risk like dietary habit, oral hygiene and other driver of microbiome of the population. Also the identification of role and factors contributing to the maintenance higher abundance and diversity of salivary microbiome, as

well as Ethiopian Unique **Cluster I** abundance in healthy controls worth important in planning prevention EC in this high incidence area of Ethiopia.

We have evaluated various mycotoxin exposure using human biomonitoring and its association with EC in order to address the limitations of earlier research that sought to evaluate mycotoxin exposure and EC using food mycotoxin incidence and EC epidemiology. Only six different mycotoxins were found in samples from healthy controls; however ten different mycotoxins were detected in plasma samples from EC patients. EC was positively correlated with exposure to multiple mycotoxins and tenuazonic acid separately. Consuming contaminated food or beverages are the primary way that people are exposed to mycotoxins. We found higher levels of TA and more mycotoxins in the plasma of EC patients in Ethiopia, even though their dietary intake was significantly reduced, which lowers their exposure level to mycotoxin.

According to toxicity studies, TA causes cytotoxicity to human intestinal epithelial cells and hepatocytes (427) and inhibits protein synthesis (426). Among other side effects, TA has been shown to induce emesis and hemorrhagic gastro-enteropathy in animal experiments (428). Additionally, it was reported that animals exposed to TA for ten months developed a precancerous lesion on their esophageal tissue. Based on this, the authors made the conclusion that extended exposure times to TA may result in progression to EC (429). Additional research further shown that when human fetal esophageal epithelium cell lines are exposed to *Alternaria* mycotoxins such alternariol and alternariol monomethyl ether, oncogenes are quickly induced and develop into tumors in mice (455). The higher frequency of TA detection and the statistically substantially higher concentration among cases suggest that TA exposure may be an etiological factor of EC, given that the dietary habits of the population in the current investigation were constant over an extended period of time. It has been shown that hazardous mycotoxins are present in a number of common food sources, including wheat, barley, teff, maize, milk, and coffee (456–458). The level of Aflatoxin and OTA contamination in the wheat sampled from the Arsi geographical area of three localities such as Hetosa, Gedeb, and Lemowas higher than the EU maximum threshold established (456). Furthermore, TA was the second most prevalent mycotoxin found in pregnant women's serum samples taken from Butajira (459), which is the nearby district of the current study area, confirming the mycotoxin's widespread distribution. Internal exposure to aflatoxin B1 and fumonisin B1 was linked to the risk of EC, and the synergistic effect of co-exposure may enhance risk, according to the results of an assessment of multiple mycotoxin exposure conducted in China utilizing a human biomonitoring approach (430). Potential

etiologic agents of mycotoxin for EC and healthy population are also at increased risk, as evidenced by Ethiopia's lack of legislation and regulation on dietary mycotoxin and the high prevalence of mycotoxin in both afflicted and healthy controls in the current study.

5.2. Conclusion

ESCC is more common in younger females, and the lower esophagus is where most cases of this histological type begin. There was no gender difference in the prevalence of EAC, indicating that no gender based exposure difference to its risk factors.. Reduced diversity of the oral microbiota, distinct community type which were functionally connected to many metabolisms, were significantly associated with EC. EC is linked to compositionally unique taxa that accurately distinguish EC cancer patients from healthy controls, indicating the potential non-invasive biomarkers for this cancer type. Additionally, low-richness case-associated taxa were found in Tanzania, Ethiopia, and Uganda's healthy populations, indicating populations at risk. TA and multiple mycotoxin exposure were strongly linked to an elevated risk of EC, with OTA being common in both healthy controls and EC patients exhibiting mycotoxin public health concerns in the district. Taken it account the population common food commodity were not changed over the time exposure to mycotoxin play role for the development of EC with other factors as well.

5.3. Recommendation

We recommend researchers, policy makers and community to better help the prevention of EC and identification of its etiology based our results and supporting previous findings.

For Researchers:

- Research on the factors that contribute to age and gender differences in the incidence of EC cancer is necessary in order to provide guidance for preventative and treatment plans.
- Identification of the dimensionality of case associated taxa and their effect on patient prognosis and survival is required using prospective longitudinal study for improving patients quality of life and management.
- Identification of factors associated with oral dysbiosis including dietary, behavioral, and cultural required that may potentially help in prevention of EC.
- The exact cause-and-effect relationship between mycotoxin exposure and the EC need to be ascertained by research using nail and hair samples as well as aductomics study, which are long-term exposure biomarkers.
- In animal model experimental research for mechanistic analysis of TA and the combined impact of mycotoxin co-exposure are required..

- A prospective cohort research is necessary in the study area to investigate the directionality of cases and the impact of mycotoxin..
- The mechanism of protection and pathogenesis of control and cases associated oral microbial taxa reacquired to be determining for prevention and treatment of EC.

For policy makers:

- Increase awareness on EC among at high risk groups in the district is required for prevention and to increase early health seeking behavior.
- Provide awareness on mycotoxins occurrence and risk and incorporate mycotoxin regulation in Ethiopian food safety policy
- Integrating oral health and hygiene in routine community health education to prevent chronic disease including EC

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Annexes

Supplementary Figures

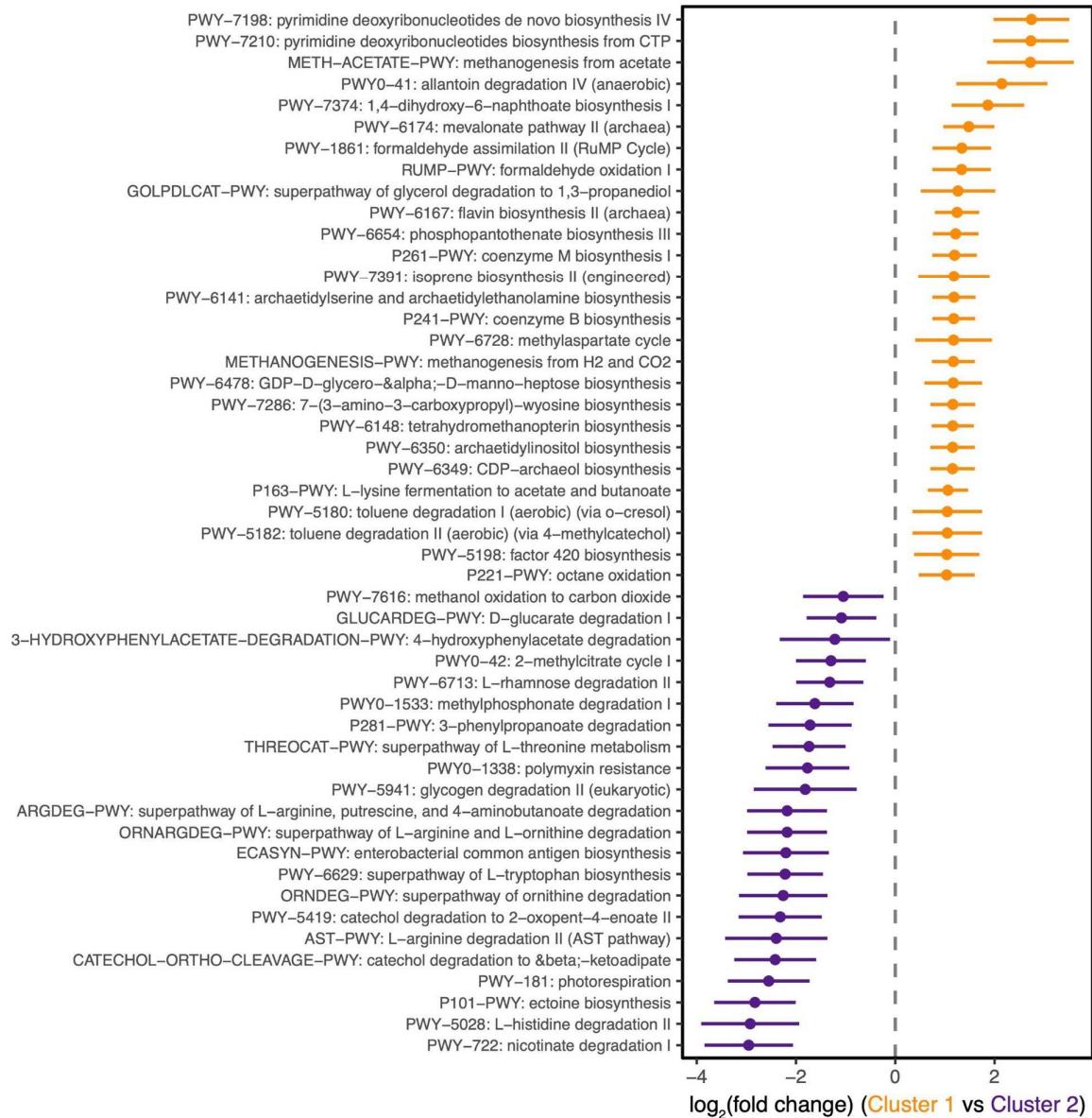


Figure S1. Healthy community clusters are inferred to be functionally distinct. Metagenomic inference (*see Methods*) uncovers an enrichment in amino acid metabolism in Cluster 2 while numerous pathways associated with archaea are enriched in community cluster2. N=108 healthy participants, N_{Cluster1}=64, N_{Cluster2}=44; FDR-corrected Welch's t-test of log₂-normalized pathway abundances. Error bars represent 95% confidence intervals. Significance was determined as FDR<0.05 and an absolute fold change >1.

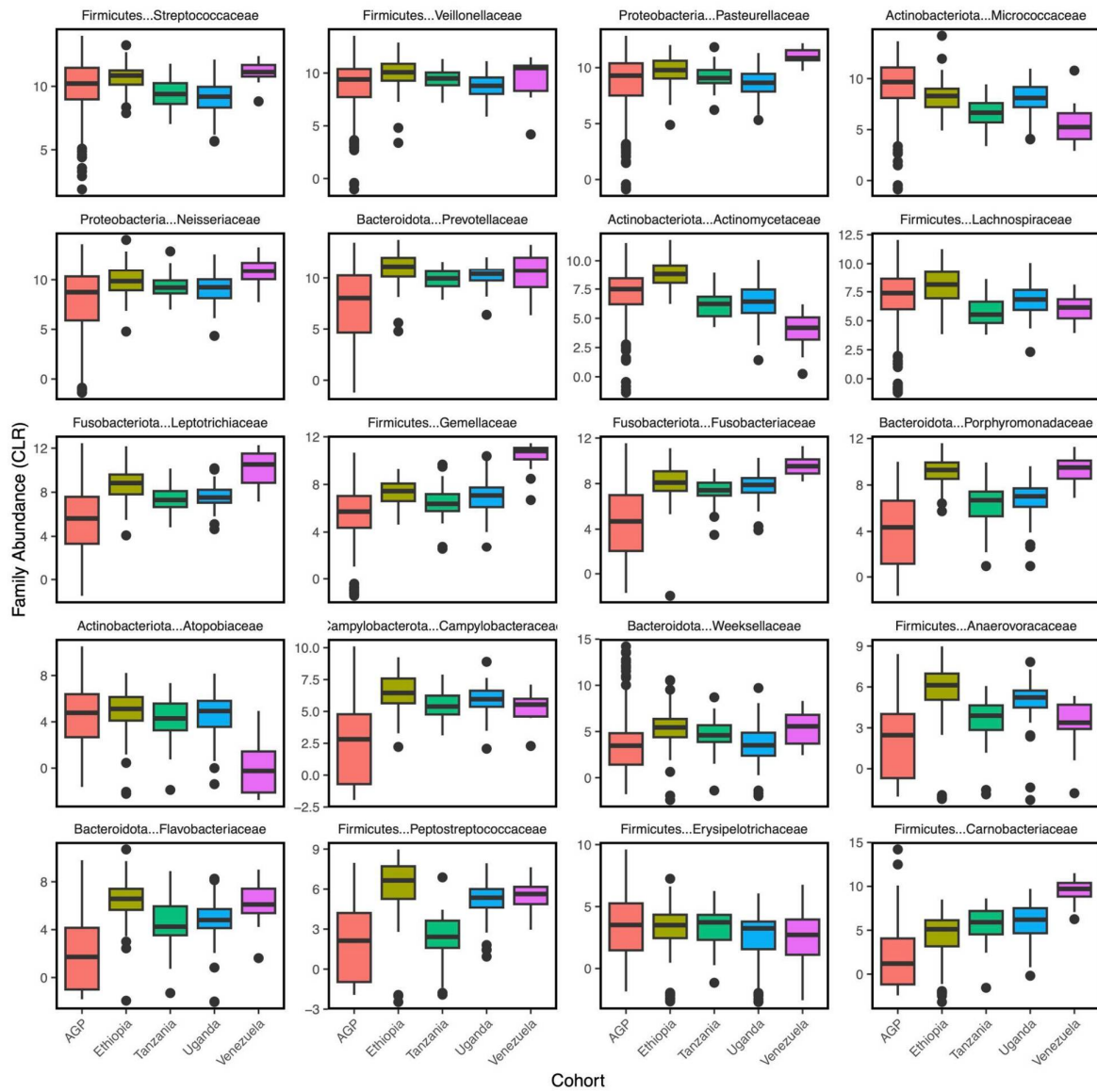


Figure S2. Abundances of the 20 most abundant bacterial families across global cohorts. Families were selected based on mean CLR abundance across all samples. $N_{AGP}=492$, $N_{Ethiopia}=107$, $N_{Tanzania}=36$, $N_{Uganda}=97$, $N_{Venezuela}=16$.

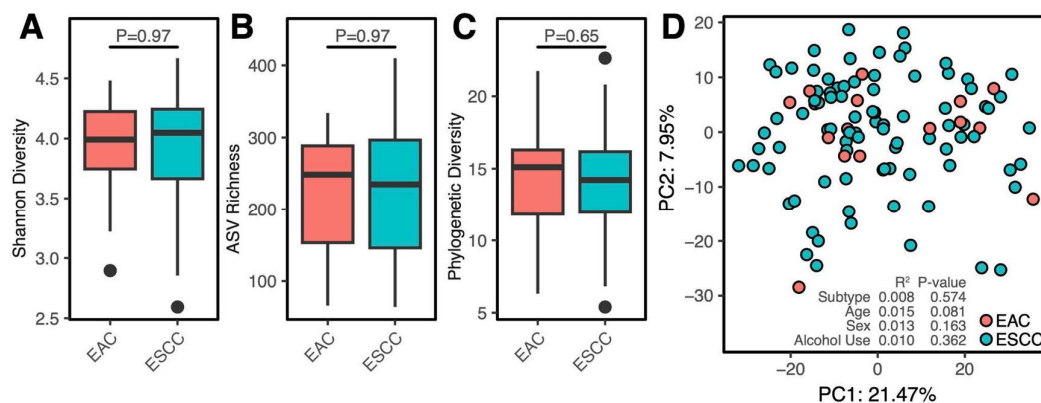


Figure S3. There is no significant difference in microbiota composition between esophageal cancer subtypes ESCC and EAC. Microbial diversity is not significantly reduced irrespective of choice of alpha diversity metric including (A) Shannon's diversity index, (B) ASV richness, and (C) phylogenetic diversity. (D) Visualization by PCoA demonstrates no significant variation in microbiome composition by cancer status which is supported by statistical analysis (inset, PERMANOVA). Statistical analysis for planes A and C by GLM with covariates of sex, age, alcohol use. Statistical analysis for panel B same with negative binomial GLM. $N_{\text{ESCC}}=87$, $N_{\text{EAC}}=16$ for all panels.

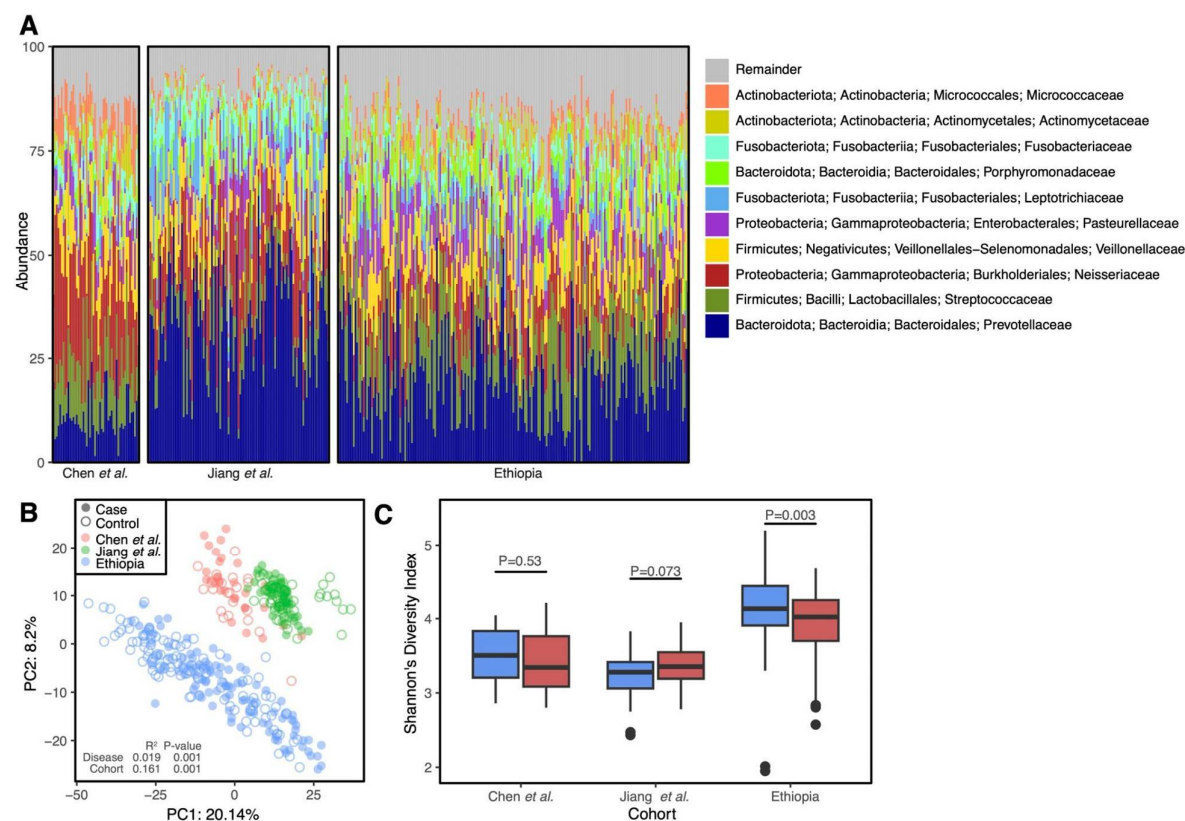
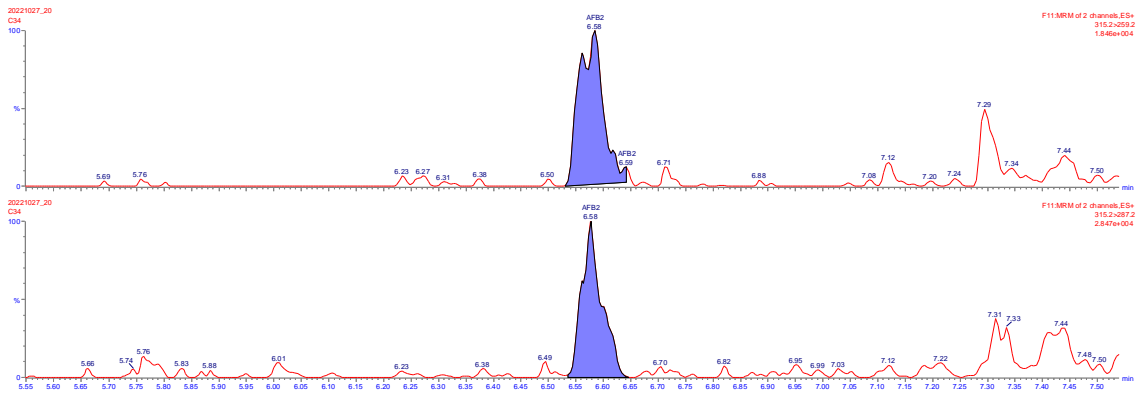


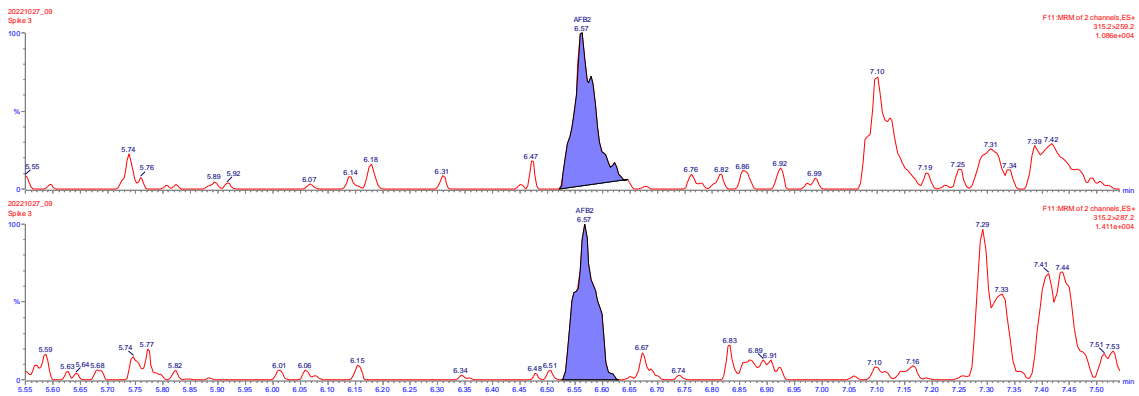
Figure S4. Esophageal cancer cohorts are unique in composition and diversity. (A) Taxonomic barplots summarized at the family level demonstrate similar presence of the major families in the oral microbiota; however, distinct differences in major families including a lower abundance of Prevotellaceae and an increase in Neisseriaceae in the Chen *et al.* cohort. (B) PCoA of CLR Euclidean distances demonstrates that cohorts are compositionally distinct explaining the major source of variation, while there is still a significant effect of cancer status (PERMANOVA table inset). (C) Chinese cohorts do not display differential alpha diversity between case and controls (Welch's t-test). In all panels: $N_{\text{Ethiopia}}=211$ ($N_{\text{cases}}=103$, $N_{\text{controls}}=108$), $N_{\text{Chen}}=52$ ($N_{\text{cases}}=31$, $N_{\text{controls}}=21$), $N_{\text{Jiang}}=109$ ($N_{\text{cases}}=56$, $N_{\text{controls}}=53$).

AFB2

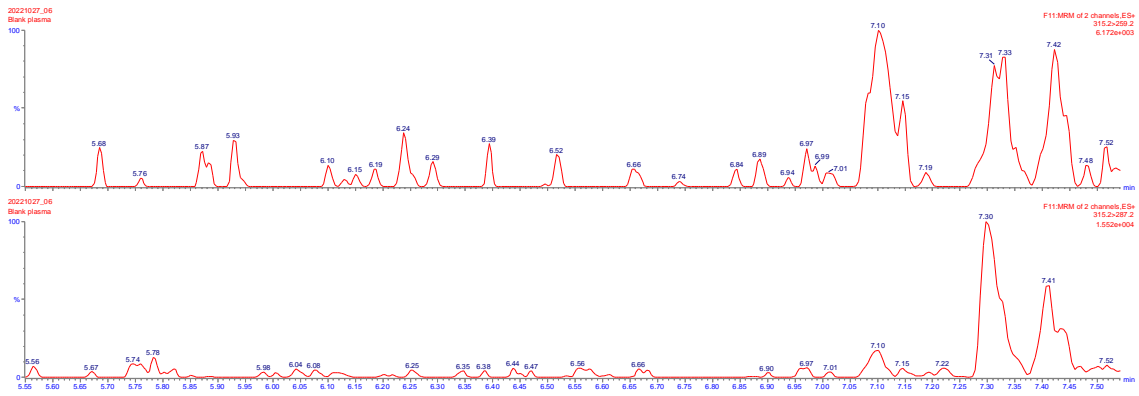
Positive plasma sample, quantified concentration 0.14 µg/L



Blank plasma spiked at 0.10 µg/L

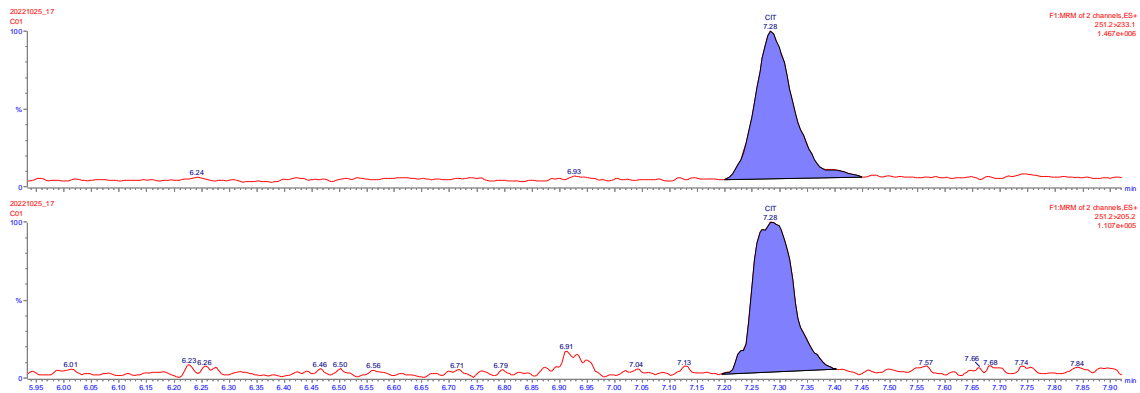


Blank plasma (non-spiked)

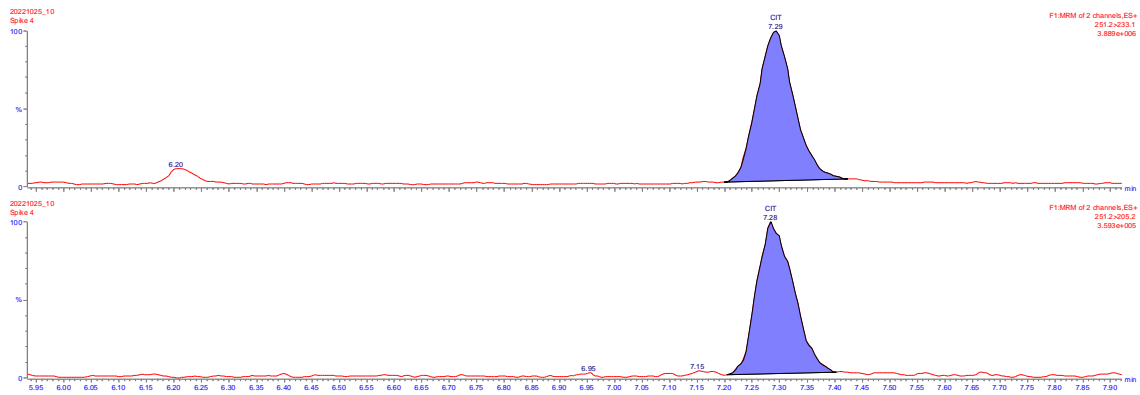


CIT

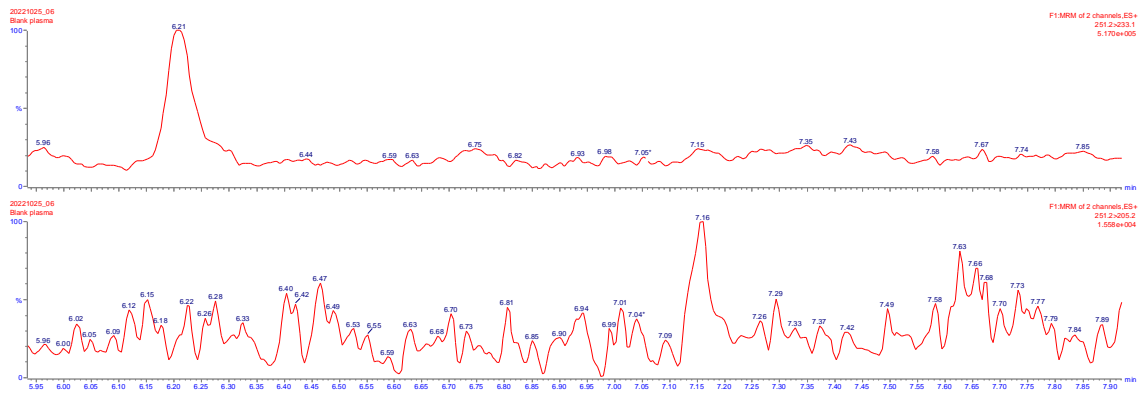
Positive plasma sample, quantified concentration 1.5 µg/L



Blank plasma spiked at 2.5 µg/L

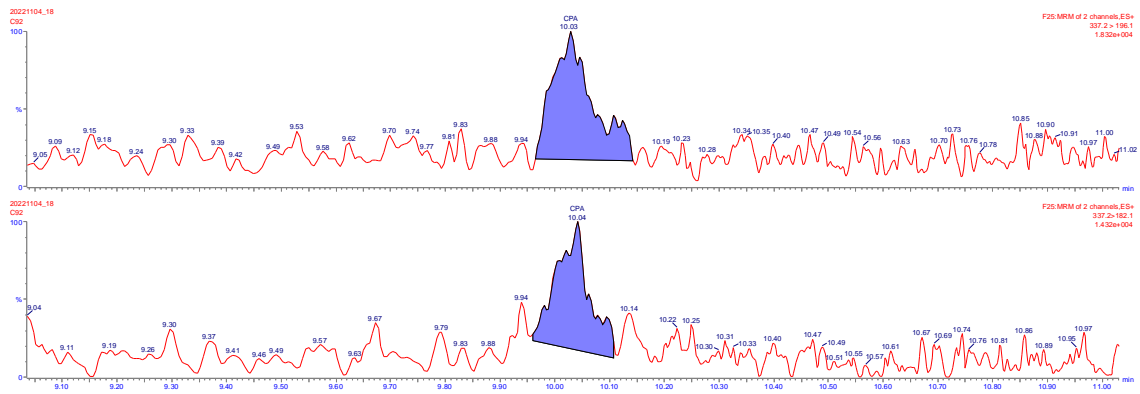


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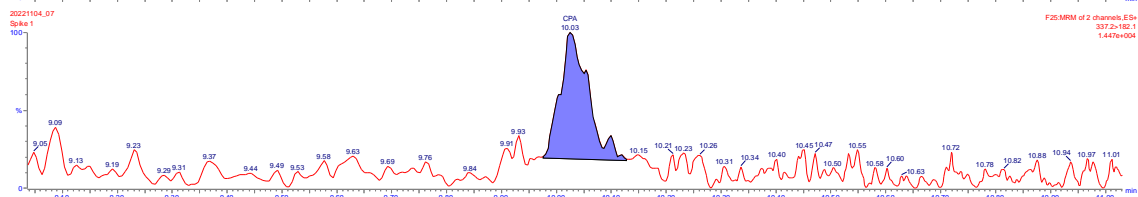
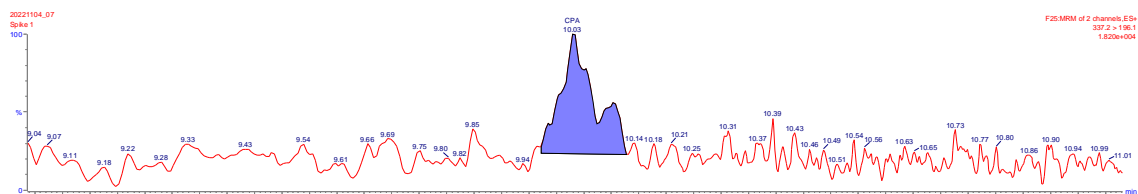


CPA

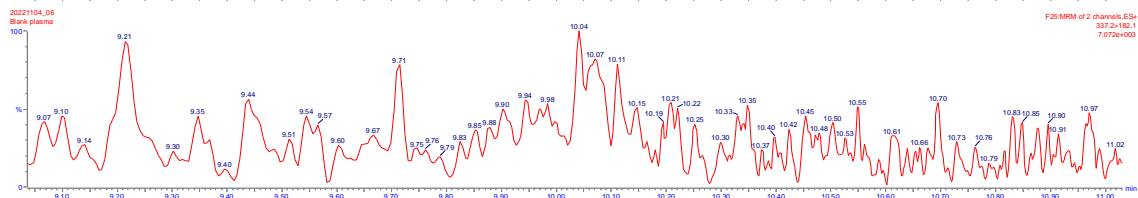
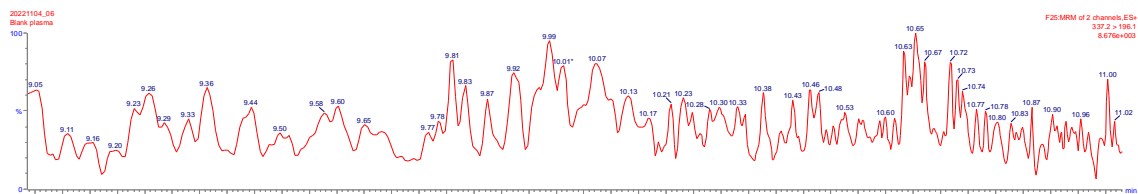
Positive plasma sample, CPA was detected but not quantified (<LLOQ; LLOQ = 0.66 µg/L)



Blank plasma spiked at 0.3 µg/L

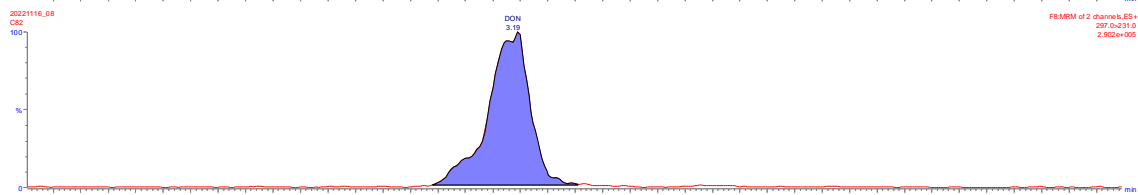
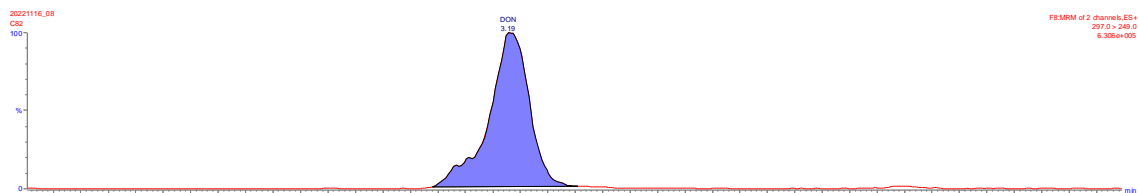


Blank plasma (non-spiked)

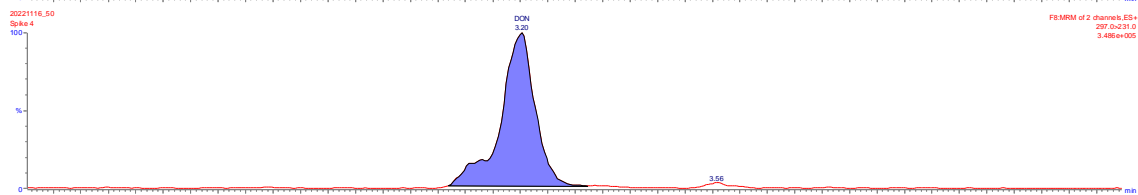
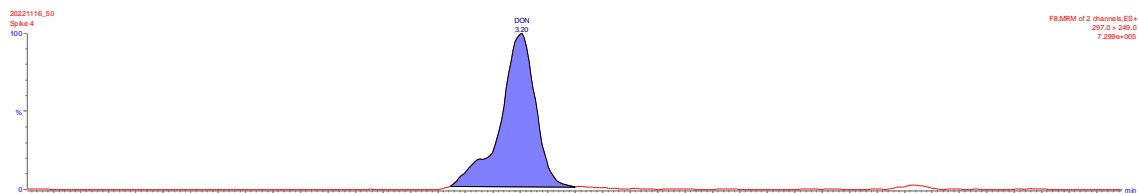


DON

Positive plasma sample, quantified concentration 8.0 µg/L



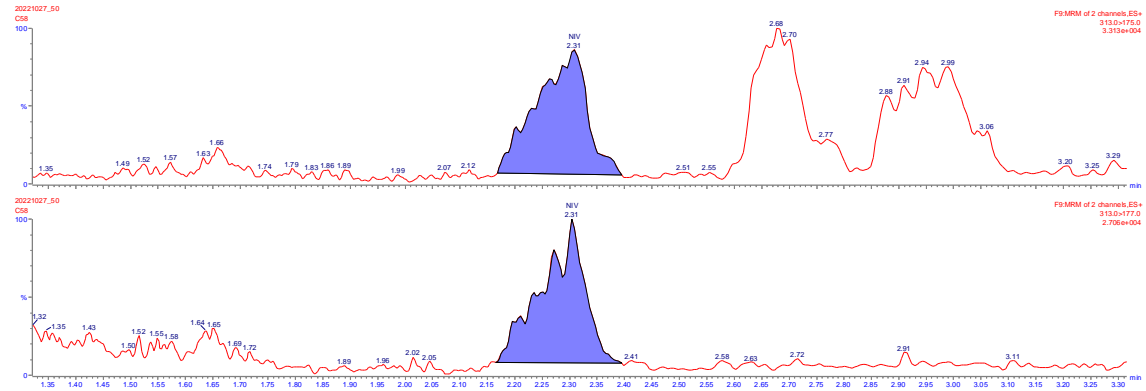
Blank plasma spiked at 10 µg/L



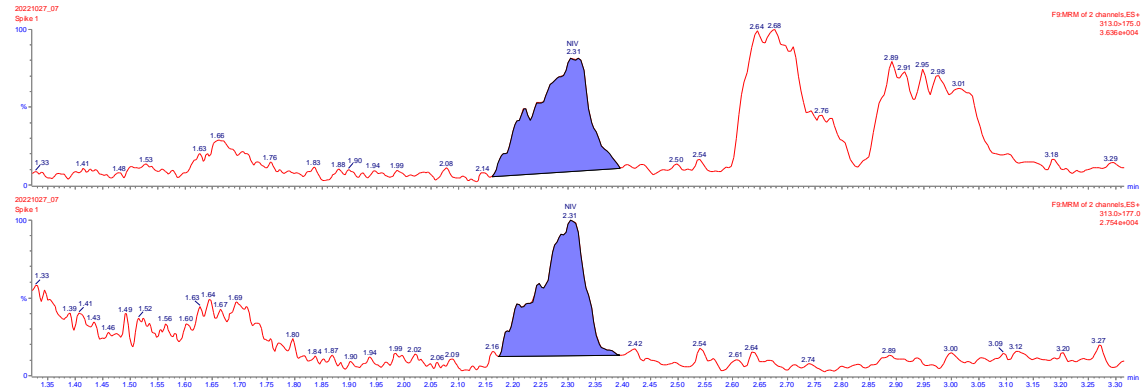
Blank plasma (non-spiked)

NIV

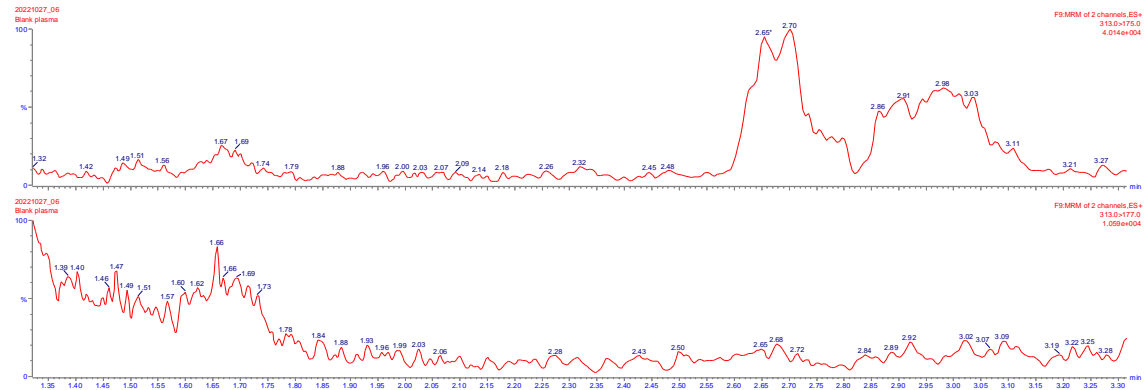
Positive plasma sample, quantified concentration 2.4 µg/L



Blank plasma spiked at 2.5 µg/L

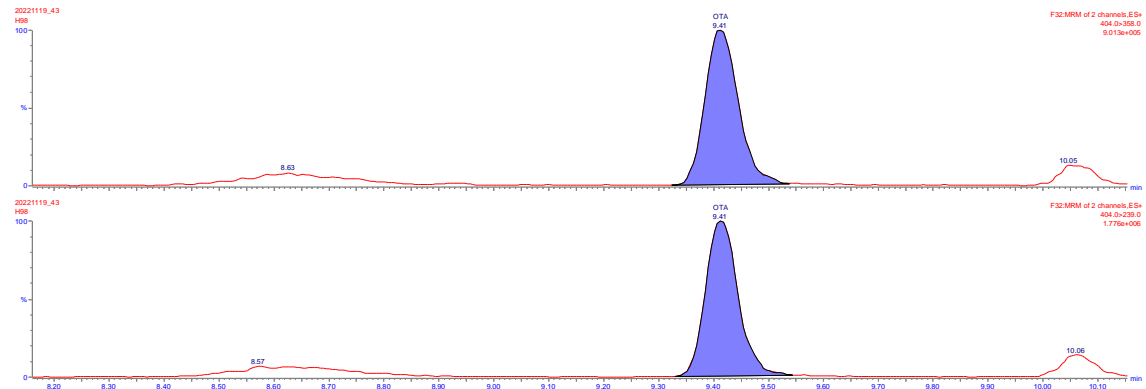


Blank plasma (non-spiked)

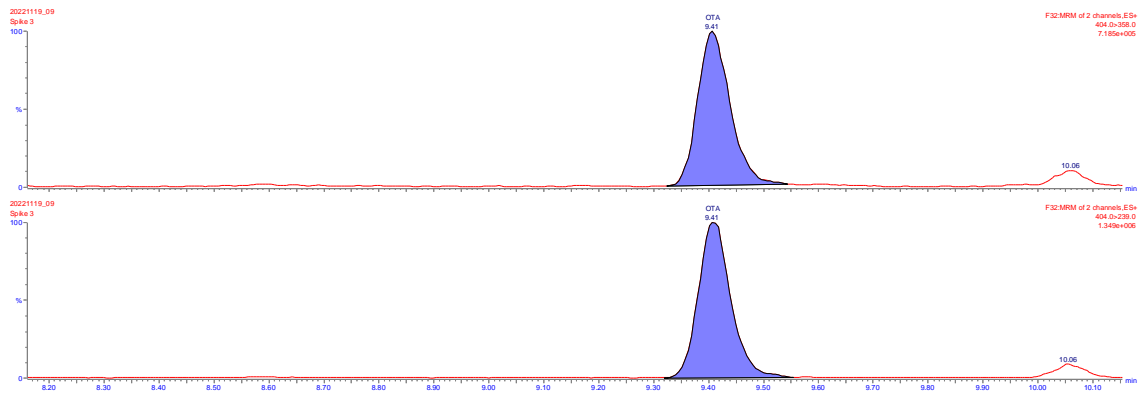


OTA

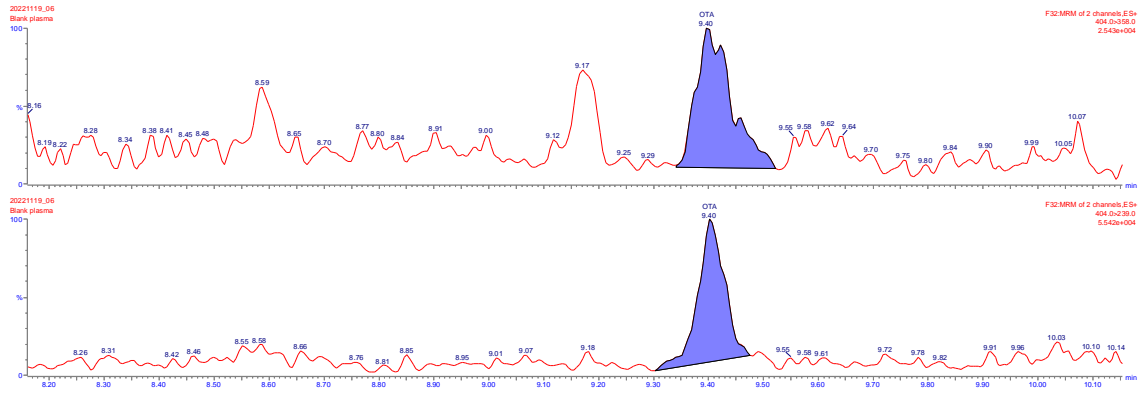
Positive plasma sample, quantified concentration 1.6 µg/L



Plasma spiked at 1.0 µg/L, total concentration 1.13 µg/L^a

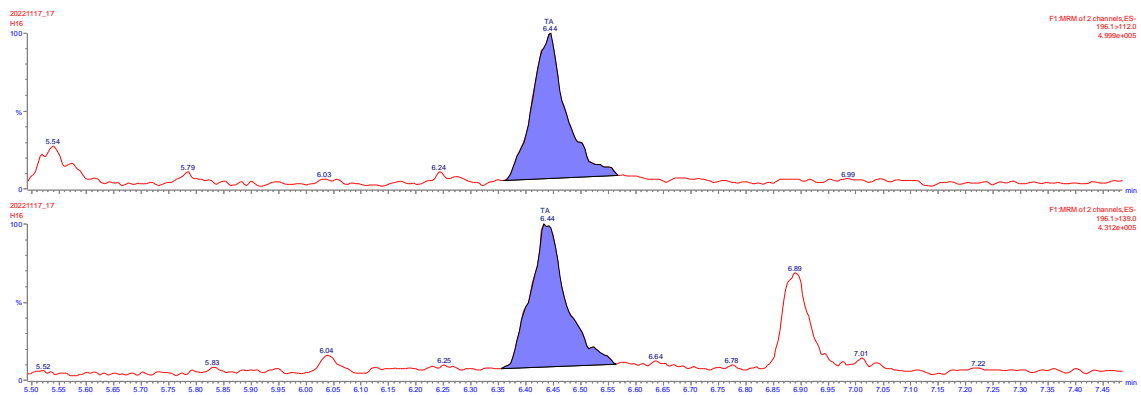


Plasma (non-spiked), calculated concentration 0.13 $\mu\text{g/L}^a$

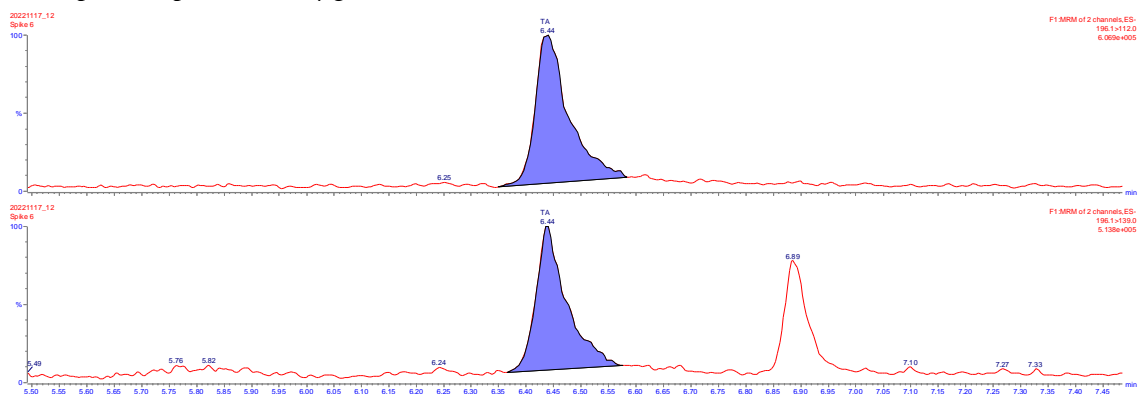


TA

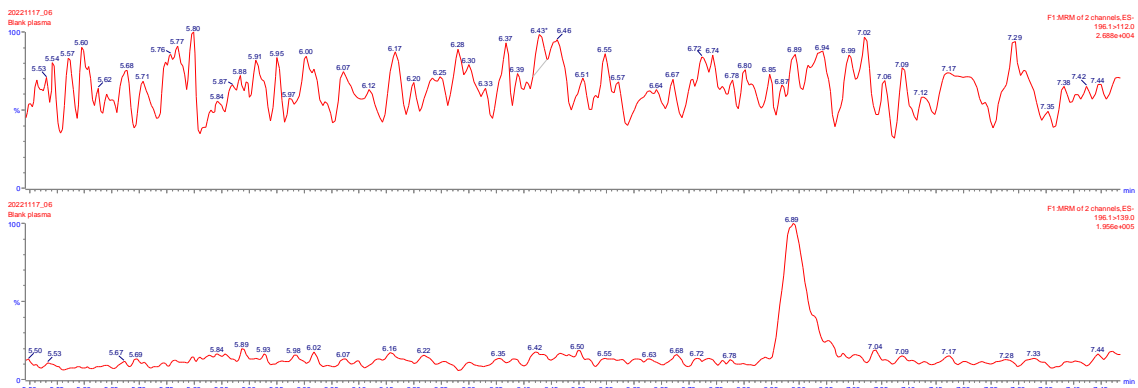
Positive plasma sample, quantified concentration 98 $\mu\text{g/L}$



Blank plasma spiked at 160 $\mu\text{g/L}$

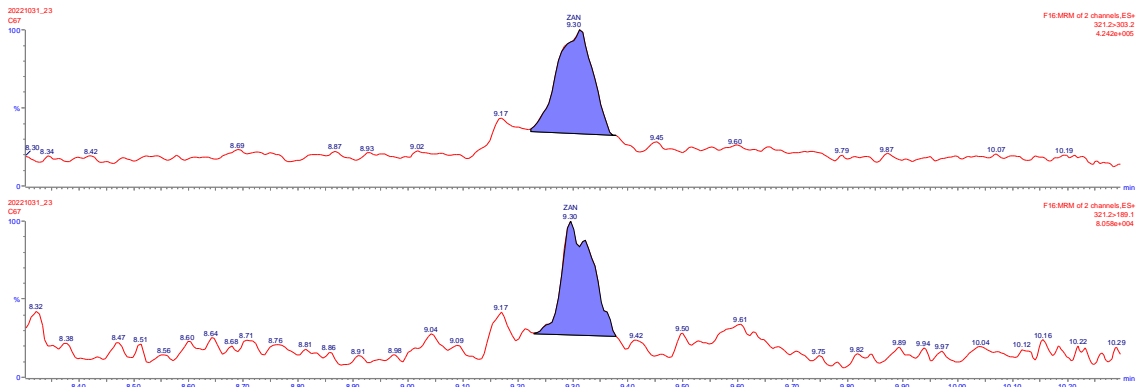


Blank plasma (non-spiked)

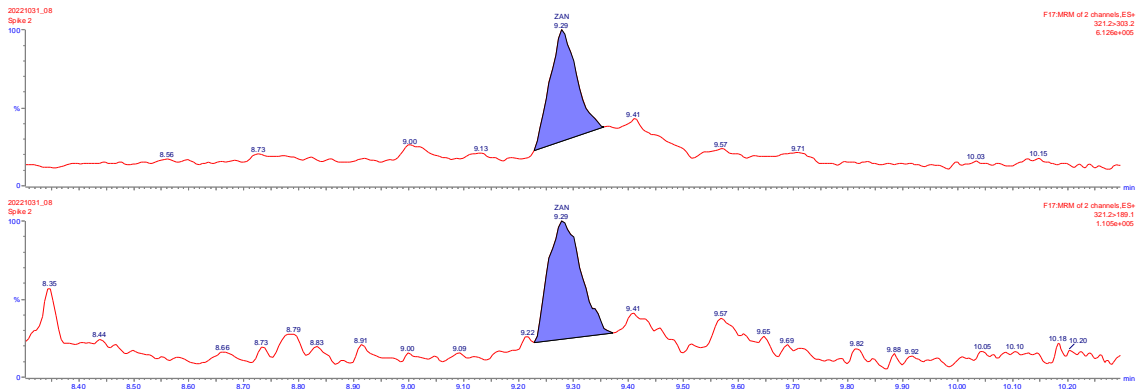


ZAN

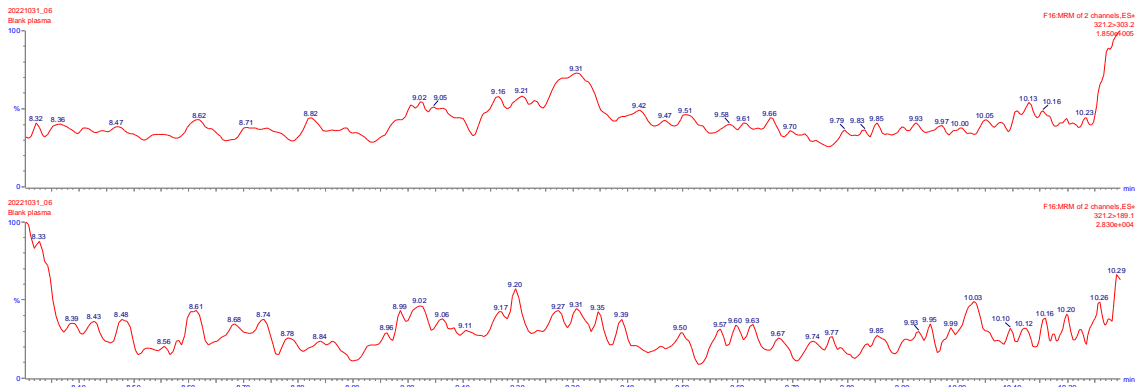
Positive plasma sample, detected but not quantified (<LLOQ; LLOQ = 0.72 µg/L)



Blank plasma spiked at 0.6 µg/L

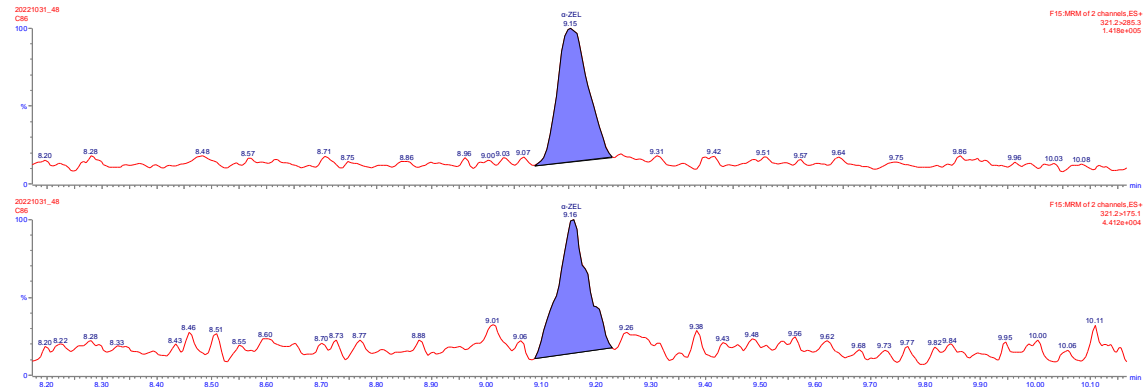


Blank plasma (non-spiked)

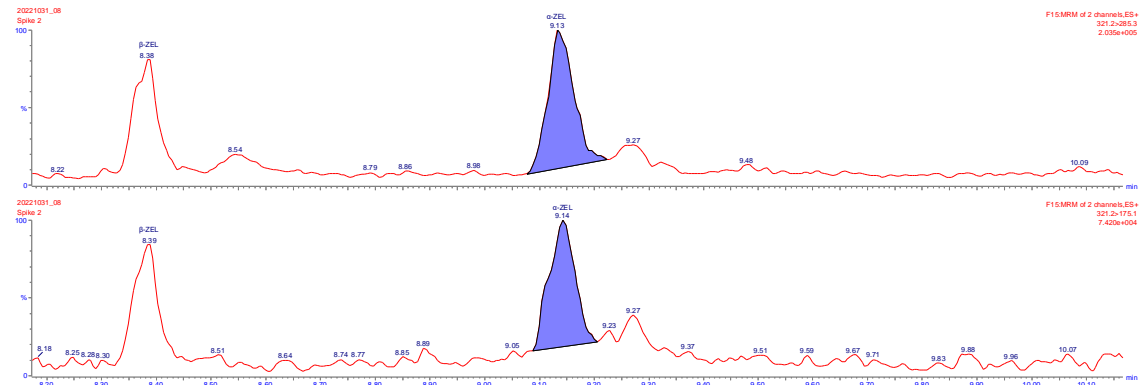


α -ZEL

Positive plasma sample, detected but not quantified (<LLOQ; LLOQ = 0.50 $\mu\text{g/L}$)



Blank plasma spiked at 0.6 $\mu\text{g/L}$



Blank plasma (non-spiked)

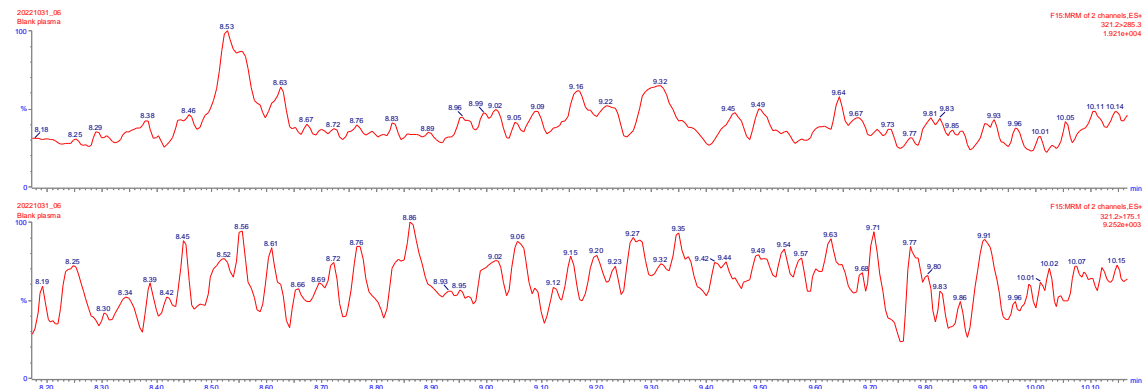
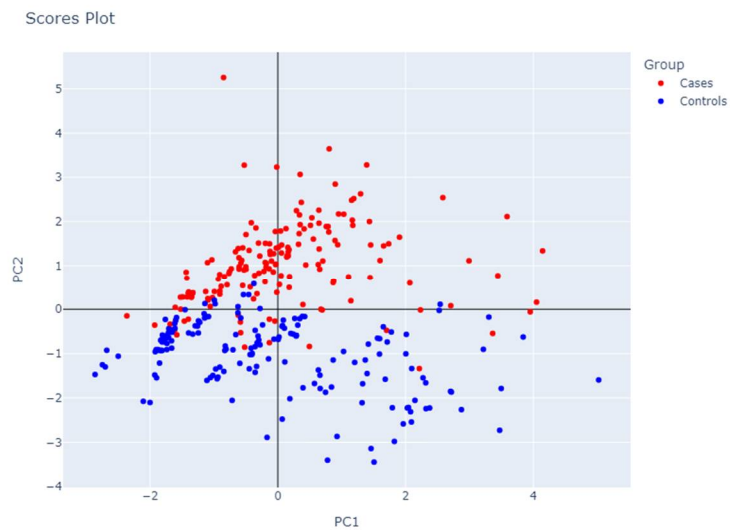


Figure S-5. Representative chromatograms of all mycotoxins detected in plasma and comparison with a blank plasma^a sample spiked with the mycotoxin standard at a similar concentration and a non-spiked blank plasma^a sample.

^a Plasma used to prepare matrix-matched calibration curves and quality controls was positive for OTA, with a calculated concentration of 0.13 $\mu\text{g/L}$.

AFB2- aflatoxin B2, CIT- citrinin, CPA- cyclopiiazonic acid, DON- deoxynivalenol, ENNB- ennatin B, NIV- nivalenol, OTA- ochratoxin A, TA- tenuazonic acid, ZAN- zearalanone, α -ZEL- α -zearalenol.

A)



B)

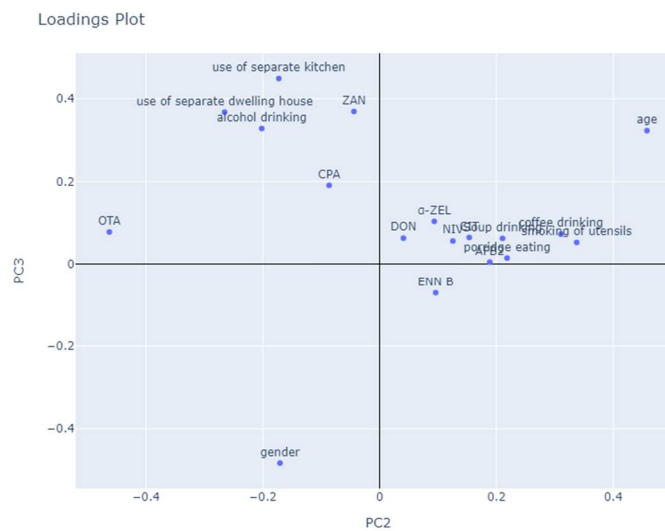
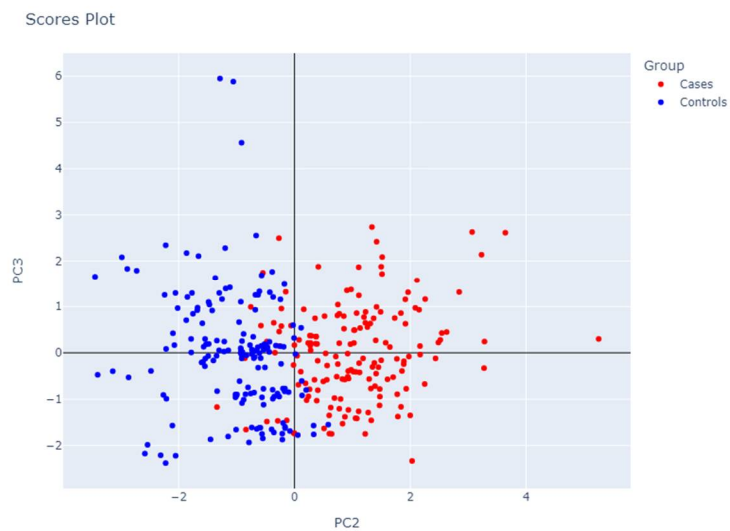
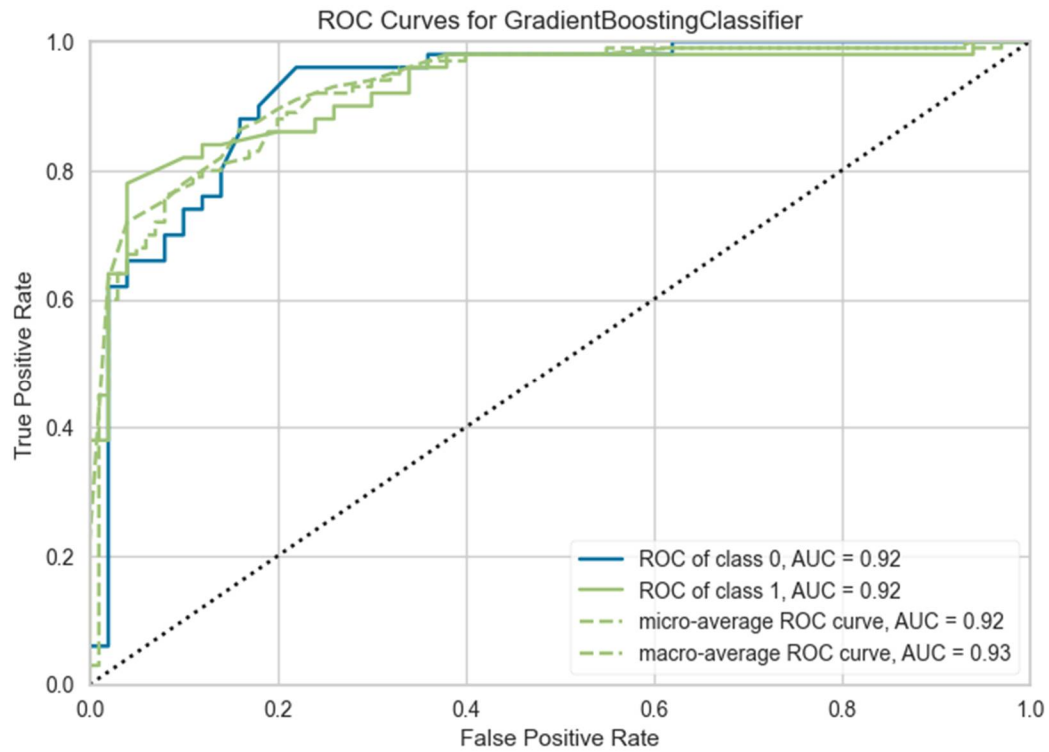


Figure S-6. Principal component analysis (PCA) for the esophageal cancer case-control study based on demographic and lifestyle variables, and mycotoxin concentrations: Scores and Loading plots for (A) PC1 vs PC2, and (B) PC2 vs PC3.

A)



B)

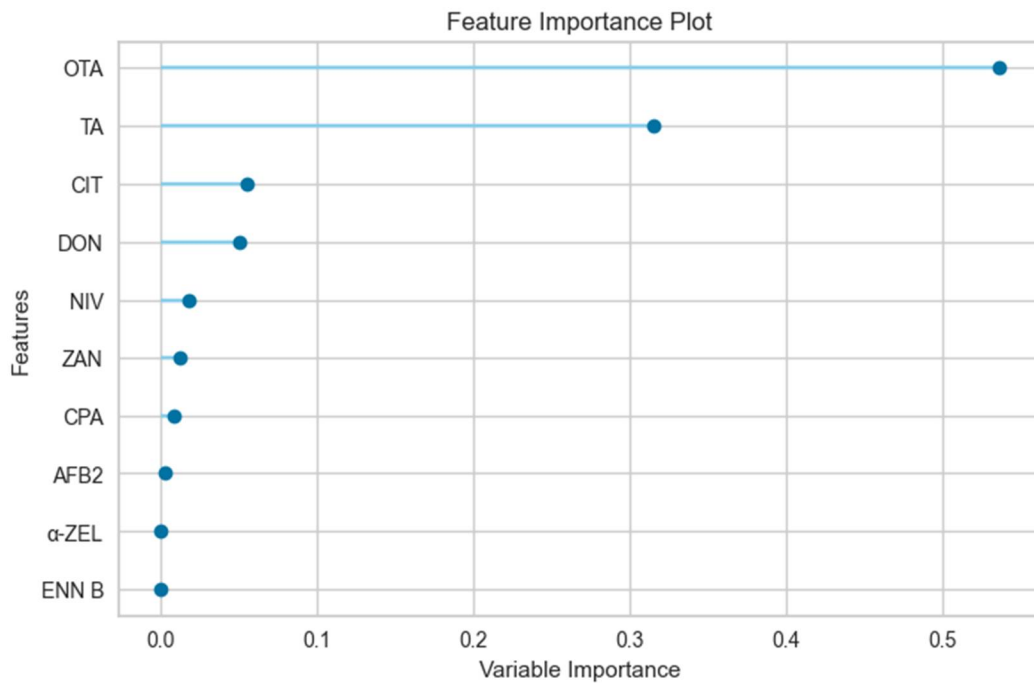


Figure S-7. Gradient Boosting Classifier model to classify esophageal cancer patients from controls based on mycotoxin concentrations: (A) Receiver Operating Characteristic curve, and (B) feature importance plot.

Supplementary tables

Table S-1. Differentially abundant genera between cases and controls.

Taxon	Estimate (log2)	P value	FDR
<i>Actinobacteriota; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium; dentium</i>	3.08	5.33E-04	9.76E-03
<i>Actinobacteriota; Actinobacteria; Actinomycetales; Actinomycetaceae; Actinomyces; israelii</i>	3.06	5.84E-04	9.39E-03
<i>Actinobacteriota; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Alloscardovia; omnicoles</i>	2.94	4.35E-04	8.00E-03
<i>Actinobacteriota; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium; breve</i>	2.82	1.91E-03	2.35E-02
<i>Proteobacteria; Gammaproteobacteria; Enterobacterales; Pasteurellaceae; Actinobacillus; NA</i>	2.81	4.86E-03	4.90E-02
<i>Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus; anginosus</i>	2.73	7.06E-04	1.15E-02
<i>Actinobacteriota; Actinobacteria; Corynebacteriales; Corynebacteriaceae; Corynebacterium; NA</i>	2.67	1.01E-02	8.35E-02
<i>Bacteroidota; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella_7; multisaccharivorax</i>	2.67	1.60E-03	1.93E-02
<i>Firmicutes; Negativicutes; Veillonellales-Selenomonadales; Veillonellaceae; Anaeroglobus; NA</i>	2.5	2.15E-03	2.78E-02
<i>Bacteroidota; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; oulorum</i>	2.32	7.50E-03	6.72E-02
<i>Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Ligilactobacillus; NA</i>	2.28	1.51E-02	9.40E-02
<i>Spirochaetota; Spirochaetia; Spirochaetales; Spirochaetaceae; Treponema; vincentii</i>	2.26	8.83E-03	6.75E-02
<i>Proteobacteria; Gammaproteobacteria; Burkholderiales; Neisseriaceae; Eikenella; corrodens</i>	2.14	4.27E-03	4.33E-02
<i>Actinobacteriota; Actinobacteria; Propionibacteriales; Propionibacteriaceae; Pseudopropionibacterium; propionicum</i>	2.13	7.16E-03	6.52E-02
<i>Firmicutes; Negativicutes; Veillonellales-Selenomonadales; Veillonellaceae; Anaeroglobus; geminatus</i>	2.07	1.49E-02	9.68E-02
<i>Firmicutes; Clostridia; Lachnospirales; Lachnospiraceae; Howardella; NA</i>	2.04	9.10E-03	6.34E-02
<i>Actinobacteriota; Coriobacteriia; Coriobacteriales; Eggerthellaceae; Slackia; exigua</i>	1.87	8.93E-03	6.36E-02
<i>Firmicutes; Negativicutes; Veillonellales-Selenomonadales; Selenomonadaceae; Centipeda; NA</i>	1.56	7.15E-03	5.92E-02
<i>Firmicutes; Negativicutes; Veillonellales-Selenomonadales; Veillonellaceae; Veillonella; NA</i>	1.55	1.51E-05	1.13E-03
<i>Actinobacteriota; Coriobacteriia; Coriobacteriales; Atopobiaceae; Atopobium; NA</i>	1.4	3.65E-03	3.79E-02
<i>Firmicutes; Negativicutes; Veillonellales-Selenomonadales;</i>	1.38	2.21E-06	4.59E-04

<i>Veillonellaceae; Veillonella; parvula</i>			
<i>Bacteroidota; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; NA</i>	1.26	3.83E-04	9.18E-03
<i>Bacteroidota; Bacteroidia; Bacteroidales; Prevotellaceae; Alloprevotella; NA</i>	1.17	2.04E-03	3.09E-02
<i>Actinobacteriota; Actinobacteria; Actinomycetales; Actinomycetaceae; Actinomyces; NA</i>	0.78	2.88E-03	3.89E-02
<i>Bacteroidota; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; shahii</i>	-1.41	7.63E-03	7.13E-02
<i>Actinobacteriota; Actinobacteria; Corynebacteriales; Corynebacteriaceae; Corynebacterium; durum</i>	-1.56	6.81E-03	6.64E-02
<i>Actinobacteriota; Actinobacteria; Micrococcales; Micrococcaceae; Rothia; dentocariosa</i>	-1.72	5.18E-03	5.57E-02
<i>Spirochaetota; Spirochaetia; Spirochaetales; Spirochaetaceae; Treponema; NA</i>	-1.82	1.73E-03	2.64E-02
<i>Campylobacterota; Campylobacteria; Campylobacterales; Helicobacteraceae; Wolinella; NA</i>	-1.97	1.42E-02	8.05E-02
<i>Actinobacteriota; Actinobacteria; Actinomycetales; Actinomycetaceae; F0332; NA</i>	-2.07	1.26E-03	1.90E-02
<i>Firmicutes; Clostridia; Lachnospirales; Defluviitaleaceae; Defluviitaleaceae UCG-011; NA</i>	-2.08	6.28E-03	4.42E-02
<i>Firmicutes; Clostridia; Lachnospirales; Lachnospiraceae; Catonella; morbi</i>	-2.11	1.23E-02	7.99E-02
<i>Firmicutes; Clostridia; Clostridia vadinBB60 group; NA; NA; NA</i>	-2.13	6.79E-03	6.06E-02
<i>Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus; intermedius</i>	-2.19	1.37E-02	8.62E-02
<i>Firmicutes; Negativicutes; Veillonellales-Selenomonadales; Selenomonadaceae; NA; NA</i>	-2.21	9.96E-03	7.80E-02
<i>Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus; sanguinis</i>	-2.23	2.13E-03	2.83E-02
<i>Actinobacteriota; Actinobacteria; Actinomycetales; Actinomycetaceae; NA; NA</i>	-2.28	3.60E-03	3.18E-02
<i>Bacteroidota; Bacteroidia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga; ochracea</i>	-2.29	4.34E-03	4.13E-02
<i>Bacteroidota; Bacteroidia; Bacteroidales; Porphyromonadaceae; Porphyromonas; gingivalis</i>	-2.37	3.40E-03	3.89E-02
<i>Bacteroidota; Bacteroidia; Sphingobacteriales; Lentimicrobiaceae; Lentimicrobium; NA</i>	-2.41	1.79E-03	2.36E-02
<i>Firmicutes; Clostridia; Peptostreptococcales-Tissierellales; Anaerovoracaceae; [Eubacterium] saphenum group; NA</i>	-2.46	3.22E-03	3.49E-02
<i>Fusobacteriota; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Oceanivirga; NA</i>	-2.54	1.15E-02	7.55E-02
<i>Proteobacteria; Gammaproteobacteria; Burkholderiales; Neisseriaceae; Neisseria; oralis</i>	-2.59	2.79E-03	3.27E-02
<i>Firmicutes; Clostridia; Peptostreptococcales-Tissierellales;</i>	-2.72	1.28E-03	1.45E-02

<i>Anaerovoracaceae; Amnipila; NA</i>			
<i>Proteobacteria; Gammaproteobacteria; Burkholderiales; Burkholderiaceae; Lautropia; NA</i>	-2.79	1.76E-06	1.95E-04
<i>Actinobacteriota; Actinobacteria; Micrococcales; Micrococcaceae; Rothia; aeria</i>	-2.89	1.34E-04	3.90E-03
<i>Fusobacteriota; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia; hofstadii</i>	-3.03	1.58E-04	4.28E-03
<i>Firmicutes; Clostridia; Lachnospirales; Lachnospiraceae; NA; NA</i>	-3.1	2.81E-05	1.37E-03
<i>Firmicutes; Clostridia; Peptostreptococcales-Tissierellales; Peptostreptococcaceae; Filifactor; alocis</i>	-3.69	4.86E-06	3.82E-04

Table S-2. Differentially abundant functional pathways (case vs control; inferred)

Pathway ID	Pathway Annotation	Estimate (log2)	SE	P-value	FDR
GOLPDLAT-PWY	superpathway of glycerol degradation to 1,3-propanediol	0.806	0.326	1.411E-02	0.089
PWY-1541	superpathway of taurine degradation	0.415	0.175	1.879E-02	0.100
PWY-6608	guanosine nucleotides degradation III	0.234	0.088	8.595E-03	0.072
PWY-5861	superpathway of demethylmenaquinol-8 biosynthesis	0.131	0.040	1.328E-03	0.024
PWY-6703	preQ0 biosynthesis	0.128	0.035	3.487E-04	0.011
PWY-5838	superpathway of menaquinol-8 biosynthesis I	0.117	0.033	4.533E-04	0.013
PWY-5897	superpathway of menaquinol-11 biosynthesis	0.107	0.033	1.515E-03	0.024
PWY-5898	superpathway of menaquinol-12 biosynthesis	0.107	0.033	1.515E-03	0.024
PWY-5899	superpathway of menaquinol-13 biosynthesis	0.107	0.033	1.515E-03	0.024
PWY-5840	superpathway of menaquinol-7 biosynthesis	0.095	0.031	2.811E-03	0.035
ASPASN-PWY	superpathway of L-aspartate and L-asparagine biosynthesis	0.078	0.032	1.662E-02	0.095
PWY-6700	queuosine biosynthesis	0.076	0.029	9.949E-03	0.078
PWY-7199	pyrimidine deoxyribonucleosides salvage	0.071	0.030	1.776E-02	0.098
PWY-6147	6-hydroxymethyl-dihydropterin diphosphate biosynthesis I	0.059	0.012	3.919E-06	0.001
PWY-7196	superpathway of pyrimidine ribonucleosides	0.039	0.014	4.323E-03	0.046

	salvage				
POLYISOP RENSYN- PWY	polyisoprenoid biosynthesis (E. coli)	0.037	0.012	2.553E-03	0.034
PWY-6125	superpathway of guanosine nucleotides de novo biosynthesis II	0.028	0.012	1.719E-02	0.096
PWY-7200	superpathway of pyrimidine deoxyribonucleoside salvage	0.027	0.010	1.206E-02	0.083
DENOVOP URINE2- PWY	superpathway of purine nucleotides de novo biosynthesis II	0.025	0.008	2.924E-03	0.036
COA-PWY	coenzyme A biosynthesis I	0.024	0.010	1.862E-02	0.100
PWY-841	superpathway of purine nucleotides de novo biosynthesis I	0.023	0.009	1.002E-02	0.078
DAPLYSIN ESYN- PWY	L-lysine biosynthesis I	-0.044	0.017	1.256E-02	0.084
GLYCOLY SIS-E-D	superpathway of glycolysis and Entner-Doudoroff	-0.064	0.021	2.732E-03	0.035
PWY4FS-8	phosphatidylglycerol biosynthesis II (non-plastidic)	-0.068	0.017	1.378E-04	0.005
PWY4FS-7	phosphatidylglycerol biosynthesis I (plastidic)	-0.068	0.017	1.378E-04	0.005
PWY-7400	L-arginine biosynthesis IV (archaebacteria)	-0.109	0.043	1.189E-02	0.083
ARGSYN- PWY	L-arginine biosynthesis I (via L-ornithine)	-0.118	0.043	7.093E-03	0.064
PWY-6969	TCA cycle V (2-oxoglutarate:ferredoxin oxidoreductase)	-0.126	0.052	1.673E-02	0.095
TCA	TCA cycle I (prokaryotic)	-0.135	0.053	1.192E-02	0.083
PWY0-1061	superpathway of L-alanine biosynthesis	-0.146	0.061	1.809E-02	0.099
P161-PWY	acetylene degradation	-0.162	0.067	1.602E-02	0.095

P23-PWY	reductive TCA cycle I	-0.171	0.071	1.658E-02	0.095
PWY-5910	superpathway of geranylgeranyldiphosphate biosynthesis I (via mevalonate)	-0.185	0.076	1.518E-02	0.093
PWY-922	mevalonate pathway I	-0.202	0.082	1.486E-02	0.092
GLUTORN-PWY	L-ornithine biosynthesis	-0.208	0.067	2.317E-03	0.033
PWY0-1261	anhydromuropeptides recycling	-0.217	0.083	9.644E-03	0.078
ARGSYNB SUB-PWY	L-arginine biosynthesis II (acetyl cycle)	-0.220	0.060	3.039E-04	0.011
PWY-2941	L-lysine biosynthesis II	-0.239	0.082	3.948E-03	0.044
REDCITCY C	TCA cycle VIII (helicobacter)	-0.261	0.103	1.214E-02	0.083
PWY0-1479	tRNA processing	-0.266	0.100	8.150E-03	0.070
P125-PWY	superpathway of (R,R)-butanediol biosynthesis	-0.320	0.111	4.451E-03	0.046
PWY-6353	purine nucleotides degradation II (aerobic)	-0.355	0.137	1.021E-02	0.078
PWY-3781	aerobic respiration I (cytochrome c)	-0.419	0.140	3.100E-03	0.037
PPGPPMET-PWY	ppGpp biosynthesis	-0.460	0.182	1.220E-02	0.083
SALVADE HYPOX-PWY	adenosine nucleotides degradation II	-0.476	0.154	2.290E-03	0.033
DENITRIFI CATION-PWY	nitrate reduction I (denitrification)	-0.478	0.161	3.388E-03	0.039
P105-PWY	TCA cycle IV (2-oxoglutarate decarboxylase)	-0.516	0.187	6.284E-03	0.059
PWY-5177	glutaryl-CoA degradation	-0.522	0.209	1.331E-02	0.085
PWY-7528	L-methionine salvage cycle I (bacteria and plants)	-0.531	0.198	8.051E-03	0.070
PWY-6590	superpathway of Clostridium acetobutylicum acidogenic fermentation	-0.564	0.174	1.398E-03	0.024

UBISYN-PWY	superpathway of ubiquinol-8 biosynthesis (prokaryotic)	-0.566	0.180	1.898E-03	0.029
PWY-7090	UDP-2,3-diacetamido-2,3-dideoxy-α-D-mannuronate biosynthesis	-0.567	0.232	1.547E-02	0.093
ALL-CHORISMATE-PWY	superpathway of chorismate metabolism	-0.569	0.166	7.183E-04	0.019
CENTFERM-PWY	pyruvate fermentation to butanoate	-0.571	0.177	1.491E-03	0.024
PWY-5855	ubiquinol-7 biosynthesis (prokaryotic)	-0.577	0.177	1.318E-03	0.024
PWY-5856	ubiquinol-9 biosynthesis (prokaryotic)	-0.577	0.177	1.318E-03	0.024
PWY-5857	ubiquinol-10 biosynthesis (prokaryotic)	-0.577	0.177	1.318E-03	0.024
PWY-6708	ubiquinol-8 biosynthesis (prokaryotic)	-0.577	0.177	1.318E-03	0.024
PWY-4361	S-methyl-5-thio-α-D-ribose 1-phosphate degradation	-0.585	0.233	1.288E-02	0.084
PWY-7527	L-methionine salvage cycle III	-0.585	0.233	1.268E-02	0.084
PWY-7094	fatty acid salvage	-0.605	0.214	5.033E-03	0.049
ENTBACSYN-PWY	enterobactin biosynthesis	-0.610	0.199	2.443E-03	0.034
PWY-5088	L-glutamate degradation VIII (to propanoate)	-0.611	0.241	1.190E-02	0.083
PWY-7031	protein N-glycosylation (bacterial)	-0.623	0.217	4.566E-03	0.046
PWY-6143	CMP-pseudamate biosynthesis	-0.648	0.224	4.280E-03	0.046
PWY-4984	urea cycle	-0.677	0.191	5.027E-04	0.014
LEU-DEG2-PWY	L-leucine degradation I	-0.687	0.242	4.954E-03	0.049
PWY-6731	starch degradation III	-0.700	0.254	6.416E-03	0.059
PWY-5920	superpathway of heme biosynthesis from glycine	-0.818	0.194	3.824E-05	0.003
GLYCOLY	superpathway of glycolysis, pyruvate	-0.885	0.201	1.744E-05	0.002

SIS-TCA-GLYOX-BYPASS	dehydrogenase, TCA, and glyoxylate bypass				
TCA-GLYOX-BYPASS	superpathway of glyoxylate bypass and TCA	-0.920	0.216	3.026E-05	0.003
GLYOXYL ATE-BYPASS	glyoxylate cycle	-0.955	0.239	8.753E-05	0.005
PWY0-42	2-methylcitrate cycle I	-0.976	0.369	8.735E-03	0.072
GLYCOL-GLYOXDE G-PWY	superpathway of glycol metabolism and degradation	-0.979	0.209	5.081E-06	0.001
PWY-5747	2-methylcitrate cycle II	-0.989	0.239	4.993E-05	0.003
METH-ACETATE-PWY	methanogenesis from acetate	-1.086	0.430	1.219E-02	0.083
PROTOCA TECHUAT E-ORTHO-CLEAVAG E-PWY	protocatechuate degradation II (ortho-cleavage pathway)	-1.275	0.279	8.652E-06	0.001
PWY-6728	methylaspartate cycle	-1.338	0.344	1.336E-04	0.005

Table S-3. Optimized UHPLC-MS/MS parameters for the analysis of mycotoxins (n = 39) and isotopically labeled internal standards (n = 7).

Mycotoxins		Retention time (min)	ESI mode	Adduct	Precursor ion (m/z)	Cone voltage (V)	Fragment ion (m/z)	Collision energy (eV)
Aflatoxins	¹³ C-AFB1	6.8	+	[M+H] ⁺	330.2	55	255.1 / 301.2*	40 / 23
	AFB1	6.8	+	[M+H] ⁺	313.1	55	241.1* / 285.1	35 / 20
	AFB2	6.6	+	[M+H] ⁺	315.2	40	259.2* / 287.2	28 / 26

	AFG1	6.2	+	[M+H] ⁺	329.1	40	215.1 / 243.1*	31 / 25
	AFG2	5.9	+	[M+H] ⁺	331.1	55	245.1* / 285.1	30 / 27
	AFM1	5.9	+	[M+H] ⁺	329.1	40	229.1* / 273.1	40 / 22
Alternaria toxins	AME	10.1	+	[M+H] ⁺	273.2	30	199.2 / 258.1*	30 / 26
	AOH	8.0	+	[M+H] ⁺	259.0	40	185.0* / 213.0	30 / 26
	¹³ C-TA	6.4	-	[M-H] ⁻	198.1	30	114.0 / 141.0*	23 / 20
	TA	6.4	-	[M-H] ⁻	196.1	30	112.0 / 139.0*	23 / 20
Beauvericin	BEA	12.6	+	[M+NH ₄] ⁺	801.5	30	244.2* / 262.2	33 / 31
Citrinin	¹³ C-CIT	7.3	+	[M+H] ⁺	264.2	30	98.05 / 246.2*	43 / 18
	CIT	7.3	+	[M+H] ⁺	251.2	30	205.2 / 233.1*	27 / 15
Cyclopiazonic acid	CPA	10.0	+	[M+H] ⁺	337.2	30	182.1 / 196.1*	23 / 18
Enniatins	ENN A	12.9	+	[M+NH ₄] ⁺	699.5	25	210.0* / 228.2	35 / 35
	ENN A1	12.9	+	[M+NH ₄] ⁺	685.5	40	210.2 / 228.2*	30 / 30
	ENN B	12.6	+	[M+NH ₄] ⁺	657.5	40	196.0* / 214.0	30 / 30
	ENN B1	12.8	+	[M+NH ₄] ⁺	671.5	30	196.2* / 210.2	31 / 30
Fumonisin	¹³ C-FB1	8.2	+	[M+H] ⁺	756.5	50	356.4 / 374.4*	40 / 35
	FB1	8.2	+	[M+H] ⁺	722.5	40	334.4* / 352.3	40 / 35
	FB2	10.2	+	[M+H] ⁺	706.4	65	336.3* / 354.3	35 / 30
	FB3	9.2	+	[M+H] ⁺	706.5	40	336.4* / 354.4	35 / 32
	HFB1	7.9	+	[M+H] ⁺	406.4	40	236.3 / 334.3*	25 / 25
Ochratoxins	OTA	9.4	+	[M+H] ⁺	404.0	40	239.0 / 358.0*	22 / 15
	OT α	5.8	+	[M+H] ⁺	257.1	20	221.1 / 239.1*	20 / 10
Roquefortin-C	ROQ-C	8.1	+	[M+H] ⁺	390.3	40	193.2* / 322.2	26 / 20
Sterigmatocystin	STC	10.0	+	[M+H] ⁺	325.1	35	281.1 / 310.1*	35 / 25
Trichothecenes A	DAS	7.0	+	[M+NH ₄] ⁺	384.3	25	247.2 / 307.2*	13 / 10
	NEO	4.4	+	[M+NH ₄] ⁺	400.3	30	185.2* / 305.2	20 / 9
	¹³ C-HT2	7.9	+	[M+NH ₄] ⁺	464.3	20	229.2 / 278.2*	13 / 12
	HT2	7.9	+	[M+NH ₄] ⁺	442.3	22	215.1* / 263.2	12 / 12

	¹³ C-T2	8.8	+	[M+NH ₄] ⁺	508.4	30	229.2 / 322.3*	20 / 15
	T2	8.8	+	[M+NH ₄] ⁺	484.3	35	215.1 / 305.2*	20 / 12
Trichothecenes B	3-ADON	5.3	+	[M+H] ⁺	339.2	35	203.2 / 231.1*	15 / 10
	DOM	4.2	+	[M+H] ⁺	281.2	30	109.3* / 137.2	15 / 13
	¹³ C-DON	3.2	+	[M+H] ⁺	312.1	30	216.2* / 263.2	15 / 10
	DON	3.2	+	[M+H] ⁺	297.0	30	231.0 / 249.0*	11 / 11
	FUS-X	4.2	+	[M+H] ⁺	355.5	16	137.1 / 247.1*	23 / 12
	NIV	2.3	+	[M+H] ⁺	313.0	30	175.0* / 177.0	17 / 16
Zearalenone	α-ZAL	8.9	+	[M+H] ⁺	323.2	20	189.1 / 305.3*	22 / 8
	α-ZEL	9.2	+	[M+H] ⁺	321.2	20	175.1 / 285.3*	25 / 12
	β-ZAL	8.1	+	[M+H] ⁺	323.3	20	189.1 / 305.3*	23 / 10
	β-ZEL	8.4	+	[M+H] ⁺	321.2	20	175.1 / 285.3*	25 / 12
	ZAN	9.3	+	[M+H] ⁺	321.2	25	189.1 / 303.2*	20 / 13
	ZEN	9.5	+	[M+H] ⁺	319.2	20	283.2* / 301.2	12 / 8

*Quantifier transition

Table S-4. Performance characteristics of the method for the determination of mycotoxins in plasma by UHPLC-MS/MS: calibration range, linearity, limit of detection (LOD), lower limit of quantification (LLOQ), accuracy and precision (CV).

Mycotoxins		Calibration range (µg/L)	R ²	LOD (µg/L)	LLOQ (µg/L)	Accuracy (%) ^a	CV ^a
Aflatoxins	AFB1	0.03-2	0.99	0.021	0.039	93	0.15
	AFB2	0.03-2	0.99	0.018	0.024	103	0.14
	AFG1	0.03-2	0.99	0.028	0.042	96	0.18
	AFG2	0.03-2	0.99	0.025	0.040	103	0.17
	AFM1	0.03-2	0.99	0.029	0.057	93	0.19
Alternaria toxins	AME	1.2-20	0.99	0.54	0.96	105	0.16
	AOH	1.2-20	0.99	0.51	0.88	103	0.15
	TA	5-400	0.99	5.0	10	94	0.16

Beauvericin	BEA ^b	-	-	0.03	-	-	-
Citrinin	CIT	0.30-20	0.99	0.11	0.46	103	0.14
Cyclopiazonic acid	CPA	0.30-20	0.98	0.18	0.66	106	0.18
Enniatins	ENN A ^b	-	-	0.03	-	-	-
	ENN A1 ^b	-	-	0.03	-	-	-
	ENN B	0.03-2	0.98	0.011	0.16	102	0.16
	ENN B1 ^b	-	-	0.03	-	-	-
Fumonisin	FB1	1.2-20	0.99	0.81	1.3	101	0.14
	FB2	1.2-20	0.99	0.66	1.0	95	0.15
	FB3	1.2-20	0.99	0.78	1.2	94	0.17
	HFB1	0.30-20	0.99	0.24	0.44	104	0.14
Ochratoxins	OTA ^c	0.13-50.13	0.99	0.020	0.070	103	0.15
	OT α	0.30-20	0.99	0.15	0.56	104	0.13
Roquefortin-C	ROQ-C	0.30-20	0.99	0.094	0.28	102	0.16
Sterigmatocystin	STC	0.30-20	0.99	0.11	0.32	104	0.19
Trichothecenes A	DAS	0.30-20	0.99	0.13	0.51	97	0.16
	NEO	0.30-20	0.99	0.16	0.48	102	0.16
	HT2	0.30-20	0.99	0.12	0.29	103	0.16
	T2	0.30-20	0.99	0.10	0.27	103	0.14
Trichothecenes B	3-ADON	0.06-20	0.99	0.036	0.12	102	0.14
	DOM	0.06-20	0.98	0.029	0.091	94	0.13
	DON	0.06-400	0.99	0.027	0.082	97	0.13
	FUS-X	0.30-20	0.99	0.28	0.72	103	0.16
	NIV	2.5-400	0.99	0.31	1.36	103	0.14
Zearalenone	α -ZAL	0.30-20	0.99	0.10	0.52	99	0.19
	α -ZEL	0.30-20	0.99	0.11	0.50	103	0.15

	β-ZAL	0.30-20	0.99	0.10	0.51	103	0.16
	β-ZEL	0.30-20	0.99	0.14	0.64	103	0.15
	ZAN	0.30-20	0.99	0.27	0.72	103	0.15
	ZEN	0.30-20	0.99	0.22	0.53	104	0.18

^a At the spiked concentration immediately above the LLOQ; ^b semiquantitative determination; ^c plasma used to prepare matrix-matched calibration curves and quality controls was positive for OTA, with a calculated concentration of 0.13 µg/L.

Table S-5. Multivariate binary logistic regression analysis for the onset of esophageal cancer based on demographic and lifestyle variables and mycotoxin exposure quartiles for cases and location-matched controls in Ethiopia.

Variables		Cases (n=166)	Controls (n=166)	Multivariate binary logistic regression analysis ¹	
				AOR (95% CI)	p-value
Continuous		Mean ± standard deviation			
Age (years)*		52±14	39±7	1.19 (1.14-1.28)	<0.001
AFB2 quartile		1.05±0.40	1.00±0	-	0.999
CIT quartile		1.25±0.84	1.02±0.23	2.58 (0.96-6.92)	0.060
CPA quartile		1.14±0.64	1.11±0.56	2.01 (0.96-4.21)	0.064
DON quartile		1.52±1.14	1.60±1.20	1.08 (0.71-1.64)	0.718
ENNB quartile		1.04±0.33	1.00±0	-	0.999
NIV quartile		1.11±0.56	1.00±0	-	0.999
OTA quartile*		1.90±1.03	3.11±0.85	0.26 (0.16-0.42)	<0.001
TA quartile*		2.67 ±0.91	2.34±1.28	1.57 (1.02-2.41)	0.040
ZAN quartile		1.04±0.33	1.16±0.68	0.65 (0.22-1.93)	0.437
α-ZEL quartile		1.04±0.33	1.00±0	-	1.000
Categorical		Frequency (n (%))			
Gender	Female	96 (57.8)	126 (75.9)	1	-
	Male	70 (42.2)	40 (24.1)	3.02 (0.84-10.8)	0.090
Alcohol drinking*	Yes	8 (4.8)	26 (15.7)	0.027 (0.003-0.246)	0.001
	No	158 (95.2)	140 (84.3)	1	-
Soup drinking	Yes	142 (85.5)	130 (78.3)	1.43 (0.31-6.67)	0.647
	No	24 (14.5)	36 (21.7)	1	-
Coffee drinking	Yes	166 (100)	149 (89.8)	-	0.998
	No	0 (0)	17 (10.2)	1	-
Porridge eating*	Yes	164 (98.8)	145 (87.3)	31.58 (1.87-533)	0.017
	No	2 (1.2)	20 (12.0)	1	-
Use of	Yes	62 (37.3)	121 (72.9)	1	-

separate dwelling house*	No	104 (62.7)	45 (27.1)	21.3 (5.84-77.4)	<0.001
Use of separate kitchen	Yes	75 (45.2)	102 (61.4)	1	-
	No	91 (54.8)	64 (38.6)	-	0.640
Smoking of utensils*	Yes	143 (86.1)	80 (48.2)	44.0 (10.3-189)	<0.001
	No	23 (13.9)	86 (51.8)	1	-

AOR- adjusted odds ratio; CI- confidence interval.

*Variables with a statistically significant difference between the case and control groups.

¹ Percentage of correct classification (cases / controls): 90.4% / 93.3%.

Table S-6. Multivariate binary logistic regression analysis for the onset of esophageal cancer based on age, gender and number of mycotoxin exposures for cases and location-matched controls in Ethiopia.

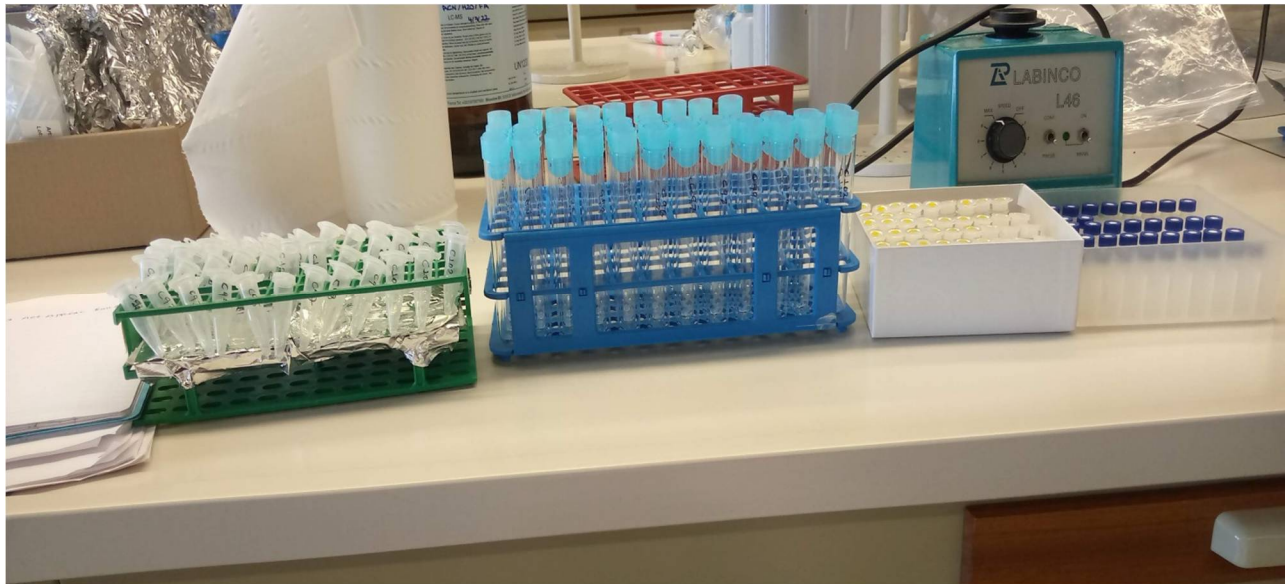
Variables		Cases (n=166)	Controls (n=166)	Multivariate binary logistic regression analysis ¹	
				AOR (95% CI)	p-value
Continuous		Mean ± standard deviation			
Age (years)*		52±14	39±7	1.15 (1.11-1.19)	<0.001
Number of mycotoxin exposures*		2.27±0.72	1.72±0.66	3.17 (2.02-4.97)	<0.001
Categorical		Frequency (n (%))			
Gender	Female	96 (57.8)	126 (75.9)	1	0.490
	Male	70 (42.2)	40 (24.1)	1.26 (0.66-2.39)	

AOR- adjusted odds ratio; CI- confidence interval.

*Variables with a statistically significant difference between the case and control groups.

¹ Percentage of correct classification (cases / controls): 74.7% / 83.1%.

Sample Lab activities pictures





Curriculum Vitae (CV)

1. Personal Information

Full name: Girma Mulisa Misgana



Addis Ababa University, Department of Microbiology, immunology and parasitology, a
PhD fellow in Medical Microbiology



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girmamulisa30@gmail.com

Sex: Male

Date of birth: 28/07/1987

Nationality: Ethiopian

1. Work Experience:

Institution	Date started serving and date of exit	Position
Adama Hospital Medical College	February 2015 to now	Assistant professor of Medical Microbiology
Adama Hospital Medical College	From September 2012 to February 2015	Lecturer
Ginnir Hospital	From October 2006 to October 2009	Junior Lab Technologist

2. Educational Background and Training:

Institution	Date entered and date completed	Instruction language	Degree/ certificate obtained
Jimma University	October 1, 2009 to June 28, 2012	English	MSc degree in Medical Microbiology
Haramaya University	October 25, 2003 to July 8, 2006	English	BSc degree in Medical Laboratory Technology

- ❖ International Clinical Micro Research Training in 2014 at St. Pauls' Hospital Millennium Medical College, Addis Ababa
- ❖ Research Result writing and Effective Oral Communication in 2015, at Ethiopian Hotel, Addis Ababa, Ethiopia

3. Personal Skills

3.1. Language proficiency

Language	Listening	Reading	Speaking	Writing
English	Proficient user	Proficient user	Independent user	Independent user
Amharic	Proficient user	Proficient user	Proficient user	Proficient user
Afan Oromo	Proficient user	Proficient user	Proficient user	Proficient user

3.2. Publications

4. **Mulisa G**, Abebe T, Gutema B, Mahmuda J, Khan MdAA, Gheit T, et al. Exploring Oesophageal Cancer in Ethiopia: Elevated Incidence in Females and Younger Cases. *Cancer Rep.* 2024 Dec;7(12):e70048.
- **Mulisa G**, Pero-Gascon R, McCormack V, Bisanz JE, Talukdar FR, Abebe T, et al. Multiple mycotoxin exposure assessment through human biomonitoring in an esophageal cancer case-control study in the Arsi-Bale districts of Oromia region of Ethiopia. *International Journal of Hygiene and Environmental Health.* 2025 Jan; 263:114466.
- **Girma Mulisa Misgana**, Ketema Abdissa, Gameda Abebe. Bacterial contamination of mobile phones of healthcare workers at Jimma University Specialized Hospital, Jimma, South West Ethiopia. *IJIC.* 2015;11(1):1-8
- **Girma Mulisa Misgana**, Tilaye W Abebe, Niguse H Geda, Mohamed S Hassen, Gameda A Ayana. Multi-drug resistant *Mycobacterium tuberculosis* and associated risk factors in the Oromia region of Ethiopia. *IJID.* 2015;11(1):1-7
- **Mulisa G**, Selassie LG, Jarso G, Shiferew T, Zewdu A, et al. (2016) Prevalence of Extended Spectrum Beta-lactamase Producing Enterobacteriaceae: A Cross Sectional Study at Adama Hospital, Adama, Ethiopia. *J Emerg Infect Dis* 1: 102. doi:10.4172/jeid.1000102
- Abebe TW, Chaka TE, **Misgana GM**, Adlo AM (2016) Determinants of Survival among Adults on Antiretroviral Therapy in Adama Hospital Medical College, Oromia Regional state, Ethiopia. *J HIV AIDS* 2(1): doi <http://dx.doi.org/10.16966/2380-5536.117>
- Shiferaw, T., Gebr-silasse, L., Mulisa, G., Zewidu, A., Belachew, F., Muleta, D., & Zemene, E. (2016). Bacterial Indoor-Air Load and its Implications for Healthcare-

Acquired Infections in a Teaching Hospital in Ethiopia. *International Journal of Infection Control*, 12(1). <https://doi.org/10.3396/ijic.v12i1.15808>

4.1.Conference papers

- Girma M. et al. Multi-drug resistant Mycobacterium tuberculosis and associated risk factors in the Oromia region of Ethiopia on the 2nd Asia-African Congress of Mycobacteriology, Isfahan, Iran-25-28th February 2017.
- Girma M et al. Multi-drug resistant Mycobacterium tuberculosis and associated risk factors in the Oromia region of Ethiopia on the 11th National TB Research Annual conference (TRAC), Dire dawa, Ethiopia-21-24th March 2016
- A seminar presentation on Serum Diagnostic Biomarkers for Multidrug-Resistant Mycobacterium tuberculosis, Adama, Ethiopia
- Girma M. et al. Prevalence of Extended Spectrum Beta-lactamase Producing *Enterobacteriaceae*: A Cross Sectional Study at Adama Hospital, Adama, Ethiopia on 26 annual conference of Ethiopian public health association Bahirdar, Ethiopia 25-30th March 2015.
- Multi-drug resistant Mycobacterium tuberculosis and associated risk factors in the Oromia region of Ethiopia on 27th annual conference of Ethiopian public health association.

4.2.Professional Member Ship

- Regular member of African Esophageal Cancer Consortium (AfrECC)
- A regular Member American Society for Microbiology
- A regular member of Ethiopian Public Health Association
- A regular Member Ethiopian Society for Microbiology

Note: Attached some training and participation certificate

International Agency
for Research on Cancer



CERTIFICATE

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

This is to certify that

Girma Misgana

has successfully completed the module

Introduction to Cancer Epidemiology

co-directed by

Dr Pietro Ferrari and Dr Laure Dossus from the Nutrition and Metabolism Branch,

during the **IARC Summer School 2023** in Lyon, France

A handwritten signature in black ink, appearing to read "Anouk Berger".

Anouk Berger
Head, Learning and Capacity Building Branch
International Agency for Research on Cancer

A handwritten signature in black ink, appearing to read "Elisabethe Weiderpass".

Elisabete Weiderpass, MD, PhD
Director
International Agency for Research on Cancer



This is a Certificate of Participation to the 4th DAAD/PAGEL Chronic Diseases Summer School on the theme:

NCD and Cancer Epidemiology in Ethiopia: Enhancing Screening Approaches for Prevention

Organized by the School of Public Health (AAU) in collaboration with MLU, Germany
Presented to

Girma MUSA Misgana

During the occasions of the 4th DAAD/PAGEL Chronic Diseases Summer School, Celebration of the 10th year of Collaboration on NCD Research between Addis Ababa University (AAU) and Martin Luther University (MLU) in Germany, and the 5th year of the establishment of the NCD Epidemiology Research Working Group at SPH/AAU

September 03, 2019, Addis Ababa, Ethiopia

Dr Adamu Addissie

Chairperson of NCD Working Group, AAU

Dr Eva Kantelhardt

Chairperson of International Health Working Group, MLU

Prof Damen Haile Mariam
Dean of School of Public Health, AAU



Accreditation No. :01AU-COHS-CPDP 0121



AHMC-CPD center

Certificate of completion

This certificate is awarded to

GIRMA MULISA MISGANA

for successful completion of training on R Project statistical computing
organized by Adama Hospital Medical College (AHMC) CPD-center from
Sept.26-28/2023.

Alem Deksis (MPH, Assis't. prof.)
AHMC CPD Director



Dr. Endashaw Abebe (MD, Internist)
V/ Provost of Adama Hospital Medical College



This is to certify

CERTIFICATE OF APPRECIACION

Present to Dr. Girma Mulisa Misgana (Oral Presentation)

at the 2st Asian African Congress of Mycobacteriology
Istahan, Iran ~ 25-28th February, 2017

The Asian~African Society of Mycobacteriology (AASM) is a nonprofit, scientific organization with the mission to foster unilateral, bilateral and multilateral networks of scientific, research and training collaborations among experts from AASM countries.

Prof. A. A. Velayati
President of Congress

Velayati

Dr. Chohamreza Forouzesh
Vice President of Congress

Chohamreza Forouzesh

Dr. Parissa Farnia
Director of Scientific Section

Parissa Farnia

Mr. Mahdi Hedayati
Director of Administrative Section

Mahdi Hedayati



MICRORESEARCH CERTIFICATE

THIS CERTIFIES THAT

Girma Mulisa

Has been awarded this certificate upon successful completion of the
International Clinical MicroResearch Training at a workshop carried
out at St Paul's Hospital Millennium Medical College,

Addis Ababa, Ethiopia

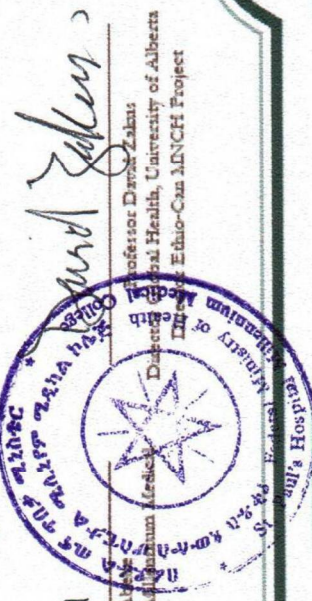
Given this 10th day of September, 2014

Dr. Zechene Alem

Dr. Zechene Alem
Provost, St Paul's Hospital Millennium Medical College
Director, International Health, University of Alberta
Ethiopia-Ethio-Can MINCH Project

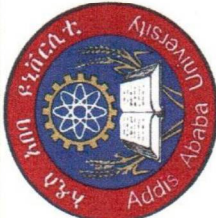
Dr. Robert Borchert

Professor Naomi MacDonald
Professor Robert Borchert
MicroResearch
Dalhousie University



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CERTIFICATE OF COMPLETION



Research Methods, Research Ethics and Grant Writing Trainings

This certificate is presented to Girma Mulisa for successfully completing intensive trainings on Research Methods, Research Ethics and Grant Writing as part of iRIM Research Capacity Building Project. The trainings were conducted at Adama Hospital Medical College from April 20 to 24, 2015 in Adama.

Dr. Sileshi Garoma

Provost, Adama Hospital Medical College



Dr. Miliard Derbew

PI, MEPI Project AAU

This training was conducted with the support of CHS-AAU and MEPI Project



Ethiopian Public Health Association

Certificate

Presented this certificate of recognition to


Girma Mulisa

for his/her contribution as a presenter of a scientific paper at the 26th
Annual Conference of the EPHA.

February, 2015


Dr Filimona Bisrat
President EPHA




Dr Hailegnaw Eshete
Executive Director EPHA

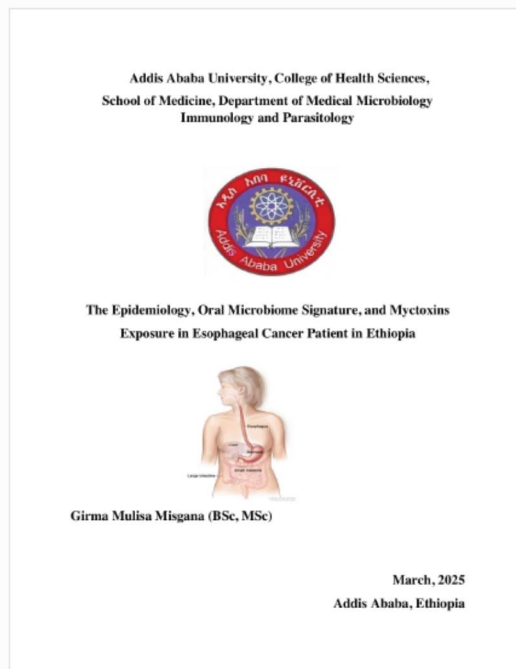


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