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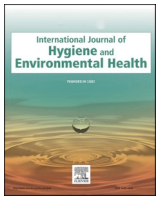

Multiple mycotoxin exposure assessment through human biomonitoring in an esophageal cancer case-control study in the Arsi-Bale districts of Oromia region of Ethiopia

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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 were 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

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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.

1. 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 (Brown et al., 2023). Cancer is increasingly a global health issue. The International Agency for Research on Cancer (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 (Bray et al., 2024). Esophageal cancer (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% (Sheikh et al., 2023). The two common histological subtypes are Esophageal Squamous Cell Carcinoma (ESCC) and Esophageal Adenocarcinoma (EAC) of which ESCC accounts for more than 85% of its histological types (Abnet et al., 2018; Morgan et al., 2022).

EC is a major public health concern worldwide, although its incidence and mortality has been found to vary significantly by region (Zhou et al., 2023). 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 (Abnet et al., 2018). 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 (Abnet et al., 2018; Liu et al., 2023; Sheikh et al., 2023). 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 (Dong and Thrift, 2017; Rustgi and El-Serag, 2014).

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 (Codipilly et al., 2018; Liang et al., 2017). 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 (Timotewos et al., 2018) with a significant increasing trend in its incidence (Wondimagegnehu et al., 2020), which is particularly high in Arsi-Bale districts of Oromia regional state (Deybasso et al., 2021a; Bulcha et al., 2018; Shewaye and Seme, 2016). Like other African countries, there is no screening program of EC in Ethiopia, leading to late diagnosis and poor survival (Middleton et al., 2021; Mwachiro et al., 2021). The treatment options available to this cancer is 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 (Alaouna et al., 2019; Ohashi et al., 2015). Exposure to aflatoxins from contaminated food, has also been associated with the development of ESCC in Africa (Brown et al., 2020). 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 (McCormack et al., 2017). 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 (Deybasso et al., 2021a; Shewaye and Seme, 2016). 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 (Shephard et al., 2000, 2007; Sun et al., 2007). 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 (Claeys et al., 2020; Marchese et al., 2018) with proven mechanism of carcinogenesis (Mace, 1997). IARC listed aflatoxins (including aflatoxin B1, B2, G1, G2 and M1) as carcinogenic to humans (Group 1) (Centre international de recherche sur le cancer, 2012) and fumonisins (including fumonisin B1 and B2) (International Agency for Research on Cancer, 2002) and ochratoxin A (International Agency for Research on Cancer, 1993) 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 (Ostry et al., 2017). In the African and Asian EC belt, common cereal crops used for food have been reported to be contaminated with mycotoxins (Alizadeh et al., 2012; Chu and Li, 1994; Ghasemi-Kebria et al., 2013; Lipenga et al., 2021; Sun et al., 2007). 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 esophageal cancer in Iran and China were associated with increased risk of EC (Alizadeh et al., 2012; Chu and Li, 1994; Sun et al., 2007).

Mycotoxin contamination has been reported to be a health concern in Ethiopia, as major agricultural food commodities were contaminated by carcinogenic mycotoxins (Ayelign and De Saeger, 2020). 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 (Deybasso et al., 2021b). Coffee, which is also reported to be contaminated with mycotoxins (Soliman, 2005), 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 (Heyndrickx et al., 2015). 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 (Lipenga et al., 2021; Sun et al., 2007), biomonitoring (Xue et al., 2019) and mechanistic experimental (Yu et al., 2021) 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.

2. Materials and methods

2.1. Study design and setting

A health care facility-based case-control study design was used. Cases were pathologically confirmed newly diagnosed and treatment-naive esophageal cancer 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 min, 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.

2.2. 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 (OTα), 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 μg/L of the 5 AFs and OTA standards and 600 μg/L of the other 36 mycotoxin standards in methanol; and (ii) containing 6 μg/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.

2.3. 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 0.03–0.50 μg/L. No additional testing was performed to characterize the EDTA plasma purchased from the Red Cross East Flanders and compare it with the EDTA plasma collected in Ethiopia.

2.4. 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 min and centrifuged at 3300 g for 15 min 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 min, 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 min 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.

2.5. 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 × 100.0 mm) from Waters with an Acquity UPLC HSS T3 VanGuard pre-column (1.8 μm, 2.1 mm id x 5 mm). The chromatographic separation has already been described by Martins et al. (Martins et al., 2019, 2020). The optimized UHPLC-MS/MS parameters for the analysis of mycotoxins are shown in **Table S-1**.

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/x2 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 (Gu et al., 2014). 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 (European Commission, 2016): 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 ±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 LLOQ = k·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% (González and Alonso, 2020). 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 (Kruve et al., 2015) (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: Accuracy (%) = 100 * measured concentration/spiked concentration (González and Alonso, 2020); 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: CV = SD/measured concentration (González and Alonso, 2020); 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-2**.

2.6. 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) (European Food Safety Authority et al., 2018) 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 LLOQ/2, and non-detects (i.e. below the LOD) were replaced by 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 (Chawla et al., 2002).

3. Results

3.1. Demographic and lifestyle variables

Table 1 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, α = 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, α = 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

Table 1
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 [b]	p-value
Continuous		**Mean ± standard deviation**				
Age (years)[a]		52 ± 14	39 ± 7	11.4	246	<0.001
Categorical		**Frequency (n (%))**		**χ^2**	**df [b]**	**p-value**
Gender[a]	Female	96 (57.8)	126 (75.9)	12.2	1	<0.001
	Male	70 (42.2)	40 (24.1)			
Established risk factors						
Alcohol drinking[a]	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[a]	Yes	166 (100)	149 (89.8)	17.9	1	<0.001
	No	0 (0)	17 (10.2)			
Porridge eating[a]	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[a]	Yes	62 (37.3)	121 (72.9)	42.4	1	<0.001
	No	104 (62.7)	45 (27.1)			
Use of separate kitchen[a]	Yes	75 (45.2)	102 (61.4)	8.8	1	0.003
	No	91 (54.8)	64 (38.6)			
Smoking of utensils[a]	Yes	143 (86.1)	80 (48.2)	54.2	1	<0.001
	No	23 (13.9)	86 (51.8)			

[a] Variables with a statistically significant difference between the case and control groups.
[b] Degrees of freedom.

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.

3.2. Mycotoxin exposure

Evidence of exposure to 10 of the different mycotoxins investigated was observed in plasma samples of the participants as shown in Fig. 1. 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-1**.

Table 2 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 (Fig. 2). 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.

3.3. 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-2**). 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) (White et al., 2014), 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 (Fig. 3). 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-3**).

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-3**). Table 3 shows the results of the final multivariate binary logistic regression analysis considering the five most important predictor variables from the Gradient Boosting Classifier model (Fig. 3-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 4 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

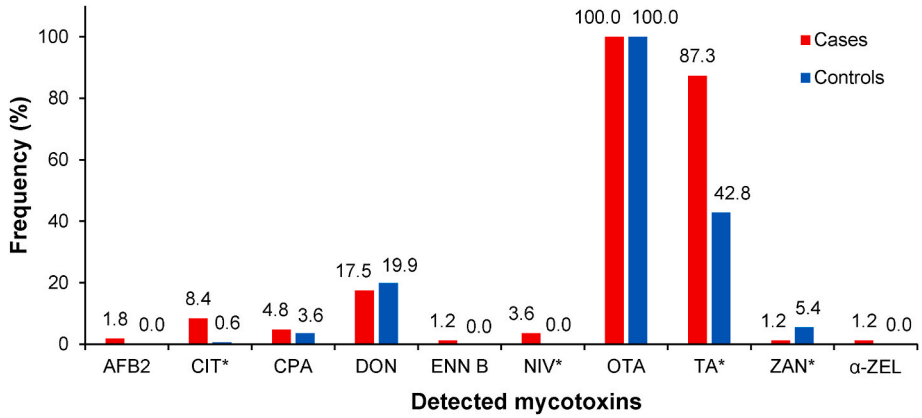

Fig. 1. Frequency of detection of mycotoxins in plasma from esophageal cancer cases and location-matched controls in Ethiopia.
*Variables with a statistically significant difference between the case and control groups. Results of the chi-square test (χ^2, p-value): AFB2- aflatoxin B2 (3.0, 0.08), CIT-citrinin (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 2
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[a]	0.020	0.070	0.28	0.73	1.7	3.8	8.0	24
TA[a]	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

[a] 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.

significantly associated with the control group.

Fig. 4 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-4**). Table 5 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.

4. Discussion

In Ethiopia, where there is a high esophageal cancer incidence and already established important risk factors for EC, i.e. alcohol consumption and tobacco use, are rare (Deybasso et al., 2021a; Shewaye and Seme, 2016), 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 (Ahmad et al., 2011; Pritchett et al., 2017), polycyclic aromatic hydrocarbons metabolites from in-door air pollution (Mwachiro et al., 2021), N-nitrosamines from traditional brews (Isaacson et al., 2015), smoking and daily use of spicy chilies and salted foods (Mmbaga et al., 2021). Dietary and various environmental exposure determinants of EC were reported from Ethiopia (Deybasso et al., 2021b). Mycotoxins are a major cause of food intoxication in Sub-Saharan Africa, where the hot and humid tropical climate favors the growth of fungi (Bankole et al., 2006). Recently, dietary exposure to mycotoxins in Nigerian mothers and infants was investigated by analyzing breast milk, complementary food and urine (Braun et al., 2022; Ezekiel, 2022). 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 (Kigen et al., 2017; Mlombe et al., 2015). 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 (Habschied et al., 2021; Heyndrickx et al., 2015).

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 (Kamangar et al., 2020; Sung et al., 2021). Gender-based variations in the incidence of EC have also been reported in Africa EC high-risk region (Middleton et al., 2018). In South Africa, EC is more common among males (Ferndale et al., 2020) whereas, a study in Sudan by Gasmelseed et al., in 2015 found a higher incidence of EC among females (Gasmelseed et al., 2015). Similarly, other studies conducted in Ethiopia (Hassen et al., 2021; Shewaye and Seme, 2016) 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 (Deybasso et al., 2021a), 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 μg/L for cases and controls, respectively (Table 2), which is higher compared to the results of mycotoxin biomonitoring studies from China (median OTA concentration was 0.16 μg/L) (Fan et al., 2020) and Italy (median OTA concentration was 0.17 μg/L) (Di Giuseppe et al., 2012), and similar to a study in Bangladesh (median OTA concentration was 0.57 μg/L) (Ali et al., 2014). In contrast, a study in Northern Spain reported a median OTA concentration in plasma of 2.60 μg/L (Arce-López et al., 2020a), 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

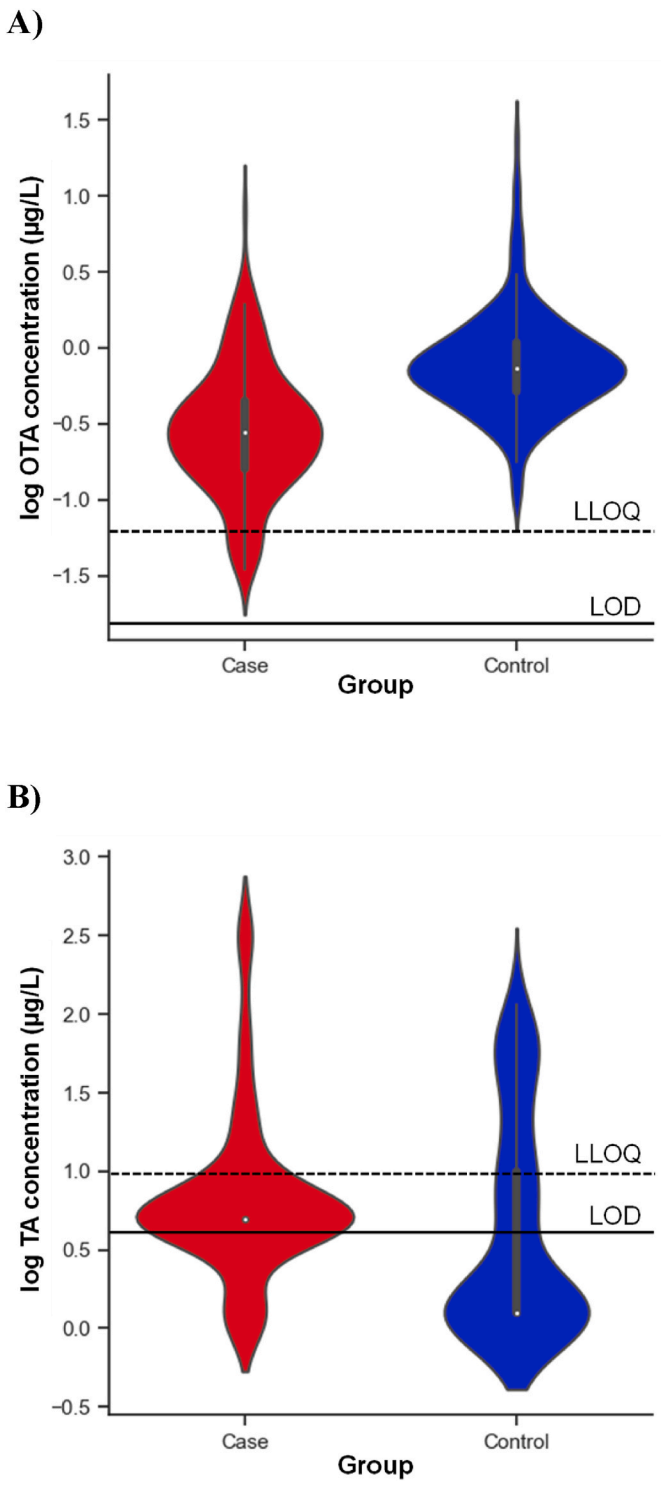

Fig. 2. Violin plots for mycotoxin exposure in the esophageal cancer case-control study: (A) ochratoxin A (OTA), and (B) tenuazonic acid (TA). Mycotoxin concentrations are indicated in logarithmic scale. The limit of detection (LOD) is indicated as a solid line. The lower limit of quantification (LLOQ) 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.

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 (Karima et al., 2010). It was also the most frequently detected mycotoxin among the healthy population of Spain with increasing frequency (Arce-López et al., 2020a; Medina et al., 2010) and among Chinese healthy volunteer individuals living in Nanjing (Fan et al., 2020).

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 (Yu and Pedroso, 2023). 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 (Deybasso et al., 2021b). Furthermore, coffee bean and its derivative products were reported to be contaminated with OTA (Benites et al., 2017) 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) (Raters and Matissek, 2008) and its long half-life in human plasma and serum (35 days) (Al-Jaal et al., 2019) 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 (International Agency for Research on Cancer, 1993), and more recent data show evidence of the carcinogenic activity and toxicity of OTA (Ostry et al., 2017). Since OTA is widely prevalent in food and feed and also commonly reported in human biomonitoring studies, *the European Food Safety Authority (*EFSA) recommended further research on OTA mechanistic toxicity and genotoxicity (Schrenk et al., 2020). An experimental study showed that exposure to OTA in a dose of 0.125 µM–0.5 mM over two time periods within 48 h was able to induce biomarkers of hypoxia and transformation in human kidney cell (Raghubeer et al., 2019). Some mechanisms including inhibition of protein synthesis, induction of oxidative stress, and DNA adduct formation were mentioned for the toxic action of OTA (Kőszegi and Poór, 2016). In addition, the ability of OTA in damaging esophageal epithelial cell by reactive oxygen species generation and oxidative DNA damage was demonstrated *in vitro* (Zhao et al., 2021). This oxidative process was well mentioned in cancer development previously (Hanahan and Weinberg, 2011; Takeshima and Ushijima, 2019). OTA is also linked to liver and kidney damage. A benchmark dose lower confidence limit ($BMDL_{10}$) of 4.73 µg/kg body weight (bw) per day for the non-neoplastic endpoint was calculated from kidney lesions observed in pigs (Schrenk et al., 2020). This $BMDL_{10}$ 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, (Arce-López et al., 2020a). 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 $BMDL_{10}$ of 14.5 µg/kg bw per day (i.e., 3208 µg/L in plasma) was calculated from kidney tumors seen in rats (Schrenk et al., 2020). 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 (Moretti and Susca, 2017). 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 (Habib et al., 2021; Saleem and El-Shahir, 2022) and cereals, mainly wheat and its products (Masiello et al., 2020; Zhao et al., 2015), and barley (Castañares et al., 2020).

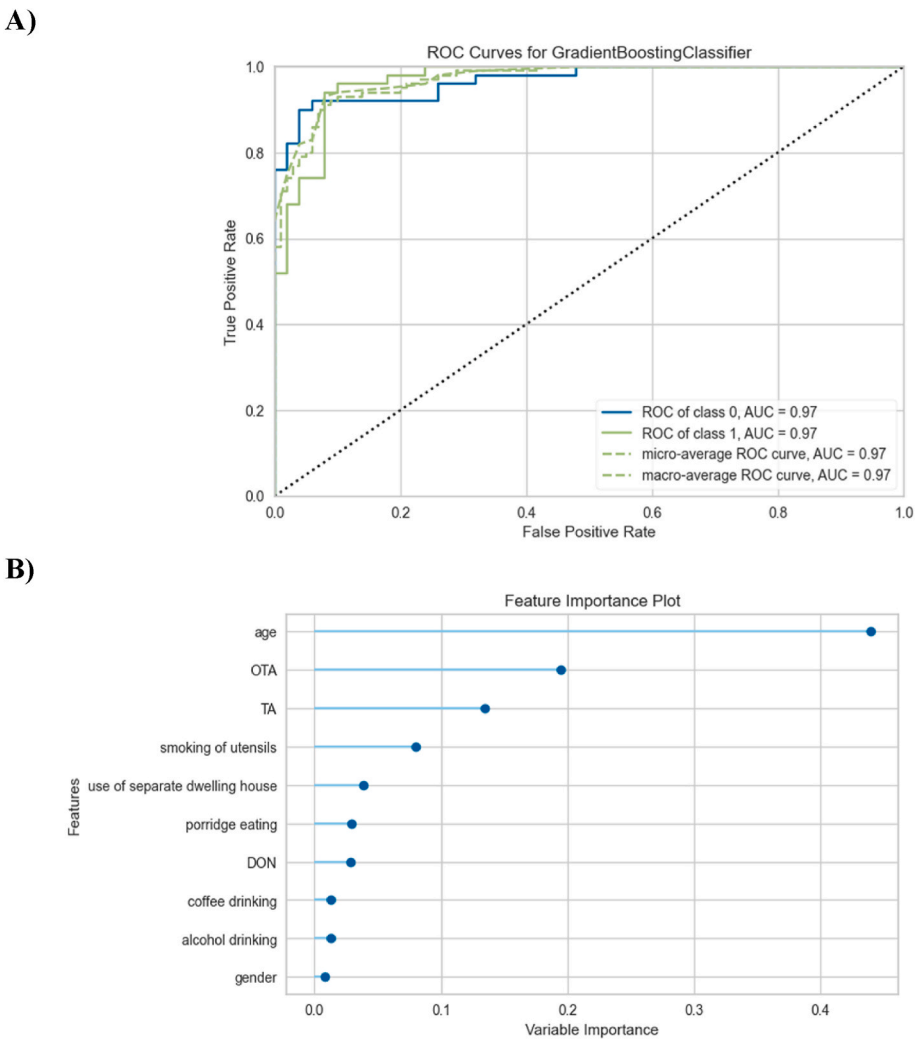

Fig. 3. Gradient Boosting Classifier model to classify esophageal cancer patients from controls based on demographic and lifestyle variables, and mycotoxin concentrations: (A) Receiver Operating Characteristic curve, and (B) feature importance plot.

A few toxicity studies showed that TA inhibits protein synthesis (Shigeura and Gordon, 1963) and induces cytotoxicity to human intestinal epithelial cells and hepatocytes (den Hollander et al., 2022). In animal trials it was observed that TA causes emesis and hemorrhagic gastro-enteropathy among other effects (Fraeyman et al., 2017). 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 (Yekeler et al., 2001). 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 (Ostry et al., 2017). 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 (Claeys et al., 2020), 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 (Xue et al., 2019). Our result is in line with another study in which CIT was detected in 90% of plasma samples (LOD 0.07 μg/L) analyzed from 104 participants in Bangladesh (Ali et al., 2018). Moreover, CIT was reported to be associated with nephropathy and promote the renal cell carcinogenesis (Tsai et al., 2023).

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, Fig. 1) 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 (Creppy et al., 2004; Radzka-Pogoda et al., 2023; Wang et al., 2020). For example, OTA co-exposure with CIT increased OTA-DNA adduct

Table 3
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 (Fig. 3-B) for cases and location-matched controls in Ethiopia.

Variables		Cases (n = 166)	Controls (n = 166)	Multivariate binary logistic regression analysis [b]	
				AOR (95% CI)	p-value
Continuous		**Mean ± standard deviation**			
Age (years)[a]		52 ± 14	39 ± 7	1.17 (1.12–1.23)	<0.001
OTA quartile[a]		1.90 ± 1.03	3.11 ± 0.85	0.28 (0.19–0.41)	<0.001
TA quartile[a]		2.67 ± 0.91	2.34 ± 1.28	1.80 (1.26–5.58)	0.001
Categorical		**Frequency (n (%))**			
Use of separate dwelling house[a]	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[a]	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.
[a] All variables are statistically significantly different between the case and control groups.
[b] Percentage of correct classification (cases/controls): 86.1%/87.3%.

Table 4
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 [b]	
			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[a]	1.93 ± 1.12	3.10 ± 0.85	0.21 (0.18–0.23)	<0.001
TA quartile[a]	2.75 ± 0.92	2.34 ± 1.28	1.88 (1.68–2.11)	<0.001

AOR-adjusted odds ratio; CI- confidence interval.
[a] Variables with a statistically significant difference between the case and control groups.
[b] 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%.

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 (Fromme et al., 2016). Since patients with upper gastrointestinal tract cancer, including EC, are unable swallow solid food due to the early obstruction of the gastrointestinal passage (Reim and Friess, 2015) 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.

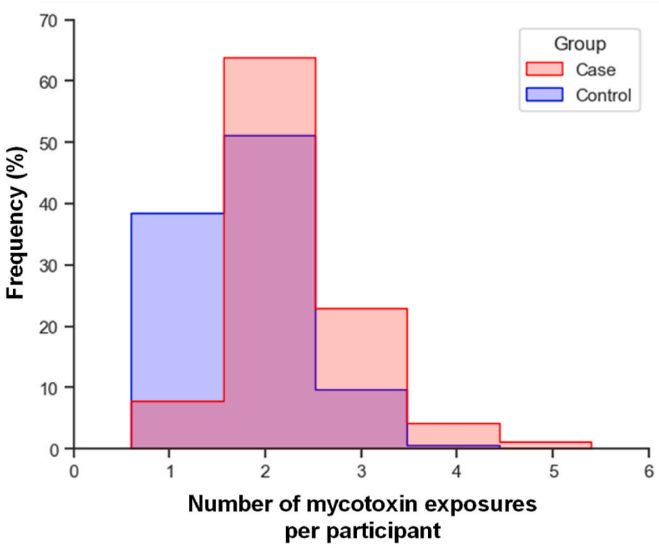

Fig. 4. Number of mycotoxin exposures per participant among esophageal cancer cases and location-matched controls in Ethiopia.

Table 5
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 [b]	
			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[a]	2.30 ± 0.72	1.73 ± 0.65	2.54 (2.10–3.07)	<0.001

AOR-adjusted odds ratio; CI- confidence interval.
[a] Variables with a statistically significant difference between the case and control groups.
[b] 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%.

4.1. 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 (Arce-López et al., 2020b), 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 (Riley et al., 2015), 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.

5. 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 polices 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.

CRediT authorship contribution statement

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.

Declaration of competing interest

All the authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2024.114466.

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