Biological monitoring and exposure assessment for mycotoxins in inhabitants of Bangladesh and Germany

Dissertation

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Citation of original publications in this dissertation

This dissertation has been written based on the following peer-reviewed original publications and some unpublished results.

1. **Ali N**, Blaszkewicz M, Manirujjaman M, Perveen R, Nahid AA, Mahmood S, Rahman M, Hossain K, Degen GH (2014) Biomonitoring of ochratoxin A in blood plasma and exposure assessment of adult students in Bangladesh. Mol Nutr Food Res 58:2219–2225
2. **Ali N**, Hossain K, Blaszkewicz M, Rahman M Mohanto NC, Alim A, Degen GH (2015a) Occurrence of aflatoxin M1 in urine from rural and urban adult cohorts in Bangladesh. Arch Toxicol (published online 2015 Sep 21) doi:10.1007/s00204-015-1601-y
3. **Ali N**, Blaszkewicz M, Alim A, Hossain K, Degen GH (2015b) Urinary biomarkers of ochratoxin A and citrinin exposure in two Bangladeshi cohorts: follow-up study on regional and seasonal influences. Arch Toxicol (published online 2015 Dec 26) doi: 10.1007/s00204-015-1654-y
4. **Ali N**, Blaszkewicz M, Degen GH (2015c) Occurrence of the mycotoxin citrinin and its metabolite dihydrocitrinone in urines of German adults. Arch Toxicol 89:573–8
5. **Ali N**, Blaszkewicz M, Mohanto NC, Rahman M, Alim A, Hossain K, Degen GH (2015d) First results on citrinin biomarkers in urines from rural and urban cohorts in Bangladesh. Mycotoxin Res 31: 9–16
6. **Ali N**, Blaszkewicz M, Nahid AA, Rahman M, Degen GH (2015e) Deoxynivalenol exposure assessment for pregnant women in Bangladesh. Toxins 7:3845–57
7. Gerding J, **Ali N**, Schwartzbord J, Cramer B, Brown DL, Degen GH, Humpf HU (2015) A comparative study of the human urinary mycotoxin excretion patterns in Bangladesh, Germany and Haiti using a rapid and sensitive LC-MS/MS approach. Mycotoxin Res 31:127–36
8. Föllmann W, **Ali N**, Blaszkewicz M, Degen GH (2016) Biomonitoring of mycotoxins in urine: Pilot study in mill workers. J Toxicol Environ Health Part A, in press
9. **Ali N**, Blaszkewicz M, Manirujjaman M, Degen GH (2016a) Biomonitoring of concurrent exposure to ochratoxin A and citrinin in pregnant women in Bangladesh. Food Chem Toxicol, submitted (date 4 Jan 2016)
10. **Ali N**, Blaszkewicz M, Degen GH (2016b) Assessment of deoxynivalenol exposure among Bangladesh and German adults. Manuscript in preparation

Summary

Mycotoxins, toxic secondary metabolites of various fungi, are frequent contaminants of crops worldwide. Prominent examples are aflatoxin B_1, ochratoxin A, fumonisins, deoxynivalenol and zearalenone, found at variable levels in diverse geographical regions. These mycotoxins are of concern, since their ingestion can cause disorders and severe diseases in animals and humans. Most developed countries enforce strict regulations to minimize dietary exposure of consumers. But, in developing countries as Bangladesh there is no regular surveillance for contaminants, and data on mycotoxins in food and feed is scarce or absent. As biomonitoring (analysis of parent compounds and metabolites in human samples) covers intake from all dietary sources, this approach was used to systematically investigate mycotoxin exposure in inhabitants of Bangladesh and compare it with the situation in other countries.

Determining urinary biomarkers of exposure by means of an LC-MS/MS based multi-mycotoxin method served to select major mycotoxins of interest, namely aflatoxin B_1 (AFB_1, hepatocarcinogen), ochratoxin A, citrinin (OTA, CIT; nephrotoxic), and deoxynivalenol (DON, emetogen, immunotoxic). Then an in-depth analysis by sensitive specific methods was conducted for AFM_1 (metabolite of AFB_1), OTA, CIT and its main metabolite dihydrocitrinon (HO-CIT), or for DON and its metabolites, to determine the concentration ranges in samples (urines) collected from several Bangladeshi cohorts and in those from a German cohort.

To gain insight into the range of mycotoxin exposure among the Bangladeshi population, urine samples were collected from cohorts in two districts of the country, and from residents of rural and urban areas with presumably some differences in food habits. Bangladesh has a subtropical monsoon climate, characterized by seasonal variations in rainfall, temperature and humidity, conditions that favour the growth of certain fungal species on crops and stored food commodities. Thus, urines were collected from cohorts in summer and winter season for mycotoxin biomarker analysis.

My thesis is based on seven original publications in peer-reviewed journals on the occurrence and ranges of suitable biomarkers of exposure for AFB_1, CIT, OTA, and DON in the Bangladeshi cohorts *(Ali et al. 2014; Ali et al. 2015a,b,c,d,e; Gerding et al. 2015)*. The included publications provide more details on study cohorts, sample collection, analytical methods and their validation, full sets of biomarker data and an in-depth interpretation of all results. The thesis presents also unpublished data on AFM_1 and DON biomarker analysis in Bangladeshi and German cohorts, and an overview of the literature on urinary biomarker data from other countries. Main findings of this first systematic biomonitoring study in the

Bangladeshi population and a German 'reference' cohort are briefly summarized below along with comments regarding possible risks associated with an exposure to these mycotoxins.

Aflatoxin B_1: In the organism AFB_1 is partly converted to the hydroxylated metabolite AFM_1 and excreted with urine (and milk). AFM_1 was not detected by HPLC-FD analysis (LOD 1.7 pg/mL) in any of the urine samples from German adults (n=50). But, AFM_1 was detected in >40% of urines from Bangladeshi cohorts in one district (n=62), and in 31% of urines from pregnant women (n=54) in another district. The latter samples contained similar or slightly lower AFM_1 levels (mean 13.9, max 142 pg/mL) than those found in the other cohort during two sampling seasons (mean 13.6, max 140 pg/mL in summer, and mean 27.7, max 190 pg/mL in winter). These novel biomarker results indicate frequent exposure to the potent hepatotoxic mycotoxin AFB_1 in the Bangladeshi population, and at levels which should raise concerns. As AFB_1 is a potent mutagenic carcinogen, no "safe" intake values can be defined, and the exposure should be kept as low as possible (ALARA principle).

Ochratoxin A and Citrinin: Both compounds are known nephrotoxins, with OTA being more potent than CIT. Absorbed OTA has a long half-life in blood of humans, since only a small fraction of circulating OTA is excreted in urine. In principle, OTA biomarker analysis can use either blood or urine samples, although non-invasive sampling is often preferred. CIT is converted to the less toxic metabolite dihydrocitrinon (HO-CIT), the major urinary metabolite in humans. Thus, the sum of both ('total CIT') served as biomarker of exposure.

The mean OTA plasma concentration (0.85 ng/mL) in Bangladeshi students (n=64) only slightly exceeds an 'overall mean world OTA serum level' of 0.7 ng/mL in apparently healthy populations. Dietary intake of OTA (mean 11.7, max 91.4 ng/kg b.w/week) calculated on the basis of plasma levels in Bangladeshi students is lower than the tolerable weekly OTA intake (120 ng/kg body weight) set by European Food Safety Agency (EFSA). This first data indicated low risks in the student group, yet further studies in other Bangladeshi cohorts were indicated, also to assess concomitant exposure to CIT.

Biomarkers for OTA and CIT exposure were present in most of the urines collected from our Bangladeshi cohorts. In residents of two areas in Rajshahi district, no regional difference was observed in urinary OTA levels, whereas sampling season had a clear impact, with higher OTA mean levels found in winter (mean 0.19, max 1.75 ng/mL urine) than in summer (mean 0.06, max 0.55 ng/mL). For CIT biomarkers more pronounced regional and seasonal variations were found among these cohorts. Their mean level of total CIT (CIT plus HO-CIT) was significantly higher in winter (mean 3.77, max 48.12 ng/mL urine) than in summer (mean 0.51, max 5.70 ng/mL) season. In both seasons, total CIT biomarker concentrations were

significantly higher in the rural than in the urban cohort. Determination of urinary OTA concentrations (mean 0.10, max 0.84 ng/mL) for pregnant women cohorts from Dhaka district and CIT biomarker analysis (total mean 0.73, max 9.40 ng/mL) confirmed a frequent concomitant exposure to these mycotoxins in the Bangladeshi population. In German adults, the urinary OTA concentration (mean 0.21, max 1.82 ng/mL) was similar to that found in the Bangladeshi adult cohorts in winter season. However, CIT biomarker levels in German urines (total mean 0.14, max 0.58 ng/mL) were clearly lower than those in all Bangladeshi samples.

The novel biomarker results for CIT and a urinary excretion rate of 36% per day were used for calculating probable daily CIT intake in all cohorts and compare these values to the preliminary tolerable daily intake (TDI) of 0.2 µg/kg bw set by EFSA. The TDI for CIT exposure was not exceeded in the German cohort, but in up to 24% of the Bangladeshi cohort. Estimates of OTA exposure from urine data are hampered by its low excretion rate (< 3%).

***Deoxynivalenol**:* DON is mainly metabolized to glucuronides and about 70% of an ingested dose is excreted in urine within a day. Overall, biomonitoring results for all Bangladeshi cohorts reveal low DON exposure, with some regional but no marked seasonal variation. The urinary DON level in pregnant women from Dhaka district (mean 0.86, max 7.16 ng/mL) was higher than in the Rajshahi cohorts (mean 0.17, max 1.78 ng/mL in summer, mean 0.16; max 1.21 ng/mL in winter). Urinary DON concentrations (mean 9.02, max 38.44 ng/mL) in German adults are significantly higher ($p<0.0001$) than in any of the Bangladeshi cohorts. However, the probable daily DON intakes for all participants in both countries were below the TDI of 1 µg/kg/d set by WHO, thereby indicating that present DON exposures are not of concern.

Conclusions: The frequent detection of AFM_1 in Bangladeshi urines clearly indicates aflatoxin exposures of concern. To minimize this health risk, further studies to identify major sources of AFB_1 intake are recommended. Exposure to CIT an OTA should be further monitored, including susceptible groups in the population (especially children), and in light of possible combined effects.

Zusammenfassung

Mykotoxine, toxische Sekundärmetaboliten verschiedener Schimmelpilzarten, treten weltweit als Kontaminanten in Getreide und anderem pflanzlichen Erntegut auf. Bekannte Beispiele sind Aflatoxin B_1, Ochratoxin A, Fumonisine, Deoxynivalenol and Zearalenon. Man findet sie in verschiedenen geographischen Regionen der Erde in unterschiedlicher Häufigkeit und Menge. Diese Mykotoxine geben Grund zur Besorgnis, weil ihre Aufnahme die Gesundheit von Mensch und Tieren beeinträchtigen und zu schweren Erkrankungen führen kann. Entwickelte Länder haben daher strikte Regelungen für Mykotoxine und Kontrollstrukturen etabliert, um eine nahrungsbedingte Aufnahme (Exposition) zu minimieren. In Entwicklungsländern wie Bangladesh gibt es noch keine solchen Maßnahmen zum Schutz der Verbraucher. Daten zur Kontamination von Lebens- und Futtermitteln mit Mykotoxinen sind daher rar oder fehlen ganz. Das Humanbiomonitoring (die Analyse von Fremdstoffen und deren Metaboliten in Körper-flüssigkeiten) spiegelt eine Aufnahme aus allen Nahrungsquellen wider. Es wurde nun genutzt, um erstmals systematisch die Mykotoxin-Exposition in der Bevölkerung in Bangladesh zu untersuchen und deren Belastung mit der in anderen Ländern zu vergleichen.

Die Analyse sog. *'biomarker of exposure'* in Urinproben mit Hilfe einer LC-MS/MS basierten Multi-Mykotoxin-Methode erlaubte es, eine Auswahl wichtiger Mykotoxine zu treffen, nämlich Aflatoxin B_1 (AFB_1, hepatotoxisch und kanzerogen), Ochratoxin A, Citrinin (OTA, CIT; nephrotoxisch) und Deoxynivalenol (DON, emetogen, immunotoxisch). Selektive Methoden - mit Anreicherung der Analyten AFM_1 (Metabolit von AFB_1), OTA, CIT und Dihydrocitrinon (HO-CIT Metabolit von CIT) sowie DON und seine Metaboliten - wurden dann zur Bestimmung der Konzentrationen in Humanproben (Urinen) von mehreren Personengruppen in Bangladesh und in einer deutschen Kohorte eingesetzt.

Zwecks Charakterisierung der Mykotoxin-Exposition in der Bevölkerung von Bangladesh, wurden dort Urine von Kohorten in zwei Distrikten gesammelt und von Bewohnern ländlicher und städtischer Regionen mit vermutlich unterschiedlichen Nahrungsgewohnheiten. Bangladesh hat ein subtropisches (Monsun-)Klima mit saisonalen Schwankungen in Niederschlag, Temperatur und Luftfeuchte. Die Bedingungen sind förderlich für das Wachstum von Schimmelpilzen auf Erntegut und eingelagerten Produkten. Daher wurden in den Kohorten jeweils im Sommer und im Winter Urinproben für das Mykotoxin-Biomonitoring gesammelt.

Meine Dissertation zur biomarker-basierten Ermittlung der Exposition gegen AFB_1, CIT, OTA und DON in Bewohnern von Bangladesh stützt sich auf 7 Originalarbeiten in referierten Fachzeitschriften (Ali *et al.* 2014; Ali *et al.* 2015a,b,c,d,e; Gerding *et al.* 2015), mit detaillierten Angaben zu Studien-Kohorten, Probensammlung, Analysenmethoden und Validierung, kompletten Biomarker-Datensets und vertieften Diskussionen aller Befunde. Die vorliegende Schrift enthält ferner bislang unpublizierte Daten zu AFM_1- und DON-Analysen in Kohorten

aus Bangladesh und Deutschland sowie Übersichten zu publizierten Mykotoxin-Daten aus anderen Ländern. Wesentliche Befunde der ersten systematischen Biomonitoring-Studie in der Bevölkerung in Bangladesh und einer deutschen Vergleichs-Kohorte werden nachfolgend kurz zusammengefasst und mögliche Risiken der Mykotoxin-Expositionen diskutiert.

***Aflatoxin B_1**:* AFB_1 wird im Organismus u.a. in den hydroxylierten Metaboliten AFM_1 umgewandelt, der in Urin (und Milch) ausgeschieden wird. AFM_1 war mit sensitiver Analytik in keiner deutschen Urinprobe (n=50) nachweisbar (LOD 1,7 pg/mL). Dagegen wurde AFM_1 in >40% der Urine (n=62) von Bewohnern eines Distrikts in Bangladesh detektiert und in 31% der Proben von Schwangeren (n=54) aus einer anderen Region. Deren Urine hatten ähnliche bzw. etwas geringere AFM_1 Konzentrationen (Mittelwert, MW 13,9; max. 142 pg/mL) als die zu zwei Zeiten gesammelten Urine der anderen Kohorte (MW 13,6; max. 140 pg/mL im Sommer und MW 27,7; max. 190 pg/mL im Winter). Die neuen Biomarker-Daten belegen die häufige Exposition der Bevölkerung in Bangladesh gegenüber dem hepatotoxischen Mykotoxin AFB_1 und zwar in einer Höhe, die als bedenklich anzusehen ist. Da AFB_1 ein potentes mutagenes Kanzerogen ist, können keine '*safe intake*' Werte definiert werden, und die Exposition mit der Nahrung sollte so gering wie möglich sein (sog. ALARA Prinzip).

***Ochratoxin A und Citrinin**:* Beide Substanzen wirken primär nierentoxisch, OTA ist dabei potenter als CIT. Aufgenommenes OTA zirkuliert relativ lang im Blut des Menschen, weil täglich nur ein kleiner Anteil davon im Urin ausgeschieden wird. OTA kann in Blut oder Urin analysiert werden, wobei nicht-invasive Probenahme oft vorzuziehen ist. CIT wird in das geringer toxische Dihydrocitrinon (HO-CIT) umgewandelt, ein Hauptmetabolit im Urin von Menschen. Die Summe beider Analyten ('total CIT') dient daher als 'biomarker of exposure'.

In Bangladesh bei Studenten (n=64) gemessene OTA Blutplasma Werte (MW 0.85 ng/mL) liegen nur gering über dem 'overall mean world OTA serum level' von 0,7 ng/mL in anderen gesunden Personengruppen. Die auf Basis der OTA Blutspiegel berechnete Aufnahmemenge der Studenten (MW 11,7, max. 91.4 ng/kg KG/Woche) ist geringer als eine von der European Food Safety Agency (EFSA) abgeleitete unschädliche Exposition für OTA, der sog. *'tolerable weekly intake'* von 120 ng/kg KG. Die Daten sprachen zwar für geringe OTA Belastungen in der Gruppe der Studenten, dennoch waren Studien in anderen Bangladesh-Kohorten angezeigt, auch um begleitende Expositionen gegenüber CIT zu ermitteln.

Biomarker für OTA- und CIT-Exposition waren in fast allen Urinen der Bangladesh Kohorten nachweisbar. Bei Bewohnern zweier Gebiete in Rajshahi fanden sich keine regionalen Unterschiede in den OTA-Urinspiegeln, aber deutliche saisonale Einflüsse, mit höheren Werten im Winter (MW 0,19; max. 1,75 ng/mL) als im Sommer (MW 0,06; max. 0.55 ng/mL). Die CIT-Biomarker Analysen dieser Kohorten zeigten ausgeprägtere regionale und saisonale Unterschiede, nämlich signifikant höhere Gesamt-CIT (CIT+HO-CIT) Konzentrationen im Winter

(MW 3,77; max. 48.12 ng/mL Urin) als im Sommer (MW 0,51; max 5.70 ng/mL) sowie in beiden Sammelperioden signifikant höhere Spiegel in der ländlichen als in der städtischen Bevölkerung. Die in Urinen einer Kohorte von Schwangeren im Dhaka Distrikt gefundene OTA-Konzentration (MW 0,10; max. 0.84 ng/mL) und CIT-Biomarker Analysen (MW 'total' 0,73; max. 9.40 ng/mL) bestätigen eine häufige und gleichzeitig bestehende Exposition der Einwohner in Bangladesh gegen beide Mykotoxine. In der deutschen Kohorte gefundene OTA Konzentrationen (MW 0,21; max. 1.82 ng/mL Urin) ähneln denen der Bangladesh-Kohorte im Winter. Dagegen liegen CIT-Biomarker-Werte in deutschen Urinen (MW 'total' 0,14; max. 0.58 ng/mL) deutlich unter den Werten aller Proben aus Bangladesh.

Auf Basis der CIT-Biomarker-Daten und einer Exkretionsrate von 35% im Urin wurde für alle Kohorten deren tägliche CIT-Aufnahme berechnet und die Werte dann mit dem von der EFSA abgeleiteten vorläufigen '*tolerable daily intake'* (TDI) von 0,2 µg/kg KG verglichen. In der deutschen Kohorte wurde der TDI für CIT Exposition nicht überschritten, aber in bis zu 24% der Individuen der Bangladesh-Kohorten. Eine Abschätzung der OTA Exposition aus Urin-Daten ist wegen der geringen Exkretionsrate (< 3%) zu unsicher und nicht sinnvoll.

Deoxynivalenol*:* DON wird überwiegend zu Glucuroniden metabolisiert, und etwa 70% einer aufgenommenen Dosis werden am Tag im Urin ausgeschieden. Insgesamt belegen die Biomonitoring-Befunde eine niedrige DON Exposition aller Bangladesh-Kohorten, zwar mit regionalen Unterschieden, doch ohne klare saisonale Einflüsse: Urinspiegel in Schwangeren im Dhaka Distrikt (DON MW 0,86; max. 7.16 ng/mL) sind höher als in den Rajshahi Kohorten (MW 0,17; max. 1.78 ng/mL im Sommer, MW 0,16; max. 1.21 ng/mL im Winter). DON Konzentrationen (MW 9,02; max. 38.44 ng/mL) in der deutschen Kohorte sind signifykant höher als in den Urinen aus Bangladesh. Die berechnete tägliche DON-Aufnahme aller Teilnehmer aus beiden Ländern lag unter dem TDI der WHO von 1 µg/kg KG. Die gegenwärtigen DON Expositionen geben somit keinen Anlass zur Besorgnis.

Schlussfolgerungen: Der häufige Nachweis des Biomarkers AFM_1 in Urinproben aus Bangladesh ist ein klares Indiz für Aflatoxin-Expositionen, die als bedenklich angesehen werden. Um gesundheitliche Risiken der Bevölkerung künftig minimieren zu können, werden Folgestudien (Nahrungsmittelanalysen) empfohlen mit dem Ziel, die Hauptquellen einer AFB_1 Aufnahme zu identifizieren. Biomonitoring für CIT- und OTA-Exposition sollte fortgesetzt und auf empfindliche Gruppen der Bevölkerung (Kinder) ausgedehnt werden, auch in Hinblick auf mögliche Kombinationseffekte.

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ADME	Absorption, distribution, metabolism and excretion
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFP_1	Aflatoxin P_1
AFQ_1	Aflatoxin Q_1
ALARA	As low as reasonably achievable
BEN	Balkan endemic nephropathy
B.W/W	Body weight/week
BMI	Body mass index
CYP	Cytochrome P450
CIT	Citrinin
HO-CIT	Dihydrocitrinone
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DOM-1	De-epoxy metabolite 1
EC	European Community
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FAO	Food and Agriculture Organization
FB_1	Fumonisin B_1
FFQ	Food frequency questionnaire
HPLC	High performance liquid chromatography
FD	Fluorescence detection
IAC	Immunoaffinity column
IARC	International Agency for Research on Cancer

JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS/MS	Liquid chromatography - tandem mass spectrometric detection
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
m/z	mass-to-charge ratio
MW	Molecular weight
NOEL	No observed effect level
OTA	Ochratoxin A
OTα	Ochratoxin alpha
PTDI	provisional tolerable daily intake
Q1 / Q3	Quadrupole 1 / Quadrupole 3
RP	Reverse phase
RSD	Relative standard deviation
SCF	Scientific Committee on Food
SD	Standard deviation
SGT	Glutathione S-transferase
S/N	Signal-to-noise ratio
SPE	Solid phase extraction
SRM	Selected reaction monitoring mode
STs	Sulfotransferases
UV	Ultraviolet
TDI	Tolerable daily intake
WHO	World health organization
β -Gluc/ArylS	β-Glucuronidase/Arylsulfatase
ZEN	Zearalenone

1. Introduction

1.1 General information on mycotoxins

Mycotoxins are toxic secondary metabolites produced by filamentous fungi that grow under a wide range of climatic conditions on agricultural commodities in the field and during storage (CAST 2003; Zöllner and Mayer-Helm 2006). Their frequent occurrence in food and feed has raised concern, since ingestion of mycotoxins can cause disorders and severe diseases in animals and humans (Bennett & Klich 2003; Fung & Clark 2004; Marin et al. 2013; Richard 2007; Rocha et al. 2014). Prominent examples of mycotoxins are aflatoxin B_1, ochratoxin A, fumonisins, deoxynivalenol and zearalenone. Several studies have demonstrated their nephrotoxic, hepatotoxic, carcinogenic, teratogenic, immunotoxic and mutagenic characteristics in animal and human. An overview on toxic properties and mechanism of action of some major mycotoxins is given in Table 1.

1.2 Estimation of human exposure to mycotoxins

Human exposure to undesirable compounds, e.g. mycotoxins, can be estimated by two complementary approaches (Fig. 1): For the *'Top-down approach*' an analysis of mycotoxin contaminants is conducted, usually for the most important (not all!) food commodities. Then data on frequency of detection and contaminant levels are combined with information on food consumption in the general population to estimate the intake. But, dietary intake for certain subgroups (vegetarians, children etc.) can differ. Thus, uncertainties remain whether all sources are covered, and the data do not allow to judge (extreme) exposures of individuals. The '*Bottom-up approach*' is based on an analysis of parent compound and/or metabolites of the contaminant of interest in body fluids of individuals in a given cohort. The blood or urine levels of the analytes (or biomarkers) reflect exposure from all sources, but depend also on the kinetics of the mycotoxin, *i.e.* its fate in the organism (ADME). Thus, validation studies are important that have demonstrated a good correlation between the dietary mycotoxin intake and the concentration of a chosen biomarker of exposure.

In short, biomonitoring i.e. analysis of mycotoxin biomarker in human body fluids provides the best approach as it covers mycotoxin intake from all dietary sources and exposure by various routes (Degen 2011). In developing countries like Bangladesh, where food contaminant data is scarce or unavailable, human biomonitoring is especially useful to investigate the human exposure to mycotoxins.

Table 1 Overview on mechanism of action and toxic properties of some major mycotoxins, frequently found as food and feed contaminants (modified table from Šegvić Klarić et al. 2012)

Mycotoxins	Fungal species	Primary events at the cellular level	Toxic properties
Aflatoxin B_1	*A. flavus,* *A. parasiticus*	Metabolic activation → AFB_1-8,9-epoxide → modification of major cell macromolecules	Hepatotoxic, immunosuppressive, carcinogenic (group 1, IARC), teratogenic, mutagenic
Citrinin	*P. citrinum* *A. ochraceus* *M. purpureus*	Affects mitochondrial permeability transition, calcium flux, and cytochrome c release from mitochondria, inhibits macromolecule biosynthesis → cell death	Nephrotoxic, hepatotoxic, genotoxic (group 3, IARC), teratogenic, immunotoxic
Deoxynivalenol	*F. culmorum,* *F. graminearum*	Inhibition of protein synthesis → cell death, disruption of cytokine regulation	Causes nausea, food refusal, vomiting, diarrhoea, immunotoxic, IARC group 3
Diacetoxyscirpenol and T2-toxin	*F. sporotrichioides* *F. poae* *F. graminearum*	Inhibition of protein synthesis → cell Death, T-2 → erythrocyte lysis, induction of lipid peroxidation, apoptosis, inhibition of mitochondrial electron transport	Alimentary toxic aleukia (inflammation of the skin, vomiting, damage to hematopoietic tissues, haemorrhagia), IARC group 3
Fumonisin B_1	*F. proliferatum,* *F. verticillioides*	Inhibition of ceramide synthase → changes in sphingolipid metabolism, → protein kinase activity, oxidative stress → oxidative damage of cell macromoleculs	Equine leukoencephalomalacia, porcine pulmonary oedema, hepatotoxic, nephrotoxic, immunosuppressive, carcinogenic (group 2B, IARC)
Ochratoxin A	*A. ochraceus,* *P. verrucosum* *P. cyclopium*	Competition with phenylalanine and inhibition of Phe-dependent enzymes, inhibition of protein and DNA synthesis, mitochondrial transport system and transport of organic anions and cations, oxidative stress, DNA damage, affects glucose metabolism and Ca2+ homeostasis	Nephrotoxic, neurotoxic, hepatotoxic, affects blood coagulation, immunotoxic carcinogenic (group 2B, IARC), teratogenic
Zearalenone	*F. culmorum* *F. graminearum*	Resembles 17β-oestradiol → binds to oestrogen receptors in mammalian target cells	Disruption of hormonal control, IARC group 3

A: Aspergillus; F: Fusarium; M: Monuscus; P: Penicillium.

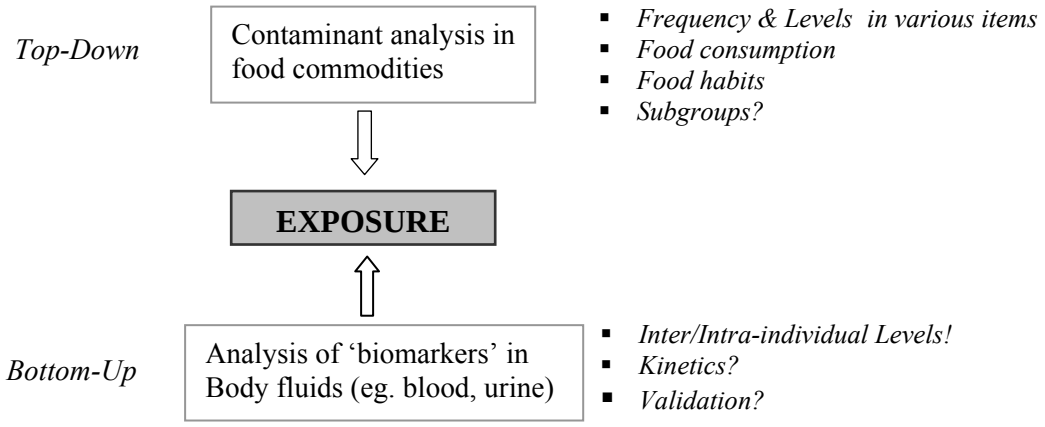

Fig. 1 An illustration of two approaches used to estimate human exposure to undesirable compounds - such as mycotoxins.

Human biomonitoring studies may require the sampling of blood for subsequent biomarker analysis. Analysis of blood plasma or serum provides useful data on internal circulating biomarker levels, but blood sampling is invasive and requires medical personal. Non-invasive sampling of body fluids (e.g. urine, breast milk or saliva) is easier to perform in field studies and often better accepted by the participants. Breast milk can be obtained only from nursing mothers, yet is of interest with respect to infant exposure to mycotoxins. Urine is the most widely chosen option for mycotoxin biomarker analysis as sampling is non-invasive and can be used for biomonitoring in all groups of the population. Suitable methods have become available to determine the presence of important mycotoxins and their metabolites in human biological fluids (Cano-Sancho et al. 2011; Turner 2012; Wild & Gong 2010).

1.3 Background on mycotoxin occurrence in Bangladesh

Bangladesh has a subtropical monsoon climate, characterized by seasonal variations in rainfall, temperature and humidity (http://en.wikipedia.org/wiki/Geography_of_Bangladesh). Three seasons are usually recognized: a hot, humid summer (32-40^{o}C) from March to June; a humid rainy monsoon season (28-35^{o}C) from Juli to October; and a warm, dry winter (15-25^{o}C) from November to February. Agriculture in Bangladesh is heavily dependent on the weather and crops are mainly produced in summer and in winter. Food stored in one season (e.g. summer) is largely consumed in the next season until new seasonal (e.g. winter) crops become available (Ali et al. 2015d). Humidity at harvest and in storage can favour the growth of a variety of mycotoxin producing moulds on agricultural products.

Various food commodities, including those consumed by the local population, such as rice and wheat as staple food, pulses, oilseeds, tuber and root crops, are susceptible to contamination by fungi and mycotoxins. Surveys in Bangladesh document the presence of aflatoxins, ochratoxin A, deoxynivalenol, fumonisin B_1 and zearalenone in plant derived food (maize, cereals, groundnuts) and feed (Dawlatana et al. 2002; Dawlatana et al. 2008). In addition to these key commodities, also consumption of eggs, meat and milk can contribute to human mycotoxin exposure when livestock has been fed with contaminated materials. With regard to the latter, reports indicate variable mycotoxin contaminations of dairy feed and forages from Bangladesh (Phillips et al. 1996; Al-Mamun et al. 2002). This contamination can result in carry-over to animal products. A more recent investigation in Bangladesh reported the presence of AFB_1 in some food commodities and in poultry feed, at levels above EU regulatory limits (Roy et al. 2013). Overall, this data inform on an occurrence of certain mycotoxins, but do not allow to conclude on the extent of human exposure (see section 1.2).

1.4 Background on four major mycotoxins (AFB_1, OTA, CIT and DON)

1.4.1 Aflatoxin B_1

1.4.1.1 Physiochemical properties of AFB_1

Aflatoxin B_1 ($C_{17}H_{12}0_6$, MW 312) is crystalline compound, soluble in moderately polar solvents such as methanol, chloroform, methanol and dimethylsulfoxide, and dissolve in water to the extent of 10-20 mg/litre. AFB_1 fluoresce under UV radiation. The crystalline form of AFB_1 is stable in absence of light and particularly UV radiation, even at temperature over 100^{o}C. Chemically AFB_1 is a highly substituted coumarin structure with a fused dihydrofurofuran moiety (Kensler et al. 2011). Aflatoxin M_1 ($C_{17}H_{12}0_7$, MW 328), one of the hydroxylated metabolites of AFB_1, is found in milk of mammals that ingested AFB_1 and also excreted in urine. Chemical structures of AFB_1 and its metabolites are depicted in Fig. 2 (p.5)

1.4.1.2 Toxicity and kinetics of AFB_1

Toxicity: Aflatoxin B_1 (AFB_1) is the most prevalent aflatoxin and a potent liver carcinogen in various species, including humans (IARC group 1). AFB_1 exerts serious toxic effects with a range of consequences: i) large doses lead to acute toxicity and death ii) chronic subletal doses induce tumors, and also impair growth and immunologic functions (EFSA 2007). Acute

toxicity of AFB_1 has been well demonstrated in experimental animals: The most susceptible species are rabbits, and ducks while chickens and rats have greater tolerance (EFSA 2007). AFM_1 the metabolite of AFB_1, has lower potency, but is also a genotoxic agent (FAO/WHO 2001). The degree of AFB_1 toxicity depends on age (young animals being more sensitive than adults), as well as sex and nutritional status.

Kinetics: Absorption of AFB_1 in the rat small intestine is a rapid process that follows first-order kinetics, with an absorption rate constant [k(a)] of 5.84 ± 0.05 h-1 (EFSA 2007). Absorbed AFB_1 reaches the liver through the portal system and is metabolised by cytochrome P450 (CYP)-dependent mono-oxygenases in reactions involving the incorporation of an atom of molecular oxygen into the substrate (EFSA 2007).

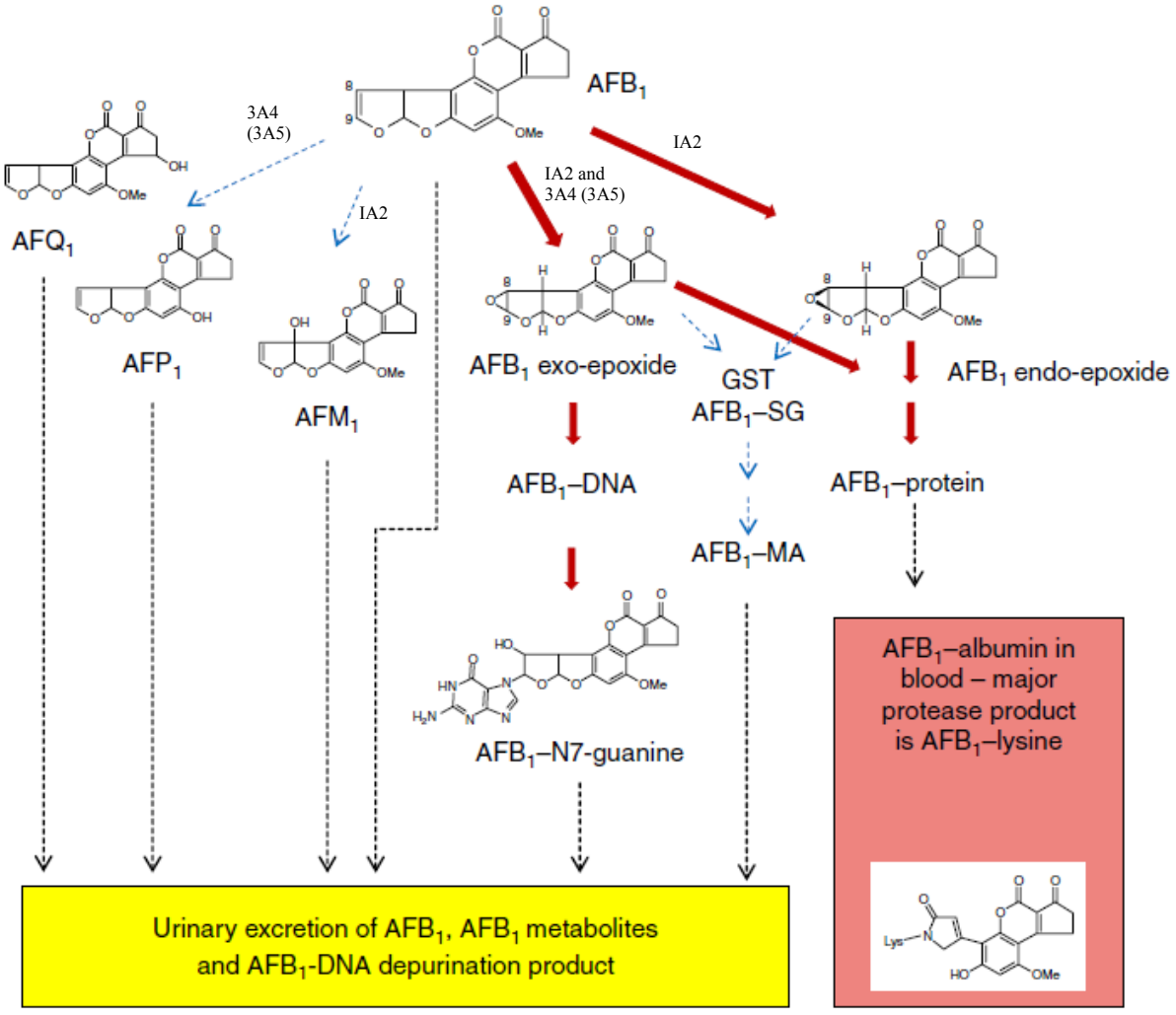

Fig. 2 Aflatoxin B_1 metabolism and biomarkers. Me, methyl; GST, glutathione S-transferase; SG, glutathione; MA, mercapturic acid; → epoxide-related toxicity pathways; ····>, non-epoxide-related toxicity pathways; - - -> excretion or blood routes (Turner et al. 2012).

The major CYP enzymes involved in human AFB_1 metabolism in liver are CYP3A4, 3A5 and 1A2 (Wild and Turner 2002), while the lipoxygenases and prostaglandin H synthase appear to be important in its biotransformation in lung (Dohnal et al. 2014). Generally, there is some diversity in the metabolism of AFB_1 among different animal species (Wu et al. 2009). AFB_1 metabolism involves several pathways that are depicted in Fig. 2.

The most critical reaction is bioactivation to (endo-, exo-) AFB_1-8,9-epoxide, highly reactive metabolite which covalently binds to DNA and induces mutations or forms adducts with proteins. The GST-catalyzed conjugation of AFB_1-8,9-epoxide is an important detoxication reaction (Degen and Neumann 1981; Hengstler et al. 1999). Other reactions are O-dealkylation to AFP_1, hydroxylation to AFM_1 and AFQ_1, or ketoreduction to AFL. Aflatoxin B_1 metabolites can serve as biomarkers of human exposure to this mycotoxin: AFM_1, AFQ_1 and AFP_1 have been detected in human urines as well as AFB_1-N7-guanine, the depurination product of the DNA adduct. Moreover, the AFB_1-albumin adduct in blood or AFB_1-lysin (Groopman et al. 2014; Kensler et al. 2011) are useful biomarkers of exposure (Fig. 2).

1.4.1.3 Human exposure to AFB_1

Humans are exposed to AFB_1 by consumption of contaminated dietary staples, including maize and groundnuts. AFB_1 contaminated commodities are a major problem in developing countries where hot and humid climate favours fungal growth and where food storage conditions are poor (Groopman et al. 2008; Williams et al. 2004; Wild and Turner 2002). Aflatoxin B_1 the most prevalent aflatoxin, is a major cause of human disease in several parts of the world including, Asia and Sub-Saharan Africa (Williams et al. 2004; Wild and Gong 2010; Kensler et al. 2011). The population in low-income countries often cannot afford to discard even visibly spoiled food or they use it as animal feed. Then, the transfer of AFB_1 and its similarly toxic metabolite AFM_1 into milk of lactating animals and in human breast milk will add to the risk of exposure for the youngest and most susceptible part of the population (Degen et al. 2013).

Several epidemiological studies investigated urinary aflatoxin M_1, aflatoxin-N7-guanine and serum aflatoxin-albumin/lysin adduct as biomarkers of AFB_1 exposure in human populations with an increased risk of hepatocellular carcinoma (Wogan et al. 2012; Turner et al. 2012). More recent study showed dose-response relationships between early (pre- and postnatal)

aflatoxin exposure and growth retardation of infants in Gambia, Benin and Togo (Turner 2013). In many biomonitoring studies, urinary AFM_1 has also served as a biomarker of AFB_1 exposure in human population in different countries (see discussion section Table 12).

1.4.2 Ochratoxin A

1.4.2.1 Physiochemical properties of OTA

Ochratoxin A ($C_{20}H_{18}O_6NCl$, MW 403) is a weak organic acid (pKa 4.4 for the carboxylic acid group, and pKa 7.3 for the phenolic group). At acidic and neutral pH, OTA is soluble in polar organic solvents (acetone, methanol, chloroform), slightly soluble in water and insoluble in petroleum ethers and saturated hydrocarbons. OTA is soluble in aqueous sodium bicarbonate solution and other alkaline solutions. In acidic and alkaline solutions, OTA shows green and blue fluorescence, respectively. The absorption maxima in ethanol (95%) are 332 nm and 212 nm; the extinction coefficient at 332 nm is 5500 M^{-1} cm^{-1} in methanol (Kuiper-Goodman & Scott 1989). The metabolite OTα ($C_{11}H_9ClO_5$, MW 256) is more polar than OTA; thus, it elutes earlier than OTA in reverse phase HPLC. Due to the dihydroiso-coumarin structure, OTα shows also fluorescence (highest at 450 nm). A pKa value for OTα is not available in the published literature; based on a structural similarity to 2-hydroxy-benzoic acid (with pKa 3.0) OTα is expected to have more acidic properties than OTA. The structure of OTA and its major metabolites are depicted in Fig. 3 (p.8).

1.4.2.2 Toxicity and kinetics of OTA

Toxicity: Kidney is the most susceptible target organ of OTA toxicity in all tested animals (Kuiper-Goodman and Scott 1989; Mally and Dekant 2009). Studies have demonstrated its carcinogenic (IARC group 2B), hepatotoxic, immunotoxic, and teratogenic effects in animals. OTA is considered as hazard for human health, and long-term human exposure to OTA may be involved in Balkan endemic nephropathy (Castegnaro et al. 2006). OTA inhibits protein synthesis indirectly, through impairing the activity of phosphophenolpyruvate carboxykinase (PEPCK). Studies in eukaryotic cell cultures and *in vivo* suggest that OTA competitively inhibits the Phe-tRNA synthase, which is the primary cause of OTA toxicity at cellular level (Creppy et al. 1979, 1980).

Kinetics: In most animal species, OTA is passively absorbed from stomach and particularly, from the proximal jejunum (Ringot et al. 2006). The absorption of OTA varies among the species: 66% in pigs, 56% in rats and rabbits and 40% in chicken (Galtier et al. 1981). Once it reaches the bloodstream, OTA (as dianion form) is bound to serum albumin, which partly explains its long-half life in human body (Il'ichev et al. 2002). The half-life of OTA in human blood is about 35.5 days, in pigs 72-120 hours and in rats 55-120 hours (Studer-Rohr et al. 2000; WHO 2001a). OTA in blood is distributed to different tissues (Kidney > liver > muscle > fat), and reabsorption, as a consequence of enterohepatic recirculation, plays an important role (Roth et al. 1988). In all species, both renal and faecal excretion play important role in plasma clearance of OTA (WHO 2001a). In kidney, OTA is mainly eliminated through tubular filtration in kidney, and faecal excretion is mainly due to biliary excretion (Ringot et al. 2006). In mammals including humans, OTA is also excreted through breast milk, and this is of importance because the milk is consumed by the young (Degen et al. 2013).

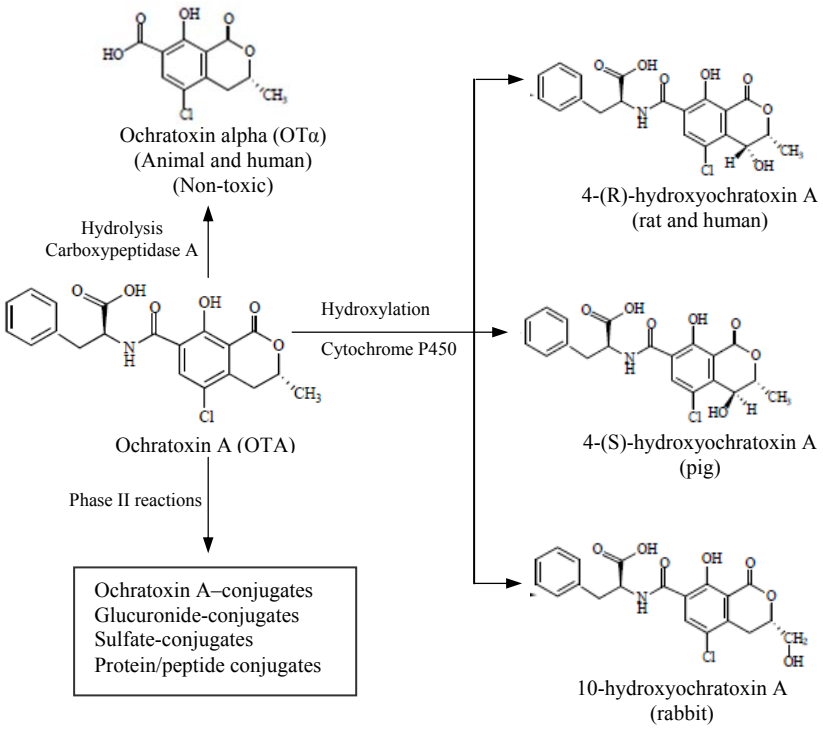

Fig. 3 Proposed metabolic pathways of ochratoxin A in animals and humans (adapted from Wu et al. 2011)

In mammalian species, OTA metabolism comprises phase I and phase II reactions (Fig. 3). Hydrolysis is the major metabolic pathway whereby a much less toxic compound OTα is produced by the cleavage of the peptide bond in OTA. Ochratoxin A hydrolysis to OTα is mediated by gastrointestinal tract enzymes (e.g. carboxypeptidase A, trypsin) and intestinal microflora (Madhyastha et al. 1992). A small fraction of absorbed OTA is converted, mainly in the liver, into 4(R/S)-hydroxy-OTA and 10-hydroxy-OTA by P450 enzymes; their toxicity is lower than that of OTA (Ringot et al. 2006). Bioactivation of OTA catalysed by cytochrome P450, prostaglandin synthase and lipooxygenase has been proposed to play a role (Pfohl-Leszkowicz and Manderville 2007), however, data supporting this view are scarce or lacking (Degen et al. 2007; Mally and Dekant 2009). Published information about phase II metabolism of OTA is limited: Glucuronide conjugates have been detected in bile of pigs and mice exposed to OTA (Roth et al. 1988, Kühn et al. 1995), whilst no conjugates were found *in vitro* with human and rat hepatocytes (Gross-Steinmeyer et al. 2002). Published information on formation of OTA conjugates in humans is contradictory: Pena et al. (2006) report indirect evidence for OTA glucuronide in human urines, but this was not confirmed in another study (Vatinno et al. 2007). More recent findings in urines of highly OTA exposed infants indicate that OTA glucuronidation can occur (Muñoz 2012). It is worth noting that the major human metabolite OTα is largely excreted in urine as conjugate (Coronel et al. 2011; Muñoz et al. 2010; Klapec et al. 2012)

1.4.2.3 Human exposure to OTA

Human are exposed through consumption of OTA contaminated foodstuffs of plant and animal origin, including cereals, pulses, raisins, spices, coffee, cacao, beer, wine, meat, meat products, or edible offal (EFSA 2006; Ostry et al. 2013). Along with OTA also citrinin, another nephrotoxic mycotoxin, has been found in various foods and in animal feed and their co-occurrence has raised concerns on possible risks for human and animal health (Peraica et al. 2008). Several biomonitoring studies have been conducted to investigate human exposure to OTA (reviewed by Scott et al. 2005; Märtlbauer et al. 2009; Duarte et al. 2011), and indicate worldwide occurrence of this mycotoxin contaminant. The occurrence and levels of OTA in biological matrices (serum/plasma and urine) of various human populations has been summarized in the discussion section (see Fig. 11 and Table 13) along with new data from the studies in Bangladeshi cohorts and a German cohort.

1.4.3 Citrinin

1.4.3.1 Physiochemical properties of CIT

Citrinin ($C_{13}H_{14}O_5$; MW 250) in solution shows fluorescence under UV light and absorption peaks at 250 nm and 333 nm (in methanol). CIT forms lemon-yellow crystals, melting point at 172^{o}C (Xu et al. 2006). CIT is sparingly soluble in water but soluble in sodium carbonate or sodium acetate, diluted sodium hydroxide, in acetonitril, methanol, ethanol and other polar organic solvents (Deshpande 2002). Solutions of CIT should be protected from light and high temperatures to avoid degradation.

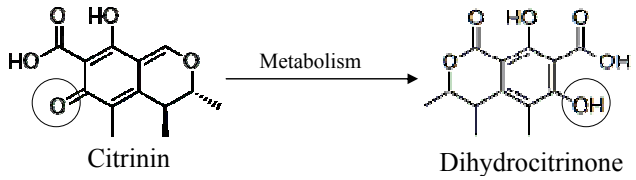

Fig. 4 Structures of citrinin and its metabolite dihydrocitrinone

1.4.3.2 Toxicity and kinetics of CIT

Toxicity: In animal studies, CIT has similar toxic properties as OTA, with kidney being also the primary target organ of CIT toxicity (Frank 1992). In animal studies, high doses of CIT can exert genotoxic, teratogenic, tumorigenic and immunotoxic effects (Frank 1992; EFSA 2012). The mycotoxin OTA is carcinogenic to rodents and has been classified as group 2B human carcinogen, whereas CIT is in group 3 due to limited evidence for carcinogenicity in experimental animals (IARC 1986, 1993).

Kinetics: Information on CIT kinetics is limited. In animal studies, after *i.v.* administration of 3 mg/kg body weight in rats, CIT was found in liver (15%) and kidney (6%); about 74% of the ingested dose was excreted in 24 h urine (Phillips et al. 1979). CIT was detected in serum after oral application to rats (Frank 1992), and there is new evidence that CIT binds to serum albumin (Poór et al. 2015). Dihydrocitrinone (HO-CIT) was first identified as minor metabolite of CIT in urines of rats (Dunn et al. 1983). In a recent study with a human volunteer who ingested a small CIT dose (45 ng/kg b.w.) about 12% of CIT was excreted in

24h urine as parent compound, but 24% as metabolite HO-CIT (Ali et al. 2015f). This observation is of interest since formation of the metabolite HO-CIT is considered as detoxication reaction (Föllmann et al. 2014). It is presently unknown which enzymes catalyze this reaction, and the ability to convert CIT to HO-CIT may vary among individuals. Thus, the sum of both analytes ('total CIT') should be considered as biomarker of exposure in human biomonitoring studies.

1.4.3.3 Human exposure to CIT

Human are exposed to CIT through mycotoxin contaminated food. Since CIT has been detected along with OTA in food and feed, their co-occurrence has raised concerns with regards to possible combined adverse effects on health of humans and animals (EFSA 2006; EFSA 2012; Ostry et al. 2013). CIT was suspected to be involved together with OTA in Balkan endemic nephropathy (Castegnaro et al. 2006; Peraica et al. 2008; Pfohl-Leszkowicz 2009). In contrast to OTA, only few biomonitoring studies have been carried out to assess CIT exposure in human populations. This is partly due to the fact that first sensitive methods for CIT biomarker analysis have been established only recently (Blaszkewicz et al. 2013). Data on CIT biomarker analyses in human urines are summarized in discussion (Table 14).

1.4.4 Deoxynivalenol

1.4.4.1 Physiochemical properties of DON

Chemically DON ($C_{15}H_{20}O_6$; MW 296.3 g/mol) is a type B trichothecene. DON crystallizes as colourless needles, has a high temperature tolerance (stable at 120ºC, moderately stable at 180ºC) and is soluble in water and in some polar solvents, including aqueous methanol, acetonitril, and ethyl acetate (EFSA 2004). The structure of DON and its metabolite DOM-1 are depicted in Fig. 5 (p. 12).

1.4.4.2 Toxicity and kinetics of DON

Toxicity: Consumption of DON contaminated feed has been associated with a number of adverse effects in animals, including feed refusal, vomiting (thus the synonym 'vomitoxin', diarrhea, dizziness and fever, (Rotter et al. 1996). Chronic exposure to DON can lead to growth faltering, immunological and neurological dysfunction (Pestka and Smolinski 2005).

Susceptibility of animals to DON can be ranked as follows: swine > mice > rats > poultry ≈ ruminants (Pestka and Smolinski 2005). The acute effects of DON in humans are similar to those seen in animals and chronic dietary DON exposure in animals' causes altered nutritional efficiency (EFSA 2013), whilst long term effects in humans were not established so far. Toxicity of DON involves inhibition of protein synthesis, and it activates a signaling pathway known as ribotoxic stress response in cells and induces apoptosis (Pestka and Smolinski 2005). Moreover, effects include altered neuroendocrine signaling, impaired gut integrity and immune function (Pestka 2010).

Kinetics: DON is rapidly absorbed and oral bioavailability is estimated to reach 55 % in pigs (Rotter et al. 1996). Organ distribution was measured in pigs only following a single intravenous injection of DON (1 mg/kg b.w.) and revealed high initial concentrations in plasma, kidney and liver (EFSA 2004). The plasma elimination half-life of DON was found to vary between 1.2 and 3.9 hours in pigs from various studies (EFSA 2004).

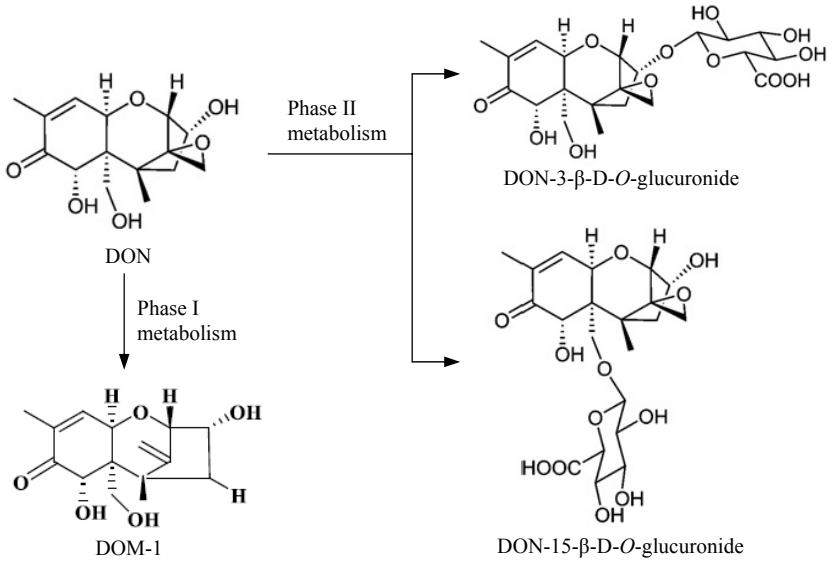

Fig. 5 Structure of DON, its metabolite DOM-1 and glucuronide isomers (modified from Warth et al. 2012)

DOM-1 is the primary metabolite (phase I metabolism) of DON found in urine and feces of DON exposed animals (Pestka and Smolinski 2005). DON is conjugated with glucuronic acid (phase II metabolism) in liver and its metabolites (DON-3ß-Glu and DON-15ß-Glu) can be found in human urine (Fig. 5). After DON intake about 68-72 % of these DON biomarkers (aglycone and conjugates) are excreted in urine within a day (Warth et al. 2013, Turner et al. 2010b). DOM-1 is also excreted as glucuronide, but only at rather low levels in human urine.

1.4.4.3 Human exposure to DON

DON is the most frequently detected mycotoxin contaminant of maize, wheat and barley in temperate regions of the world (WHO 2001b; EFSA 2013). Human are exposed to DON through consumption of various contaminated food commodities. DON biomarker occurrence and levels have been analyzed in urines from the general population of some Asian, European, African and American countries (see discussion section Table 15).

1.5 Scope and aims of this work

To protect consumers, developed countries have established regulations and limit values for the most important mycotoxins in food and feed in order to minimize dietary exposure. However, in developing countries like Bangladesh there is no regular surveillance for contaminants, although there is now awareness of this deficit (Islam & Hoque 2013). Data available from small surveys indicate the presence of some mycotoxins in certain crops and in animal feed in Bangladesh (Dawlatana et al. 2002; Dawlatana et al. 2008; Roy et al. 2013), but are insufficient to assess mycotoxin exposure in the population. As biomonitoring (analysis of parent compounds and/or metabolites in human samples) covers intake from all dietary sources, this approach was used to investigate mycotoxin exposure in inhabitants of Bangladesh and compare it with the situation in Germany and other countries.

My thesis project on biomonitoring was designed and conducted with specific aims. These aims were accomplished by a stepwise strategy: An initial determination of analyte patterns ('screening') in urines by means of an LC-MS/MS based multi-mycotoxin method for biomarkers of exposure served to select four major mycotoxins of interest, namely aflatoxin B_1 (hepatocarcinogen), ochratoxin A and citrinin (nephrotoxins), and deoxynivalenol (emetogen, immunotoxic). Then an in-depth analysis by more sensitive tailored methods was conducted for AFM_1 (metabolite of AFB_1), OTA and metabolite OTα, CIT and metabolite HO-CIT, and DON and its metabolites, to determine the concentration ranges in samples (urines) collected from Bangladeshi cohorts and in those from a German cohort.

Another aim of this study was to gain insight into the variability of mycotoxin exposure among the Bangladeshi population. Therefore, urines were collected from cohorts in two districts of the country, and residents of rural and urban areas with presumably some

differences in food habits. Moreover, Bangladesh has a subtropical monsoon climate, characterized by clear seasonal variations in rainfall, temperature and humidity, conditions which favour the growth of certain fungal species on crops and stored food commodities. Thus, urines were collected from two cohorts in summer and winter season for mycotoxin biomarker analysis. Finally, biomarker-based estimates of mycotoxin exposure ranges in Bangladeshi and German cohorts were compared in a broader context with data reported for other populations of the world and also discussed with regard to possible risks associated with an exposure to the mycotoxins examined.

2. Materials and methods

2.1 Standards, chemicals and reagents

Chloroform, Methanol (HPLC grade), isopropanol, acetic acid (96%), sodium hydrogen carbonate ($NaHCO_3$) and phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany). Acetonitrile and methanol (LC-MS grade) were from Promochem (Wesel, Germany). OTA (purity >98%) was obtained from Sigma–Aldrich (Taufkirchen, Germany), and OTα standard (purity 98.9%) from Biopure (Tulln, Austria). CIT standard material (purity >98%) was purchased from Sigma-Aldrich (Taufkirchen, Germany). The CIT metabolite dihydrocitrinone HO-CIT (purity 98.9%) was from AnalytiCon Discovery GmbH (Potsdam, Germany). AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1 standard solution was from Sigma-Aldrich Chemie (Taufkirchen, Germany). The β-glucuronidase/arylsulfatase (β-Gluc/ArylS) enzyme from *Helix pomatia* was purchased from Roche (Mannheim, Germany). The aflatoxin M_1 ELISA kits were purchased from Helica Biosystems Inc., Santa Ana, CA 92704, USA. IAC columns i.e. AflaTest® WB^{SR}, $AflaM_1$ HPLC, CitriTest® and DON Test WB HPLC were purchased from Ruttmann, Hamburg, Germany.

2.2 Study populations and sampling areas

Bangladesh: In total 164 adult urines were collected (n = 69 in summer May 2013, n = 95 in winter February 2014) from residents of a rural (Mongol Para, Puthia) and an urban area (Rajshahi University region) of Rajshahi district in Bangladesh, among which there were 62 participants enrolled in both sampling periods (Ali et al. 2015b). In between February to

March 2014 (winter) urine samples were also collected from pregnant women (n = 54) of a rural and a suburban area of Savar region in the Dhaka district of Bangladesh (Ali et al. 2015e). In June 2013, blood samples (n = 64) were collected from healthy bachelor students at Gonoshasthaya Samaj Vittik Medical College and Hospital, Savar, Dhaka-1344, Bangladesh (Ali et al. 2014). The sampling sites are indicated in Fig. 6. All the participants were informed about the study and a written consent was obtained from them prior to inclusion in the study. The participants were of good health and they were asked to fill a short questionnaire for anthropometric information (age, gender, height, and weight), occupation and daily, weekly and normal food habits. Foods consumed by the study subjects two days prior of sample collection were also recorded in the questionnaire.

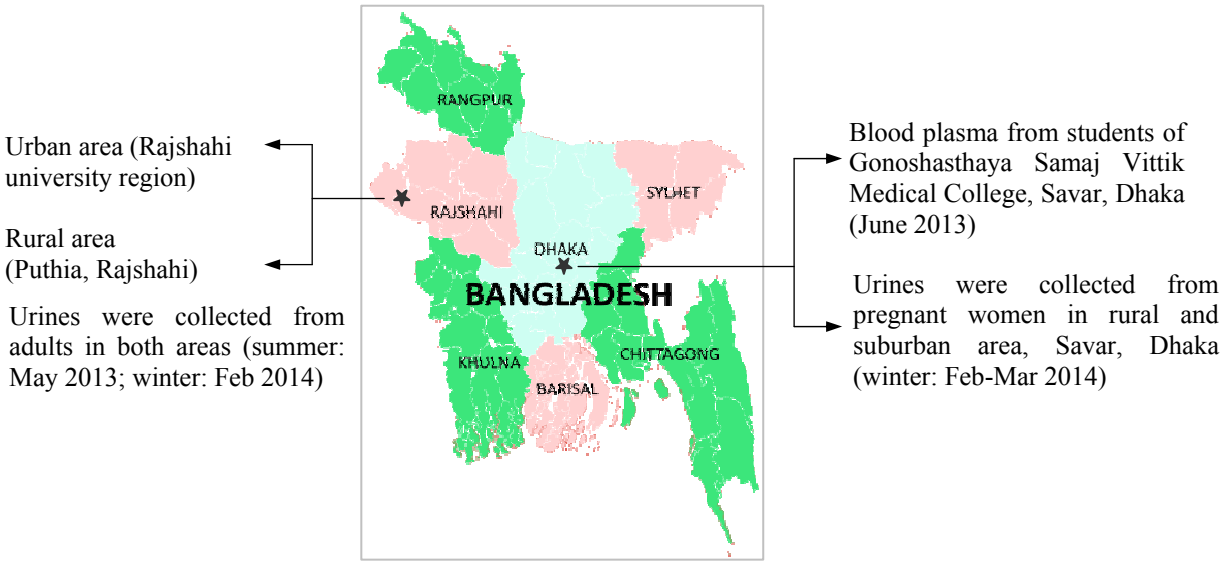

Fig. 6 Map of Bangladesh and selected sampling sites in Rajshahi and Dhaka district. Source: https://www.google.de/search?q=map+of+bangladesh

Approximately 50 mL first morning urine samples were collected into a non-sterile disposable container and blood samples (5-7 ml) were from each individual by venipuncture into EDTA containing tubes. The collected samples were first stored at -20^{o}C at Rajshahi University and sent on dry ice to IfADo, Dortmund for subsequent biomarkers analysis. Urinary creatinine was determined by a modified Jaffe method (Blaszkewicz and Liesenhoff 2012) to control the differences of urine dilution between individual spot urines. This study was approved by the Institute of Biological Sciences of Rajshahi University, Rajshahi-6205, Ethics Committee of

Gonoshasthaya Samaj Vittik Medical College, Dhaka-1344, Bangladesh and by the Institutional Internal Review Board of IfADo, Dortmund, Germany.

Germany: In 2013 (July-August) IfADo employees (n = 50) who participated gave their written consent before inclusion in the study. First morning urine samples were collected into a non-sterile disposable container (approximately 80 mL) and immediately stored at -20^{o}C until biomarkers analysis. All the volunteers were asked to fill a short questionnaire for their baseline anthropometric information (age, gender, height, weight and occupation).

2.3 Demographic information of the study populations

Bangladesh: Among the rural and urban adult cohorts in Rajshahi district, the number of male and female participants were equal. The mean age of our volunteers was 39 ± 11 years (range 22–60 years) with no significant difference in gender or regions. The average body mass index (BMI) of the urban cohort was significantly higher (23.8 ± 3.2 kg/m^2) than that of the rural (21.4 ± 2.6 kg/m^2) cohort (Ali et al. 2015b). The mean age of the pregnant women cohort in Dhaka district was 25 ± 5 years ranging from 18 to 36 years with no significant difference between rural and suburban residents. The average BMI for all subjects was 20.1 ± 3.0 kg/m^2. Volunteers of the rural cohort had significantly higher BMI (21.2 ± 2.3 kg/m^2) than those in the sub-urban (18.6 ± 3.2 kg/m^2) cohort (Ali et al. 2015e). For student cohort in Dhaka, the mean age was 20.2 ± 0.8 years and mean BMI was 22.0 ± 3.3 kg/m^2 (Ali et al. 2014). All the participants consumed typical Bangladeshi food. Details on baseline characteristics of all cohorts are given in above publications.

Germany: Among 50 volunteers, 54% were female, and 46% were male participants. The mean age of all participants was 38.5 years, with no significant difference between females and males. The average BMI for all subjects was 25.2 ± 3.8 kg/m^2. The participants consumed typical mixed German food. They are all employees at IfADo (scientists, technicians, office workers, etc.) residing in Dortmund or nearby urban settlements. Details on basic anthropometric characteristics of this cohort are given in Ali et al. 2015c.

2.4 Biomarker analysis

An LC-MS/MS based multi-mycotoxin method suited to detect 23 analytes was applied for an initial determination of patterns of biomarkers in urines collected from cohorts living in three

different continents (Gerding et al. 2015). Based on occurrence of certain mycotoxin biomarkers, *i.e.* AFM_1, OTA, CIT and DON metabolites in urines from a Bangladeshi and/or a German cohort, then 'tailored' methods were used to determine biomarker occurrence and concentration ranges in all study cohorts from Bangladesh and a German 'reference' cohort. Analysis by 'tailored' methods involves sample clean-up to enrich urinary analytes and in some cases also enzymatic hydrolysis of glucuronide/sulfate metabolites. It is thus more labor-intensive than the 'dilute-and-shoot' approach applied for the initial screening, but also more sensitive thereby resulting in higher frequencies of 'positive detects' which facilitates subsequent statistical analysis, *e.g.* for group comparisons.

2.4.1 Aflatoxin M_1

2.4.1.1 AFM_1 determination by ELISA

Aflatoxin M_1 (AFM_1) levels in urine were determined using enzyme-linked immunosorbent assay (ELISA) according to the procedure specified in the method protocol. In brief, both the AFM_1 standards (provided in the kit) and the urine samples were diluted with distilled water (1:20 or 1:5); 100 µL of each were mixed with 200 µL assay buffer. Then 100 µL of this mixture was transferred to antibody coated microtiter well, and the plate incubated at RT for 1 hour. An automated microplate washer (Tecan Hydro Flex, Salzburg, Austria) was then used for washing the plate with washing solution (supplied with the kit). AFM_1 conjugate (100 µL) was added to each well and incubated at RT for 15 min; then the plate was washed again with washing solution to remove the unbound conjugate. Substrate reagent (100 µL) was added to each well and the colour reaction was allowed to proceed in the dark for 15 min at RT. Then stop solution (100 µL) was added to the wells to terminate the enzyme reaction, and within 15 min the absorbance was measured at 450 nm by a microplate reader (Tecan Genios®, Salzburg, Austria). The absorption intensity is inversely proportional to AFM_1 concentration in the samples. The AFM_1 level in the samples was calculated from concurrent standard curves using AFM_1 standard solution for each plate. All urine samples were analyzed in duplicate at two different dilutions (1:5 and 1:20) and average values were calculated. For method validation, blank urines (from Germany) were spiked with different concentrations (20 to 60 pg/mL) of AFM_1 standard; the recovery rate was in the range of 85 to 115%, and the method detection limit (MDL) was determined to be 30 pg/mL. More details have been described in Ali et al. 2015a.

2.4.1.2 AFM_1 determination by HPLC-FD

Sample preparation: Immunoaffinity column (AflaTest® WB^{SR} and $AflaM_1$ HPLC: Vicam® from Ruttmann, Hamburg, Germany) were used for urine clean-up and enrichment of the analyte. In brief, urine samples were thawed and centrifuged at 3500 g for 10 min at 20°C. Then 10 mL of urine was adjusted with 1 N hydrochloric acid or 1 M sodium hydroxide to pH between 5.5 to 7.0, followed by a centrifugation step as described above. The sample was then loaded on AflaTest® WB^{SR} or $AflaM_1$ HPLC at a flow rate of 1 drop/s. Columns were washed twice with 10 mL distilled H_2O, then AFM_1 was eluted (flow rate 1 drop/s) with 2 mL of methanol. The eluate was evaporated to dryness under a stream of nitrogen at 45°C, and the residue was dissolved in 500 µL of acetonitrile/water (25:75), vortexed and centrifuged at 12000 rpm for 3 min prior to HPLC-FD analysis. Thus the enrichment factor was 20.

HPLC–FD analysis: AFM_1 analyses were carried out with a HPLC Shimadzu system consisting of two LC-10AS pumps, RF-10Axl fluorescence detector, SIL-10AD Vp auto injector, CBM-20A communication module, and Shimadzu LC solution software. A Microsorb-MV 100 C_{18} column (150mm×4.6mm, 5 µm; R0086200D5, Agilent Technologies, 76337 Waldbronn, Germany) fitted with a C_{18} metaguard column (10x4.6 mm, Microsorb A104MG) was used for separation of the analytes at a column temperature of 25°C. Chromatographic separation was obtained by an isocratic system with a mobile phase of 25% acetonitrile and 75% water. The flow rate was 1 mL/min and the injection volume was 80 µL. The fluorescence detector was set at 360 nm excitation and 440 nm emission wavelengths. The retention time for AFM_1 was 7.9.

Validation of parameters: The calibration curve with pure standards in mobile phase showed linearity in the range of 50-5000 pg/mL for the analyte with coefficient of determination (R^2) of 0.9998. Analysis of AFM_1 spiked urine yielded similar data for linearity in the range of LOD–1000 pg/mL. The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the lowest quantity of analyte that can be clearly distinguished from the background (LOD; S/N = 3) or quantified (LOQ; S/N ≥ 10). For AFM_1, the LOD was 1.7 pg/mL and LOQ was 5 pg/mL. Recovery assays were performed in urine with no measurable background of AFM_1. Recovery of analyte was assessed at three concentration levels in spiked samples (n = 4) with mean value of 90% for AflaTest® WB^{SR} and 88% for $AflaM_1$

HPLC columns (Table 2). The intra-day and inter-day repeatability at a spike concentration of 50 pg/mL (n = 10) were determined using also the mycotoxin-free urine, and showed acceptable precisions for the measurements (Table 3). Chromatograms of spiked urine with AFM_1 standard and a natural AFM_1 contaminated urine sample are presented in Fig. 7.

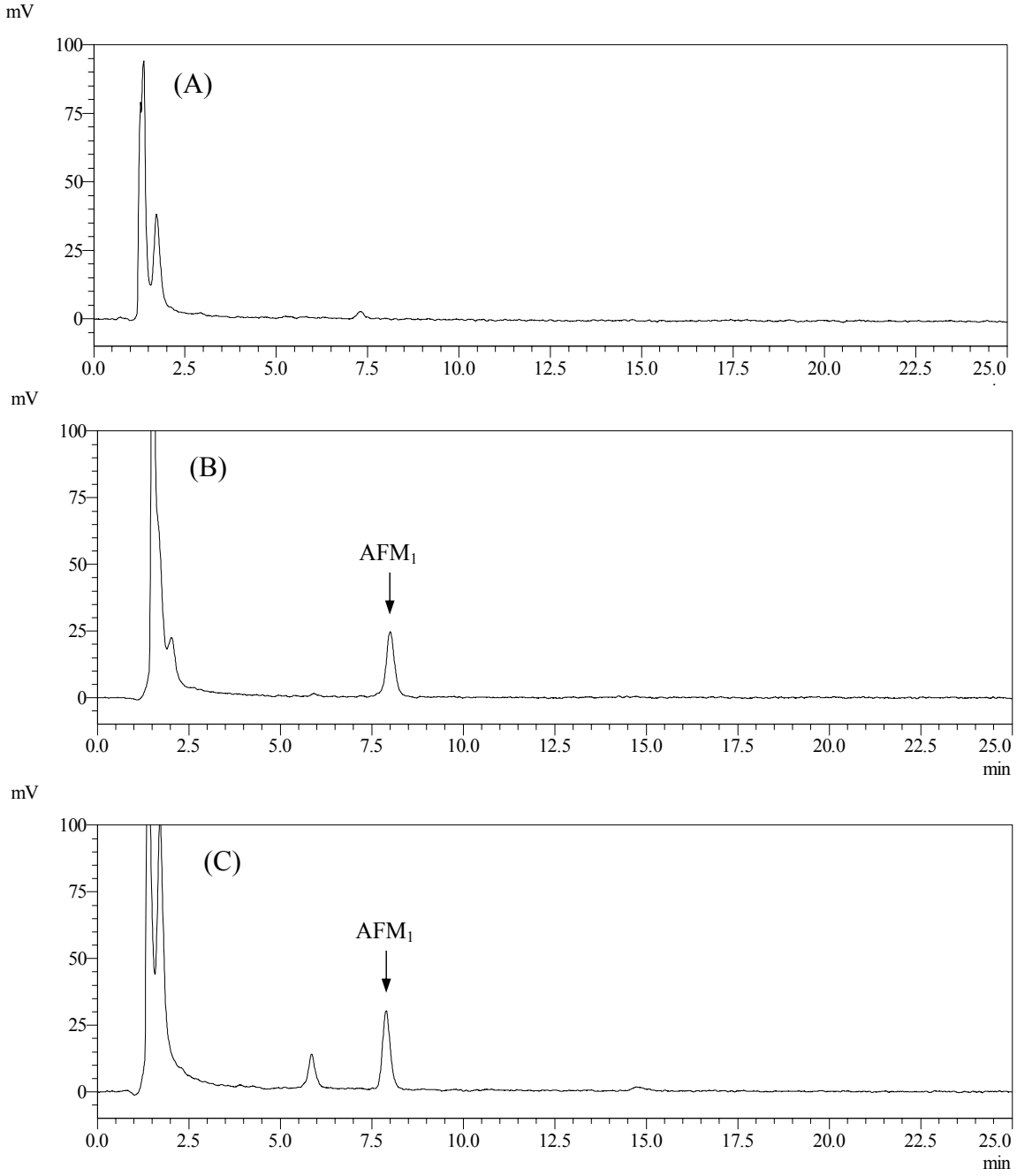

Fig. 7 HPLC chromatograms of (A) a blank urine (B) a urine spiked urine with AFM_1 standard at 50 pg/mL (C) a naturally AFM_1-contaminated urine sample (56 pg/mL) from rural male volunteer.

Table 2 Recovery of AFM_1 using two types of IAC columns in human urine

Spike level (pg/mL)	AflaTest(R) WB^{SR}		$AflaM_1$ HPLC	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
10 (n = 4)	92	4.2	88	6.2
20 (n = 4)	88	5.2	86	4.8
100 (n = 4)	90	3.4	90	4.2

Table 3 Intra-day and inter-day assay repeatability for AFM_1 in urine using two types of IAC column

Spike level (pg/mL)	AflaTest(R) WB^{SR}			$AflaM_1$ HPLC		
	Mean ± SD (pg/mL)	Recovery (%)	RSD (%)	Mean ± SD (pg/mL)	Recovery (%)	RSD (%)
Intra-day (50) n = 10	0.46 ± 0.02	92	4.3	0.43 ± 0.03	86	6.9
Inter-day (50) n = 10	0.44 ± 0.03	88	6.8	0.45 ± 0.02	90	4.4

2.4.2 Ochratoxin A

OTA and its metabolite OTα were measured by a validated method (Muñoz et al. 2010) which involves liquid–liquid extraction (LLE). Briefly, 3 mL urine or 0.5 mL plasma sample was enzymatically treated by adding 250 µL or 100 µL hydrolysis buffer (pH 5.0) and 40 µL and 10 µL of β-Gluc/ArylS and incubation at 37^{o}C overnight, respectively. Then, the hydrolyzed urine or plasma sample was mixed with 3 mL of 1% $NaHCO_3$, and pH was adjusted to 2.8-3.0 with 1M H_3PO_4. Then 3 mL or 2 mL chloroform/isopropanol (97:3) mixture was added to hydrolyzed urine and plasma samples respectively, and kept on a rotary mixer for 20 min, followed by centrifugation at 4500 rpm for 20 min at 20^{o}C. The aqueous upper layer was discarded and 2 mL (for urine) or 1 mL (for plasma) of the organic layer was transferred with a pipette to a vial and evaporated to dryness under a stream of nitrogen at 45^{o}C. The residue was dissolved in 500 µL or 250 µL of methanol/water (1:1) respectively. The vial was vortexed, centrifuged and the extract was filtered through a 0.45 µm pore size Teflon syringe filter (WICOM, Germany) before HPLC-FD analysis. Then analysis of OTA and its metabolite OTα was carried out by high performance liquid chromatography with fluorescence detection (Ali et al. 2014; Ali et al. 2015b).

2.4.3 Citrinin

Urinary citrinin and its metabolite dihydrocitrinon (HO-CIT) were measured after immunoaffinity column (IAC) cleanup for enrichment of analytes according to Blaszkewicz et al. (2013): Five mL of urine was diluted with 5 mL of 1 mM acetic acid in water and mixed for 15 min on a rotary shaker. The sample was then loaded on a CitriTest® column at a flow rate of 1 drop/s. The column was washed twice with 5 mL distilled water, then CIT and its metabolite HO-CIT were eluted (flow rate 1 drop/s) using 4 mL of methanol. The eluate was evaporated to dryness under a stream of nitrogen at 40°C, and the residue was dissolved in 500 µL methanols, vortexed and filtered through a 0.45 µm pore size Teflon syringe filter (WICOM GmbH, Germany). The urine extract was then measured by a validated LC/MS-MS method as described elsewhere in more detail (Blaszkewicz et al. 2013; Ali et al. 2015b,c).

2.4.4 Deoxynivalenol

Urine samples were hydrolyzed as described above (section 2.4.2) to convert glucuronides of DON and DOM-1 to their aglycone form. Then clean-up and enrichment of analytes was performed by immunoaffinity column (IAC) extraction with DONTest™ (Ali et al. 2015e). Briefly, the column was rinsed with 1 mL of water and then the entire hydrolyzed urine sample was loaded on a DON Test™ column at a flow rate of 1 drop/sec. The column was washed with 5 mL distilled water, then DON was eluted (flow rate 1 drop/sec) from the column using 2 mL of methanol. The elute was evaporated to dryness under a stream of nitrogen at 45°C; the residue was dissolved in 500 µL water/methanol (90:10), vortexed and filtered through a 0.45 µm pore size Teflon syringe filter. Thus, the enrichment factor was 6. Then, DON and its metabolite DOM-1 were measured in urine extracts by a validated LC/MS-MS method as described elsewhere in more detail (Ali et al. 2015e).

2.4.5 Food consumption data

All Bangladeshi participants were asked to fill out a food frequency questionnaire (FFQ) (see Appendix) to record their daily, weekly and normal food habits and two days of food consumption prior to urine sample collection. The FFQ asked for intake of typical food

consumed by Bangladeshi people: staple food, mainly cereals such as rice, wheat, maize, and lentils as the major pulse. Chicken meat, eggs, milk and milk-based products were also included, as well as coffee and beverages. Among all foodstuffs only rice is regularly consumed by the participants one to three times in a day. Beer and wine, items of relevance for OTA intake in Europe (EC/SCOOP 2002), are usually not consumed in Bangladesh and coffee is generally consumed only by a few urban people. Assessment of food consumption was done using the score 1–4 (1 = do not consume at all, 2 = 1 time daily, 3 = 2 times daily and 4 = 3 times daily).

2.4.6 Statistical analysis

All the data was analyzed using the software IBM SPSS Statistics version 22. Descriptive analysis was used to determine mean, median and interquartile ranges of the analytes. Differences of the analytes' concentrations between the male-female cohorts, regions and seasons were analyzed by independent sample t-test. Spearman correlation coefficient (two-tailed) was used to assess the correlation of analytes with food consumption, age, gender and body mass index (BMI). One-way ANOVA was used to compare analyte concentrations in the rice consumption groups. Box plots were used to describe analyte concentration by region and season. A level of alpha 0.05 was assigned for statistical significance. More details on statistical analysis have been described in my publications (Ali et al. 2014, Ali et al. 2015a,b,c).

3. Results

3. 1 Aflatoxin M_1 as biomarker of AFB_1 exposure

3.1.1 Comparison of ELISA and HPLC-FD in AFM_1 analysis

The collected urines have been analyzed by both, an ELISA assay (MDL 30 pg/mL) and an HPLC-FD (LOD 1.7 pg/mL; LOQ 5.0 pg/mL) method. An initial analysis of 95 samples by ELISA indicated the frequent detection (46%) of AFM_1 in urines (range LOD–348 pg/mL) from rural and urban residents in Bangladesh (Ali et al. 2015a). When subsequently analysed by HPLC-FD, the detection frequency for AFM_1 was similar (42%), although the mean and maximal concentrations (Table 4) were somewhat lower in all urines than those found by ELISA. Notably, there were several urines in which AFM_1 was not detectable by ELISA, but detected by HPLC-FD analysis or *vice versa*. A good correlation (p<0.01) was found between the two methods when a number of urines (n = 62) were analyzed by both methods (Fig. 8). Apparently, our ELISA analysis overestimates the AFM_1 biomarker concentration; this can be due to cross reactivity with other aflatoxins present and/or possible matrix effects.

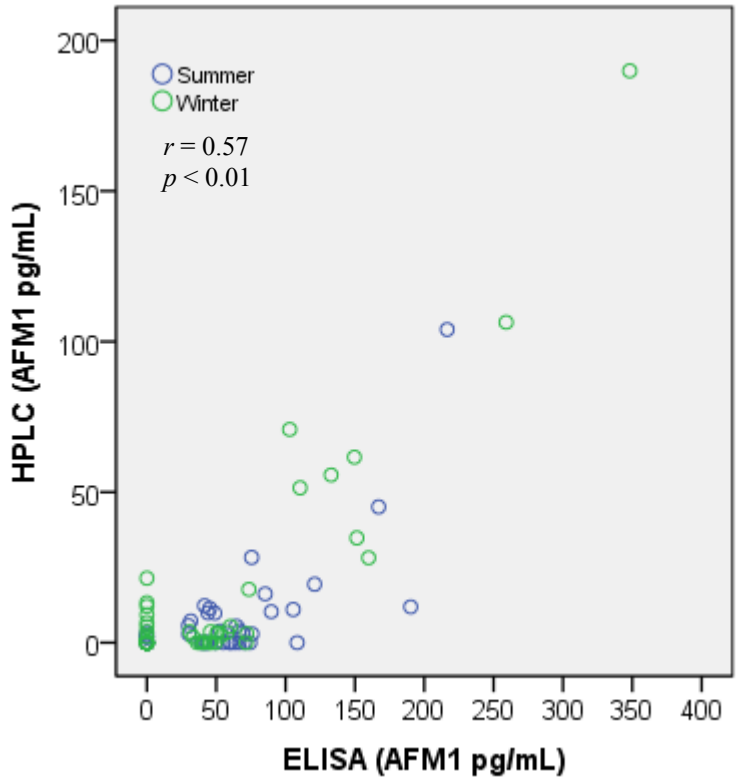

Fig. 8 Comparison of ELISA and HPLC measurements of urinary AFM_1 concentration. Spearman correlation coefficient (two-tailed) was used to assess the correlation between the two measurements.

ELISA is the most widely used screening method for measuring AFM_1 (as biomarker for human AFB_1 exposure). But, we consider HPLC-FD analysis more reliable as this method consists of a sample clean-up by IAC to enrich the analyte and provides better (more precise) quantification. Therefore, here only the more reliable HPLC-FD data are presented and in results and discussion section.

3.1.2 Bangladesh

Rural and urban adult cohort (Rajshahi): AFM_1 was detected in 40% of all urine samples at a range of 1.7–104 pg/mL in summer and 42% of samples at a range of 1.8–190 pg/mL in winter season (Table 4). The mean level of urinary AFM_1 was higher in winter (27.7 ± 42.6 pg/mL) than in summer (13.5 ± 21.2 pg/mL) season, and a significant difference (p<0.05) was observed at the mean AFM_1 level between the rural (40.4 ± 48.3 pg/mL) and the urban (3.6 ± 1.2 pg/mL) cohort (Table 4 and Fig. 9).

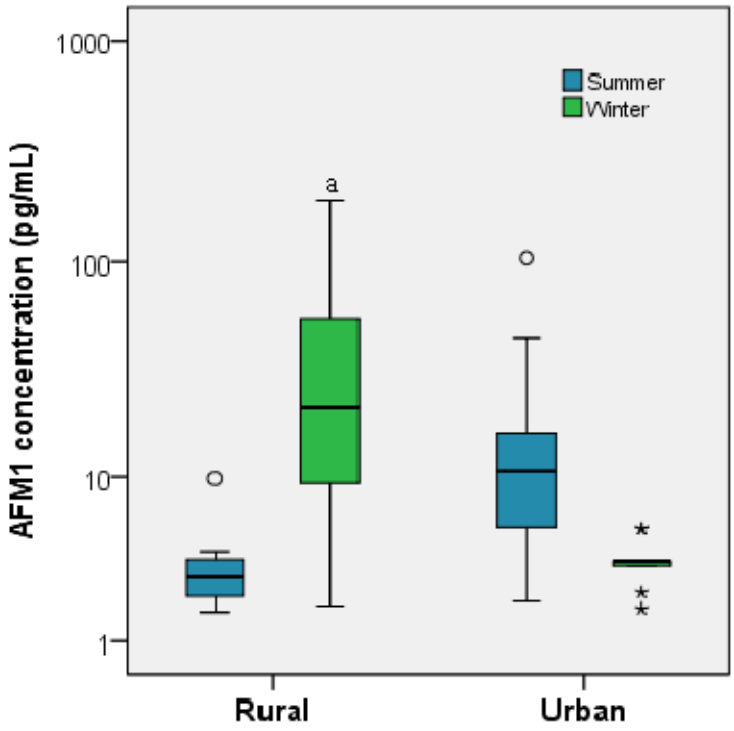

Fig. 9 Urinary level of AFM_1 in the rural and urban cohorts of Rajshahi district in summer and winter season. Only positive samples (≥ LOD) were considered for box plot preparation. [a]p<0.05 when compared to urban cohort in winter season, p-value obtained from independent sample t-test.

Table 4 Urinary levels of AFM_1 in rural and urban adult cohorts in Rajshahi district of Bangladesh

Cohorts	Gender	n	Summer				Winter			
			Positive n (%)	Mean ± SD (pg/mL)	Median (range) (pg/mL)	Mean ± SD (pg/mg crea)	Positive n (%)	Mean ± SD (pg/mL)	Median (range) (pg/mL)	Mean ± SD (pg/mg crea)
Rural	Men	15	5 (33)	2.6 ± 0.9	2.6 (1.7–4.0)	9.3 ± 5.0	10 (67)	46.3 ± 60.2	19.5 (1.9–189.9)	49.4 ± 44.6
	Women	15	2 (13)	6.6 ± 4.5	6.6 (3.4–9.8)	42.7 ± 38.6	7 (47)	32.0 ± 25.8	28.2 (3.1–70.8)	45.1 ± 38.1
	All	30	7 (23)	3.7 ± 2.8	2.9 (1.7–9.8)	18.8 ± 23.0	17 (57)	40.4 ± 48.3[a]	21.4 (1.9–189.9)	47.6 ± 40.8[b]
Urban	Men	16	9 (56)	26.1 ± 32.0	11.4 (2.9–104.0)	41.2 ± 28.6	2 (12)	4.5 ± 1.3	4.5 (3.6–5.4)	6.6 ± 0.7
	Women	16	9 (56)	8.4 ± 5.6	7.2 (2.0–19.4)	31.9 ± 21.6	7 (44)	3.4 ± 1.1	3.4 (1.8–5.4)	5.2 ± 2.5
	All	32	18 (56)	17.2 ± 24.1	10.6 (2.0–104.0)	36.5 ± 25.0	9 (28)	3.6 ± 1.2	3.6 (1.8–5.4)	5.5 ± 2.3
Both-cohorts	Men	31	14 (45)	17.7 ± 27.7	7.9 (1.7–104.0)	29.8 ± 27.6	12 (39)	39.4 ± 56.8	13.5 (1.9–189.9)	42.2 ± 43.6
	Women	31	11 (35)	8.1 ± 5.2	7.2 (2.0–19.4)	33.9 ± 23.2	14 (45)	17.7 ± 23.0	4.5 (1.8–70.8)	25.2 ± 33.2
	All	62	25 (40)	13.5 ± 21.2	7.2 (1.7–104.0)	31.6 ± 25.4	26 (42)	27.7 ± 42.6	8.0 (1.8–189.9)	33.1 ± 38.5

Positive sample refer to urines containing the analyte ≥ limit of detection (LOD: 1.7 pg/mL). Only positive samples were considered during calculation of mean and median values. [a]$p<0.05$, [b]$p<0.05$ when compared to urban cohort in winter season, p- value obtained from independent sample t-test.

Urinary AFM_1 levels did not show significant associations with consumption of certain types of food by the participants (Table 5). When the major food item rice was categorized into three groups and compared by one-way ANOVA, the higher mean AFM_1 biomarker levels were observed in the high rice consumption group only in winter season (Table 6).

Table 5 Correlation between urinary AFM_1 levels and last two days of food consumption

Food items	Summer		Winter	
	Correlation (*r)*	*p*-value	Correlation (*r)*	*p*-value
Rice	0.051	0.808	0.341	0.088
Wheat/maize	0.338	0.090	0.056	0.635
Lentil	-0.245	0.239	-0.128	0.389
Milk and Milk products	-0.198	0.344	-0.123	0.549
Chicken meat	0.201	0.336	-0.153	0.454
Groundnut	0.082	0.697	-0.114	0.325

Food consumption assessment was done using the score 1 – 4 (1 = do not consume at all, 2 = 1 time daily, 3 = 2 times daily and 4 = 3 times daily. Positive samples (≥LOD) were considered for correlation analysis. *P-value* obtained from Spearman's correlation coefficient (two-tailed).

Table 6 Urinary AFM_1 biomarker levels (pg/mL) and last two days of rice consumption

Group	Summer			Winter		
	n	Mean ± SD	Range	n	Mean ± SD	Range
Low	3	10.1 ± 6.1	1.7–16.2	2	3.6 ± 1.8	1.7–5.40
Medium	12	15.1 ± 24.4	1.8–104.0	11	23.4 ± 16.4	1.8–51.8
High	10	12.6 ± 8.7	1.7–28.3	13	34.8 ± 56.6	1.9–189.9

Low = 1 time/day, Medium = 2 times/day and High = 3 times/day rice consumption. Positive samples (≥ LOD) were considered for calculation. One way ANOVA used for analysis of biomarker level among the groups.

Pregnant women cohort (Dhaka): AFM_1 was detected in 31% of these urine samples, at a range of 1.7–141.5 pg/mL (Table 7). The mean level of urinary AFM_1 was higher in the suburban (30.2 ± 54.7 pg/mL) than in the rural (13.9 ± 33.3 pg/mL) participants (Table 7 and Fig. 10) The mean AFM1 concentration (13.9 ± 33.3 pg/mL) was similar with the level found

in adult cohorts of Rajshahi district in summer season. Urinary AFM_1 levels did not show significant associations with consumption of certain types of food by the participants.

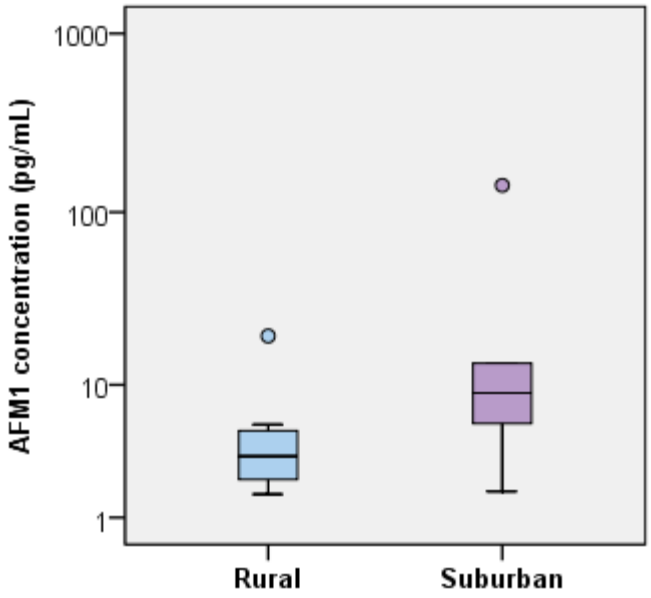

Fig 10. Urinary level of AFM_1 in pregnant women from Dhaka district of Bangladesh. Only positive samples (≥ LOD) were considered for box plot preparation.

Table 7 Urinary levels of AFM_1 among pregnant women in Dhaka district of Bangladesh

Cohorts	n	Positive n (%)	Mean ± SD pg/mL	Median (range) pg/mL	Mean ± SD pg/mg crea
Rural	32	11 (34)	5.0 ± 5.1	3.4 (1.7–19.6)	6.6 ± 4.7
Suburban	22	6 (27)	30.2 ± 54.7	9.3 (1.8–141.5)	70.4 ± 143.3
Both cohorts	54	17 (31)	13.9 ± 33.3	4.8 (1.7–141.5)	29.1 ± 86.1

Positive sample refer to urines containing the analyte ≥ limit of detection (LOD 1.7 pg/mL). Only positive samples were considered during calculation of mean and median values.

3.1.3 Germany

AFM_1 was not detected in any of the urine samples from the German cohort. This was expected, since the food consumed by the German population is usually free from AFB_1 contamination.

3.2 Ochratoxin A biomarker analysis

3.2.1 Bangladesh

Student cohort (Dhaka): OTA and its metabolite OTα were detected in 64 (100%) and 61 (95%) of all plasma samples collected from adult students of Gonoshasthaya Samaj Vittik Medical College and Hospital, Dhaka, Bangladesh (Ali et al. 2014). The average levels of OTA and OTα were 0.85 ± 1.05 ng/mL (range: 0.20–6.63 ng/mL) and 0.28 ± 0.15 ng/mL (range: LOD–0.79 ng/mL), respectively. A slightly higher OTA plasma level (0.92 ± 1.09 ng/mL) was found in male than in female students (0.78 ± 1.02 ng/mL). Also OTα mean levels were higher in male (0.33 ± 0.16 ng/mL) than female (0.23 ± 0.13 ng/mL) students. Based on OTA plasma concentrations, the dietary OTA intake was calculated by means of the Klaassen equation (conversion factor 1.97). The mean daily dietary intake of OTA for the Bangladeshi students was 11.7 ng/kg bw per week (Ali et al. 2014) which was markedly lower than the tolerable intake value of 120 ng/kg bw/week proposed by EFSA (2006).

Rural and urban adult cohort (Rajshahi): In summer OTA and OTα were in 71% and 53% in summer and in winter 98% and 62%, respectively (Ali et al. 2015b). No significant difference was found at the mean level of OTA between the rural and urban cohorts in summer. However, in rural cohort a significant difference ($p<0.05$) was found for the mean OTA level between the summer (0.05 ± 0.08 ng/mL) and winter (0.28 ± 0.52 ng/mL) season. The mean level of OTA for all participants (both cohorts) was significantly higher ($p<0.01$) in winter (0.19 ± 0.38 ng/mL) than summer (0.06 ± 0.10 ng/mL) season (Ali et al. 2015b). The metabolite OTα was present in summer urine at about 4-fold higher levels than OTA and 2-fold lower than the parent compound in winter. The ratio of OTA to OTα in the male and female subgroups of both cohorts varied considerably. The rural cohort with higher urinary levels of parent compound OTA apparently excreted also more of the non-toxic metabolite.

Pregnant women cohort (Dhaka): The OTA and OTα detection frequency were 91% and 41% in the rural and 95% and 45% in the suburban participants, respectively (Ali et al. 2016a). A significant difference ($p<0.05$) was found at the mean level of OTA between the rural (0.06 ± 0.07 ng/mL) and suburban (0.15 ± 0.19 ng/mL) cohorts (Ali et al. 2016a), whilst the mean level of OTα was quite similar between the cohorts. The ratio of OTA to OTα in both cohorts varied between individuals.

3.2.2 Germany

The OTA and OTα detection frequency were 100% and 78% in all urine samples, respectively. The mean level of OTA was slightly higher in male (0.24 ± 0.37) than female (0.18 ± 0.26) participants (Table 8). For OTα, a significant difference (p<0.05) was found between male (2.16 ± 3.60) and female (0.62 ± 0.97) volunteers (Table 8). The ratio of parent compound to it metabolite was quite variable between male female cohorts. In German adults, the urinary OTA concentration (mean 0.21, max 1.82 ng/mL) was similar to that found in the Bangladeshi adult cohorts in winter season.

Table 8 Levels of OTA and its metabolite OTα in urines from German adults

	n	Positive n (%)	Mean ± SD (ng/mL)	Median (range) (ng/mL)	Mean ± SD (ng/mg creatinine)
OTA					
Men	23	23 (100)	0.24 ± 0.37	0.14 (0.04–1.82)	0.15 ± 0.14
Women	27	27 (100)	0.18 ± 0.26	0.11 (0.02–1.42)	0.18 ± 0.27
All	50	50 (100)	0.21 ± 0.31	0.12 (0.02–1.82)	0.17 ± 0.22
OTα					
Men	23	21 (91)	2.16 ± 3.60[a]	0.97 (nd–14.25)	1.18 ± 1.78
Women	27	18 (67)	0.62 ± 0.97	0.16 (nd–3.65)	0.87 ± 2.10
All	50	39 (78)	1.33 ± 2.63	0.21 (nd–14.25)	1.01 ± 1.94

Positive sample refer to urines containing the analyte ≥ LOD; nd: level below LOD. For calculation of mean and median values, those samples that contained analyte concentration below LOD were assigned a value of one-half the detection limit. [a]p<0.05 when compared to women, p-value obtained from independent sample t-test.

3.3 Citrinin biomarker analysis

3.3.1 Bangladesh

Rural and urban adult cohort (Rajshahi): The detection frequency for CIT and HO-CIT in summer samples was 95% and 71%, and in winter 92% and 97%, respectively (Ali et al 2015b). A significant difference (p<0.05 and p<0.01) was found at the mean level of CIT and HO-CIT between the rural (0.14 ± 0.22 ng/mL, 0.78 ± 1.33 ng/mL) and the urban (0.06 ± 0.08 ng/mL, 0.08 ± 0.08 ng/mL) cohort. In winter season, only the mean level of HO-CIT showed a significant difference (p<0.001) between the rural (5.95 ± 1.63 ng/mL) and the urban (0.60 ± 1.02 ng/mL) cohort. Regarding seasonal differences, in both cohorts a significant difference (p<0.05) was found for CIT and HO-CIT levels between summer and winter season (Ali et al 2015b). The metabolite HO-CIT was present at about 5-fold higher levels than the parent compound in both seasons, indicative of HO-CIT as useful additional biomarker of citrinin

exposure. The ratio of CIT to HO-CIT in the male and female subgroups of both cohorts varied considerably. Total citrinin (CIT + HO-CIT) varies by region, being significantly higher in the rural than in the urban cohort, and a significant difference between summer (mean 0.51, max 5.70 ng/mL) and winter (mean 3.77, max 48.12 ng/mL urine) also indicates a different exposure by season (Ali et al 2015b). A provisional daily intake for CIT was calculated, and exceeded a preliminary TDI value set by EFSA 2012 (0.2 µg/kg/d) in 10% and 24% of participants in summer and winter, respectively (Table 9).

Table 9 Provisional daily intake (PDI) of citrinin (µg/kg b.w.) calculated from urine biomarker concentrations (see Methods section in Ali et al. 2015b)

Cohort and season	n	Mean ± SD µg/kg b.w	Median µg/kg b.w	Range µg/kg b.w	Exceeding TDI[c], n (%)
Bangladesh					
Adults (Rajshahi)					
Summer	62	0.043 ± 0.099	0.014	0.002–0.506	3 (10)
Winter	62	0.304 ± 0.776	0.043	0.002–4.663	15 (24)
Pregnant women (Dhaka)	54	0.102 ± 0.216[a]	0.041	0.004–1.088	3 (6)
Germany, adults	50	0.023 ± 0.017[b]	0.020	0.010–0.100	0 (0)

[a] $p<0.05$ when compared to adult cohort in winter season, [b] $p<0.001$ when compared to adult cohort in winter season and pregnant women cohort . *P-value* obtained from independent sample t-test.
[c]Preliminary TDI 'no concern for nephrotoxicity in humans' at 0.2 µg/kg b.w. per day (EFSA 2012).

Pregnant women cohort (Dhaka): The detection frequencies for CIT and HO-CIT were 84% and 84% in the rural and 91% and 86% in the suburban participants, respectively (Ali et al. 2016a, submitted). The mean levels of CIT and HO-CIT were about 2-fold higher in the rural (0.42 ± 1.20 ng/mL, 0.55 ± 1.04 ng/mL, respectively) than the suburban residents (0.15 ± 0.13 ng/mL, 0.23 ± 0.18 ng/mL, respectively). The ratio of CIT to HO-CIT in both groups varied between individuals. By and large, the HO-CIT metabolite concentration was about 1.5-fold higher than that of the parent compound CIT in the Dhaka cohort (Ali et al. 2016a, submitted). The mean concentration of total CIT biomarker (mean 0.73, max 9.40 ng/mL) is similar to the level found in adult cohorts of Rajshahi district in summer season. The preliminary TDI value was exceeded in 6 % of these study participants which is lower than that observed in rural and urban adult cohorts of Rajshahi district in winter season (Table 9).

3.3.2 Germany

CIT and its metabolite HO-CIT were detected in 82% and 84% of the urine samples (Ali et al. 2015c). A significant difference (p<0.05) was found in the mean CIT urine concentration between the male (0.04 ± 0.02 ng/mL) and female (0.03±0.02) subgroup whereas no significant difference was observed for the metabolite HO-CIT or total CIT (Ali et al. 2015c). The level of HO-CIT was about 3-fold higher than that of the parent compound indicating that this metabolite is a useful (additional) biomarker of citrinin exposure in human biomonitoring studies. The mean urine concentration for total CIT (mean 0.14, max 0.58 ng/mL) in the German cohort was clearly lower than in any of the Bangladeshi cohorts. The provisional daily CIT intake among the German adults was much lower than in all Bangladeshi cohorts and no individual exceeded the preliminary TDI value set by EFSA 2012.

3.4 Deoxynivalenol biomarker analysis

3.4.1 Bangladesh

Rural and urban adult cohorts (Rajshahi): DOM-1 was not detected in any of the urines whilst DON was detectable in 27% of urines collected in summer and in 31% of urines in winter season (Ali et al. 2016b; manuscript in preparation). There was no significant difference at the mean urinary level of DON (Table 10) between summer (0.17 ± 0.25 ng/mL) and winter (0.16 ± 0.18 ng/mL) season. A regional difference between the rural and urban residents was also not significant. The biomarker-based estimated daily DON intake was 6.3 ± 9.1 ng/kg bw in summer and 6.0 ± 6.1 ng/kg bw in winter season; the highest DON intake was 65.4 ng/kg bw (Table 11). All individuals had an estimated daily DON intake far below the tolerable daily intake (TDI) value of 1000 ng/kg bw/day (WHO 2001b).

Pregnant women cohort (Dhaka): DOM-1 was not detected in any of the samples whilst DON was detectable in 52% of the urines, at concentrations ranging from 0.18 to 7.16 ng/mL (Ali et al. 2015e). The average urinary DON level (0.86 ± 1.57 ng/mL) in the Dhaka cohort was significantly higher (p<0.01) than that in the Rajshahi district cohorts (Table 10). Accordingly, also the provisional daily DON intake (mean 52.5 ± 94.4, maximum 461 ng/kg bw) was significantly higher (p<0.01) than that of our cohorts in Rajshahi district (Table 11). Yet, all of the pregnant women participants had an estimated daily DON intake clearly below the tolerable daily intake (TDI) of 1000 ng/kg bw/day (WHO 2001b).

3.4.2 Germany

DON and its metabolite DOM-1 were detected in 100% and 40% of all urine samples, respectively (Ali et al. 2016b, manuscript in preparation). The mean level of urinary DON and DOM-1 was slightly higher in men (9.71 ± 7.82 ng/mL; 0.22 ± 0.20 ng/mL, respectively) than women (8.44 ± 5.97 ng/mL; 0.19 ± 0.22 ng/mL, respectively) participants, but this difference did not reach statistical significance. Overall, the mean level of urinary DON was significantly higher (p<0.0001) among German adults than the Bangladeshi adults (Table 10).

Table 10 Occurrence of DON and DOM-1 in urines from Bangladesh and Germany (Excerpted from Table 1 and 2; Ali et al. 2016b, manuscript in preparation and Ali et al. 2015e)

Cohort and season	n	DON			DOM-1		
		Positive n (%)	Mean ± SD (ng/mL)	Range (ng/mL)	Positive n (%)	Mean ± SD (ng/mL)	Range (ng/mL)
Bangladesh							
Adults (Rajshahi)*							
Summer	62	17 (27)	0.17 ± 0.25	nd–1.78	0	nd	nd
Winter	62	19 (31)	0.16 ± 0.18	nd–1.21	0	nd	nd
Pregnant women (Dhaka)	54	28 (52)	0.86 ± 1.57[a]	nd–7.16	0	nd	nd
Germany, adults	50	50 (100)	9.02 ± 6.84[b]	1.06–38.44	20 (40)	0.21 ± 0.21	nd–0.73

*Rural and urban residents. Positive refer to urines containing the analyte ≥ LOD, nd: level below LOD.
[a]p<0.01 when compared to the Rajshahi cohort and [b]p<0.0001 when compared to all cohorts in Bangladesh.

Table 11 Provisional daily intake (PDI) of DON (ng/kg b.w.)* among the participants in Bangladesh and Germany (Excerpted from table 3 and 4; Ali et al. 2016b and Ali et al. 2015e)

Cohort and season	n	Mean ± SD ng/kg b.w.	Median ng/kg b.w.	Range ng/kg b.w.
Bangladesh				
Adults (Rajshahi)				
Summer	62	6.3 ± 9.1	3.4	2.3–65.4
Winter	62	6.0 ± 6.1	3.5	2.3–39.8
Pregnant women (Dhaka)	54	52.5 ± 94.4[a]	11.2	3.1 – 460.8
Germany, adults	50	267.6 ± 199.0[b]	211.5	36.0–975.0

*Dietary DON intake was calculated based on urinary DON concentrations, adjusted for 24 h urine volume, assuming a 68% clearance rate and individual body weights (details in experimental section Ali et al. 2015e).

[a]$p<0.01$ when compared to adult cohorts of Rajshahi and [b]$p<0.0001$ when compared to all cohorts in Bangladesh.

The biomarker-based estimated daily DON intake (267.6 ± 199.0; maximum 975 ng/kg bw) in the German cohort was significantly higher ($p<0.0001$) than in all cohorts from Bangladesh (Table 11). Nevertheless, no German individual had an estimated daily DON intake above the tolerable daily intake (TDI) of 1000 ng/kg bw/day (WHO 2001b).

3.5 Correlation between food consumption pattern and urinary biomarker levels

Based on FFQ information, possible correlations were analyzed between food consumption in the Bangladeshi cohorts and their urinary biomarker levels for AFM_1, OTA, CIT and DON. Intake of certain Bangladeshi food items rice, wheat/maize, lentil soup, chicken meat, milk and milk based products and coffee did not show significant associations with the urinary biomarker levels. However, the higher mean level of AFM_1, OTA and total CIT biomarker levels were observed in the more rice consumption group compared to medium and low rice consumption group (Table 6 and Ali et al. 2015b).

4. Discussion

In Bangladesh, data on food contaminants are scarce, as there is no surveillance on the occurrence of mycotoxins in food commodities (Islam and Hoque 2013). In such a situation, the analysis of mycotoxin biomarkers in human samples (*e.g.* urines) is a valuable approach to gain more insights into human exposure to certain mycotoxins. Human biomonitoring is the best strategy to investigate exposure from all sources, either in the general population or in specific population groups (Cano-Sancho et al. 2010; Degen 2011; Turner et al. 2012). Our initial studies screened by ELISA for the presence of AFM_1 in urines of Bangladeshi adults (Ali et al. 2015a) and an LC-MS/MS multibiomarker method revealed the frequent exposure to other mycotoxins (OTA, CIT and DON) in the Bangladeshi and German population (Gerding et al. 2015). Based on these findings sensitive tailored methods with enrichment of analytes were then used to quantify mycotoxin biomarker concentrations in urines collected from the Bangladeshi population and a German 'reference' cohort.

This is the first systematic investigation on biomarkers for four major mycotoxins in rural, suburban and urban residents of two different districts (Rajshahi and Dhaka) in Bangladesh along with regional and seasonal variations and/or possible gender differences in exposure. Moreover, mycotoxin biomarker levels and ranges for the Bangladeshi population were compared with those determined in a German cohort. The new biomarker data are discussed here in a broader context, first with regard to some variables (gender, region and season) and possible sources of dietary mycotoxin intake among the Bangladeshi cohorts. Secondly, urinary biomarker levels are compared with published data from other parts of the world. Finally, biomarker-based estimates of exposure are discussed with regard to possible risks for humans, by comparing them to 'tolerable intake values' derived by competent authorities on the basis of toxicities for a given mycotoxin.

4.1 Aflatoxin M_1 as biomarker of AFB_1 exposure

Effects of variables (gender, region and season) on biomarker levels

In rural and urban adult cohorts of Rajshahi district (North-West part of Bangladesh) male participants had often higher mean level of urinary AFM_1 than female participants (Table 4). Yet, as biomarker levels varied considerably among the individuals, statistical data analysis did not show significant gender differences. In a regional comparison, the mean AFM_1 levels

were higher in the urban than in the rural residents in summer season, but the reverse situation was observed in winter season (Fig. 9). There was no difference in detection frequency; yet, the mean AFM_1 level of both cohorts was about two fold higher in winter (27.7 ± 42.6 pg/mL) than in summer (13.5 ± 21.2 pg/mL) season (Table 4). AFM_1 was also frequently detected in urines of pregnant women residents from a rural or suburban area in Dhaka district (Central part). Their mean concentration (13.9 ± 33.3 pg/mL, Table 7) is similar to the AFM_1 levels observed in the Rajshahi cohorts in summer season. On the other hand, the suburban residents in Dhaka have AFM_1 urine levels resembling those seen in Rajshahi cohorts in winter. Some variability in urinary AFM_1 levels among the cohorts by region and season, may be related to differences in eating habits and/or the well known fact that aflatoxin contamination of crops can be quite heterogenous and vary with environmental conditions (Ali et al. 2015a).

Overall, our biomarker data indicate a frequent exposure to AFB_1 in the Bangladeshi population. A recent study with analysis of the AFB_1-lysine adduct in human blood serum also showed rather high AFB_1 exposure in the population of this country (Groopman et al. 2014). Due to few data on aflatoxin food contamination (Roy et al. 2013), it is as yet unclear which food commodities are main contributors to mycotoxin exposure in Bangladesh. In Asian countries, likely sources of AFB_1 intake are rice and spices for preparing curries and groundnuts, as discussed previously in more detail (Ali et al. 2015a).

In contrast to the data from Bangladesh, AFM_1 was not detected in any of the urines from the German cohort. This result is expected as food consumed by the German population is usually free from AFB_1 contamination due to strict regulations for this carcinogenic mycotoxin and regular controls in food commodities.

Comparison of AFM_1 biomarker data with other countries

In several studies of human populations on different continents, urinary AFM_1 served as biomarker of exposure to AFB_1. Although different methods for urinary AFM_1 analysis were used with different limits of detection, the reported mean concentrations and ranges provide an orientation for aflatoxin exposure in Asian, African, European and South American populations. Details on the occurrence and levels of urinary AFM_1 in different populations are summarized in Table 12.

Table 12 Occurrence of AFM_1 in urines from cohorts in different countries

Country, cohort	Positive n (%)	Mean (range) pg/mL	pg/mg creatinine	Method	LOD/LOQ (pg/mL)	Reference
Asia						
Bangladesh, adults						
Summer	25/62 (40)	13.5 (1.7–104)	31.6	HPLC-FD	1.7/5.0	Ali et al. 2016 unpub.
Winter	26/62 (42)	27.7 (1.8–190)	33.1			
Bangladesh						
Pregnant women	17/54 (31)	13.9 (1.7–142)	29	HPLC-FD	1.7/5.0	Ali et al. 2016 unpub.
Bangladesh, adults	44/95 (46)	40 (31–348)	130	ELISA	30/NS	Ali et al. 2015a
China						
Pregnant women	430/512 (64)	51.5 (<LOD–4900) [gm]	na	ELISA	10/NS	Lei et al. 2013
Adults male	14/ (16)	50.3 (<LOD–3500) [gm]				
China, adults	142/300 (47)	40 (10–330)	na	HPLC-FD	5/NS	Mykkänen et al. 2005
China, adults	78/145 (54)	9.6 (LOD–243)	na	HPLC	3.6/NS	Sun et al. 1999
China, adults	18/49 (37)	NS(170–5200)	na	HPLC	70 (all AFs)	Qian et al. 1994
China, adults	NS	NS (0–3200)	na	ELISA HPLC	10/NS NS/NS	Zhu et al. 1987
Malaysia, adults	22/22 (100)	42.1 (28.9–154.7)	na	ELISA	24/NS	Sabran et al. 2012a
Malaysia, adults	98/160 (61)	23.4 (<LOD–74.7)	na	ELISA	11/NS	Sabran et al. 2012b
Europe						
Czech Republic, adults (1997-1998)	118/205 (58)	Na	0.39 (0.02–19.22)	ELISA	0.125 pg/mL	Ostry et al. 2005
Germany, adults	0/50 (0)	nd	nd	HPLC-FD	1.7/5.0	Ali et al. 2016 unpub.
Italy, adults and children	3/52 (6)	68 (20–0.146)	na	LC-MS/MS[b]	NS/20	Solfrizzo et al. 2014
Africa						
Egypt, pregnant women	44/93 (47)	na	5.48 (4.12–7.28)[gm] 19.7 (14.8–26.3)[gp]	HPLC-FD	4/NS	Piekkola et al 2012
Egypt, children	4/50 (8)	5.5 (5.0–6.2) 2.7[gm]	na	HPLC-FD	5/NS	Polychronaki et al. 2008
Ghana, adults	83/91 (91)	na	1800 (<LOD–11562)	HPLC-FD	10/NS	Jolly et al. 2006
Guinea, children	32/50 (64)	97 (8.0–801) 16.3[gm]	na	HPLC-FD	5/NS	Polychronaki et al. 2008
Ivory Coast, adults	40/99 (40)	na (nd–14.10)	na	LC-MS/MS[b]	0.06/NS	Kouadio et al. 2014
Nigeria, adults, adolescent and children	17/120 (14)	300 (LOD–1500)	na	LC-MS/MS[b]	50/150	Ezekiel et al. 2014.
America						
Brazil, adults	39/64 (61)	na	1.2 (0.19–12.7)	UPLC-MS	0.07/0.25	Jager et. 2014
Brazil, adults	45/69 (65)	5.9 (LOD–39.9)	na	HPLC-FD	0.6/1.8	Romero et al. 2010
Haiti, adults and children	11/142 (8)	60 (<LOD–140)	60	LC-MS/MS[b]	2.5/10	Gerding et al. 2015

na: not available, nd: not detected, NS: not stated, [gm]:geometric mean, [gp]: geometric mean for positive samples, [b]multi-biomarker approach

In Asia, AFM_1 has been detected quite frequently in urines from China and Malaysia. The mean level and range of urinary AFM_1 (mean 23 and 42 pg/mL, range 11–155 pg/mL) in two Malaysian cohorts (Sabran et al. 2012a,b) was close to the levels observed among the Bangladeshi cohorts (Table 12). In China, many studies have been conducted, often in regions with widespread dietary aflatoxin exposure. Early studies reported only the ranges but no mean values for urinary AFM_1, namely Zhu et al. 1987 (LOD–3200 pg/mL) and Qian et al. 1994 (170–5200 pg/mL). Clearly lower AFM_1 levels were reported in two other studies, with a mean of 9.6 (LOD–243) pg/mL (Sun et al. 1999) or 40 (range 10–330) pg/mL (Mykkänen et al. 2005). Two more recent studies in China found again a wider range of urinary AFM_1 levels: Lei et al. (2013) reported a mean of 51.5 (range LOD–4900) pg/mL) in a male cohort and a mean of 50.3 (range LOD-3500) pg/mL in pregnant women in China. By and large, the average aflatoxin exposure in the Bangladeshi population appears to be of a similar magnitude as that found in studies in Malaysia (Sabran et al. 2012a,b) and some studies in China (Sun et al. 1999; Mykkänen et al. 2005), although maximal AFM_1 biomarker levels in Bangladeshi adults were lower than in several Chinese cohorts.

In Africa, the highest urinary AFM_1 levels were found in Ghana in adults (mean 1800, range: LOD–11562 pg/mg creatinine; Jolly et al. 2006), and in Sierra Leone in children (ranges in two seasons of 100–124000 and 500-374000 pg/mL; Jonsyn-Ellis 2000). More recent data for children and adolescents in Nigeria (mean 300, range: LOD–1500 pg/mL; Ezekiel et al. 2014) and infant urines from Guinea (mean 97, range 8–801 pg/mL; Polychronaki et al. 2008) reveal that AFB_1 exposure continues to be significant in several sub-Saharan African countries.

In Europe, very low AFM_1 levels (mean 0.39, range 0.02–19 pg/mg creatinine) were found in a large (n = 205) Czech cohort (Ostry et al. 2005). Urines from Germany (n = 101; n = 50) and Belgium (n = 32) had no measurable AFM_1 levels (Gerding et al. 2014, 2015; Huybrechts et al. 2015), and only 3 of 52 urine samples (6%) in Southern Italy contained AFM_1 (mean 68, range 20–146 pg/mL; Solfrizzo et al. 2014). In South America, two studies in Brazil reported AFM_1 mean levels of 1.2 (range 0.25–6.9) pg/mg creatinine (Jager et al. 2014) and 5.9 (range 1.8–39.9) pg/mL urine (Romero et al. 2010), *i.e.* comparative low biomarker levels.

The AFM_1 urine levels found in the Bangladeshi cohorts and the rather frequent detection clearly raise concerns regarding their exposure to potent carcinogenic aflatoxins. This is further discussed in sections 4.5 and 4.6.

4.2 Ochratoxin A biomarker

Ochratoxin A has been analyzed in a) blood plasma from a student cohort (Dhaka) and b) in urines from adult cohorts (Rajshahi) and pregnant women cohort (Dhaka) in Bangladesh.

a) Blood biomarker analysis

The OTA level in plasma was higher in male than female students which may be due to often higher consumption of food in males. The mean plasma OTA concentrations in the Bangladeshi student cohort (0.85 ng/mL; Ali et al. 2014) is higher than values reported for adults in Pakistan (mean 0.3 ng/mL; Aslam et al. 2012) and for Japanese adults (mean 0.07 ng/mL; Ueno et al. 1998). The mean OTA concentration in Bangladeshi students only slightly exceeds what has been termed the "overall mean world OTA serum level" of 0.7 ng/mL for apparently healthy people (Fig. 11).

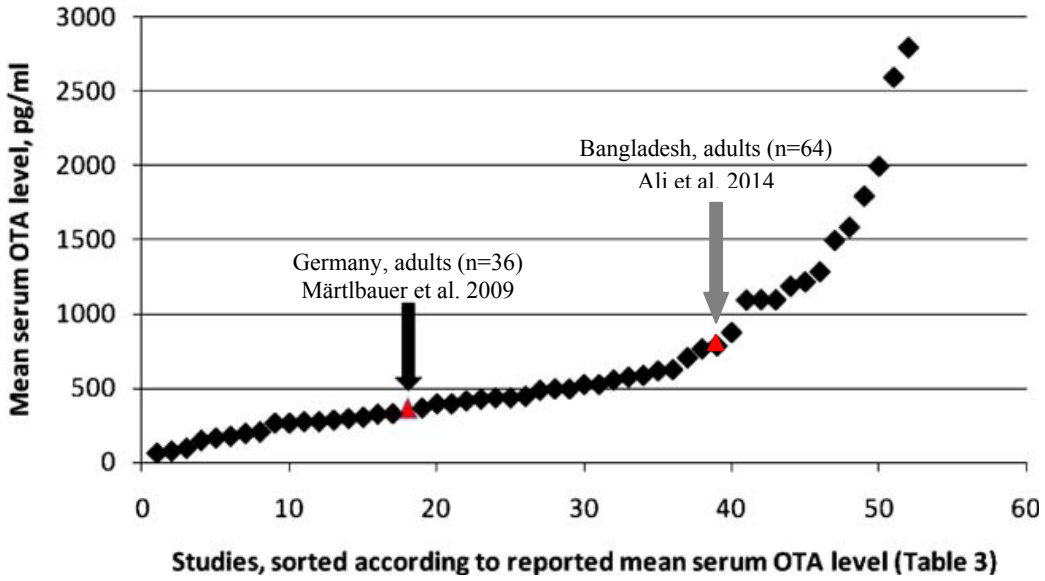

Fig. 11 Mean reported OTA serum levels (52 datasets) from healthy populations in different countries of the world (Märtlbauer et al. 2009) with new data from the Bangladeshi student cohort.

Blood plasma/serum OTA levels can be converted by means of the Klaassen equation to dietary mycotoxin intake (see section 3.2.1). The dietary OTA intake estimates for the Bangladesh cohort (mean 1.67 ng/kg b.w. per day; Ali et al. 2014) are lower than the tolerable intake value of 17 ng/kg b.w. per day set by EFSA (2006). But a few subjects exceed the limit value of 4 ng/kg b.w./day proposed by Health Canada (Kuiper-Goodman et al. 2010).

b) Urine biomarker analysis

Effects of variables (gender, region, season) on OTA levels

In urines of the adult cohorts in Bangladesh and Germany, male participants often had slightly higher mean OTA concentrations than females, although the difference was no statistically significant. Also in other studies conducted in Europe, a gender difference was not observed (Duarte et al. 2009, 2010; Manique et al. 2008; Coronel et al. 2011).

Among the Bangladeshi adults from Rajshahi district, the mean OTA urine level was higher in the rural than in the urban cohort in winter, whilst the level was similar in summer season (Ali et al. 2015b). As region has apparently a lower impact than season, the mean urine OTA level for both cohorts in winter (0.19 ± 0.38 ng/mL) and summer (0.06 ± 0.10 ng/mL) season are presented in Table 10 (below) for a comparison with biomarker data from other countries. In pregnant women from Dhaka district, OTA mean urine concentrations were similar, in this case higher in the suburban (0.15 ± 0.19 ng/mL) than the rural (0.06 ± 0.07 ng/mL) cohort (Ali et al. 2016a, submitted). The variability in urine OTA levels observed among all Bangladeshi cohorts may be related to some differences in eating habits or seasonal changes in temperate and humidity suitable for growth of OTA producing fungi on stored crops (Ali et al. 2015b). Several *Aspergillus* and *Penicillium* species are known to produce OTA and/or the structurally related mycotoxin CIT under various environmental conditions (Frisvad et al. 2006; Stoll et al. 2013; Schmidt-Heydt et al. 2015). In line with this, the biomarker results from my study indicate frequent co-occurrence of OTA and CIT in urines in both sampling seasons, and a significant correlation between OTA and total CIT in winter (Ali et al. 2015b).

Comparison of OTA biomarker data with other countries

The new OTA biomarker data for Bangladesh and German cohorts can be compared with data from several studies carried out in other countries on different continents. Although the studies applied various methods for biomarker analysis with different detection limits, the reported mean concentrations and ranges provide an orientation on the biomarker levels in these populations. The occurrences and levels of OTA in urines from different populations are summarized in Table 13.

Table 13 Occurrence of OTA and its metabolite in urines from cohorts in different countries

Country, cohort	Positive n (%)	Mean ± SD (range) ng/mL	Mean ± SD (range) ng/mg creatinine	Method	LOD/LOQ (ng/mL)	Reference
Asia						
Bangladesh, healthy adults						
Summer						
OTA	50/69 (72)	0.06 ± 0.10 (nd–0.55)	0.16 ± 0.20	LLE, HPLC-FD	0.01/0.02	Ali et al. 2015b
OTα	36/69 (52)	0.20 ± 0.77 (nd–6.10)	0.50 ± 1.12		0.01/0.02	
Winter						
OTA	94/95 (99)	0.17 ± 0.32 (nd–1.75)	0.22 ± 0.38			
OTα	56/95 (59)	0.16 ± 0.74 (nd–7.13)	0.16 ± 0.46			
Bangladesh, pregnant women						
OTA	50/54 (93)	0.10 ± 0.14 (nd–0.84)	0.16 ± 0.16	LLE, HPLC-FD	0.01/0.02	Ali et al. 2016a, subm.
OTα	23/54 (43)	0.07 ± 0.14 (nd–0.76)	0.14 ± 0.33		0.01/0.02	
Thailand	1/60 (2)	<LOQ	<LOQ	LC-MS/MS[b]	0.05/0.17	Warth et al. 2014
Europe						
Belgium						
Adults	84/239 (35)	0.03 ± na (0.003–0.37)	0.04	LC-MS/MS[b]	0.001/0.003	Heyndrickx et al. 2015
Children	79/155 (51)	0.08 ± na (0.004–3.68)	0.08			
Belgium, adults	22/32 (69)	0.009 ± na (0.003–0.033)	na	LC-MS/MS[b]	0.001/0.003	Huybrechts et al. 2015
Croatia, pregnant women						
OTA	23/40 (58)	0.13 ± 0.25 (nd–1.86)	0.17 ± 0.22	HPLC-FD	0.016/0.053	Klapec et al. 2012
OTα	40/40 (100)	1.73 ± 1.65 (0.11–7.57)	2.14 ± 2.20		0.019/0.065	
Germany, healthy adults (Dortmund: 2013)						
OTA	50/50 (100)	0.21 ± 0.31 (0.02–1.82)	0.21 ± 0.42	LLE, HPLC-FD	0.01/0.02	Ali et al.2016 unpub.
OTα	39/50 (78)	1.33 ± 2.64 (nd-14.25)	1.01 ± 1.94			
Germany, healthy adults (Dortmund: 2013)	15/50 (30)	0.04 ± 0.01 (<LOQ–0.08)	0.03 ± na	LC-MS/MS[b]	0.01/0.075	Gerding et al. 2015
Germany, healthy adults (Dortmund: 2008)						
OTA	13/13 (100)	0.07 ± 0.05 (0.02–0.29)	na	LLE, HPLC-FD	0.02/0.05	Muñoz et al. 2010
OTα	13/13 (100)	2.88 ± 2.24 (0.49–7.12)	na			
Hungary, healthy adults	54/88 (61)	0.013 ± na (0.006–0.065)	na	IAC, HPLC	0.004/0.006	Fazekas et al. 2005
Italy, healthy adults (Puglia, southern Italy: 2011)	52/52 (100)	0.144 ± 0.312 (LOQ–2.13)	na	IAC, UPLC-MS	0.006/NS	Solfrizzo et al. 2014

na: not available, nd : not detected, NS: not stated, [b]multi-biomarker approach, LLE: Liquid-liquid extraction, IAC: Immunoaffinity column clean up

Table 13 continued.

Country, cohort	Positive n (%)	Mean ± SD (range) ng/mL	Mean ± SD (range) ng/mg creatinine	Method	LOD/LOQ (ng/mL)	Reference
Portugal, healthy adults (Nationwide, 6 regions: 2007,2008)	77/95 (81)	0.016 ± 0.008 (nd–0.040)	na	IAC, HPLC-FD	0.002	Duarte et al. 2012
Summer	83/95 (87)	0.022 ± 0.015 (nd-0.071)	na			
Winter						
Portugal, healthy individuals (Nationwide: 2007)	143/155 (92)	0.022 ± na (nd–0.069)	na	IAC, LC-FD	0.008	Duarte et al. 2010
Portugal, healthy individuals (Lisbon: 2007)	31/43 (72)	0.026 ± 0.017 (nd–0.071)	na	IAC, HPLC-FD	0.008	Duarte et al. 2009
Portugal, healthy individuals (Coimbra: 2005, 2006)	13/30 (43)	0.019 ± 0.041 (nd–0.208)	Na	IAC, HPLC-FD	na/0.007	Manique et al. 2008
Portugal, healthy adults (Coimbra: 2004)	42/60 (70)	0.038 ± na (0.021–0.105)	na	IAC, HPLC-FD	na/0.02	Pena et al. 2006
Spain, healthy individuals (Valencia: 2005)	25/31 (81)	0.032 ± 0.031 (nd–0.124)	na	IAC, HPLC-FD	na/0.007	Manique et al. 2008
Spain, healthy adults (Lleida: 2009)	9/72 (12)	0.237 ± 0.181 (nd–0.562)	na	LLE, HPLC-FD	0.034/0.112	Coronel et al. 2011
OTA	43/71 (60)	0.441± 0.508 (nd–2.894)			0.023/0.076	
OTα						
Sweden, healthy adults	128/252 (51)	0.46 ± 0.57 (na)	0.73 ± 1.43	UPLC-MS	na	Wallin et al. 2015
Turkey, healthy individuals	209/233 (90)	na	14.34 ± na	HPLC-FD	0.006/0.018	Akdemir et al. 2010
UK, healthy individuals	46/50 (92)	0.013 ± na (nd–0.058)	na	IAC, HPLC-FD	na	Gilbert et al. 2001
Africa						
Ivory Coast, adults	37/99 (37)	na (nd–0.42)	na	LC-MS/MS[b]	0.01/NS	Kouadio et al. 2014
Nigeria, adults and children	34/120 (28)	0.2 ± 0.1 (nd–0.6)	na	LC-MS/MS[b]	0.05/0.15	Ezekiel et al. 2014
Cameroon, infants	70/220 (32)	0.2 ± na (0.04–2.4)	na	LC-MS/MS	0.03/0.06	Ediage et al. 2013
Sierra Leone, boys and girls						
Rainy season	47/190 (25)	na (0.5–72)	na	HPLC-FD	0.20/NS	Jonsyn-Ellis 2000
Dry season	63/244 (26)	na (0.07–148)				
America						
Haiti, adults and children	47/142 (33)	0.11 ± 0.04 (<LOQ–0.22)	0.09 ± n.a	LC-MS/MS	0.01/0.075	Gerding et al. 2015
Chile, Infants						
Months 2	18/22 (83)	0.05 ± 0.04 (LOD–0.10)	na	LLE, HPLC-FD	0.03/0.05	Muñoz et al. 2014
Months 4	18/22 (83)	0.04 ± 0.02 (LOD–0.07)	na			
Months 6	22/22 (100)	0.05 ± 0.01 (0.04–0.05)	na			

na: not available, nd : not detected, NS: not stated, [b]multi-biomarker approach, LLE: Liquid-liquid extraction, IAC: Immunoaffinity column clean up.

In Asia, there are no biomonitoring data for OTA to which the Bangladesh results can be compared to: A study in Thailand that applied multi-mycotoxin analysis (without enrichment of analytes) detected OTA only in 1 of 60 urine samples (Warth et al. 2014). Yet, it is of interest to compare OTA data for the Bangladeshi cohorts to those of some cohorts in Europe: The levels of urinary OTA in pregnant women (mean 0.10 ± 0.14, range nd–0.84 ng/mL) in Bangladesh are slightly lower than those found in pregnant women in Croatia (mean 0.13 ± 0.25 ng/mL, range nd–1.86 ng/m; Klapec et al. 2012). The OTA urines levels found in German adults (mean 0.21 ± 0.31, range 0.02–1.82 ng/mL) resemble those of the Bangladeshi cohorts from Rajshahi district in winter season (Table 8).

Within Europe, there are differences in OTA urine levels between countries which point to variable OTA exposures. For example, the OTA levels found in adults in Southern Italy (mean 0.144 ± 0.312, range LOQ–2.13 ng/mL; Solfrizzo et al. 2014), and in Spain (mean 0.237 ± 0.181, range 0.057–0.562 ng/mL; Coronel et al. 2011) were both lower than urine OTA concentrations found in Swedish adults (mean 0.46 ± 0.57 ng/mL; Wallin et al. 2015). Relatively low OTA levels were found in urines of Belgian adult cohorts (mean 0.028, range 0.002–0.368 ng/mL; Heyndrickx et al. 2015) and in the Portuguese population (mean 0.019 ± 0.012, range nd–0.071; Duarte et al. 2012).

In Africa, OTA was found in urines of Nigerian adults and children (mean 0.2 ± 0.1, range nd–0.6 ng/mL; Ezekiel et al. 2014), and in Cameroon infants (mean 0.2, range 0.04–2.4 ng/mL; Ediage et al. 2013). The highest urinary OTA levels were previously reported in children in Sierra Leone in dry (range 0.07–148 ng/mL) and in rainy (range 0.6–72.2) season (Jonsyn-Ellis 2000). In America, OTA was detected in urines of children and adults in Haiti (mean 0.06, range <LOQ–0.83 ng/mL; Gerding et al. 2015) and in breastfed Chilean infants (mean 0.05–0.16, range LOD–0.43 ng/mL; Muñoz et al. 2014).

Biomarker data compiled here (Table 13) and in a previous review (Duarte et al. 2011) indicate that OTA contamination of food is quite common on different continents, albeit mycotoxin intake can vary considerably. Since OTA excreted in urine reflects only a small fraction of an ingested dose, a conversion of urinary OTA levels to mycotoxin intake is highly uncertain (Degen 2015; and below section 4.5).

In addition to OTA, the nontoxic metabolite OTα is also excreted in human urines, and often at considerably higher levels than the parent mycotoxin (Table 13). In my present study, the mean value of urinary OTα was clearly higher in German adults (1.33 ± 2.63 ng/mL) than in

the adult cohorts (summer 0.21 ± 0.81 ng/mL, winter 0.09 ± 0.15 ng/mL) or pregnant women (0.07 ± 0.14 ng/mL) in Bangladesh. High mean levels of urinary OTα were also reported in previous studies in German adults (2.88 ± 2.24 ng/mL; Muñoz et al. 2010), in Spanish adults (0.44 ± 0.51 ng/mL; Coronel et al. 2011) and in Croatian pregnant women (1.73 ± 1.65 ng/mL; Klapec et al. 2012). At present, it is unclear whether the presence of higher OTα levels in urines from Europe is indicative of more efficient detoxication of OTA to OTα and/or related to dietary differences between the Bangladeshi and European population (who consume more milk products that can contain OTα). Thus, further research is needed to elucidate the relation of ingested OTA and excreted OTA plus its main metabolite OTα in humans, and inter-individual differences in metabolism abilities should also be considered (Ali et al. 2015b).

4.3 Citrinin biomarker

Effects of variables (gender, region, season) on biomarker levels

Male participants in Bangladesh and Germany often had higher mean levels of CIT in urines than females although this difference was not significant (Ali et al. 2015b; Ali et al. 2015c). Among the adult cohorts in Rajshahi district, urine levels of CIT and its metabolite HO-CIT were significantly higher in the rural than the urban cohort in summer season; for total CIT (sum of CIT and HO-CIT) a significant difference was observed between the rural and urban cohort in both seasons (Ali et al. 2015b). In pregnant women from Dhaka, CIT and HO-CIT levels were about 2-fold higher in the rural (0.42 ± 1.20 ng/mL; 0.55 ± 1.04 ng/mL, respectively) than the suburban (0.15 ± 0.13 ng/mL; 0.23 ± 0.18 ng/mL) residents (Ali et al. 2016a, submitted). Moreover, in Rajshahi district the mean levels of CIT biomarkers in both cohorts were significantly higher in winter (CIT 0.59 ± 0.98 ng/mL; HO-CIT 3.18 ± 8.49 ng/mL) than in summer (CIT 0.10 ± 0.17 ng/mL; HO-CIT 0.42 ± 0.98 ng/mL) season (Ali et al. 2015b).

Regional variations observed in CIT biomarker levels may be related to differences in food habits and/or an additional occupational exposure in rural Bangladeshi cohorts who are active in grain farming (Ali et al. 2015b). The more pronounced difference in biomarker levels between summer and winter samples (of the same participants) may be related to seasonal changes in temperature/humidity suitable for growth of CIT producing fungi on crops and stored food (see above section 4.2).

Table 14 Occurrence of CIT and its metabolite in urines from cohorts in different countries

Country, cohort	Positive n (%)	Mean ± SD (range) ng/mL	Mean ± SD (range) ng/mg creatinine	Method	LOD/LOQ (ng/mL)	Reference
Asia						
Bangladesh, adults						
Summer						
CIT	59/62 (95)	0.10 ± 0.17 (nd–1.22)	0.36 ± 0.60	LC-MS/MS[a]	0.02/0.05	Ali et al. 2015b
HO-CIT	44/62 (71)	0.42 ± 0.98 (nd–5.39)	1.52 ± 4.4		0.05/0.10	
Winter						
CIT	57/62 (92)	0.59 ± 0.98 (nd–5.03)	0.97 ± 1.76			
HO-CIT	60/62 (97)	3.18 ± 8.49 (nd–46.44)	3.94 ± 9.07			
Bangladesh, pregnant women						
CIT	47/54 (87)	0.31± 0.93 (nd–6.93)	0.51± 0.99	LC-MS/MS[a]	0.02/0.05	Ali et al. 2016a, subm.
HO-CIT	46/54 (85)	0.42 ± 0.82 (nd–5.53)	0.65 ± 0.69		0.05/0.10	
Europe						
Belgium, adults						
CIT	140/239 (59)	0.06 ± na (0.002–1.40)	0.07 ± na	LC-MS/MS[b]	0.001/0.003	Heyndrickx et al. 2015
HO-CIT	14/120 (12)	0.75 ± na (0.14–2.12)	0.74 ± na		0.01/0.03	
Belgium, children						
CIT	112/155 (72)	0.03 ± na (0.002–0.39)	0.04 ± na	LC-MS/MS[b]	0.001/0.003	Heyndrickx et al. 2015
HO-CIT	7/124 (6)	0.55 ± na (0.26–0.89)	0.81 ± na		0.01/0.03	
Belgium, adults						
CIT	19/32 (59)	0.02 ± na (0.002–0.12)	na	LC-MS/MS[b]	0.001/0.003	Huybrechts et al. 2015
HO-CIT	21/32 (66)	0.03 ± na (0.01–0.21)	na		0.01/0.03	
Germany, adults						
CIT	41/50 (82)	0.03 ± 0.02 (nd–0.07)	0.03 ± 0.03	LC-MS/MS[a]	0.02/0.05	Ali et al. 2015c
HO-CIT	42/50 (84)	0.10 ± 0.10 (nd–0.51)	0.10 ± 0.11		0.05/0.10	
Germany, adults						
HO-CIT	14/50 (28)	0.12 ± 0.02 (<LOQ–0.33)	0.09 ± na	LC-MS/MS[b]	0.02/0.05	Gerding et al. 2015
America						
Haiti, Children and adults						
HO-CIT	20/142 (14)	0.49 ± 0.95 (LOQ–4.34)	0.28 ± na	LC-MS/MS[b]	0.02/0.05	Gerding et al. 2015

nd: not detected, na: not available, [a]Immunoaffinity column clean up and targeted method, [b]multi-biomarker method.

Comparison of CIT biomarker data with other countries

Compared to OTA, biomonitoring data for CIT is limited and comparatively few studies have been conducted so far (Table 14). All studies applied LC-MS/MS based methods for biomarker analysis: In my analyses of Bangladeshi and German urines (Ali et al. 2015b,c and 2016a, submitted) IAC clean-up was used for analyte enrichment; studies by others used direct "dilute and shoot" multi-biomarker analyses. The occurrence and concentrations of CIT biomarkers in urines of different populations are listed in Table 14.

CIT biomarker concentrations in urines from all Bangladeshi cohorts (adults and pregnant women) are clearly higher than those determined for German adults (CIT mean 0.03 ± 0.02, range nd–0.08 ng/mL and HO-CIT mean 0.10 ± 0.10, range nd–0.51 ng/mL; Ali et al. 2015c). Similar levels as in German urines have been reported for Belgian adult cohorts (CIT mean 0.026, range 0.002–0.117 ng/mL and HO-CIT mean 0.035, range 0.013–0.210 ng/mL, Huybrechts et al. 2015; or CIT mean 0.057, range 0.002–1.398 ng/mL and HO-CIT mean 0.752, range 0.143–2.117 ng/mL, Heyndrickx et al. 2015). Another study detected the metabolite HO-CIT in urines (mean 0.49 ± 0.95, range <LOQ–4.34 ng/mL) from Haitian children and adults and at higher levels than those found in Germany (Gerding et al. 2015).

In all studies that determined both analytes, the metabolite HO-CIT was found to be present at clearly higher average levels than the parent compound CIT, although the ratios in individual urines were quite variable. This is of interest since formation of the metabolite HO-CIT is considered as detoxication reaction (Föllmann et al. 2014). It is presently unknown which enzymes catalyze this reaction, and the ability to convert CIT to HO-CIT may vary among individuals. Thus, it is indicated to measure CIT as well as its metabolite in biomonitoring studies, and consider the sum of both analytes as biomarker of exposure to this nephrotoxic mycotoxin (Ali et al. 2015b).

4.4 Deoxynivalenol biomarker

Effects of variables (gender, region, season) on biomarker levels

DON urine concentrations were slightly higher in male participants of the Bangladeshi and German cohorts than in female which is probably due to lower food consumption in the latter. Some regional but no seasonal differences in biomarker levels were observed among the

Bangladeshi adult cohorts. In pregnant women from Dhaka, the DON urine level was higher (mean 0.86 ± 1.57, range nd–7.16 ng/mL; Ali et al. 2015e) than in the Raishahi cohorts sampled in summer (mean 0.17 ± 0.25, range nd–1.78 ng/mL) and winter (mean 0.16±0.18, range nd–1.21 ng/mL) season (Table 10). Despite some variation among the Bangladeshi cohorts, their DON urine levels were far lower than those determined in the German cohort (Table 10), or in other European populations (see below, Table 15).

Geographical differences in exposure are likely due to environmental conditions (weather and climate in Asian countries like Bangladesh is different from Europe) that favour the contamination of crops in the field by DON-producing *Fusarium* species (Nielsen et al. 2011). Moreover, a small pilot study in the United Kingdom reported annual variations in DON biomarker levels in two years of different *Fusarium* prevalence (Gratz et al. 2014).

Comparison of DON biomarker data with other countries

Biomonitoring studies on DON occurrence have been conducted in several countries on different continents; the results are summarized in Table 15.

In Asia, levels of DON in urines from Chinese adults reported by Meky et al. 2003 (high risk area: mean 37, range 4–94 ng/mL; low risk area: mean 12, range 4–18 ng/mL) and later on by Turner et al. 2011a (mean 4.8, range nd–29.9 ng/mL) are clearly higher than those determined in our study in the Bangladeshi population.

In Europe, biomonitoring studies conducted in several countries indicate a variable, but frequent dietary DON exposure: This is reflected by DON urine levels reported for German adults (mean 3.38, range 2.48–17.34 ng/mL; Gerding et al. 2014), Belgian adults (mean 3.9, range 0.5–129.8 ng/mL; Heyndrickx et al. 2015), Spanish children and adults (mean 23.3 ng/mg crea; Rodríguez-Carrasco et al. 2014), Swedish adults (mean 5.38 ng/mL; Wallin et al. 2015), UK adults reported by Gratz et al. 2014 (Year 1: mean 7.1, range 0.3–27.5 ng/mL; Year 2: mean 13.5, range 0.3–28.6 ng/mL) and Turner et al. 2011b (mean 2.4, range 0.5–9.3 ng/mL) and UK pregnant women (mean 10.3 ng/mg crea, range 0.5–116.7 ng/mL; Hepworth et al. 2012). With regard to the latter group, it is worth noting that the urinary DON concentration in pregnant women in Bangladesh is significantly lower (Ali et al. 2015e).

Table 15 Occurrence of DON and its metabolite DOM-1 in urines from cohorts in different countries

Country, cohort	Positive n (%)	Mean ± SD (range) ng/mL	Mean ± SD (range) ng/mg creatinine	Method	LOD/LOQ (ng/mL)	Reference
Asia						
Bangladesh, adults						
Summer	17/62 (27)	0.17 ± 0.25 (nd–1.78)	0.64 ± 0.68	LC-MS/MS[a]	0.16/0.30	Ali et al. 2016b pre.
Winter	19/62 (31)	0.16 ± 0.18 (nd–1.21)	0.27 ± 0.27			
Bangladesh, pregnant women	28/54 (52)	0.86 ± 1.57 (nd–7.16)	2.14 ± 4.74	LC-MS/MS[a]	0.16/0.30	Ali et al. 2015e
China						
High-risk area	11/11 (100)	37.0 ± na (4.0–94.0)	na	HPLC-MS[a]	4.0/NS	Meky et al. 2003
Low-risk area	4/4 (100)	12.0 ± na (4.0–18.0)	na			
China, women	58/60 (97)	4.8 ± na (nd–29.9)	5.9 ± na	LC-MS/MS[a]	NS/0.5	Turner et al. 2011a
Europe						
Belgium, adults	89/239 (37)	3.9 ± na (0.5–129.8)	6.1 ± na	LC-MS/MS[b]	0.2/0.5	Heyndrickx et al. 2015
Belgium, children	109/155 (70)	5.2 ± na (0.5–32.5)	5.5 ± na	LC-MS/MS[b]	0.2/0.5	Heyndrickx et al. 2015
Belgium, adults	23/32 (72)	0.44 ± na (nd–3.0)	na	LC-MS/MS[b]	0.2/0.5	Huybrechts et al. 2015
Croatia, pregnant women	31/40 (76)	18.3 ± na (nd–275)	na	LC-MS/MS	4/13	Šarkanj et al. 2013
France, adults						
DON	75/76 (99)	na (0.5–28.8)	Na	LC-MS/MS[a]	NS/0.5	Turner et al. 2010a
DOM-1	26/76 (34)	na (0.02–2.8)			NS/0.06	
Germany, adults (Dortmund)						
DON	50/50 (100)	9.02 ± 6.84 (1.06–38.44)	10.50 ± 16.46	LC-MS/MS[a]	0.16/0.30	Ali et al. 2016b pre.
DOM-1	20/50 (40)	0.21 ± 0.21 (nd–0.73)	0.20 ± 0.23		0.10/0.20	
Germany, adults (Dortmund)	8/50 (16)	2.0 ± na (<LOQ–na)	2.0 ± na	LC-MS/MS[b]	0.4/4	Gerding et al. 2015
Germany, adults (Dortmund)						
DON	30/30 (100)	7.15 ± 4.10 (0.85–14.63)	6.30 ± 3.12	LC-MS/MS[a]	0.15/0.30	Föllmann et al. 2015, in press
DOM-1	15/30 (50)	0.10 ± 0.06 (LOD–0.23)	0.09 ± 0.06		0.10/0.20	
Germany, adults (Munster)	30/101 (29)	3.38 ± na (2.48–17.34)	5.30	LC-MS/MS[b]	0.4/4	Gerding et al. 2014
Italy, adults	50/52 (96)	11.89 ± 10.05 (nd–67.36)	Na	UPLC-MS/MS[b]	NS/1.5	Solfrizzo et al. 2014
Spain, children and adults						
DON	37/54 (68)	na	23.3 ± na	GC-MS/MS[b]	0.12/0.25	Rodríguez-Carrasco et al. 2014
DOM-1	2/54 (3.7)	na	2.75 ± na		0.25/0.50	
Sweden, adults						
DON	158/252 (63)	5.38 ± 12.3 (na)	7.02 ± 24.4	LC-MS/MS[b]	NS/1.5	Wallin et al. 2015
DOM-1	20/252 (8)	2.32 ± 2.68 (na)	1.56 ± 1.26		NS/9.9	
UK, adults						
DON Year 1	15/15 (100)	7.1 ± 8.0 (0.3–27.5)	7.2 ± 6.0	LC-MS/MS[a]	NS/0.1	Gratz et al. 2014
DON Year 2	15/15 (100)	13.5 ± 12.0 (0.3–28.6)	19.7 ± 11.8			

nd: not detected, na: not available, NS: not stated, [a]Immunoaffinity column clean up and targeted method, [b]multi-biomarker approach, [gm] geometric mean.

Table 15 Continued.

Country, cohort	Positive n (%)	Mean ± SD (range) ng/mL	Mean ± SD (range) ng/mg creatinine	Method	LOD/LOQ (ng/mL)	Reference
UK, adults						
DON	23/34 (68)	2.4 ± na (0.5–9.3)	na	LC-MS/MS[a]	NS/0.5	Turner et al. 2011b
DOM-1	1/34 (3)	na	na		NS/0.06	
UK, pregnant women	85/85 (100)	0.5–116.7	10.3 ± na[gm]	LC-MS/MS[a]		Hepworth et al. 2012
Africa						
Egypt, pregnant women						
DON	63/93 (68)	na	2.8 ± na (0.5–59.9)	HPLC-MS[a]	NS/0.5	Piekkola et al. 2012
DOM-1	2/93 (2)	na	na		NS/0.06	
Ivory Coast, adults	21/99 (21)	na (nd–10.0)	na	LC-MS/MS[b]	0.8/NS	Kouadio et al. 2014
South Africa, women	7/53 (13)	14.0 ± na (na)	21.3± na	LC-MS/MS[b]	4/13	Shephard et al. 2013
Cameroon, children	160/220 (73)	2.22 ± na (na)	na	LC-MS/MS[b]	0.04/NS	Ediage et al. 2013
America						
Haiti, adults and children	24/142 (17)	3.2 ± 2.0 (<LOQ–16.9)	3.6 ± na	LC-MS/MS[b]	0.4/4.0	Gerding et al. 2015

nd: not detected, na: not available, NS: not stated, [a]Immunoaffinity column clean up and targeted method, [b]multi-biomarker approach, [gm] geometric mean.

In Africa, fewer studies were conducted: Urinary DON concentrations have been reported for Egyptian pregnant women (mean 2.8, range 0.5–59.9 ng/mg crea; Piekkola et al. 2012), for South African women (mean 14 ng/mL; Shephard et al. 2013), for Cameroon children (mean 2.2 ng/mL; Ediage et al. 2013) and Ivory Coast adults (up to 10 ng/mL; Kouadia et al. 2014). In America, DON has been detected also in urines from Haitian children and adults (mean 3.2, range <LOQ–16.9 ng/mL; Gerding et al. 2015). By and large, the reported biomarker data are similar to DON levels found in Europeans.

In our survey, DOM-1, the deepoxy metabolite of DON, was not detected in any urines from the Bangladeshi population. So far, human data on the occurrence of this detoxication product are limited (Table 15). DOM-1, was present at low levels in urines from German, Spanish, Swedish, UK adults, Egyptian pregnant women and French farmers, and only found in some (not all) individuals of a given cohort.

4.5 Biomarker-based exposure estimates and tolerable intake values for mycotoxins

Mycotoxin contamination of food commodities cannot be completely avoided, yet regulations (maximum residual levels) have been set in many countries to protect the consumer. Scientific advisory committees have also derived tolerable daily intake (TDI) values for certain mycotoxins (*e.g.* DON, OTA, CIT) based on toxicological risk assessments (WHO 2001ab; EFSA 2006, 2012). Maximum levels for total aflatoxin contamination permitted in human food in Europe are 4 to 15 ppb (ng/g) depending on the commodity (EC 2006). For mutagenic carcinogens like AFB_1, and its metabolite AFM_1, maximum levels are set at a level which is as low as reasonably achievable. This has to be distinguished from a TDI for other mycotoxins (with a defined NOAEL) for which ‘safe’ doses for humans can be derived.

With regard to the question whether exposure in certain cohorts exceeds a given TDI, urine biomarker data may be used for estimating the probable daily intake, if enough information on kinetics and urinary excretion rates in humans is available. In general, mycotoxin intake estimates on the basis of urine biomarker concentrations involve some degree of uncertainty, as usually spot urines or first morning voids are analyzed rather than 24h urines.

In the case of OTA, calculation of dietary intake is highly uncertain since only a very small fraction of OTA (about 2%) is excreted in urine per day in humans (Studer-Rohr et al. 2000), and data on temporal variability in humans and possible dose-related effects on renal OTA

excretion are scarce (Degen 2015). Therefore, a probable daily intake was not estimated based on the urinary OTA levels (Ali et al. 2015b). But, blood plasma or serum OTA concentrations can be converted to mycotoxin intake (exposure), and served here to assess the exposure in a student cohort in Bangladesh in relation to TDI values set for OTA (see section 4.2).

Estimating exposure to CIT on the basis of urine data is possible, due to the rather high fraction of total CIT biomarker excretion (36%) in urine (Ali et al. 2015f). This excretion rate has been applied to calculate 'provisional daily intakes' for CIT of all Bangladeshi cohorts (Table 9, p.30). Their exposure was then compared to the TDI for CIT of 0.2 µg/kg bw ('no concern for nephrotoxicity in human') derived by EFSA (2012). This TDI for CIT was exceeded in 10% and 24% of the Rajshahi persons, in summer and winter, respectively, and in 6% of pregnant women from Dhaka. In German adults, the estimated CIT intake was lower than in Bangladeshi adults and no single German participant exceeded the TDI value.

A high fraction (about 70%) of ingested DON is excreted in human urine within a day (Turner et al 2010b; Warth et al. 2013). Urine biomarker data thus served to estimate daily DON intake in all cohorts of this study (Table 11, p.32): DON exposure was significantly higher among German adults (mean 0.27, max 0.98 µg/kg bw) than in all Bangladeshi residents (Rajshahi cohorts sampled in two seasons and the Dhaka pregnant women cohort). No individual exceeded the provisional maximum tolerable daily intake for DON of 1 µg/kg bw (WHO 2001b). This indicates that dietary DON exposure in the population of both countries is presently not of concern. On the other hand, when DON biomarker data compiled in Table 15 for cohorts in other European countries are converted to dietary intake values, the TDI for DON would be exceeded in parts of these populations.

4.6 Possible sources of dietary mycotoxin intake

Since data on AFB_1, OTA, CIT and DON contamination of food commodities in Bangladesh are scarce or totally unavailable, we made an attempt to correlate the individual biomarker levels in Bangladeshi cohorts with the consumption frequency for certain food categories. No significant association was observed between urinary biomarker levels and consumption of various foods prior to urine sampling. On the other hand, when consumption of the major food item rice was further categorized (as low, medium or high), the higher mean urine level for AFM_1, OTA and CIT were often observed in the high rice consumption group (Table 6 p.26; Ali et al. 2015b; Ali et al. 2016a, submitted; Ali et al 2016b, manuscript in preparation).

In Bangladesh rice is often consumed with curries prepared with several spices. Contaminated rice, but also spices may be sources of AFB_1 intake in the Bangladeshi population considering occurrence data from other Asian countries. In this context, a recent analysis for aflatoxins in chilli samples from Sri Lanka is of interest, where 67% of the samples exceeded the EU regulatory limit (Yogendrarajah et al. 2014). Also a study on the presence AFB_1 in pooled samples of 8 commonly consumed food commodities in Bangladesh reported that mean AFB_1 levels in 5 of 8 food commodities were above EU regulatory limits (Roy et al. 2013). The authors discussed limitations of their study (seasonal variations in contamination not captured, food types not analyzed). We fully agree that future efforts to analyse aflatoxin contamination in Bangladeshi foodstuffs are needed, in order to identify major sources and reduce their impact on aflatoxin exposure (Ali et al. 2015a).

Regarding OTA and CIT, contaminated rice and spices are considered as sources of exposure in the Bangladeshi population (Ali et al. 2014; 2015b). This view is supported by data from other Asian countries where these mycotoxins have been detected in rice samples and spices (*e.g.* Nguyen et al. 2007; Lai et al. 2015; Majeeda et al. 2013; Yogendraja et al. 2014). Yet, other commodities, not yet analysed for CIT and/or OTA, may also contribute to exposure.

DON is mainly a contaminant of wheat, maize or oat grains and derived products (EFSA 2013). These commodities are consumed much more frequently in Europe than in Bangladesh, where rice is the main staple food. In line with this, my new data for DON biomarker analysis in German and Bangladeshi cohorts (Table 10, section 3.4.2) indicate clearly higher DON exposure in Germany. Moreover, the rather low DON urine levels in the Bangladeshi cohorts indicate that there are no other relevant dietary sources for DON.

5 Conclusions

Results of this first biomarker-based assessment of mycotoxin exposure in the Bangladeshi population document a frequent intake of the carcinogenic contaminant aflatoxin B_1 (AFB_1) and also concomitant exposure to the nephrotoxic compounds ochratoxin A and citrinin (OTA and CIT). These findings raise concerns, primarily with regard to AFB_1 exposure, since the biomarker (urinary AFM_1) levels found in the Bangladeshi cohorts are significant, also in relation to biomarker data for other countries where this mycotoxin is already recognized as a problem for public health. In Bangladesh, surveillance of aflatoxin contamination in foods is clearly needed, first to identify major sources of exposure and then to reduce their impact.

The levels for biomarkers of exposure to CIT and OTA in Bangladeshi cohorts are often higher than those found in several European studies. When biomarker-based exposure estimates are compared to existing 'tolerable daily intake' (TDI) values for CIT or OTA, the present exposure in Bangladeshi cohorts does not exceed the TDI for OTA, whilst up to 24% of the adult study participants have dietary CIT exposures above the TDI. Although not of immediate concern, exposure to CIT and OTA should be further monitored, including susceptible groups in the population (especially children), and in light of possible combined effects.

DON biomarker levels in all Bangladeshi cohorts are clearly lower than those determined in the German cohort. Since the calculated daily DON intake of all individuals in both countries is below the TDI for this mycotoxin, present DON exposures are not of concern.

6 References

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Leibniz Research Center for Working Environment and Human Factors
Faculty of Chemistry and Chemical Biology
Technical University of Dortmund, Germany
Project title: Biological monitoring and exposure assessment for mycotoxins in inhabitants of Bangladesh and Germany

12/2005–12/2006 M.Sc. in Biochemistry and Molecular Biology
Thesis title: Plasma Cholinesterase activity as a biomarker for arsenicosis in humans
Rajshahi University, Rajshahi-6205, Bangladesh
Result: First class (Grade A: Top 5%)
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01/2002–12/2005 B.Sc. in Biochemistry and Molecular Biology
Rajshahi University, Rajshahi-6205, Bangladesh
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PROFESSIONAL EXPERIENCE

05/2010–06/2012 Teaching Assistant
Department of Biochemistry, Gono University, Dhaka-1344, Bangladesh
Task: Teaching of undergraduate students and helped in research work at the department

03/2009–5/2010 Research assistant
Project: Health effects in people exposed to arsenic in Bangladesh
Department of Biochemistry and Molecular Biology
Rajshahi University, Rajshahi, Bangladesh
Task: Helped in laboratory management and performed experiments

AWARDS AND HONORS

06/2012–3/2016 DAAD Scholarship for doctoral studies in Germany
09/2015 Travel Bursary Award, Eurotox congress 2015, Porto, Portugal
01/2010 Membership of Graduate Biochemist Association (GBA), Bangladesh
1/2006 Undergraduate Merit Scholarship 2006, Rajshahi University, Bangladesh

PUBLICATIONS

Original papers/Peer-reviewed

1. **Ali N**, Blaszkewicz M, Alim A, Hossain K, Degen GH. Urinary biomarkers of ochratoxin A and citrinin exposure in two Bangladeshi cohorts: follow-up study on regional and seasonal influences. Arch Toxicol (published online 2015 Dec 26) doi: 10.1007/s00204-015-1654-y
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11. Karim MR, Salam KA, Hossain E, Islam K, **Ali N,** Haque A, Saud ZA, Yeasmin T, Hossain M, Miyataka H, Himeno S, Hossain K. Interaction between chronic arsenic exposure via drinking water and plasma lactate dehydrogenase activity. Sci Total Environ 2010, 409: 278–83
12. **Ali N,** Hoque MA, Haque A, Salam KA, Karim MR, Rahman A, Islam K, Saud ZA, Khalek MA, Akhand AA, Hossain M, Mandal A, Karim MR, Miyataka H, Himeno S, Hossain K. Association between arsenic exposure and plasma cholinesterase activity: a population based study in Bangladesh. Environ Health 2010, 9: 36
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Manuscript in submission/preparation

1. **Ali N**, Blaszkewicz M, Manirujjaman M, Degen GH. Biomonitoring of concurrent exposure to ochratoxin A and citrinin in pregnant women in Bangladesh. Food Chem Toxicol 2016, submitted (date 4 Jan 2016)
2. **Ali N**, Blaszkewicz M, Degen GH. Assessment of deoxynivalenol exposure among Bangladesh and German adults. Manuscript in preparation 2016

Appendix 1: Informed consent in Bangla

সম্মতি পত্র

আমরা রাজশাহী বিশ্ববিদ্যালয়ের প্রাণরসায়ন ও অনুপ্রান বিজ্ঞান বিভাগের একদল গবেষকবৃন্দ । আমরা মাইকটক্সিন (ছত্রাকবিষ) এর উপর একটি গবেষণা পরিচালনা করতে চেষ্টা করছি ।

আমরা প্রতিনিয়ত যে খাদ্য গ্রহন করি তার মাধ্যমে বিভিন্ন মাইকটক্সিন (ছত্রাকবিষ) আমাদের শরীরে প্রবেশ করে । পরবর্তী সময়ে এই ছত্রাক বিষ আমাদের শরীরে বিভিন্ন ধরনের জটিল অসুখ সৃষ্টি করে । আমাদের শরীরে কি পরিমান ছত্রাক বিষ বিদ্যমান এবং প্রসূতি মাতার ক্ষেত্রে কি পরিমান ছত্রাক বিষ নবজাতকের শরীরে স্থানান্তরিত হয় তার সঠিক তথ্য জানা নেই । আমরা এই গবেষণার মাধ্যমে তা জানার এবং কিভাবে প্রতিরোধ করা যায় তার চেষ্টা করব ।

আপনি এই গবেষণা কাজে সহযোগিতা বা অংশগ্রহণে রাজী থাকলে গবেষণা সংশ্লিষ্ট আপনাকে কিছু ব্যক্তিগত প্রশ্ন করব এবং আপনার কাছ থেকে জৈব নমুনা (প্রাপ্তবয়স্কদের ক্ষেত্রে মুত্র, মা ও শিশুর মুত্র, এবং প্রয়োজনে বিশেষে প্রাপ্তবয়স্কদের রক্ত) সংগ্রহ করব । এইসব নমুনা সংগ্রহে কোনো ঝুঁকি নেই এবং এর ফলে আপনার কন শারীরিক সমস্যার সৃষ্টি হবে না । সংগ্রহকৃত নমুনা সমূহ আমাদের নিজস্ব ল্যাবরেটরী এবং বিদেশে আমাদের সহযোগী ল্যাবরেটরীতে পরীক্ষার জন্য পাঠানো হবে । পরীক্ষার ফলাফলসমূহ উপযুক্ত জার্নালে প্রকাশ করা হবে ।
আপনার কাছে থেকে সংগ্রহকৃত তথ্য এর পূর্ণ গোপনীয়তা রক্ষা করা হবে । গবেষণায় আপনার অংশগ্রহন বাধ্যতামূলক নয় । আপনি যে কোন মুহূর্তে এই গবেষণায় অংশগ্রহন থেকে বিরত থাকতে পারেন এবং এজন্য আপনাকে কোন প্রকার অসুবিধায় পড়তে হবে না । এই গবেষণা বিষয়ে আপনার মনে কোন প্রশ্নের উদয় হলে যোগাযোগ করুনঃ

ডঃ মোঃ খালেদ হোসেন
অধ্যাপক
প্রাণরসায়ন ও অনুপ্রাণ বিজ্ঞান বিভাগ
রাজশাহী বিশ্ববিদ্যালয়
মোবাঃ ০১৭২৬ ৮৮৪০৪৬

আমি এই গবেষণার উদ্দেশ্য সম্পর্কে অবহিত হয়ে এতে অংশগ্রহণ করতে করতে ইচ্ছুক।

.....
Tস্বাক্ষর T এবং তারিখ

T

Appendix 2: Participant information and food frequency questionnaire (FFQ)
(**Confidential)**

Project Title: Biological monitoring and exposure assessment for mycotoxins in inhabitants of Bangladesh

Participant ID : …….
Place and Date of sample collection: ……..

PERSONAL INFORMATIONS *(Please mark √ in case of Multiple Choice Questions)*

1. Name of the participant: ……
2. Age: …… (years)
3. Sex : i) Male ii) Female
4. Body weight: …… (kg)
5. Body height: ……. (cm)
6. Marital status: i) Yes ii) No
7. Smoking habits: i) Yes ii) No
8. Occupation: ……
9. Education level: i) No ii) Primary iii) Secondary iv) Higher secondary v) Graduate
10. Socioeconomic status: i) Low ii) Medium iii) Upper medium iv) High
11. Monthly income (Taka): About…….
12. Living area : i) Rural ii) Suburban urban iii) Urban

FOOD HABITS AND FOOD FREQUENCY QUESTIONNAIRES (FFQ)

13. Food consumption by the study subject per day? i) Two ii) Three iii) More
14. Food consumption by the study subject: i) Homemade ii) Restaurant iii) Both
15. How many times have you eaten the following diets **last two days** (please mark √ where appropriate in below)

Food items	**6 Times**	**5 Times**	**4 Times**	**3 Times**	**2 Times**	**1 Time**	**0 Time**
Rice							
Bread (ready made from market)							
Roti/ porota (hand made)							
Noodles/pasta							
Pulses							
Vegetables							
Milk							
Milk products							
Fish							
Egg							
Chicken							
Beef							
Dry fruits							
Soft drinks							
Fruits juice							
Tea							
Coffee							
Ground nut							

16. How often consume the following food by the subjects (please mark √ where appropriate)

Name	3 times a day	2 times a day	1 time a day		Once a week	2-4 days a week	5-6 days a week	Once a month
Cereals								
Rice								
Bread (Ready made from market)								
Roti/ Porota (Hand made)								
Nodules/Pasta								
Pulses								
Vegetables								
Ground nut (Badam)								
Animal products								
Fish								
Chicken								
Beef								
Milk								
Milk products (Cheese, curd…)								
Beverages/Fruits								
Tea								
Coffee								
Soft drinks								
Dry fruits (Date, raisin…)								
Fruits juice								

17. Any visible malnutrition observed? i) Yes ii) No. If yes. Please note……..
18. Have you suffered from any severe diseases? i) Yes ii) No If yes, please note…….
19. Have you taken the antibiotics during the last week? i) Yes ii) No

Please note that the provided information is confidential and it will be used only for research purposes. Thanks for your participation and cooperation.

…….
Name & Signature of the participant:

……
Name & Signature of the investigator (s):

Appendix 3: Informed consent in German

Probandeninformation

Hintergrund :

Mit Lebensmitteln werden auch geringe Mengen von Substanzen aufgenommen, die als unerwünschte Begleitstoffe angesehen werden. Dazu zählen auch Mykotoxine. Man weiss, dass in Deutschland von erwachsenen Personen üblicherweise mit der Nahrung aufgenommene Mengen aber kein gesundheitliches Risiko darstellen.
Nun soll untersucht werden, in welchen Konzentrationen die Substanzen in Urin vorkommen. Ziel ist es, eine neue empfindliche Methode für den Nachweis von Mykotoxinen in Urin zu erproben.

Ablauf :

Für die oben genannten Untersuchungen werden Urinproben benötigt und zwar etwa 50 - 100 ml pro Teilnehmer. Bitte sammeln Sie - gleich nach dem Aufstehen - einen Morgen -Urin in dem bereit gestellten Behälter.
Jedes Probengefäss bitte mit einem ausgefüllten Klebeetikett kennzeichen (Name und Datum der Urinspende) und dann mit diesem Blatt in der ZE Analytik abgeben.
Ferner bitten wir um das Ausfüllen eines kurzen Fragebogen (separat) in dem Nahrungsgewohnheiten erfasst werden.

Datenschutz:

Es werden im Rahmen der Studie keine personenbezogenen Daten an Dritte weitergegeben. Die Anonymität aller Urin-Spender wird gewahrt.

Einverständniserklärung

Ich habe die Probandeninformation verstanden und bin mit der Abgabe einer Urinspende einverstanden.
Meine Probe wird dem ***IfADo*** (Leibniz Institut für Arbeitsforschung an der TU Dortmund) unentgeltlich für eine wissenschaftliche Studie zur Verfügung gestellt.
Ich habe verstanden, dass

a) die Abgabe der Probe keine Risiken für mich darstellt;
b) ich meine Teilnahme an der Studie jederzeit und ohne Angabe von Gründen widerrufen kann, ohne dass mir daraus evt. Nachteile erwachsen;
c) die Ergebnisse der Analysen publiziert werden können, aber ohne Nennung meines Namens oder anderer Hinweise auf meine Identität.

Ferner versichere ich, dass meine Einwilligung zur Teilnahme freiwillig und ohne jede weitere Verpflichtung erfolgt.

Dortmund, den …….. ……..
(Name und Unterschrift)

Reprint of peer-reviewed original publications

Publication 1

Biomonitoring of ochratoxin A in blood plasma and exposure assessment of adult students in Bangladesh

Ali N, Blaszkewicz M, Manirujjaman M, Perveen R, Nahid AA, Mahmood S, Rahman M, Hossain K, Degen GH (2014). Mol Nutr Food Res 58: 2219–2225

Contribution statement

NA and GHD conceived study idea, its design and funding. NA had the responsibility to establish collaboration with scientists in Bangladesh and co-arranged the sample collection; he performed biomarker analysis, statistical data analysis and interpretation and drafted the manuscript. MB provided the instrumental facilities and contributed to final editing of the manuscript. MM, RP, AAN, MR and KH supported *the* collection of plasma samples and questionnaires, aided in FFQ data interpretation. GHD interpreted the data and co-wrote the manuscript with NA.

RESEARCH ARTICLE

Biomonitoring of ochratoxin A in blood plasma and exposure assessment of adult students in Bangladesh

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Scope: Ochratoxin A (OTA), a mycotoxin known for its nephrotoxic, immunotoxic, and carcinogenic effects in animals, deserves attention due to its widespread occurrence as food and feed contaminant. Studies in many countries report the presence of OTA in human blood plasma or serum at variable levels. However, no biomonitoring study has been carried out in so far, and also food analysis data are insufficient to assess OTA exposure.

Methods and results: Therefore, 64 blood samples were collected from healthy university students (32 female, 32 male) in Bangladesh for biomarker analysis. OTA and its metabolite ochratoxin alpha were determined in the plasma samples by a validated method using HPLC-fluorescence analysis. After liquid–liquid extraction, OTA was detected in all plasma samples (100%) at a range of 0.20–6.63 ng/mL and ochratoxin alpha was detected in 95% of the samples at 0.10–0.79 ng/mL. The OTA mean level in plasma of males (0.92 $\pm$ 1.09 ng/mL) and females (0.78 $\pm$ 1.02) were not significantly different. Statistical analysis of food consumption data for the participants, provided in a food frequency questionnaire, did not reveal a significant association between OTA level in plasma and their intake of typical staple foods (rice, wheat, maize, and lentil).

Conclusion: The dietary intake of OTA (mean 11.7, max 91.7 ng/kg b.w./wk) calculated on the basis of plasma concentration in Bangladeshi students was lower than the tolerable weekly OTA intake (120 ng/kg b.w./wk) set by EFSA. Nonetheless, further biomonitoring is recommended in cohorts from other parts of the country that may have higher mycotoxin exposure than the present group.

Keywords:
Bangladesh / Exposure / Ochratoxin A / Ochratoxin alpha / Plasma

1 Introduction

Ochratoxin A (OTA), a mycotoxin produced by a number of *Aspergillus* and *Penicillium* species, is found worldwide as contaminant in different foods and feeds [1]. Due to its carcinogenic, nephrotoxic, hepatotoxic, immunotoxic, and teratogenic effects in animals, OTA is considered as hazard for human health. The kidney is apparently the most susceptible target organ, and long-term human exposure to OTA may be involved in Balkan endemic nephropathy [2]. Regulatory bodies in many countries have set maximal contaminant levels in food commodities in order to limit dietary OTA exposure [3–5].

Human OTA exposure can be assessed by two major approaches: The mycotoxin contaminant analysis in foods combined with data of food consumption and dietary habits are used to estimate dietary OTA intake of the general population or subgroups [3, 5]. Another approach to assess human exposure is the analysis in blood (plasma or serum) and to

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Abbreviations: OTA, ochratoxin A; **OT**α, ochratoxin alpha

convert internal OTA levels by means of the Klaassen equation to dietary intake values [6–8]. The biomarker approach is particularly useful as it covers mycotoxin exposure by all routes (oral, dermal, inhalation), and it provides orientation in situations where concentration data on contaminant levels in food are scarce or not available [9, 10]. As this is the case for Bangladesh (see below), biomonitoring was chosen as the best strategy to investigate human exposure.

In the context of biomarker analysis in human blood and/or urine, it is important to consider the toxicokinetics, i.e. the long half-life of OTA in human blood ($t_{1/2}$ 35 days) as well as OTA metabolism [11]. Thus, OTA blood levels will integrate exposure over longer periods, while biomarker analysis in urine apparently better reflects day-to-day variations in exposure of adults and infants [7, 12, 13]. The main metabolite of OTA, formed by hydrolysis in the gastrointestinal tract, is ochratoxin alpha (OTα). This metabolite is more polar than OTA and considered as detoxication product [11]. In humans, OTα undergoes further phase II metabolism and is excreted with urine, often at higher levels than OTA [14–16]. OTα may thus serve as additional biomarker of mycotoxin exposure and/or indicator for the ability of humans to detoxify OTA [16].

Data for OTA plasma or serum levels of "healthy" persons from 30 countries indicate a wide range of exposures, but such data are not yet available for those countries with the highest population numbers (e.g. China, India, the United States) and for several developing countries [8]. When we recently analyzed OTA in blood plasma of two cohorts from Pakistan, the median levels (0.19 ng/mL) were comparable to those reported for the general population in the European Union [17]. In Bangladesh, the occurrence of OTA in plant-derived foods (maize, cereals, groundnuts) and feeds has been reported [18, 19]. But this survey data from only two investigational studies are insufficient to estimate typical human dietary exposure to the mycotoxin. Therefore, the objectives of the study were to analyze OTA and its metabolite OTα in blood plasma and assess the exposure of adults (university students) in Dhaka, Bangladesh.

2 Materials and methods

2.1 Collection of blood samples

Sixty-four blood samples (32 female and 32 male) were collected from healthy students who studied at the first year of honours of MBBS (Bachelor of Medicine and Bachelor of Surgery), Gonoshasthaya Samaj Vittik Medical College and Hospital, Savar, Dhaka-1344, Bangladesh. Collection of samples was done in June 2013. All participants gave their written consent before inclusion in the study, which was approved by the Ethics Committee of the Medical College. Blood samples (5–7 mL) were collected from each individual by venipuncture into EDTA containing tubes. Blood was centrifuged at 3000 rpm (about 2500 × *g*) for 15 min for isolation of plasma. The plasma samples were then stored at −20°C and mailed to IfADo at Dortmund for subsequent analysis of OTA. All participants were asked to report their daily and weekly eating habits in a short food questionnaire and some individual data (age, gender, height, and body weight) were also recorded in the form.

2.2 Standards and reagents

Methanol, chloroform, isopropanol, acetic acid (96%), sodium hydrogen carbonate ($NaHCO_3$), and phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany). The solvents used to compose the mobile phase were of HPLC grade. OTA (purity >98%) was obtained from Sigma–Aldrich (Taufkirchen, Germany), and OTα standard (purity 98.9%; 11.9 μg/mL acetonitrile) from Biopure (Tulln, Austria). OTA was dissolved in methanol and calibrated spectrophotometrically at 333 nm using the molar (M) extinction coefficient of 6400 M^{-1} cm^{-1}. The working standard solutions were prepared weekly as dilutions in methanol/water (1:1, v/v) in a range from 0.10 to 10 ng/mL. The β-glucuronidase/arylsulfatase (β-Gluc/ArylS) enzyme from *Helix pomatia* (specific activity 5.5 U/mL β-glucuronidase, 2.6 U/mL arylsulfatase at 37°C) was purchased from Roche (Mannheim, Germany).

2.3 Enzymatic hydrolysis of samples

To cleave conjugates of the analytes, 100 μL hydrolysis buffer (pH 5.0) and 10 μL of β-Gluc/ArylS enzyme was added to 0.5 mL plasma sample and incubated at 37°C for overnight before liquid–liquid extraction of OTA and its metabolite OTα [16].

2.4 Extraction of plasma

The liquid–liquid extraction method [16] was used for sample extraction with minor modifications. The enzyme-treated plasma sample (0.5 mL) was mixed with 3 mL of 1% $NaHCO_3$, and pH was adjusted to 2.8–3.0 with 1 M H_3PO_4. Then, 2 mL of a mixture chloroform/isopropanol (97:3) was added and mixed by a rotary mixer for 20 min. The mixture was centrifuged at 4500 rpm for 20 min at 20°C. In some cases, a second centrifugation was required for complete separation of the protein layer. The aqueous upper layer was discarded and 1 mL of the organic layer was transferred with an automatic pipette to a vial and evaporated to dryness under a stream of nitrogen at 45°C. The residue was dissolved in 250 μL of methanol/water (1:1). The vial was vortexed, and the extract was filtered through a 0.45 μm pore size Teflon syringe filter (WICOM, Germany) before HPLC analysis.

2.5 HPLC analysis with fluorescence detection

Analyses were carried out with a HPLC Shimadzu system consisting of two LC-10AS pumps, RF-10Axl fluorescence

detector, SIL-10AD Vp auto injector, CBM-20A communication module, and Shimadzu LC solution software. A Nucleosil 100 column (C18, 250 × 3 mm, 5 μm; Macherey & Nagel, Düren, Germany) was used for separation of the analytes at a column temperature of 40°C. Flow rate was 0.8 mL/min and the injection volume was 80 μL. The mobile phases were: as phase A acetic acid 2%/methanol (66:34, v/v), and phase B methanol/isopropanol (90:10, v/v). The stepwise gradient was: 0–15 min 5% B, 15–16 min 5–40% B, 16–30 min 40% B, 30–31 min 40–95% B, 31–33 min 95% B, 33–34 min 95–5% B, and reequilibration 34–45 min 5% B. Retention times for OTα and OTA were about 11 and 27 min, respectively. The fluorescence detector was set at 333 nm excitation and 450 nm emission wavelengths.

2.6 OTA intake assessment

The daily dietary mycotoxin intake was calculated from the mean OTA levels in plasma according to the Klaassen equation: $K_0 = Cl_p \times C_p / A$ in the version used earlier by others [6, 8]: $K_0 = 0.99 \times C_p / 0.5 = 1.97 \times C_p$, where K_0 is the continuous dietary intake (ng/kg b.w./day); Cl_p is the plasma clearance (0.99 mL/kg b.w./day); C_p is the plasma concentration of OTA (ng/mL), and A is the toxin bioavailability, estimated at 50%.

2.7 Food consumption data

All participants were asked to fill a questionnaire about their daily and weekly food habits. The food frequency questionnaire asked for intake of typical food consumed by Bangladeshi people: staple foods, mainly cereals such as rice, wheat, maize, and lentils as the major pulse. Chicken meat, eggs, milk, and milk based products were also included, as well as beverages. Beer, coffee, and wine, items of relevance for OTA intake in Europe [20], are usually not consumed in Bangladesh.

2.8 Statistical analysis

Descriptive statistics are presented as means ± SD, medians, and interquartile ranges. Box plots are used to describe distribution of OTA by gender. Differences of the baseline characteristics and OTA or OTα concentration between males and females were analyzed by independent sample *t*-test. Spearman correlation coefficient was used to assess the correlation between food consumption and OTA plasma concentration. A level of alpha 0.05 was assigned for statistical significance. Statistical analyses were performed using the software SPSS Statistics version 22.

3 Results

3.1 Validation parameters

The calibration curve was linear in the range of 0.1–10 ng/mL, and the coefficients of determination (R^2) were 0.99 for both OTA and OTα. The recoveries of analytes from matrix were assessed at three spike levels in plasma, with mean values of 92 and 71% for OTA and OTα, respectively (Table 1). Intraday repeatability, tested for all spiking levels at the same day of analysis, showed RSD of 3.1 for OTA and 5.8 for OTα. Reproducibility was determined by interday assays on three different days at a level of 0.5 ng/mL for both analytes; the RSD was 4.3 for OTA and 7.4 % for OTα. The LOD and LOQ were determined as S/N of 3 and >10, respectively. For OTA and OTα, the LOD and LOQ were 0.05 and 0.10 ng/mL in plasma.

3.2 Baseline characteristics of the participants

In total 64 subjects participated in the study, half of them were female ($n = 32$) and the other half were male ($n = 32$) students. Individual and anthropometric characteristics of the participants are presented in Table 2. The mean age of the female students was 19.8 ± 0.6 years (range: 18–21 years), and 20.5 ± 0.8 years (range: 18–21 years) for males. All volunteers were healthy and free from any chronic diseases. The average BMI for all subjects was 22.0 ± 3.3 kg/m^2. Males (22.7 ± 3.7 kg/m^2) had slightly higher BMI than females (21.4 ± 2.7 kg/m^2), but the difference was not significant.

Table 1. Recovery levels and RSD for OTA and OTα in plasma after liquid–liquid extraction

Spike level (ng/mL)	OTA		OTα	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
0.10 ($n = 3$)	97	4.6	83	3.3
0.25 ($n = 3$)	94	1.6	69	7.3
1.00 ($n = 3$)	86	3.1	60	6.8

Table 2. Baseline characteristics of the study subjects

Gender	*n*	Age (years)		BMI (kg/m^2)	
		Mean ± SD	Range	Mean ± SD	Range
Female	32	19.8 ± 0.6	18–21	21.4 ± 2.7	16.0–27.3
Male	32	20.5 ± 0.8	18–21	22.7 ± 3.7	18.0–35.9
All	64	20.2 ± 0.8	18–21	22.0 ± 3.3	16.0–35.9

Table 3. Levels of OTA and OTα (ng/mL) in plasma according to gender

	n	Positive samples (%)	Mean ± SD	Median	Range
OTA					
Female	32	32 (100)	0.78 ± 1.02	0.41	0.20–5.08
Male	32	32 (100)	0.92 ± 1.09	0.69	0.22–6.63
All	64	64 (100)	0.85 ± 1.05	0.57	0.20–6.63
OTα					
Female	32	29 (91)	0.23 ± 0.13	0.19	<LOD–0.55
Male	32	32 (100)	0.33 ± 0.16[a)]	0.27	0.13–0.79
All	64	61 (95)	0.28 ± 0.15	0.25	<LOD–0.79

a) $p < 0.05$ when compared to female, *p*-value obtained from independent sample *t*-test. Positive sample refer to plasma containing the analyte ≥LOD.

3.3 Plasma levels of OTA and OTα

OTA and OTα were detected in 64 (100%) and 61 (95%) of all plasma samples (Table 3). Only samples with an analyte concentration ≥LOD were considered as positive samples. For calculation of mean and median values, those samples that contained OTA and OTα below the limit of detection (0.05 ng/mL) were assigned a value of one half the detection limit. The mean value of OTA and OTα were 0.85 ± 1.05 ng/mL (range: 0.20–6.63 ng/mL) and 0.28 ± 0.15 ng/mL (range: LOD–0.79 ng/mL), respectively. Male students had slightly higher OTA plasma levels (0.92 ± 1.09 ng/mL) than female students (0.78 ± 1.02 ng/mL), but the difference was not significant, while a significant difference ($p < 0.01$) was observed for OTα between male (0.33 ± 0.16 ng/mL) and female (0.23 ± 0.13 ng/mL) students. Since the data were not normally distributed (Fig. 1), the comparison of plasma concentrations is also presented in a box plot, with median values and interquartile ranges (Fig. 2).

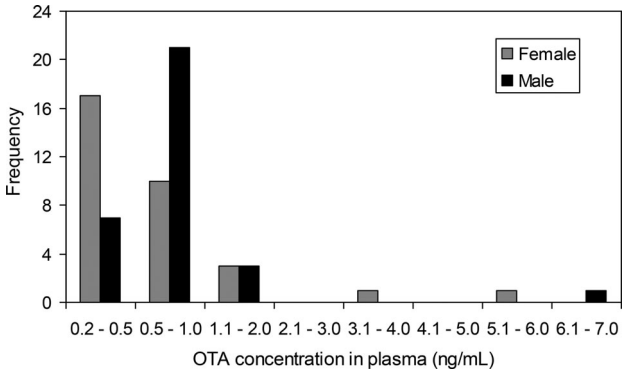

Figure 1. Distribution of OTA levels in plasma samples. Frequency test was used for the calculation of distribution.

Table 4. OTα:OTA ratio in the gender groups

	n	Mean ± SD	Median	Range
Female	29	0.83 ± 0.59	0.76	0.12–2.55
Male	32	0.80 ±0.58	0.65	0.09–2.59
All	61	0.82 ±0.58	0.71	0.09–2.59

Only positive samples considered for calculation. Analytes concentration expressed as 1 nM OTA = 0.40 ng/mL and 1 nM OTα = 0.25 ng/mL.

Of interest is also the ratio between OTA and OTα, the detoxication product of the parent mycotoxin. Ratios were calculated from nanomolar plasma concentrations after adjustment for the molecular weights (OTA 403 g/mol, OTα 255 g/mol). The mean ratio for all students was 0.82 ± 0.58 (range: 0.09–2.59) (Table 4), with no significant difference between males (0.80 ± 0.58) and females (0.83 ± 0.59). Their mean ratio OTα / OTA of <1 in blood plasma is considerably lower than that calculated from data for German adults (average nanomolar ratio OTα / OTA about 5) [16]. The lower level of OTα in blood of Bangladeshi students may be indicative of

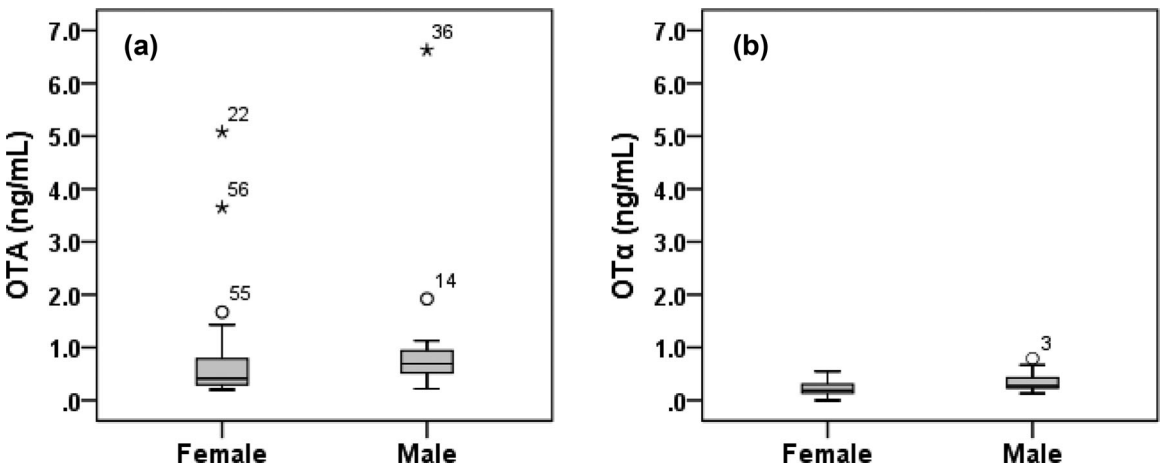

Figure 2. Comparison of plasma biomarker levels between males and females. Boxplot of concentration for (a) OTA and (b) OTα in the gender groups. The outliers were labeled by subject number.

Table 5. Daily dietary intake of OTA (ng/kg b.w.) based on OTA plasma levels

	n	Mean ± SD	Median	Range per day
Female	32	1.54 ± 2.0	0.81	0.39–10.0
Male	32	1.81 ± 2.1	1.36	0.43–13.1
All	64	1.67 ± 2.0	1.12	0.39–13.1

Table 6. Correlation (*r*) between food consumption and OTA concentration in plasma

Foods	Correlation (*r*)	*p*-value
Rice	0.24	0.843
Maize/wheat	−0.14	0.283
Lentil	0.17	0.191
Chicken meat	0.26	0.836
Milk and milk products	0.14	0.263

Assessment of food consumption was done using the score 1–4 (1 = do not consume at all, 2 = 1 time daily, 3 = 2 times daily, 4 = 3 times daily. *p*-value obtained from Spearman's correlation coefficient (two-tailed).

less efficient detoxication of OTA by gut bacteria. However, other explanations cannot be ruled out, e.g. higher intake of OTα in German adults with milk and milk products.

3.4 OTA intake estimate of Bangladeshi students

The dietary mycotoxin intake was calculated from the OTA plasma concentration by means of the Klaassen equation (conversion factor 1.97). When expressed as ng/kg b.w./day, the mean *daily* dietary intake of OTA was 1.67 ng/kg b.w. (Table 5) or multiplied by 7 days, 11.7 ng/kg b.w./wk. Taking into account also the high end OTA plasma concentration value (6.6 ng/mL), then dietary intake was 13.1 ng/kg b.w./day or 91.4 ng/kg b.w./wk. The average OTA exposure of male and female students is thus markedly lower than the tolerable intake value of 120 ng/kg b.w./wk proposed by EFSA [3].

3.5 Correlation between OTA plasma concentrations and food intake

Based on information provided in the food frequency questionnaires, possible correlations were analyzed between food consumption (graded 1–4) and OTA plasma levels in the student cohort. The food items rice, wheat/maize, lentils, chicken meat, milk, and milk based products were considered in Spearman correlation analysis (Table 6). Consumption of rice, the main staple food in Bangladesh, did not show a significant correlation ($p = 0.84$) with the plasma OTA level of the subjects. No significant correlations were also observed between OTA in plasma and other foods such as wheat/maize ($p = 0.28$), lentils ($p = 0.19$), chicken meat ($p = 0.84$), or milk and milk based products ($p = 0.26$).

4 Discussion

This is the first study that examined OTA blood levels and exposure to this mycotoxin in Bangladeshi subjects. Comparing our new data with those from studies carried out in other countries of South or North Asia these results are of interest: The mean and median plasma OTA concentrations in our cohort (0.85 and 0.57 ng/mL) are higher than OTA values reported for adults in Pakistan (mean 0.3 and median 0.19 ng/mL) [17] and for Japanese adults (mean 0.07 ng/mL) [21]. The maximum OTA level in our cohort (Table 3) was also higher than in the latter two studies. The mean Bangladeshi value of 0.85 ng/mL only slightly exceeds what has been termed the overall mean "world OTA serum level" of 0.7 ng/mL [8], but dietary OTA exposure in Bangladesh appears to be much lower than in several African countries.

According to previous work that estimated mycotoxin exposure [6, 8, 22], a conversion factor of 1.97 was applied to convert blood plasma OTA concentrations to dietary intake: The daily dietary OTA intake estimates for the Bangladesh cohort (Table 5) are lower than the tolerable intake value of 120 ng/kg b.w./wk set by EFSA, i.e. 17 ng/kg b.w./day [3]. But a few subjects exceed the limit value of 4 ng/kg b.w./day proposed by Health Canada [5]. The fact that everyone is apparently exposed to OTA (100% incidence of positive samples) raises the question of mycotoxin contamination in foods and feedstuffs in Bangladesh.

Surveillance results from 1993 to 1995 indicated a frequent and high contamination of groundnuts, maize, and poultry feed with aflatoxin B_1, yet low AFB_1 levels in pulses, rice, or wheat [18]. Analysis by a HPTLC method with a reported LOD $\leq$10 μg/kg revealed a moderate OTA contamination in 7% of the maize samples (4.5 μg/kg) and a "trace amount" (2 μg/kg) in one of 95 wheat samples; OTA was not detected in 98 rice or five pulses samples [18]. A more recent analysis of OTA in maize (50 storage samples and 70 samples collected from different markets) reported more frequent contamination in market samples, with 40% incidence and OTA levels ranging from 1 to 117 μg/kg [19]. Although maize is mainly produced as ingredient of poultry feed, it is also increasingly consumed as human food in snacks or other food items. But, data on the occurrence of OTA in the staple foods rice, lentils, millet, or wheat are not yet available for Bangladesh, although food safety is an emerging issue in this country [23]. Bangladesh is a major producer of rice, ranking 6th in the world, after China, India, Indonesia, Vietnam, and Thailand in 2012 (FAO; Available from http://faostat.fao.org/site/339/default.aspx). There are reports on OTA occurrence in Korean polished rice (2.1–6.0 ng/g in 8/88 samples) [24] and in rice from Vietnam (0.1–2.8 ng/g in 35/100 samples) [25]. The mycotoxin contamination of rice is usually lower than in wheat or corn [26]. But, contamination of rice grains with mycotoxin producing fungi in various parts of India, also West-Bengal, the neighbouring province of Bangladesh, raise some concern on the presence of OTA and other mycotoxins, for example citrinin, in this key commodity [27].

Possibly due to the small number of subjects in the study ($n = 64$), we did not observe any significant correlation between OTA levels in plasma and the food consumption data for five categories, i.e. rice, wheat/maize, lentils, chicken meat, milk, and milk products (Table 6). Rice and wheat are most frequently consumed foods in Bangladesh. As wheat and maize flour are often mixed together in food preparation, it was difficult to separate them in our analysis. Among pulses, lentils are most frequently consumed in Bangladesh and often used to prepare soups. Chicken meat was included because of the possible carry-over of OTA from poultry feed [28]. Although OTA contamination of milk and milk based products from Europe are usually very low [20], this category was included for comparison in the Spearman correlation analysis. Other items, known to contribute significantly to OTA intake in Europe, i.e. wine, coffee, and beer [20], are hardly consumed by the Bangladeshi population.

In conclusion, it can be stated that:

(i) Published information on OTA food contamination in Bangladesh was insufficient to estimate human exposure to this mycotoxin; thus biomarker analysis was introduced.

(ii) This first biomonitoring study did not indicate a high dietary OTA intake by the Bangladeshi student cohort. Since the student cohort of young healthy adults from an urban region may not reflect the situation of adults who reside in other areas of Bangladesh, further biomonitoring studies are indicated.

(iii) Such studies should also assess exposure to additional mycotoxins of interest in Bangladeshi people, applying biomarker analysis in urine and/or blood samples.

(iv) The ratio of OTα to OTA in plasma of Bangladeshi adults (OTα/OTA <1) differs from that observed in German adults (OTα/OTA >5). This is of interest as it may indicate less efficient formation of the nontoxic metabolite of OTA in the gastrointestinal tract and/or differences in OTα exposure with food.

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The authors have declared no conflict of interest.

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Reprint of peer-reviewed original publications

Publication 2

Occurrence of aflatoxin M_1 in urine from rural and urban adult cohorts in Bangladesh

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Contribution statement: NA contributed to the study concept and design, obtained samples, performed the biomarker analysis, data analysis and drafted the manuscript. KH supervised the urine sampling and contributed to the concept. MB provided the instrumental facilities and contributed to final editing of the manuscript. MR, NCM and AA supported in sample collection and questionnaire. GHD conceived the study idea, contributed to data interpretation and final editing of the manuscript.

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BIOLOGICS

Occurrence of aflatoxin M_1 in urines from rural and urban adult cohorts in Bangladesh

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Abstract Aflatoxins are important mycotoxins produced by *Aspergillus flavus* and *A. parasiticus,* moulds which contaminate mainly grains and nuts, especially in hot and humid climate. Presence of aflatoxin B_1 (AFB_1), the most toxic one and a potent hepatocarcinogen, has been reported in food and feed in Bangladesh and raised concerns about mycotoxin exposure in the population. Biomonitoring provides the best approach to assess human exposure from various sources and by all routes. Part of the ingested AFB_1 is converted in the body to aflatoxin M_1 (AFM_1), a metabolite that has served as biomarker of AFB_1 exposure, as it is excreted in urine, and thus enables non-invasive sampling, a relevant aspect in field studies. This investigation measured the AFM_1 concentration in urines collected from adult residents of a rural ($n = 52$) and an urban ($n = 43$) area in the Rajshahi district of Bangladesh. The urinary levels of AFM_1 were determined by enzyme-linked immunosorbent assay. AFM_1 was detected in 46 % of all urine samples at a range of 31–348 pg/mL. The median and mean concentration of AFM_1 in urine was 61 and 80 ± 60 pg/mL, respectively. A significant difference ($p < 0.05$) was found at the mean level of AFM_1 between the rural (99 ± 71 pg/mL) and urban (54 ± 15 pg/mL) cohort. Urinary AFM_1 levels did not show significant correlations with food frequency data or age, gender and body mass index of the participants. Among them, the highest mean AFM_1 level (101 ± 71 pg/mL) was observed in the 50–60 years age group. In conclusion, detection frequency and urinary AFM_1 levels in the Bangladeshi adults support concerns regarding their dietary exposure to AFB_1. These first data warrant further biomarker-based studies in children and in cohorts of other parts of the country.

Keywords Aflatoxin B_1 · Aflatoxin M_1 · Biomarker · Exposure · Mycotoxins

Abbreviations

AFB_1	Aflatoxin B_1
AFM_1	Aflatoxin M_1
ELISA	Enzyme-linked immunosorbent assay

Introduction

Aflatoxins (B_1, B_2, G_1, G_2) are a group of highly toxic secondary metabolites produced by *Aspergillus* species (*A. flavus* and *A. parasiticus*) which contaminate various crops, primarily in hot and humid climate (Gourama and Bullerman 1995; CAST 2003). Acute and chronic exposure to aflatoxin B_1 (AFB_1), the most prevalent aflatoxin and a potent hepatocarcinogen in various species, is a major cause of human disease in several African and some Asian countries [see reviews by Williams et al. (2004), Wild and Gong (2010), Kensler et al. (2011) and Dohnal et al. (2014)]. Epidemiological investigations applied established biomarkers of exposure (urinary aflatoxin M_1, aflatoxin-N7-guanine, serum aflatoxin-albumin/lysin adduct) and confirmed that AFB_1 contamination of the food supply puts an exposed population at increased risk of developing hepatocellular

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carcinoma (Wogan et al. 2012; Turner et al. 2012). More recent studies [reviewed by Turner (2013)] demonstrate also clear dose–response relationships between early (pre- and post-natal) aflatoxin exposure and growth retardation of infants in Gambia, Benin and Togo.

Aflatoxin contamination of dietary staples, including maize and groundnuts, often at high levels, is a major problem in developing countries where the tropical climate favours mould growth and where storage conditions for crops are poor (Williams et al. 2004; Wild and Turner 2002). The population in low-income countries often cannot afford to discard even visibly spoiled food or they use it as animal feed. Then, the transfer of AFB_1 and its similarly toxic metabolite AFM_1 into milk of lactating animals and in human breast milk will add to the risk of exposure for the youngest and most susceptible part of the population (Degen et al. 2013; Turner 2013).

To protect consumers, most developed countries have established regulations and limit values (maximum permissible levels) for several major mycotoxins in food, including aflatoxins (EC 2006; van Egmond et al. 2007). However, not everywhere a strict control for mycotoxins is enforced, as indicated, for instance, by the Rapid Alert System for Food and Feed (RASFF) and numerous notifications on aflatoxins in imported nuts, spices or figs (http://ec.europa.eu/food/safety/rasff/index_en.htm).

In Bangladesh, there is no regular control of food commodities for mycotoxins, although there is now awareness of this deficit (Islam and Hoque 2013). One survey has detected AFB_1 (by HPTLC) in plant-derived food and feed collected between 1993 and 1995, with the highest levels in groundnuts and maize, and at lower levels in pulses, wheat and rice (Dawlatana et al. 2002). A more recent study in Bangladesh (Roy et al. 2013) investigated the presence of AFB_1 in pooled samples of eight commonly consumed food commodities (rice, lentils, wheat flour, dates, betelnut, red chilli powder, ginger and groundnuts) and in poultry feed. The mean levels of AFB_1 exceeded the US or EU regulatory limits in five of eight food commodities tested. The authors (Roy et al. 2013) discuss a number of limitations of their study (e.g. seasonal variations in contamination not captured, food types not analysed) and then recommend further studies that utilize biomarkers of exposure in humans to improve assessment of exposure and risk due to aflatoxins in Bangladesh.

Indeed, when food contaminant data are scarce, as in the case of AFB_1, analysis of biomarkers in human body fluids provides a better approach as biomonitoring covers mycotoxin intake from all dietary sources and exposure by various routes (Degen 2011). A recent study with analysis of the AFB_1-lysine adduct in human blood serum indicates a rather high risk of exposure for the population in Bangladesh (Groopman et al. 2014). This biomarker-based approach requires invasive sampling (blood taken by medical personal); yet, non-invasive sampling of body fluids (such as urine, breast milk or saliva) is easier to perform in field studies and often better accepted by the participants.

AFM_1, a hydroxylated metabolite of AFB_1, present in mycotoxin exposed individuals, has been analysed in human blood, breast milk and urine samples from several countries (see Leong et al. 2012; Degen et al. 2013; Sabran et al. 2012a, b). As AFM_1 levels in urine are strongly correlated with aflatoxin intake in chronically exposed persons (Zhu et al. 1987), there is confidence in the use of urinary AFM_1 measures as exposure assessment tool (Turner 2013; Mitchell et al. 2013). Therefore, we have now investigated the occurrence of urinary AFM_1 in two adult cohorts in Bangladesh.

Materials and methods

Study population and sampling

Ethical permission for the biomonitoring study was granted by the Institute of Biological Sciences of Rajshahi University, Rajshahi-6205, Bangladesh, and it was approved by the Institutional Internal Review Board of *IfADo*, Germany. This study recruited 95 participants in the Rajshahi district of Bangladesh. Urine samples were collected from adult residents of a rural ($n = 52$) and an urban ($n = 43$) area in February 2014 (winter season). The participants were informed about the study, and a written consent was obtained from all of them prior to inclusion in the study. About 50 mL of first morning urine sample was collected into a non-sterile disposable container from each of the 95 recruited participants prior to consumption of food and water on that day. The urine samples were first stored at −20 °C at Rajshahi University and sent on dry ice to *IfADo,* Dortmund for subsequent biomarker analysis. Urinary creatinine was measured by a modified Jaffe method (Blaszkewicz and Liesenhoff-Henze 2012) to account for variability in urine concentration between individual samples. All participants were of good health, and they were asked to fill in a short questionnaire for anthropometric information (age, gender, height and weight) and occupation (Ali et al. 2015). Food habits were recorded in another questionnaire (see below).

Food consumption data

All participants were asked to fill in a questionnaire with a focus on the last 2 days of food consumption prior to urine sampling and in addition to record their regular food habits. The food frequency questionnaire (FFQ) asked for intake of typical food items consumed by Bangladeshi people:

Table 1 Regional distribution and demographic characteristics of the participants

Characteristics	Rural	Urban	All
Subjects (*n*)	52	43	95
Gender (*n*)			
Men	25	22	47
Women	27	21	48
Age (years)			
Mean ± SD	39.3 ± 11.9	34.9 ± 8.6	37.3 ± 10.7
Range	18–60	24–58	18–60
Occupation *n*, (%)			
Men			
Farmers	21 (84)	0	21 (45)
Office worker	0	20 (91)	20 (43)
Student	1 (4)	2 (9)	3 (6)
Others	3 (12)	0	3 (6)
Women			
Housewives	26 (96)	15 (71)	41 (85)
Office workers	0	2 (10)	2 (4)
Student	1 (4)	4 (19)	5 (11)
BMI (kg/m^2)			
Mean ± SD	21.7 ± 3.4	23.8 ± 3.2*	22.6 ± 3.4
Range	14.3–32.0	18.2–32.9	14.3–32.9
Creatinine (g/L)			
Mean ± SD	0.70 ± 0.39	0.87 ± 0.62	0.78 ± 0.51

SD standard deviation

* $p < 0.001$ as compared with rural cohort, p value obtained from independent sample t test

staple foods, mainly cereals such as rice, wheat, maize and lentils as the major pulses. Consumption frequency was graded 1–4 (see Table 4). Chicken meat, eggs, groundnuts, milk and milk-based products were also included. Among all foodstuffs, only rice is regularly consumed one to three times in a day by the majority of participants. Milk is only consumed by a small number of participants on a regular basis. Information about consumption of dates, betelnut and spices were not recorded in the questionnaire.

AFM_1 analysis in urine

AFM_1 levels in urine were determined using enzyme-linked immunosorbent assay (ELISA). The aflatoxin M_1 ELISA kits were purchased from Helica Biosystems Inc., Santa Ana, CA 92704, USA. The urine samples were centrifuged at 3200 g for 5 min, and supernatant was then used for AFM_1 determination according to the procedure specified in method protocol. In brief, both the AFM_1 standards (provided in the kit) and the urine samples were diluted with distilled water (1:20 or 1:5); 100 µL of each was mixed with 200 µL assay buffer. Then, 100 µL of this mixture was transferred to antibody-coated microtiter well, and the plate incubated at RT for 1 h. An automated microplate washer (Tecan Hydro Flex, Salzburg, Austria) was then used for washing the plate with washing solution (supplied with the kit). AFM_1 conjugate (100 µL) was added to each well and incubated at RT for 15 min; then, the plate was washed again with washing solution to remove the unbound conjugate. Substrate reagent (100 µL) was added to each well, and the colour reaction was allowed to proceed in the dark for 15 min at RT. Then stop solution (100 µL) was added to the wells to terminate the enzyme reaction, and within 15 min, the absorbance was measured at 450 nm by a microplate reader (Tecan Genios®, Salzburg, Austria). The absorption intensity is inversely proportional to AFM1 concentration in the samples. The AFM_1 level in the samples was calculated from concurrent standard curves using AFM_1 standard solution for each plate. All urine samples were analysed in duplicate at two different dilutions (1:5 and 1:20), and average values were calculated. For method validation, blank urines (from Germany) were spiked with different concentrations (20–60 pg/mL) of AFM_1 standard; the recovery rate was in the range of 85–115 %, and the method detection limit (MDL) was determined to be 30 pg/mL.

Statistical analysis

Statistical analysis of data was performed using the software IBM SPSS Statistics version 22. Descriptive analysis was used to determine mean, median and interquartile ranges of the analyte. Differences of the baseline characteristics and the analyte concentration between the male–female cohort and regions were analysed by independent sample t test. All the participants are categorized into four age groups according to frequency test, and the AFM_1 levels within the groups were compared using ANOVA test. Spearman correlation coefficient (two-tailed) was used to assess the correlation between food consumption and urinary AFM_1 concentration and also used to assess the correlation of analyte level with age, gender and body mass index (BMI). A level of alpha 0.05 was assigned for statistical significance.

Results

Demographic characteristics of the study cohorts

The demographic information on the cohorts is summarized in Table 1. Of the 95 participants, 51 % were female and 49 % were male. The mean age of all participants was 37.3 ± 10.7 years (range 18–60 years) with no significant difference between gender and regions. The average body mass index (BMI) of the urban cohort was significantly higher (23.8 ± 3.2 kg/m^2) than in the rural cohort

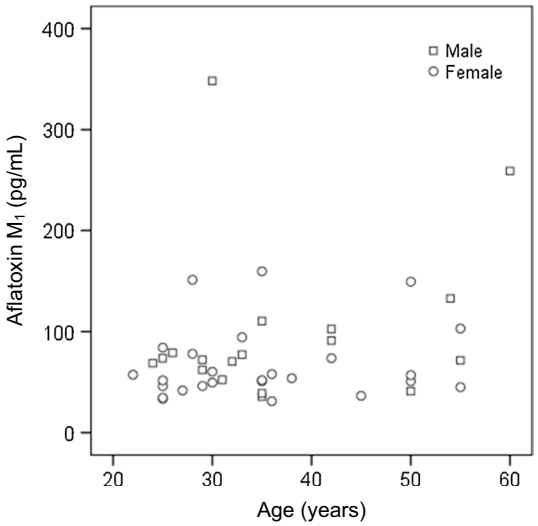

Fig. 1 Distribution of urinary AFM_1 concentrations in participants by age and gender. Only positive samples (≥30 pg/mL) are represented in the graph ($n = 44$)

(21.7 ± 3.4 kg/m^2). There was no significant difference in urinary creatinine levels of the rural and urban cohort. The rural people are mainly farmers or farm workers involved in grain production; housewives in rural areas often also take part in farm work. The urban residents are university employees or work at home (housewives). All participants consumed the typical Bangladeshi staple food rice and to a lesser extent roti (whole wheat flatbread); these are generally consumed with vegetables, lentils, fish, poultry and beef. They use several types of spices (red chilli powder is the most common one) for preparation of their daily food. There are also some differences in eating habits between the two cohorts (see discussion section).

Levels of AFM_1 in the rural and urban cohort

The distribution of AFM_1 concentrations in urine samples grouped according to age and gender is depicted in Fig. 1. Urine samples containing AFM_1 levels at or above the method detection limit (30 pg/mL) are considered as positive samples. The overall AFM_1 detection frequency is 46 %, with 50 % positives in the rural and 42 % in the urban cohort. The mean, median and range of urinary AFM_1 concentrations and also the creatinine-adjusted mean AFM_1 concentrations of all groups are given in Table 2. The range of AFM_1 was 31–348 pg/mL (or 20–533 pg/mg creatinine), with median and mean values of 61 and 80 ± 60 pg/mL, respectively, in all urine samples. The mean AFM_1 values in urines of the rural cohort (99 ± 71 pg/mL, 164 ± 98 pg/mg creatinine) were higher than in the urban cohort (54 ± 15 pg/mL or 83 ± 48 pg/mg creatinine), and this difference reached statistical significance ($p < 0.05$; $p < 0.01$; Table 2).

Table 2 AFM_1 levels in urines according to gender and region

Cohort subgroup	*n*	Positive	Median (range)	Mean ± SD	
		n (%)	(pg/mL)	(pg/mL)	(pg/mg creatinine)
All subjects	95	44 (46)	61 (31–348)	80 ± 60	130 ± 90
Rural					
Female	27	14 (52)	65 (34–160)	82 ± 43	181 ± 123
Male	25	12 (48)	84 (39–348)	118 ± 93	144 ± 58
All	52	26 (50)	75 (34–348)	99 ± 71[a]	164 ± 98[b]
Urban					
Female	21	12 (57)	50 (31–84)	50 ± 14	80 ± 46
Male	22	6 (27)	65 (35–79)	61 ± 16	88 ± 55
All	43	18 (42)	52 (31–84)	54 ± 15	83 ± 48

[a] $p < 0.05$ and [b] $p < 0.01$ when compared to urban, p value obtained from independent sample t test. The mean and median value of AFM_1 were calculated based on positive samples (≥30 pg/mL)

Further statistical data analysis revealed no significant correlations for urinary AFM_1 concentration with gender, age and BMI of the study participants. The highest mean value of urinary AFM_1 (101 ± 71 pg/mL or 174 ± 88 pg/mg creatinine) was observed in the 50–60 years age group (Table 3). The body mass index of the participants showed a negative trend of correlation with AFM_1, but at an insignificant level (data not shown).

Correlation between urinary AFM1 concentration and food intake

From information provided in the food frequency questionnaires, possible correlations were analysed between urinary AFM_1 levels and food consumption (graded 1–4) in the cohorts. The food items rice, wheat/maize, lentils, chicken meat, groundnut, milk and milk-based products were considered in Spearman correlation analysis (Table 4). Consumption of rice, the main staple food in Bangladesh, showed a positive trend of correlation ($p = 0.09$) with the urinary AFM_1 level of the subjects. No significant correlations were observed between AFM_1 in urine and other foods such as wheat/maize ($p = 0.53$), lentils ($p = 0.52$), chicken meat ($p = 0.51$) or milk and milk-based products ($p = 0.18$) and groundnut ($p = 0.37$).

Discussion

This is the first study that reports AFM_1 levels in urines from rural and urban residents of Bangladesh. The frequent detection of AFM_1 (46 %)[1] in the 95 urine samples

[1] Percentage of positive samples based on the method detection limit of 30 pg/mL; detection frequency can vary depending on methods and detection limits.

Table 3 AFM_1 levels in urines according to age groups of the participants

Age (years)	*n*	Positive	Mean ± SD	
		n (%)	(pg/mL)	(pg/mg creatinine)
18–29	23	15 (61)	65 ± 29	104 ± 59
30–39	35	16 (46)	84 ± 78	136 ± 118
40–49	17	4 (24)	76 ± 29	109 ± 31
50–60	20	9 (45)	101 ± 71	174 ± 88

Mean value of AFM_1 calculated for the positive samples (≥30 pg/mL). AFM_1 levels within the groups were calculated using ANOVA test

Table 4 Correlation (*r*) between last 2 days food consumption and urinary AFM_1 concentration

Food item	Correlation (*r*)	*p* value
Rice	0.257	0.093
Wheat/maize	0.076	0.534
Lentil	−0.064	0.523
Chicken meat	0.065	0.512
Milk and milk products	−0.206	0.181
Groundnut	−0.138	0.371

Assessment of food consumption was done using the score 1–4 (1 = do not consume at all, 2 = 1 time daily, 3 = 2 times daily and 4 = 3 times daily). Positive samples (≥30 pg/mL) were considered for correlation analysis. *P* values obtained from Spearman's correlation coefficient (two-tailed)

(Table 2) and the range of concentrations detected (31–348 pg/mL or 20–533 pg/mg creatinine) clearly indicate a significant aflatoxin exposure of the adult Bangladeshi population. To put this finding into perspective, our new data can be compared with urinary AFM_1 biomarker data from studies carried out in several countries on different continents. Although different methods for urinary AFM_1 analysis were used with different limits of detection, the reported mean concentrations and ranges provide an orientation for aflatoxin exposure in African, Asian, European and South American populations.

Studies in Brazil reported AFM_1 mean levels of 1.2 (range 0.25–6.9) pg/mL (Jager et al. 2014) and 5.9 (range 1.8–39.9) pg/mL urine (de Romero et al. 2010). These values are clearly lower than those in the Bangladeshi cohorts. In Europe, very low AFM_1 levels (mean 0.39, range 0.02–19 pg/mg creatinine) were found in a large ($n = 205$) Czech cohort (Ostry et al. 2005). Urines from Germany ($n = 101$; $n = 50$) and Belgium ($n = 32$) had no measurable biomarker levels (Gerding et al. 2014, 2015; Huybrechts et al. 2014), and only 3 of 52 urine samples (6 %) in Southern Italy contained AFM_1 (mean 68, range 20–146 pg/mL; Solfrizzo et al. 2014). In urines from various Asian countries, AFM_1 has been detected quite frequently: in two Malaysian cohorts, mean levels of 23.4 and 42.1 pg/mL, with ranges of 11–154.7 ng/mL, were reported (Sabran et al. 2012a, b). These AFM_1 concentrations are lower than those observed in our Bangladeshi cohorts (Table 2).

Many studies have been conducted in China, often in regions with widespread dietary aflatoxin exposure. Some reports do not mention mean values for urinary AFM_1 levels, but provide ranges LOD to 3200 pg/mL (Zhu et al. 1987) or 170–5200 pg/mL (Qian et al. 1994). About tenfold lower maximum levels were reported in two other studies, with a mean of 9.6 (LOD to 243) pg/mL (Sun et al. 1999) or 40 (range 10–330) pg/mL (Mykkänen et al. 2005). But two more recent studies found again a wider range of urinary AFM_1 levels: Lei et al. (2013) reported a mean of 51.5 (range LOD to 4900) pg/mL in a male cohort and a mean of 50.3 (range LOD to 3500) pg/mL in pregnant women. A similar extent of exposure was observed in intervention studies conducted by Tang et al. (2008) who applied a series of AFB_1 biomarkers, including AFM_1: the range observed over 3 months in the group with no intervention was 0.24–1276.2 pg/mg creatinine and mean AFM_1 levels of 59.4, 61.7 and 78.7 pg/mg creatinine. The creatinine-adjusted values in our Bangladesh cohort give a mean of 130 and a range of 20–533 pg/mg creatinine (Table 2). Thus, their average aflatoxin exposure appears to be of a similar magnitude as that found in several studies in China, although maximal AFM_1 biomarker levels in Bangladeshi adults were lower than in Chinese individuals.

In Africa, the highest urinary AFM_1 levels were found in Ghana in adults (mean 1800, range LOD to 11,562 pg/mg creatinine; Jolly et al. 2006) and in Sierra Leone in children (ranges in two seasons of 100–124,000 and 500–374,000 pg/mL; Jonsyn 2000). More recent data for children and adolescents in Nigeria (mean 300, range LOD to 1500 pg/mL; Ezekiel et al. 2014) and infant urines from Guinea (mean 97, range 8–801 pg/mL; Polychronaki et al. 2008) reveal that AFB_1 exposure continues to be significant in several sub-Saharan African countries.

Since aflatoxin contamination of food is known to be highly variable, one can expect that urinary excretion of its metabolite AFM_1 by individuals consuming such food will be also quite variable (vide supra). With a biological half-life of about 1 day, the urinary excretion of AFM_1 biomarker would reflect dietary AFB_1 intake averaged over 1–3 days (Cheng et al. 1997). Therefore, the food frequency questionnaire for our Bangladeshi cohort focused on consumption of various items during the last 2 days prior to urine collection, yet it recorded also regular dietary habits. This information was used to look for correlations with urinary biomarker levels in our cohorts: a trend for a positive correlation was only observed between urinary AFM_1 levels and rice consumption during the last

2 days (Table 4). The higher levels of AFM_1 in residents of the rural area (Table 2) may be related to regularly higher consumption of rice by the rural people than the urban people. The reported AFB_1 contamination of rice samples from Bangladesh was comparatively low (Dawlatana et al. 2002; Roy et al. 2013), yet it is the main staple food, and higher AFB_1 levels were found in parboiled rice in India (Reddy et al. 2009) and in rice in central Vietnam, with clear seasonal effects (Nguyen et al. 2007). However, other sources cannot be excluded. Rice is usually consumed with curries prepared with spices, mainly red chilli powder. Chilli samples from Bangladesh and Sri Lanka often contain AFB_1 at levels exceeding EU regulatory limits (Roy et al. 2013; Yogendrarajah et al. 2014). Whilst consumption of spices was not been recorded in our food questionnaire, other items such as groundnuts, known as a source of AFB_1 (Dawlatana et al. 2002; Roy et al. 2013), were only rarely consumed by our cohort (during the last 2 days only once by 6 of 95 persons). Thus, it is presently not possible to put a tag on those food items which contribute mostly to aflatoxin exposure in the Bangladeshi cohort.

AFM_1 constitutes only a small part of the total metabolized AFB_1 (Neal et al. 1998) and a urinary excretion rate of about 2 % of an ingested dose (Zhu et al. 1987). Nonetheless, the urinary AFM_1 biomarker data clearly indicate a frequent exposure to aflatoxins in Bangladesh, and at levels similar to those found in other Asian countries (China, Malaysia, vide supra). This new finding and the recent study of another (long-term) biomarker of exposure (aflatoxin B_1-lysine albumin adduct) in blood of Bangladeshi pregnant women and their children raise concerns (Groopman et al. 2014). Therefore, analysis of AFB_1 contamination in food should be continued, also for commodities such as maize and parboiled rice which were not included in the recent survey (Roy et al. 2013).

Moreover, as Bangladesh has a subtropical monsoon climate, characterized by clear seasonal variations in rainfall, temperature and humidity (http://en.wikipedia.org/wiki/Geography_of_Bangladesh) and since aflatoxin contamination of crops is quite heterogenous and varies with environmental conditions, further human biomonitoring is indicated. Follow-up biomarker studies in adults, with urine sampling in the summer season, and also in other parts of the population (pregnant women and infants) will provide better insights regarding exposure to a highly toxic mycotoxin.

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Compliance with ethical standards

Conflict of interest None of the authors have any conflict of interest to declare.

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Publication 3

Urinary biomarkers of ochratoxin A and citrinin exposure in two Bangladeshi cohorts: follow-up study on regional and seasonal influences

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Contribution statement

NA co-conceived study idea, designed study, co-arranged the sample collection, performed the analysis of all biomarkers and statistical data analysis, data interpretation and drafted the manuscript. MB supervised the instrumental analysis and contributed to final editing of the manuscript. AA and KH supported the sample collection and aided in FFQ data interpretation. GHD co-conceived study idea, interpreted the data and co-wrote the manuscript.

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ANALYTICAL TOXICOLOGY

Urinary biomarkers of ochratoxin A and citrinin exposure in two Bangladeshi cohorts: follow-up study on regional and seasonal influences

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Abstract Biomonitoring studies can provide valuable insights into human mycotoxin exposure, especially when food contaminant data are scarce or unavailable as in Bangladesh. First biomonitoring data in Bangladeshi adults indicated exposure to the nephrotoxic mycotoxins ochratoxin A (OTA) and citrinin (CIT). This led us to conduct a follow-up study with analysis of urinary biomarkers for both CIT and OTA to investigate regional and seasonal influences on mycotoxin exposure in two Bangladeshi cohorts. In total, 164 urines were collected ($n = 69$ in summer, $n = 95$ in winter) from residents of a rural and an urban area, among which there were 62 participants enrolled in both sampling periods. Most urines had detectable biomarker levels (OTA, CIT and its metabolite dihydrocitrinone, HO-CIT), with more or less pronounced differences with regard to season and region. In both cohorts, OTA was found at a mean level of 0.06 ± 0.10 ng/mL urine (range 0.01–0.55 ng/mL) in summer and a mean of 0.19 ± 0.38 ng/mL (range 0.01–1.75 ng/mL) in winter season. A season difference was significant in the rural cohort, but not in the urban cohort, and slightly higher mean OTA levels in the rural compared to the urban cohort were only observed in winter urines. CIT biomarkers showed more pronounced variations, with a CIT mean of 0.10 ± 0.17 ng/mL (range 0.02–1.22 ng/mL) and HO-CIT mean of 0.42 ± 0.98 ng/mL (range 0.02–5.39 ng/mL) in summer, and CIT mean of 0.59 ± 0.98 ng/mL (range 0.05–5.03 ng/mL) and HO-CIT mean of 3.18 ± 8.49 ng/mL (range 0.02–46.44 ng/mL) in winter urines of both cohorts. In both seasons, total CIT biomarker concentrations were significantly higher in the rural cohort than in the urban cohort. A provisional daily intake for CIT was calculated and exceeded a preliminary value set by EFSA (0.2 µg/kg/d) in 10 and 24 % of participants in summer and winter, respectively. No significant correlations were found between urinary biomarker levels and intake of certain types of food, except for a positive trend for higher rice consumption. Our results in the Bangladeshi population indicate frequent co-exposure to nephrotoxic mycotoxin food contaminants that vary by season and region.

Keywords Biomarkers · Citrinin · Exposure · Mycotoxins · Ochratoxin A · Urine

Abbreviations

CIT	Citrinin
HO-CIT	Dihydrocitrinone
OTA	Ochratoxin A
OTα	Ochratoxin alpha

Introduction

Ochratoxin A (OTA) and citrinin (CIT) are secondary fungal metabolites produced by some *Aspergillus* and *Penicillium* species (Frisvad et al. 2006). These mycotoxins can

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be found in various foodstuffs and in animal feed, and their co-occurrence has raised concerns for human and animal health (Ostry et al. 2013). Both mycotoxins are nephrotoxic to animals, although CIT is less potent than OTA (EFSA 2006, 2012). OTA is carcinogenic to rodents and has been classified as group 2B human carcinogen, while CIT is in group 3 due to limited evidence for carcinogenicity in experimental animals (IARC 1986, 1993). Chronic OTA exposure has been associated with Balkan endemic nephropathy, although other etiologic agents including CIT are also discussed (Castegnaro et al. 2006; Peraica et al. 2008; Pfohl-Leszkowicz 2009).

Co-occurrence of these mycotoxins has been found in cereals from Balkan endemic nephropathy regions of Bulgaria (Petkova-Bocharova et al. 1991; Vrabcheva et al. 2000a, b), in grains in Germany (Meister 2003; Schmidt et al. 2003) and in rice in India and Vietnam (Nguyen et al. 2007; Reddy et al. 1983). While many surveys have been conducted on OTA contamination of human foods (EC/SCOOP 2002), data on CIT occurrence in foodstuffs are still limited and insufficient to reliably estimate CIT exposure in the human population (EFSA 2012).

When food contaminant data are scarce which is often the case in developing countries, analysis of mycotoxin biomarkers in human body fluids (biomonitoring) can provide useful insights into human exposure (Duarte et al. 2011; Turner et al. 2012). Bangladesh, a developing country in subtropical South Asia, has a climate characterized by distinct seasonal variations in rainfall, temperature and humidity. This hot and humid climate makes a suitable environment for the growth of molds on food and feed in Bangladesh, and mycotoxin contamination of food commodities may vary between seasons. Occurrence of OTA has been reported in plant-derived foods (maize, cereals and groundnuts) and in feeds in Bangladesh (Dawlatana et al. 2002, 2008), but data on CIT in food or feed are unavailable.

A recent biomonitoring study indicated the presence of OTA in blood plasma of adult Bangladeshi students at levels similar to those found in Germany (Ali et al. 2014). First analysis of CIT biomarkers in urines from adult people in Bangladesh indicated a frequent exposure, higher than in Germany (Ali et al. 2015b). However, information on seasonal and regional variations and a possible co-exposure to both nephrotoxic mycotoxins in the population of Bangladesh is still lacking.

Therefore, in total 164 urine samples were collected on two occasions ($n = 69$ in summer and $n = 95$ in winter) from residents of a rural and an urban area of Bangladesh, among which there were 62 participants who enrolled in both sampling periods. Their urine samples were analyzed by two sensitive methods for targeted analysis of OTA (Muñoz et al. 2010) and CIT (Blaszkewicz et al. 2013) biomarkers in human urine. These methods have proved to be useful for analysis of urines from Chile, Germany and Bangladesh (Muñoz et al. 2014; Ali et al. 2015a, b). In addition to the parent compounds OTA and CIT, their main metabolites ochratoxin alpha (OTα) and dihydrocitrinone (HO-CIT) were analyzed also, since the often higher metabolite levels in urine may increase sensitivity and thereby facilitate exposure monitoring (Muñoz et al. 2010; Coronel et al. 2011; Klapec et al. 2012; Ali et al. 2015c).

Materials and methods

Chemicals and materials

Methanol (HPLC grade), chloroform, isopropanol, acetic acid (96 %), sodium hydrogen carbonate ($NaHCO_3$) and phosphoric acid (85 %) were purchased from Merck (Darmstadt, Germany). Acetonitrile and methanol (LC–MS grade) were from Promochem (Wesel, Germany). OTA (purity >98 %) was obtained from Sigma-Aldrich (Taufkirchen, Germany) and OTα standard (purity 98.9 %; 11.9 µg/mL acetonitrile) from Biopure (Tulln, Austria). OTA was dissolved in methanol and calibrated spectrophotometrically at 333 nm using the molar (M) extinction coefficient of 6400 M^{-1} cm^{-1}. CIT standard material (5 mg, purity >98 %) was purchased from Sigma-Aldrich (Taufkirchen, Germany); CIT solutions in methanol were calibrated at 321 nm using the absorptivity $\varepsilon = 5490$ M^{-1} cm^{-1} (Molinie et al. 2005). The CIT metabolite dihydrocitrinone (HO-CIT) [$C_{13}H_{14}O_6$; (3R,4S)-6,8-dihydroxy-3,4,5-trimethyl-1-oxo-3,4-dihydro-1H-iso-chromene-7-carboxilic acid; purity 98.9 %] was from AnalytiCon Discovery GmbH (Potsdam, Germany). For OTA and OTα, the working standard solutions were prepared weekly as dilutions in methanol/water (1:1, v/v) in a range from 0.10 to 10 ng/mL. For CIT and HO-CIT, working standard solutions were prepared weekly from a stock solution of CIT (200 µg/mL acetonitrile) and HO-CIT (500 µg/mL acetonitrile), by dilution in methanol in a range of 1–20 ng/mL. The β-glucuronidase/arylsulfatase (β-Gluc/ArylS) enzyme from *Helix pomatia* (specific activity 5.5 U/mL β-glucuronidase, 2.6 U/mL arylsulfatase at 37 °C) was purchased from Roche (Mannheim, Germany).

Cohorts and collection of urines

In May 2013 (summer season), urine samples were collected from 69 volunteers of which 32 were residents of a rural area (Mongol Para, Puthia) and 37 of an urban area (Rajshahi University region) in the Rajshahi District of Bangladesh. In February 2014 (winter season), 95 urine samples (52 rural and 43 urban participants) were collected

from the same areas. There were 62 volunteers who provided urines in both sampling rounds. Urine donors were informed about the study, and a written consent was obtained from all participants prior to inclusion in the study. The participants were of good health, and they were asked to fill a short questionnaire for anthropometric information (age, gender, height and weight), occupation and food habits. First morning urine samples were collected into a non-sterile disposable container (approximately 50 mL) in both collection periods. Urine samples were first stored at −20 °C at Rajshahi University and sent on dry ice to IfADo, Dortmund, for subsequent biomarkers analysis. To control for differences of urine dilution between individual spot urines, creatinine was also measured by a modified Jaffe method (Blaszkewicz and Liesenhoff-Henze 2012). This study was approved by the Institute of Biological Sciences of Rajshahi University, Rajshahi-6205, Bangladesh, and by the Institutional Internal Review Board of IfADo.

Collection of food consumption data

All participants were asked to fill a questionnaire about their daily, weekly and normal food habits. Foods consumed by the study subjects 2 days prior of sample collection were also recorded in the questionnaire. The food frequency questionnaire (FFQ) asked for intake of typical food consumed by Bangladeshi people: staple foods, mainly cereals such as rice, wheat, maize and lentils as the major pulse. Chicken meat, eggs, milk and milk-based products were also included, as well as coffee and beverages. Among all foodstuffs, only rice is regularly consumed by the participants one to three times in a day. Beer and wine, items of relevance for OTA intake in Europe (EC/SCOOP 2002), are usually not consumed in Bangladesh, and coffee is generally consumed only by a few urban people. Assessment of food consumption was done using the score 1–4 (1 = do not consume at all, 2 = 1 time daily, 3 = 2 times daily and 4 = 3 times daily).

Sample preparation and chromatographic analysis

Urinary concentrations of OTA and CIT biomarkers were analyzed by validated methods (Muñoz et al. 2010; Blaszkewicz et al. 2013) which apply liquid–liquid extraction for OTA, and immunoaffinity column cleanup for CIT extraction. Briefly, for OTA prior to extraction, 3 mL urine sample was enzymatically treated by adding 250 µL hydrolysis buffer (pH 5.0) and 40 µL of β-Gluc/ArylS and incubation at 37 °C overnight. Then, the hydrolyzed urine sample was mixed with 3 mL of 1 % $NaHCO_3$, and pH was adjusted to 2.8–3.0 with 1 M H_3PO_4. Then 3 mL chloroform/isopropanol (97:3) mixture was added and kept on a rotary mixer for 20 min, followed by centrifugation at 4500 rpm for 20 min at 20 °C. The aqueous upper layer was discarded, and 2 mL of the organic layer was transferred with a pipette to a vial and evaporated to dryness under a stream of nitrogen at 45 °C. The residue was dissolved in 500 µL of methanol/water (1:1). The vial was vortexed and centrifuged, and the extract was filtered through a 0.45-µm pore size Teflon syringe filter (WICOM, Germany) before HPLC analysis. For CIT and HO-CIT extraction, urine aliquots were diluted with the same volume of 1 mM acetic acid and then loaded on a CitriTest® column (Vicam® purchased from Ruttmann, Hamburg, Germany) for cleanup and enrichment of the analytes; further details of the procedure are described elsewhere (Ali et al. 2015b).

Analysis of OTA and its metabolite OTα was carried out by high-performance liquid chromatography with fluorescence detection (Ali et al. 2014), and CIT and its metabolite HO-CIT were measured by liquid chromatography with tandem mass spectrometry (Ali et al. 2015b). Spiked samples were used to define the minimum detectable level: The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the lowest quantity of analyte that can be clearly distinguished (LOD) from the background (S/N = 3) or quantified (LOQ; S/N ≥ 6). The LOD and LOQ for OTA and OTα in urine were 0.01 and 0.02 ng/mL, respectively. For CIT and HO-CIT, the LOD was 0.02 and 0.05 ng/mL, and the LOQ was 0.05 and 0.10 ng/mL, respectively. Recovery assays were performed in urine with no measurable background of the analytes. For OTA and OTα, recovery of analytes was assessed at three concentration levels in triplicate spiked samples with mean values of 97 and 68 %, respectively. For CIT and HO-CIT, recovery of analytes was assessed at three concentration levels in triplicate spiked samples with mean values of 79 and 82 %, respectively. The intraday repeatability and inter-day repeatability at a spike concentration of 0.25 ng/mL ($n = 6$) were determined using also mycotoxin-free urine, and showed acceptable precisions for both OTA and CIT and their metabolites at these low concentrations.

CIT intake assessment

The estimation of CIT intake among the participants was performed based on results of urinary total CIT (CIT + HO-CIT) analysis. The following equation was used to assess the provisional daily intake (PDI) of CIT among the participants.

$$\mathrm{PDI}\left(\frac{\mu g}{kg}\text{body weight}\right) = C \times \frac{V}{W} \times \frac{100}{E}$$

where C = biomarker concentration (µg/L), V = daily urine excretion (L), W = body weight (kg) and E = excretion rate (%). In the calculation, urinary output was considered as 1.5 L per day and the urinary total CIT daily excretion

rate as 36 % of an ingested dose (Ali et al. 2015c). Individual body weight was used during the calculation of PDI.

Statistical analysis

Statistical analysis of data was performed using the software IBM SPSS Statistics version 22. Descriptive analysis was used to determine mean, median and interquartile ranges of the analytes. Those samples containing analyte levels below the limit of detection (LOD) were assigned a value of one-half the detection limit for calculation of mean and median values, since this is considered a better approach to estimate average concentrations for left censored data rather than assigning a value of zero to measurements below a detection limit (Hornung and Reed 1990). Differences of the analytes' concentrations between the male–female cohorts, regions and seasons were analyzed by independent sample *t* test. Spearman correlation coefficient (two-tailed) was used to assess the correlation of analytes with food consumption, age, gender and body mass index (BMI). One-way ANOVA was used to compare analyte concentrations in the rice consumption groups. A level of alpha 0.05 was assigned for statistical significance. Box plots were used to describe analyte concentration by region and season. The box represents the central data of distribution where upper and lower limits of the box indicate 25th and 75th percentiles (first quartile or Q1 and third quartile or Q3, respectively), and the median value is presented as a line inside the box.

Results

Demographic characteristics of the study cohorts

Demographic characteristics of the two cohorts are summarized in Table 1. One-half of the participants enrolled in both samples collection were male and the other half female. Among all participants, 30 (15 males, 15 females) were inhabitants of a rural and 32 (16 males and 16 females) of an urban area. The mean age of our volunteers was 39 ± 11 years (range 22–60 years) with no significant difference in gender or regions. The average body mass index (BMI) of the urban cohort was significantly higher (23.8 ± 3.2 kg/m^2) than that of the rural cohort (21.4 ± 2.6 kg/m^2). There were no significant differences in urinary creatinine levels between rural and urban cohorts, yet a significant difference was observed between summer (0.43 ± 0.51 g/L) and winter (0.78 ± 0.51 g/L) season. The higher level of urinary creatinine found in winter may be related to seasonal differences in food/drinking habits and/or collection of first morning void urine which is more concentrated. The rural people are mainly farmers or farm workers involved in grain production; housewives in rural areas often also take part in farm work. The urban residents are university employees or work at home (housewives). All participants consumed the typical Bangladeshi staple food rice and, to a lesser extent, roti (whole wheat flatbread); these are generally consumed with vegetables, lentils, fish, poultry and beef.

Table 1 Regional distribution and demographic characteristics of the participants

Characteristics	Rural cohort	Urban cohort	All
Subjects (*n*)	30	32	62
Gender (*n*)			
Men	15	16	31
Women	15	16	31
Age (years)			
Mean ± SD	41 ± 12	36 ± 9	39 ± 11
Range	22–60	24–57	22–60
Occupation, *n* (%)			
Men			
Farmers	11 (73)	0	11 (35)
Office worker	0	16 (100)	16 (52)
Student	1 (7)	0	1 (3)
Others	3 (20)	0	3 (10)
Women			
Housewives	15 (100)	11 (69)	26 (84)
Office workers	0	2 (12)	2 (6)
Student	0	3 (19)	3 (10)
BMI (kg/m^2)			
Mean ± SD	21.4 ± 2.6	23.8 ± 3.2[a]	22.6 ± 3.2
Range	16.2–27.3	18.9–32.9	16.2–32.9
Creatinine (mg/L)[c]			
Summer	388 ± 407	412 ± 468	400 ± 436
Winter	719 ± 405	871 ± 664[b]	798 ± 554

[a] $p < 0.001$ when compared to rural cohort and [b] $p < 0.05$ when compared to summer

[c] Value presented as mean ± SD

Urinary levels of OTA and OTα by cohorts and seasons

Urines with analyte concentrations at or above the limit of detection (LOD) were considered as positive samples. The distribution of urinary levels of ochratoxin A and its metabolite OTα in the rural and urban cohort are shown in Fig. 1. Overall, in summer the OTA and OTα detection frequency was 71 and 53 % and in winter 98 and 62 %, respectively (Table 2). Urinary levels of OTA and OTα in the two cohorts and male–female subgroups in summer and winter are given in Table 2. For OTA, no significant difference was found between the rural and urban cohorts in summer. However, in rural cohort a significant difference

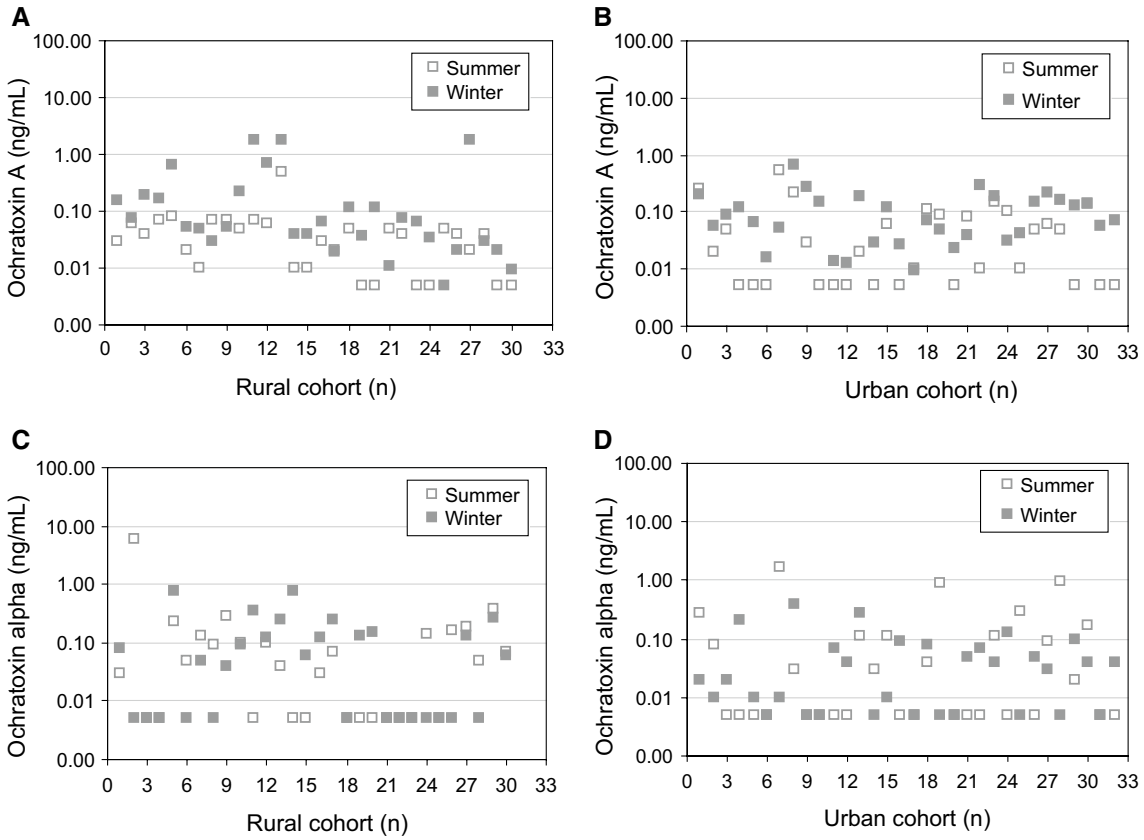

Fig. 1 Distribution of ochratoxin A and ochratoxin alpha in the rural (**a**, **b**) and urban (**c**, **d**) cohort in summer and winter season. According to serial number on the x-axis, the first half are men and second half are women participants in both cohorts. Log-transformed values are presented in the graph

($p < 0.05$) was found for the mean OTA level between the summer (0.05 ± 0.08 ng/mL) and winter (0.28 ± 0.52 ng/mL) season. The mean level of OTA for all subjects (both cohorts) was significantly higher ($p < 0.01$) in winter (0.19 ± 0.38 ng/mL) than in summer (0.06 ± 0.10 ng/mL) season (Table 2).

The metabolite OTα was present in summer urine at about fourfold higher levels than OTA (mainly due to one OTα value of 6.10 ng/mL) and twofold lower than the parent compound in winter. The ratio of OTA to OTα in the male and female subgroups of both cohorts varied considerably (Fig. 1). The rural cohort with higher urinary levels of parent compound OTA apparently excreted also more of the non-toxic metabolite (Table 2). As depicted in Fig. 3a, excretion of OTA is significantly higher ($p < 0.05$) in winter than in summer season in the rural area. Statistical analysis revealed no significant correlation of urinary biomarkers with age, gender and BMI of the study subjects. The biomarker levels for all subjects enrolled in summer ($n = 69$) and in winter ($n = 95$) are presented in Supplementary Table 1.

Urinary levels of CIT and HO-CIT by cohorts and seasons

The distribution of urinary levels of CIT and its metabolite HO-CIT in the rural and urban cohort are shown in Fig. 2. Urines with analyte concentrations at or above the limit of detection (LOD) were considered as positive samples. Overall, in summer the CIT and HO-CIT detection frequency was 95 and 71 % and in winter 92 and 97 %, respectively (Table 3). Urinary CIT and HO-CIT levels of the two cohorts and of subgroups in two seasons are given in Table 3. In summer, a significant difference ($p < 0.05$ and $p < 0.01$) was found for the mean level of CIT and HO-CIT between the rural (0.14 ± 0.22, 0.78 ± 1.33 ng/mL) and the urban (0.06 ± 0.08, 0.08 ± 0.08 ng/mL) cohort. In winter season, only the mean level of HO-CIT showed a significant difference ($p < 0.001$) between the rural (5.95 ± 1.63 ng/mL) and the urban (0.60 ± 1.02 ng/mL) cohort. On the other hand, in both cohorts a significant difference ($p < 0.05$) was found for CIT and HO-CIT levels between summer and winter season (Table 3).

Table 2 Levels of OTA and its metabolite OTα in urines according to gender, region and season

Gender	*n*	Summer				Winter			
		Positive *n* (%)	Mean ± SD (ng/mL)	Median (range) (ng/mL)	Mean ± SD (ng/mg crea)	Positive *n* (%)	Mean ± SD (ng/mL)	Median (range) (ng/mL)	Mean ± SD (ng/mg crea)
Rural cohort									
OTA									
Men	15	15 (100)	0.08 ± 0.11	0.06 (0.01–0.48)	0.21 ± 0.23	15 (100)	0.39 ± 0.58	0.15 (0.03–1.75)	0.53 ± 0.76
Women	15	9 (60)	0.03 ± 0.02	0.02 (nd–0.05)	0.11 ± 0.09	14 (93)	0.16 ± 0.43	0.03 (nd–1.72)	0.21 ± 0.42
All	30	24 (80)	0.05 ± 0.08[a]	0.04 (nd–0.48)	0.16 ± 0.18	29 (97)	0.28 ± 0.52	0.06 (nd–1.75)	0.37 ± 0.63
OTα									
Men	15	10 (67)	0.48 ± 1.56	0.05 (nd–6.10)	0.77 ± 1.98	10 (67)	0.17 ± 0.26	0.06 (nd–0.79)	0.20 ± 0.26
Women	15	8 (53)	0.08 ± 0.11	0.03 (nd–0.38)	0.33 ± 0.35	7 (47)	0.08 ± 0.09	0.01 (nd–0.27)	0.20 ± 0.39
All	30	18 (60)	0.28 ± 1.10	0.04 (nd–6.10)	0.55 ± 1.42	17 (57)	0.12 ± 0.20	0.05 (nd–0.79)	0.20 ± 0.33
Urban cohort									
OTA									
Men	16	8 (50)	0.08 ± 0.15	0.01 (nd–0.55)	0.11 ± 0.15	16 (100)	0.13 ± 0.17	0.08 (0.01–0.69)	0.14 ± 0.13
Women	16	12 (75)	0.06 ± 0.05	0.05 (nd–0.15)	0.21 ± 0.24	16 (100)	0.11 ± 0.08	0.07 (0.01–0.30)	0.16 ± 0.12
All	32	20 (62)	0.07 ± 0.11	0.02 (nd–0.55)	0.16 ± 0.20	32 (100)	0.12 ± 0.13	0.07 (0.01–0.69)	0.15 ± 0.12
OTα									
Men	16	7 (44)	0.15 ± 0.43	0.01 (nd–1.72)	0.27 ± 0.52	12 (75)	0.07 ± 0.12	0.02 (nd–0.39)	0.10 ± 0.17
Women	16	8 (50)	0.16 ± 0.30	0.01 (nd–0.95)	0.61 ± 1.20	10 (62)	0.04 ± 0.04	0.04 (nd–0.13)	0.07 ± 0.09
All	32	15 (47)	0.16 ± 0.36	0.01 (nd–1.72)	0.44 ± 0.93[b]	22 (69)	0.06 ± 0.09	0.03 (nd–0.39)	0.09 ± 0.13
Both cohorts									
OTA									
All	62	44 (71)	0.06 ± 0.10[c]	0.03 (nd–0.55)	0.16 ± 0.19	61 (98)	0.19 ± 0.38	0.06 (nd–1.75)	0.25 ± 0.45
OTα									
All	62	33 (53)	0.21 ± 0.81[d]	0.03 (nd–6.10)	0.49 ± 1.18	39 (62)	0.09 ± 0.15	0.04 (nd–0.79)	0.14 ± 0.25

Positive sample refer to urines containing the analyte ≥ limit of detection (LOD); *nd* is level below LOD. Samples that contained analyte levels below LOD are assigned a value of one-half of LOD during calculation of mean and median values

[a] $p < 0.05$, [b] $p < 0.05$, [c] $p < 0.01$ and [d] $p < 0.05$ when compared to winter season

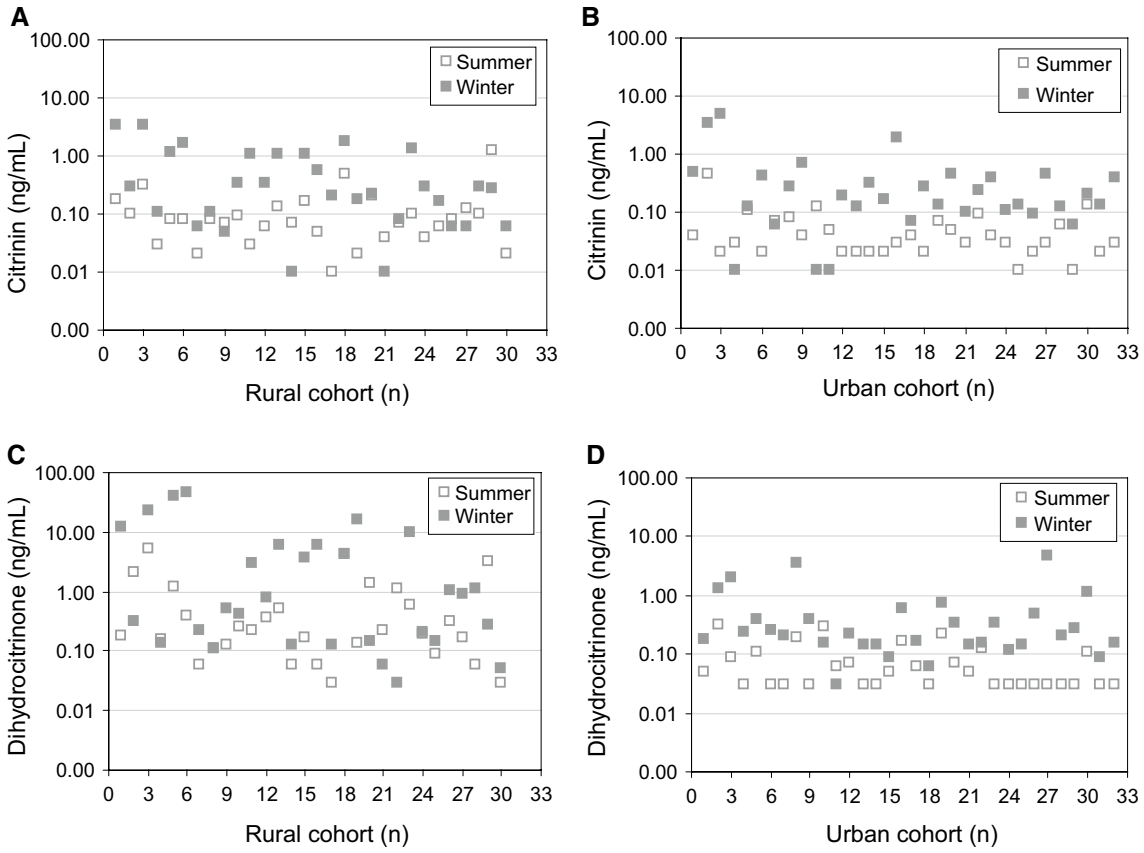

Fig. 2 Distribution of citrinin and dihydrocitrinone in the rural (**a**, **b**) and urban (**c**, **d**) cohort in summer and winter season. According to serial number on the x-axis, the first half are men and second half are women participants in both cohorts. Log-transformed values are presented in the graph

In urines from both seasons, the metabolite HO-CIT was present at about fivefold higher levels than the parent compound, indicative of HO-CIT as useful additional biomarker of citrinin exposure. Although the ratio of CIT to HO-CIT in the male and female subgroups of both cohorts varied considerably (Fig. 2), the rural cohort with higher urinary levels of CIT (parent compound) apparently excretes also more of the metabolite (Fig. 2; Table 3). Urinary total citrinin (CIT + HO-CIT) is significantly higher in the rural cohort than in the urban cohort of Bangladeshi adults, and a significant difference between summer and winter season also indicates a different exposure by season (Fig. 3b). The biomarker levels for all subjects enrolled in summer ($n = 69$) and in winter ($n = 95$) are presented in Supplementary Table 2. Statistical analysis revealed no significant correlation of urinary biomarkers with age, gender and BMI of the study subjects.

Co-occurrence and correlation of OTA with total CIT

Considering all positive samples (≥LOD), the presence (co-occurrence) of both OTA and total CIT biomarkers in urine was 68 % in summer and 97 % in winter season, respectively (Table 4). A significant positive co-relationship was found between OTA and total CIT in the rural cohort ($p < 0.05$) and also for all subjects ($p < 0.01$) in winter season. The higher percentage of co-occurrence and significant positive correlation between two biomarkers may be related to the higher levels of OTA and total CIT in winter urines and/or the presence of fungi on stored crops which may produce both mycotoxins under certain climatic conditions.

Biomarker-based CIT intake estimates for the Bangladeshi cohorts

The daily intake of CIT was calculated based on a urinary excretion rate of total CIT (36 %) over 24 h as observed in a human volunteer (Ali et al. 2015c). When expressed as µg/kg b.w./day, the mean daily intake of CIT was 0.043 ± 0.099 and 0.304 ± 0.776 µg/kg b.w./day in the summer and winter season, respectively (Table 5). The estimated daily intake was significantly higher in the rural cohort than in the urban cohort, both in summer ($p < 0.01$) and in winter ($p < 0.05$) season (Table 5). This can be compared with the preliminary

Table 3 Levels of CIT and its metabolite HO-CIT in urines according to gender, region and season

Gender	*n*	Summer				Winter			
		Positive *n* (%)	Mean ± SD (ng/mL)	Median (range) (ng/mL)	Mean ± SD (ng/mg crea)	Positive *n* (%)	Mean ± SD (ng/mL)	Median (range) (ng/mL)	Mean ± SD (ng/mg crea)
Rural cohort									
CIT									
Men	15	15 (100)	0.10 ± 0.07	0.08 (0.02–0.31)	0.34 ± 0.35	14 (93)	0.94 ± 1.13	0.34 (nd–3.51)	1.33 ± 1.78
Women	15	14 (93)	0.17 ± 0.31	0.07 (nd–1.22)	0.72 ± 1.07	14 (93)	0.37 ± 0.50	0.21 (nd–1.77)	0.88 ± 1.48
All	30	29 (97)	0.14 ± 0.22[a]	0.08 (nd–1.22)	0.53 ± 0.80 [g]	28 (93)	0.66 ± 0.91	0.28 (nd–3.51)	1.11 ± 1.63
HO-CIT									
Men	15	15 (100)	0.76 ± 1.40	0.22 (0.06–5.39)	1.46 ± 1.92	15 (100)	9.17 ± 15.35	0.77 (0.11–46.44)	10.09 ± 15.59
Women	15	13 (87)	0.80 ± 1.30	0.19 (nd–4.39)	4.17 ± 8.41	14 (93)	2.72 ± 4.71	0.27 (nd–16.12)	4.36 ± 6.91
All	30	28 (93)	0.78 ± 1.33[b]	0.20 (nd–5.39)	2.81 ± 6.15[h]	29 (97)	5.95 ± 11.63[j]	0.65 (nd–46.44)	7.23 ± 12.20[k]
Urban cohort									
CIT									
Men	16	16 (100)	0.07 ± 0.11	0.04 (0.02–0.45)	0.23 ± 0.27	13 (81)	0.83 ± 1.43	0.24 (nd–5.03)	1.36 ± 2.62
Women	16	14 (88)	0.04 ± 0.03	0.03 (nd–0.13)	0.18 ± 0.12	16 (100)	0.21 ± 0.14	0.13 (0.06–0.46)	0.34 ± 0.30
All	32	30 (90)	0.06 ± 0.08[c]	0.03 (nd–0.45)	0.20 ± 0.21	29 (91)	0.52 ± 1.05	0.18 (nd–5.03)	0.85 ± 1.90
HO-CIT									
Men	16	10 (62)	0.10 ± 0.09	0.06 (nd–0.31)	0.37 ± 0.35	15 (94)	0.62 ± 0.94	0.23 (nd–3.60)	0.74 ± 0.99
Women	16	6 (38)	0.06 ± 0.05	0.03 (nd–0.22)	0.25 ± 0.16	16 (100)	0.57 ± 1.12	0.19 (0.06–4.64)	0.98 ± 2.07
All	32	16 (50)	0.08 ± 0.08[d]	0.04 (nd–0.31)	0.31 ± 0.27	31 (97)	0.60 ± 1.02	0.21 (nd–4.64)	2.86 ± 1.60
Both cohorts									
CIT									
All	62	59 (95)	0.10 ± 0.17[e]	0.05 (nd–1.22)	0.36 ± 0.60[i]	57 (92)	0.59 ± 0.98	0.21 (nd–5.03)	0.97 ± 1.76
HO-CIT									
All	62	44 (71)	0.42 ± 0.98[f]	0.10 (nd–5.39)	1.52 ± 4.43	60 (97)	3.18 ± 8.49	0.27 (nd–46.44)	3.94 ± 9.07

Positive sample refers to urines containing the analyte ≥ limit of detection (LOD); *nd* is level below LOD. Samples that contained analyte levels below LOD are assigned a value of one-half of LOD during calculation of mean and median values

[a] $p < 0.01$, [b] $p < 0.05$, [c] $p < 0.05$, [d] $p < 0.01$, [e] $p < 0.01$, [f] $p < 0.05$ and [i] $p < 0.05$ when compared to winter season. In summer [a] $p < 0.05$, [b] $p < 0.01$, [g] $p < 0.05$, [h] $p < 0.05$ and in winter [j] $p < 0.01$, [k] $p < 0.05$ when compared to urban cohort

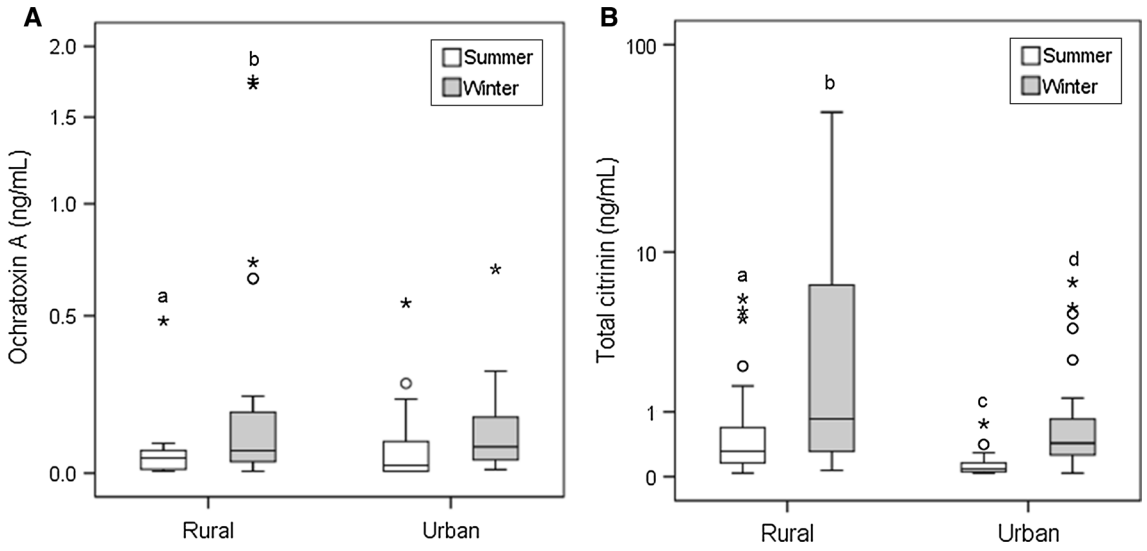

Fig. 3 Urinary biomarker levels of OTA (**a**) and total CIT (**b**) in the rural and urban cohorts in summer and winter season. In **a**, significant difference found between *a* and *b* ($p < 0.05$). In **b**, significant difference found between *a* and *b* ($p < 0.05$), *c* and *d* ($p < 0.01$), *a* and *c* ($p < 0.01$) and *b* and *d* ($p < 0.05$)

Table 4 Co-occurrence and correlation of ochratoxin A with total citrinin

Cohort	*n*	Summer		Winter	
		Co-occurrence *n* (%)	Correlation (*r*)	Co-occurrence *n* (%)	Correlation (*r*)
Rural	30	23 (77)	0.249	29 (97)	0.443*
Urban	32	19 (59)	0.024	31 (97)	0.223
All	62	42 (68)	0.114	60 (97)	0.333**

p value obtained from Spearman's correlation coefficient (two-tailed)

Positive samples (≥LOD) are considered for the calculation. * $p < 0.05$, ** $p < 0.01$

Table 5 Provisional daily intake (PDI) of citrinin (µg/kg b.w.) calculated from urine biomarker concentrations

Cohort	Summer					Winter				
	n	Mean ± SD	Median	Range	Exceeding TDI[c], *n* (%)	*n*	Mean ± SD	Median	Range	Exceeding TDI[c], *n* (%)
Rural	30	0.079 ± 0.134[a]	0.022	0.003–0.506	3 (10)	30	0.550 ± 1.064[b]	0.073	0.005–4.663	11 (37)
Urban	32	0.009 ± 0.009	0.005	0.002–0.049	0 (0)	32	0.073 ± 0.109	0.026	0.002–0.461	4 (12)
All	62	0.043 ± 0.099	0.014	0.002–0.506	3 (10)	62	0.304 ± 0.776	0.043	0.002–4.663	15 (24)

See 'Methods' section and text for further explanations

[a] $p < 0.01$, [b] $p < 0.05$ when compared to urban cohort. *p* value obtained from independent sample *t* test

[c] Preliminary TDI 'no concern for nephrotoxicity in humans' at 0.2 µg/kg b.w. per day (EFSA 2012)

tolerable daily intake for CIT of 0.2 µg/kg body weight per day discussed by EFSA (2012) ('no concern for nephrotoxicity in humans'). There were 10 % participants in summer and 24 % participants in winter who had a CIT exposure above the tolerable daily intake proposed by EFSA (2012), and the highest calculated intake in one person was 4.66 µg/kg b.w./day (Table 5).

Correlation between biomarker levels in urine and food consumption pattern

Based on information provided in the food frequency questionnaires (FFQ), possible correlations were analyzed between food consumption (graded 1–4) in the cohorts and urinary biomarkers level of OTA, CIT and their

Table 6 Correlation between urinary biomarker levels and last 2 days of food consumption

Food items	Summer		Winter	
	Correlation *r* (*p* value)		Correlation *r* (*p* value)	
	OTA	Total CIT	OTA	Total CIT
Rice	0.07 (0.56)	0.25 (0.07)	0.03 (0.85)	0.16 (0.20)
Wheat/maize	−0.05 (0.56)	−0.20 (0.12)	−0.07 (0.59)	−0.21 (0.10)
Lentil	0.01 (0.92)	−0.21 (0.09)	0.19 (0.13)	−0.07 (0.56)
Milk and milk products	−0.05 (0.71)	−0.21 (0.09)	0.19 (0.12)	0.02 (0.88)
Chicken	−0.04 (0.08)	−0.15 (0.14)	−0.03 (0.80)	0.06 (0.64)
Coffee	−0.17 (0.18)	−0.14 (0.26)	−0.12 (0.35)	−0.09 (0.45)

Assessment of food consumption is done using the score 1–4 (1 = do not consume at all, 2 = 1 time daily, 3 = 2 times daily and 4 = 3 times daily. Positive samples (≥LOD) are considered for correlation analysis

p values obtained from Spearman's correlation coefficient (two-tailed)

Table 7 Urinary biomarkers level and last 2 days of rice consumption

Group	*n*	Summer		Winter	
		Mean ± SD		Mean ± SD	
		OTA (ng/mL)	Total CIT (ng/mL)	OTA (ng/mL)	Total CIT (ng/mL)
Low	8	0.04 ± 0.05	0.08 ± 0.06	0.11 ± 0.07	0.41 ± 0.18
Medium	27	0.05 ± 0.07	0.54 ± 1.38	0.11 ± 0.14	2.34 ± 5.66
High	27	0.07 ± 0.13	0.62 ± 0.93*	0.30 ± 0.54	5.59 ± 12.10

Low = 1 time/day, medium = 2 times/day and high = 3 times/day rice consumption

* $p < 0.05$ when compared to low consumption group. One-way ANOVA used for analysis

metabolites. Using Spearman correlation analysis, intake of the food items rice, wheat/maize, lentil soup, chicken meat, milk and milk-based products and coffee did not show significant associations with the urinary biomarker levels (Table 6). However, by one-way ANOVA analysis, the highest OTA and total CIT biomarker levels were observed in the high rice consumption group compared to medium and low rice consumption group (Table 7). The biomarker levels with respect to consumption of other food commodities are presented in Supplementary Table 3.

Discussion

When food contaminant data are scarce or missing as in Bangladesh (Islam and Hoque 2013), analysis of mycotoxin biomarkers in human urines (biomonitoring) is a valuable approach to gain more insights into human exposure to certain mycotoxins. Based on findings from a pilot study (Ali et al. 2015b) and a screening that used multitoxin analysis in urines (Gerding et al. 2015), the present investigation focused on the two nephrotoxic mycotoxins CIT and OTA. This is the first study in rural and urban residents of Bangladesh that investigated their exposure to OTA and CIT along with regional and seasonal influences on urinary biomarker levels and/or possible gender differences. Sensitive targeted methods of analysis enabled detection of OTA, CIT and their main metabolites (OTα and HO-CIT) in most urines collected in summer and winter season (Tables 2, 3). The data are discussed first with regard to variables (gender, region, season) and possible sources of dietary mycotoxin intake in our cohorts. Then biomarker levels in Bangladeshi urines are compared with those reported for other countries. Finally, possibilities and limitations to relate biomarker-based exposure estimates to existing tolerable daily intake values for CIT and OTA are addressed.

Effects of variables (gender, region, season, dietary habits) on biomarker levels

Gender Females often had lower mean levels of OTA and CIT biomarkers in urines collected in both seasons, except for a higher CIT level in the rural cohort in summer (Tables 2, 3). But, statistical data analysis did not reveal significant differences in biomarker levels between the male and female subgroups. This is not surprising in light of considerable interindividual variability in biomarker levels (Figs. 1, 2). For urinary OTA, a lack of significant difference between male and female participants was also observed in several studies carried out in Europe (Duarte et al. 2009, 2010; Manique et al. 2008; Coronel et al. 2011). Such data are not yet available for CIT biomarkers.

Region Overall, the mean biomarker levels were higher in the rural than in the urban cohort in both seasons (Fig. 3). For OTA, the difference between the two cohorts was statistically not significant. Yet, CIT and HO-CIT levels were significantly higher in the rural than in the urban cohort in summer season (Table 3), and when considering total CIT (the sum of CIT and its metabolite HO-CIT), a significant difference was observed between the cohorts in both seasons (Fig. 3). At present, it is unclear whether this biomarker result reflects a difference in food habits and/or an additional occupational exposure in the rural cohort: They are mainly farmers involved in grain production, while urban people mainly work in offices or at home (Table 1). There are also some differences in eating habits between these cohorts. Rural people mainly depend on home-cooked food, while urban people also take restaurant food occasionally beside the homemade food. The urban people eat less rice than the rural people (more details in Ali et al. 2015b).

Season The biomarker levels for both nephrotoxic mycotoxins were significantly higher in winter (OTA 0.19 ± 0.38 ng/mL; CIT 0.59 ± 0.98 ng/mL) than in summer (OTA 0.06 ± 0.10 ng/mL; CIT 0.10 ± 0.17 ng/mL) season (Tables 2, 3). The clear effect of season on urinary biomarkers is not only observed for study participants who provided urines in both sampling periods ($n = 62$; Fig. 3; Tables 2, 3), but also seen in the larger cohorts ($n = 69$ in summer, $n = 95$ in winter; Supplementary Table 1 and 2). This seasonal variation may be related to the following factors: Bangladesh has a subtropical monsoon climate with wide seasonal variations in rainfall, temperature and humidity, while regional climatic differences in this flat country are minor (http://en.wikipedia.org/wiki/Geography_of_Bangladesh). Crops are mainly produced in summer and in wintertime, and harvested crops from one season are largely consumed in the next season until new ones become available. The climate with mostly high humidity can favor the growth of various mycotoxin-producing fungi on crops in the field or in stored food commodities in Bangladesh. Several *Aspergillus* and *Penicillium* species are known to produce OTA and/or CIT under various environmental conditions (Frisvad et al. 2006; Stoll et al. 2013; Schmidt-Heydt et al. 2015). The biomarker results from our study indicated frequent co-occurrence of OTA and CIT in both sampling periods, and a significant positive correlation was observed between OTA and total CIT in urine in winter season (Table 4).

Possible sources of dietary mycotoxin intake

Data on CIT and OTA contamination of food commodities in Bangladesh are not available, yet we made an attempt to correlate the individual biomarker levels with the consumption frequency for certain food categories in our cohort. There was no significant correlation between urinary biomarker levels and consumption of several food items during the last 2 days (Table 6). However, the mean levels of OTA and total CIT were considerably higher in the high rice consumption group when we categorized the entire cohort by rice consumption frequency during the last 2 days (Table 7) or by regular food habit records (data not shown). Consumption of other foodstuffs such as wheat/maize, lentil soup, milk products, chicken meat and coffee did not show significant correlations with mycotoxin biomarker levels in urine (Supplementary Table 3).

Rice is staple food in Bangladesh and often consumed with curries prepared with several spices. Both rice and spices may be sources of CIT and OTA intake in our cohorts, considering mycotoxin occurrence data from other countries: CIT and/or OTA have been detected in rice from India and Vietnam (Reddy et al. 1983; Nguyen et al. 2007) and also in rice from China, Pakistan and Canada (Lai et al. 2015; Majeeda et al. 2013; Bansal et al. 2011). OTA and CIT were also found in different spices commonly marketed in India (Saxena and Mehrotra 1989; Jeswal and Kumar 2015) and in chilies in Sri Lanka (Yogendrarajah et al. 2014). Such information on mycotoxin contamination of rice and spices marketed in Bangladesh is not available, and also other food items may contribute to exposure.

Comparison of biomarker levels in Bangladesh and other countries

Our new results for Bangladesh can be compared with OTA and CIT biomarker data from studies carried out in other countries on different continents. Although these studies often applied various methods for biomarker analysis with different detection limits, the reported mean concentrations and ranges can provide an orientation for OTA and CIT urinary levels found in these populations. As urine data for OTA have been reviewed earlier (Coronel et al. 2011; Duarte et al. 2011), the following comparison will only focus on some more recent studies.

OTA biomarkers In the present study, the mean level of OTA was 0.06 ± 0.10 (range <LOD–0.55) in summer, but 0.19 ± 0.38 (range <LOD–1.75) ng/mL in winter urines of both Bangladeshi cohorts (Table 2). Lower or similar levels of OTA were found in urines of Belgian cohorts (in adults: mean 0.028, range 0.002–0.368 ng/mL, in children: mean 0.079, range 0.003–3.683 ng/mL; Heyndrickx et al. 2015) and in two German cohorts (mean 0.04 ± 0.01, range <LOQ–0.0.08 ng/mL; Gerding et al. 2015; and mean 0.07 ± 0.05, range 0.02–0.29 ng/mL; Muñoz et al. 2010). Relatively, low OTA levels were also found in the Portuguese population (mean 0.019 ± 0.012, range nd–0.071 reported by Duarte et al. 2012, and mean 0.022, range nd–0.069 in Duarte et al. 2010). Higher levels of urinary OTA were found in adults in Southern Italy

(mean 0.144 ± 0.312, range LOQ–2.13 ng/mL; Solfrizzo et al. 2014) and in Spain (mean 0.237 ± 0.181, range 0.057–0.562 ng/mL; Coronel et al. 2011). A recent study in Swedish adults reported even higher average levels of urinary OTA (mean 0.46 ± 0.57 ng/mL; Wallin et al. 2015). Some data are also available for non-European countries: Urinary OTA was detected in children and adults in Haiti (mean 0.06, range <LOQ–0.83 ng/mL; Gerding et al. 2015) and in breastfed Chilean infants (mean 0.05–0.16, range LOD–0.43 ng/mL; Muñoz et al. 2014). OTA was found in urines of Nigerian adults and children (mean 0.2 ± 0.1, range nd–0.6 ng/mL; Ezekiel et al. 2014) and in Cameroon infants (mean 0.2, range 0.04–2.4 ng/mL; Ediage et al. 2013). The highest urinary OTA levels were previously reported in children in Sierra Leone in dry (range 0.07–148 ng/mL) and in rainy (range 0.6–72.2) season (Jonsyn 2000). This study found also rather high levels of 4-hydroxy-OTA (4R-OH-OTA) in urines from boys and girls. The same metabolite was detected, yet not quantified, in urines of Bulgarian adults (Castegnaro et al. 2006). However, as 4R-OH-OTA was not detectable in an earlier study of BEN patients by the same group, or in other studies, its role as additional biomarker of OTA exposure is unclear (Castegnaro et al. 1991).

Cleavage of the peptide bond in OTA by the intestinal microflora produces the nontoxic metabolite OTα that is then excreted mainly as glucuronide and often present in urine at clearly higher amounts than OTA parent compound (Muñoz et al. 2010; Coronel et al. 2011; Klapec et al. 2012). In Bangladeshi adults, OTα (Table 2) was found in summer (mean 0.21 ± 0.81, range nd–6.10 ng/mL) and in winter urine (mean 0.09 ± 0.15, range nd–0.79 ng/mL). Their mean OTα levels are lower compared to those found in Spanish (0.44 ± 0.51, range 0.06–2.89 ng/mL; Coronel et al. 2011) and in German adults (mean 2.88 ± 2.24, range 0.49–7.12 ng/mL; Muñoz et al. 2010) or in Croatian pregnant women (mean 1.73 ± 1.65, range 0.11–7.57 ng/mL; Klapec et al. 2012). As yet it is unclear whether the presence of higher OTα levels in European samples is indicative of more efficient detoxication of OTA to OTα and/or related to dietary differences between Bangladeshi and European cohorts. Thus, we agree with conclusions of Coronel et al. (2011) that further research is needed to elucidate the relation between ingested OTA and excreted OTA and metabolites in humans and that inter-individual differences in metabolism abilities should also be considered.

CIT biomarkers Compared to OTA, the biomonitoring for CIT is far less developed, and therefore, fewer biomarker data from other countries are as yet available. Concentrations of CIT biomarkers in urines from Bangladeshi adults (see Table 3 for CIT and HO-CIT mean values and ranges and Fig. 3 for total CIT) are clearly higher than those reported for German adults (CIT mean 0.03 ± 0.02, range nd–0.08 ng/mL and HO-CIT mean 0.10 ± 0.10, range nd–0.51 ng/mL; Ali et al. 2015a) and those for Belgian adult cohorts (CIT mean 0.026, range 0.002–0.117 ng/mL and HO-CIT mean 0.035, range 0.013–0.210 ng/mL, Huybrechts et al. 2015; or CIT mean 0.057, range 0.002–1.398 ng/mL and HO-CIT mean 0.752, range 0.143–2.117 ng/mL (Heyndrickx et al. 2015). Another recent study by Gerding et al. (2015) with analysis of the metabolite HO-CIT (but not parent compound) confirmed clearly higher levels in Bangladeshi (winter) urines (mean 2.75 ± 8.43, range <LOQ–58 ng/mL) than in Haitian (mean 0.49 ± 0.95, range <LOQ–4.34 ng/mL) and German samples (mean 0.12 ± 0.02, range <LOQ–0.33 ng/mL). Overall, these biomarker data indicate notable differences in citrinin exposure between the Bangladeshi and other populations.

All studies, including the present one, report often clearly lower concentrations of CIT than its metabolite HO-CIT in urine, while average and inter-individual ratios of CIT/HO-CIT urine levels are apparently quite variable (Ali et al. 2015a, b; Heyndrickx et al. 2015; Huybrechts et al. 2015). This observation is of interest since the formation of the metabolite HO-CIT is considered as detoxication reaction (Föllmann et al. 2014). It is presently unknown which enzymes catalyze this reaction, and the ability to convert CIT to HO-CIT may vary among individuals. Thus, it is indicated to measure CIT as well as its metabolite in biomonitoring studies, and consider the sum of both analytes as biomarker of exposure to this nephrotoxic mycotoxin.

In short, biomonitoring studies indicate more or less pronounced differences in OTA and CIT exposure between populations of several countries. Their urinary biomarker levels reflect probably a different degree of mycotoxin contamination of local food commodities and also differences in food consumption patterns.

Biomarker-based exposure estimates and tolerable intake values for OTA and CIT

Mycotoxin contamination of food commodities cannot be completely avoided, yet regulations (maximum residual levels) have been set in many countries to protect the consumer, and Scientific Advisory Committees have derived tolerable daily intake (TDI) values for certain mycotoxins based on toxicological risk assessments (e.g., EFSA 2006, 2012). With regard to the question whether exposure in certain cohorts exceeds a given TDI, urine biomarker data may be used for estimating the probable daily intake, if enough information on kinetics and urinary excretion rates in humans is available. Below, we discuss uncertainties that exist when converting urine biomarker concentrations for OTA and CIT to amounts of mycotoxin intake.

Overall, a good correlation was reported between urine OTA levels in UK adults and dietary OTA consumption (Gilbert et al. 2001) and, more recently, also between infant OTA intake with breast milk and OTA concentrations in babies' urines (Muñoz et al. 2014). But, the relation between ingested OTA dose and excreted OTA amounts can vary between individuals (Castegnaro et al. 2006), possibly due to inter-individual differences in metabolism as discussed already above. Moreover, one has to keep in mind that only a very small fraction of OTA is excreted: In a human volunteer who ingested ^{3}H-OTA, about 5 % of the ^{3}H-dose was excreted over 24 h in urine, and only part of the radioactivity (between 42 and 54 %) in urine extracts was unmetabolized OTA (Studer-Rohr et al. 2000). This indicates a human urinary OTA excretion rate of 2 % or less per day at the given exposure, a value similar to about 2.5 % OTA excretion found in 24 h urines of piglets fed with OTA (Gambacorta et al. 2013). Mycotoxin intake estimates on the basis of urine OTA concentrations thus involve a high degree of uncertainty, also as usually spot urines or first morning voids (not 24 h urines) are analyzed, and data on temporal variability in humans and possible dose-related effects on renal OTA excretion are scarce (Degen 2015). For this reason, we and others refrain so far from 'translating' OTA urine levels to probable daily intake values. On the other hand, blood is a suitable matrix for OTA biomonitoring, and in several studies the mean OTA levels in plasma or serum have served to estimate daily dietary OTA intake, based on the Klaassen equation (Duarte et al. 2011; Märtlbauer et al. 2009).

For CIT, there is now information on kinetics in a human volunteer who ingested a small dose (45 ng/kg b.w.): Urine analysis during the next 24 h revealed a cumulative excretion of about 36 % of the CIT dose (12 % as parent compound, 24 % as HO-CIT; Ali et al. 2015c). This value for excretion can be used now for converting the total biomarker concentrations to mycotoxin intake (probably daily intake, calculated as described in the Methods section), and was applied here to assess CIT exposure in the Bangladeshi cohort (Table 5). Their probable daily intake (PDI) for CIT was then compared to the tolerable daily intake value (TDI) for CIT of 0.2 µg/kg bw ('no concern for nephrotoxicity in human') derived by EFSA (2012). The TDI value of 0.2 µg/kg b.w./day proposed by EFSA (2012) was exceeded in 10 % of all study participants in summer and 24 % in winter, and more than 20-fold in one person with a calculated CIT intake of >4 µg/kg b.w. The uncertainty in estimates of probably daily intake for CIT is much lower than in the case of OTA, due to the rather high fraction of CIT biomarker excretion in urine. Finally, OTA being a more potent nephrotoxin than CIT (EFSA 2006, 2012), the observed frequent co-exposure to both mycotoxins (Table 4) in Bangladeshi cohorts raises some concern.

Conclusions

The results of this biomonitoring study indicate a variable but frequent exposure to the two nephrotoxic mycotoxins CIT and OTA in the Bangladeshi population. Biomarker data analysis revealed season, region and food consumption patterns as determinants of mycotoxin exposure in our cohorts. Their urinary levels for CIT and OTA biomarkers are often higher than those found in several European studies. The new data point to the need to identify major sources of CIT and OTA intake in Bangladesh, and conduct also biomonitoring studies in population groups which may be at increased risk such as children and pregnant women.

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Compliance with ethical standards

Conflict of interest None of the authors have any conflict of interest to declare.

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Publication 4

Occurrence of the mycotoxin citrinin and its metabolite dihydrocitrinone in urines of German adults

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Contribution statement

NA and GHD co-conceived study idea and designed study. NA had the responsibility to collect urine samples, performed the biomarker analysis, statistical data analysis and drafted the manuscript. MB supervised the LC-MS/MS analysis and its optimization and contributed to final editing of the manuscript. GHD supervised the data interpretation and co-wrote the manuscript with NA.

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TOXICOKINETICS AND METABOLISM

Occurrence of the mycotoxin citrinin and its metabolite dihydrocitrinone in urines of German adults

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Abstract As data on food contamination with the mycotoxin citrinin (CIT) are scarce, a recently developed method for biomarker analysis (Blaszkewicz et al. in Arch Toxicol 87:1087–1094, 2013) was applied to investigate CIT exposure of German adults. CIT and its human metabolite dihydrocitrinone (HO-CIT) were determined in urine samples from a group of 50 healthy adults ($n = 27$ females and $n = 23$ males). After cleanup by immunoaffinity (CitriTest®) columns, extracts were analyzed by LC–MS/MS. The mycotoxin and its major metabolite HO-CIT were detected in 82 and 84 % of all urine samples, at concentrations ranging from 0.02 (limit of detection, LOD) to 0.08 ng/mL for CIT, and 0.05 (LOD) to 0.51 ng/mL for HO-CIT. Median urine analyte levels in the cohort were 0.03 (CIT) and 0.06 ng/mL (OH-CIT) or adjusted to creatinine 20.2 ng/g crea (CIT) and 60.9 ng/g crea (HO-CIT), respectively. Except for higher urinary CIT levels in males, differences between subgroups were not significant. This first biomarker analysis indicates widespread and variable exposure to CIT in German adults, and conversion of ingested mycotoxin to its less toxic metabolite HO-CIT, which may serve as biomarker of exposure in addition to the parent compound.

Keywords Citrinin · Dihydrocitrinone · Exposure · Mycotoxins · Urine

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Introduction

Citrinin [$C_{13}H_{14}O_5$; IUPAC Name: (3*R*,4*S*)-8-hydroxy-3,4,5-trimethyl-6-oxo-4,6-dihydro-3H-isochromene-7-carboxylic acid] is a polyketide mycotoxin produced by several fungi of the genera *Penicillium, Aspergillus* and *Monascus* (Frisvad et al. 2006). In animal studies, CIT has similar toxic properties as ochratoxin A (OTA), another nephrotoxic mycotoxin contaminant in food commodities (Frank 1992; Flajs and Peraica 2009). This and co-occurrence of CIT and OTA in food and feed have raised concerns on possible risks for human and animal health (EFSA 2006, 2012; Ostry et al. 2013). Both mycotoxins have been detected in cereals from Bulgarian villages with a history of Balkan endemic nephropathy (BEN) and in pooled food of Serbian BEN families (Petkova-Bocharova et al. 1991; Vrabcheva et al. 2000a, b; Pfohl-Leszkowicz et al. 2007), in grains in Germany (Schmidt et al. 2003), as well as in breakfast cereals and in wheat samples from France (Molinié et al. 2005; Pfohl-Leszkowicz et al. 2007), and in rice in Vietnam (Nguyen et al. 2007). In contrast to many surveys on OTA contamination of human foods (EC/SCOOP 2002), data on CIT occurrence in commodities are scarce: Up to now, there are no legal requirements for food surveillance (Flajs and Peraica 2009), except for a recent Commission Regulation (EU No. 212/2014) with regard to maximum levels of CIT in food supplements based on rice fermented with red yeast *Monascus purpureus*. Moreover, CIT analysis in food or feed is not trivial (Schmidt et al. 2003; Xu et al. 2006). Thus, the available data, recently reviewed by EFSA (EFSA 2012), are insufficient to reliably estimate CIT exposure in the human population.

Two complementary approaches can be used to assess human exposure, as exemplified for OTA (Castegnaro et al. 2006; Duarte et al. 2011; EC/SCOOP 2002): Analysis of

mycotoxin concentrations in food commodities combined with food intake estimates for the general population or population subgroups (external exposure) and/or analysis of suitable biomarkers in human body fluids (biomonitoring) to assess internal exposure. Biomonitoring covers human exposure from various sources and by all routes and is the best tool to investigate occurrence and ranges of mycotoxins in different populations (Degen 2011; Duarte et al. 2011; Leong et al. 2012; Malir et al. 2012; Turner 2010). This "bottom-up" approach can provide first insights when food analysis data are scarce or insufficient, as in the case of CIT. A recently developed method for targeted analysis of CIT and its metabolite dihydrocitrinone (HO-CIT) in human blood plasma and urine have been applied in a small pilot study that indicated the frequent presence of measurable biomarker levels in these matrices (Blaszkewicz et al. 2013). This method now served to analyze CIT and HO-CIT in urine samples from 50 healthy German adults, with the aim to establish concentration ranges that result from dietary mycotoxin intake in this cohort, and for future comparison with results for cohorts from other countries.

Materials and methods

Standards and reagents

CIT standard material (5 mg, purity >98 %) was purchased from Sigma-Aldrich (Taufkirchen, Germany); CIT solutions in methanol were calibrated at 321 nm using the molar absorptivity $\varepsilon = 5{,}490\ M^{-1}\ cm^{-1}$ (Molinié et al. 2005). The CIT metabolite dihydrocitrinone [$C_{13}H_{14}O_6$; (3*R*,4*S*)-6,8-dihydroxy-3,4,5-trimethyl-1-oxo-3,4-dihydro-1H-isochromene-7-carboxilic acid; purity 98.9 %] was from AnalytiCon Discovery GmbH (Potsdam, Germany). Working standard solutions of CIT and HO-CIT were prepared weekly from a stock solution of CIT (200 µg/mL acetonitrile) and HO-CIT (500 µg/mL acetonitrile), by dilution in methanol in a range of 1–20 ng/mL. Acetonitrile, methanol (LC–MS grade) and acetic acid (96 %) were purchased from Merck (Darmstadt, Germany).

Urine sample collection

Fifty healthy IfADo employees (27 females and 23 males) volunteered to participate and gave their written consent before inclusion in the study. First, morning urine samples were collected into a non-sterile disposable container (approximately 80 mL) between July and August 2013. All samples were immediately stored at −20 °C until analysis. Volunteers were asked to fill a short questionnaire for anthropometric information (age, gender, height, weight and occupation).

Creatinine analysis

Creatinine was determined in urine samples to correct for differences in urine dilution between individual spot urines by a modified Jaffe method (Blaszkewicz and Liesenhoff 2012). Analysis of creatinine was carried out with a plate reader, Tecan Genios® (Salzburg, Austria). In the results section, measured CIT and HO-CIT concentrations in urine are also given as ng/g creatinine.

Sample extraction

Urine sample cleanup and enrichment used immunoaffinity column (IAC) extraction with CitriTest® columns (Vicam®, purchased from Ruttmann, Hamburg, Germany) as described by Blaszkewicz et al. (2013): 5 mL of urine was diluted with 5 mL of 1 mM acetic acid in water and mixed for 15 min on a rotary shaker. The sample was then loaded on a CitriTest® column at a flow rate of 1 drop/s. The column was washed twice with 5 mL distilled water; then, CIT was eluted (flow rate 1 drop/s) from the column using 4 mL of methanol. The eluate was evaporated to dryness under a stream of nitrogen at 40 °C, and the residue was dissolved in 500 µL methanol, vortexed and filtered through a 0.45-µm pore size Teflon syringe filter prior to LC–MS/MS analysis.

LC–MS/MS analysis

Liquid chromatography with tandem mass spectrometry was used to measure CIT and its metabolite HO-CIT in urine samples by the method of Blaszkewicz et al. (2013). Detection was performed with a 1200-L Quadrupole MS/MS equipped with an electrospray ionization (ESI) source and a Prostar® Varian HPLC system and Varian MS Workstation version 6.9.1 data system (Agilent Technologies, Germany). The following settings were used: nitrogen as drying gas (19 psi), gas temperature 200 °C and argon used as collision gas (2.0 mTorr). Chromatographic separation was carried out at 21 °C on a Nucleosil® 100-5 C18 HD 125 × 3 mm column with ammonium formate 1 mM in water (mobile phase A) and ammonium formate 1 mM in methanol (mobile phase B) as eluents in the following gradient: 0–1 min 20 % B, 1–3 min 20–75 % B, 3–11 min 75 % B, 11–12 min 75–100 % B, 12–14 min 100 % B (column wash), 14–14.5 min 100–20 % B, and from 14.5 to 20 min 20 % B (re-equilibration). The flow rate was 0.2 mL/min. ESI–MS/MS was executed by multiple reaction monitoring (MRM) in negative ion mode. The specific transitions of precursor ion and product ion were as follows: 249.0 → 204.7 m/z (loss of the carboxyl group) and 249.0 → 176.7 for CIT. The optimized collision energy (CE) was 15 and 19 eV, respectively. For

Table 1 Recovery of CIT and HO-CIT in human urine after cleanup with IAC

Spike level (ng/mL)	CIT		HO-CIT	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
0.05 ($n = 3$)	80	13.3	80	1.5
0.5 ($n = 3$)	76	13.2	82	11.4
1.0 ($n = 3$)	73	14.8	95	1.2
2.0 ($n = 3$)	69	7.0	87	6.0

HO-CIT, the transitions of precursor and product ions were 265.0 → 221.0 and 265.0 → 176.7 m/z with an optimized CE of 16 and 20 eV, in that order. The retention time for CIT and its metabolite HO-CIT was 9.3 and 8.7 min.

Statistical analysis

Descriptive statistics are presented as mean ± SD or medians and inter-quartile ranges. For calculation of mean and median values, those samples that contained CIT and HO-CIT below the LOD were assigned a value of one-half the detection limit. Differences in urinary CIT and HO-CIT concentrations between males and females were analyzed by independent-sample *t* test. Correlation between CIT and HO-CIT with age and body mass index was assessed by Spearman's correlation coefficient (two-tailed). Box plots were used to describe analytes by gender groups. The box represents the central data of distribution where upper and lower limits of the box indicate 25th and 75th percentiles (first quartile or Q1 and third quartile or Q3, respectively), and the median value is presented as a line inside the box. In the box plot, outlier values are more than 1.5 times the inter-quartile range (IQR) and the Q1 and Q3, respectively. Outlier values mark as circle are more than 1.5 times the IQR away from Q1 and Q3 and mark as asterisk are three times the IQR away from Q1 and Q3. All analyses were performed using software of IBM SPSS Statistics 22.

Results

Validation parameters for CIT and HO-CIT analysis

The calibration curve with pure standards in mobile phase was linear in the range of 0.5–20 ng/mL, and the coefficients of determination (R^2) were 0.995 and 0.998 for CIT and HO-CIT, respectively. Analysis of spiked urine extracts yielded similarly good correlations at low concentrations. Spiked samples were used to define the minimum detectable level: The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the lowest quantity of analyte that can be clearly distinguished (LOD) from the background (S/N = 3) or quantified (LOQ; S/N ≥ 6). For CIT, the LOD was 0.02 and LOQ was 0.05 ng/mL. For HO-CIT, the LOD and LOQ were 0.05 and 0.10 ng/mL, respectively. Recovery assays were performed in a urine with no measurable background of CIT and HO-CIT: Recovery of analytes were assessed at four concentration levels in triplicate spiked samples with mean values of 75 and 86 % for CIT and HO-CIT, respectively (Table 1). The same CIT- and HO-CIT-free urine was applied to determine the intra-day and inter-day repeatability (Table 2) at a spike concentration of 0.5 ng/mL ($n = 6$).

Characteristics of the urine donor cohort

Of the 50 subjects in this study, 54 % were females and 46 % were males. The basic anthropometric characteristics of the cohort are given in Table 3. The mean age of all participants was 38.5 years, with no significant difference between females and males. The average body mass index

Table 2 Intra-day and inter-day assay repeatability for CIT and HO-CIT in urine

Spike level (ng/mL)	CIT			HO-CIT		
	Mean ± SD	Recovery (%)	RSD (%)	Mean ± SD	Recovery (%)	RSD (%)
Intra-day ($n = 6$) 0.5 ng/mL	0.39 ± 0.05	78	13.3	0.41 ± 0.03	82	7.7
Inter-day ($n = 6$) 0.5 ng/mL	0.38 ± 0.05	75	13.1	0.40 ± 0.05	80	12.6

Table 3 Basic anthropometric characteristics of the participants

Gender	*n*	Age (years)		BMI (kg/m^2)		Creatinine (g/L)
		Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
Female	27	38.7 ± 12.7	20–65	24.8 ± 3.8	20–35	1.1 ± 0.5
Male	23	38.3 ± 14.0	18–62	25.6 ± 3.9	19–35	1.6 ± 1.1
All	50	38.5 ± 13.2	18–65	25.2 ± 3.8	19–35	1.3 ± 0.9

Table 4 Descriptive statistics of CIT and HO-CIT in urines according to gender

	n	Positive samples (%)	Mean ± SD (ng/mL)	Median (range) (ng/mL)	Mean ± SD (ng/g creatinine)	Median (range) (ng/g creatinine)
CIT						
Female	27	20 (74)	0.03 ± 0.02	0.02 (nd–0.07)	29.4 ± 26.7	20.6 (nd–120.0)
Male	23	21 (91)	0.04 ± 0.02[a]	0.04 (nd–0.08)	39.9 ± 44.6	19.8 (nd–190.5)
All	50	41 (82)	0.03 ± 0.02	0.03 (nd–0.08)	34.2 ± 36.0	20.2 (nd–190.5)
HO-CIT						
Female	27	21 (78)	0.10 ± 0.10	0.05 (nd–0.43)	103.5 ± 107.7	63.3 (nd–480.0)
Male	23	21 (91)	0.11 ± 0.11	0.07 (nd–0.51)	100.0 ± 115.8	55.3 (nd–548.4)
All	50	42 (84)	0.10 ± 0.10	0.06 (nd–0.51)	102.0 ± 110.4	60.9 (nd–548.4)

Positive sample refer to urines containing the analyte ≥ LOD; nd, level below LOD

For calculation of mean and median values, those samples that contained CIT and HO-CIT below LOD were assigned a value of one-half the detection limit

[a] $p < 0.05$ when compared to female, p value obtained from independent-sample t test

(BMI) for all subjects was 25.2 ± 3.8 kg/m^2. Males had slightly higher BMI than females, but the difference was not significant. All urine donors were healthy and free from any chronic diseases. Their average urinary creatinine concentration was 1.3 ± 0.9 g/L. The participants consumed typical mixed German food (grain-based products, potatoes and vegetables, milk and milk-based products, eggs, meat and meat-products); three were vegetarians. They are all employees at IfADo (scientists, technicians, office workers, etc.) residing in Dortmund or nearby urban settlements.

Occurrence and levels of CIT and HO-CIT

Urine samples containing analyte concentration at or above the limit of detection (LOD) were considered as positive samples: CIT was detected in 41 (82 %), and HO-CIT was detected in 42 urine samples (84 %) (Table 4). The distribution of CIT and HO-CIT concentrations in all urines is shown in Fig. 1. Samples with CIT and HO-CIT below the LOD were assigned a value of one-half the detection limit for the distribution graph and for the calculation of mean and median values. Descriptive statistics for CIT and HO-CIT urinary levels of all study subjects and of gender groups are given in Table 4, as concentration (ng/mL) and also adjusted for urinary creatinine content (ng/g). A significant difference was found for the CIT concentration between the male and female subgroup ($p = 0.020$), whereas no significant difference was observed for the metabolite HO-CIT or the sum of both, i.e., "total CIT" excreted (Fig. 2). Citrinin and its metabolite had no significant correlation with age and BMI of the study subjects.

Interestingly, the metabolite was present in urines of the German adults at about threefold higher levels than the parent compound, suggesting that HO-CIT may be a useful additional biomarker of citrinin exposure. On the other hand, the ratio of CIT to OH-CIT in individuals which excrete both analytes at readily measurable concentrations varies considerably between 0.75 and 11 (data not shown).

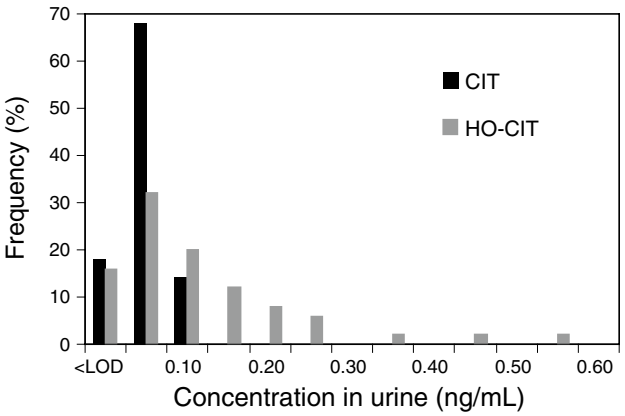

Fig. 1 Distribution of CIT and HO-CIT levels in urine samples. Frequency test was used for the calculation of distribution

Discussion

This is the first study that examined the occurrence of CIT and its metabolite in urines from a cohort of German adults. The method of Blaszkewicz et al. (2013) with IAC cleanup and analysis by LC–MS/MS allowed frequent detection of CIT and HO-CIT in the 50 urine samples (82 and 84 %, respectively). A study with a different sample cleanup method for subsequent multi-mycotoxin analysis reported one CIT-positive sample in 40 urines from Belgium (Njumbe Ediage et al. 2012). This is not in contrast to our result in German urines, since the CIT detection levels of both methods differ considerably (LOD 2.88 ng/mL;

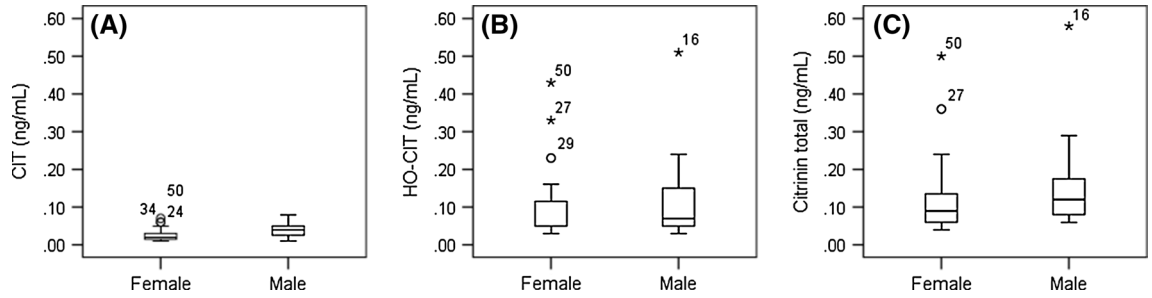

Fig. 2 Comparison of urinary biomarker levels between males and females. Box plot of concentrations for (**a**) CIT (**b**) HO-CIT and (**c**) Citrinin total (parent compound and metabolite) in the gender groups. The outliers are labeled by subject number. Only the CIT urinary concentration (**a**) shows a significant difference ($p = 0.02$) between males and females, but not the metabolite or the total concentration (**b**, **c**)

Njumbe Ediage et al. 2012 versus LOD 0.02 ng/mL; Blaszkewicz et al. 2013 and this study). The detection frequencies in our study with German urines are still higher than those reported recently for urine samples from $n = 29$ Belgian adults (59 % for CIT and 66 % for HO-CIT) analyzed by a direct multi-mycotoxin method: The average urinary levels for CIT (26 pg/mL, range 2–117 pg/mL) and for HO-CIT (35 pg/mL, range 13–210 pg/mL) found in the Belgium study (Huybrechts et al. 2014) are slightly lower than those measured in the present study for German adults (Table 4).

Together these novel findings on the occurrence of CIT and its metabolite in urine indicate a frequent exposure to this mycotoxin in the population of two western European countries. No other biomonitoring data for CIT are available so far, except for preliminary results from two cohorts in Bangladesh (Ali et al. 2014), where one cohort has higher urine levels than the German volunteers. At present, it is not possible to convert urine biomarker levels to dietary CIT intake since kinetic studies in humans have not been conducted, which allow to estimate bioavailability and renal clearance. This supports the view of EFSA that additional research on CIT is needed (EFSA 2012).

It is worth noting that HO-CIT was present in urines of all adult German subjects on at about threefold higher levels on average than the parent compound, suggesting that the metabolite may be a useful additional biomarker of mycotoxin exposure. But, the ratio for CIT/OH-CIT levels in urine can be quite variable among individuals (0.75–11). Recent data show that HO-CIT is much less toxic than CIT, suggesting that formation of this metabolite can be considered as detoxication reaction (Föllmann et al. 2014). HO-CIT has been first identified in urine of rats, albeit only as minor metabolite of CIT (Dunn et al. 1983). Thus, kinetics of CIT could differ between rodents and humans, an aspect of interest for a toxicological risk assessment. Presently, studies are underway to gain further insights into the disposition of CIT in humans: First data show consistently higher levels of CIT and HO-CIT in blood than in urine (Degen et al. 2014). Finally, it should be kept in mind that OTA, another nephrotoxic mycotoxin, is also often detected in humans (Duarte et al. 2011; EFSA 2006; Malir et al. 2012; Pfohl-Leszkowicz et al. 2007).

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Conflict of interest None of the authors have any conflict of interest to declare.

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Publication 5

First results on citrinin biomarkers in urines from rural and urban cohorts in Bangladesh

Ali N, Blaszkewicz M, Mohanto NC, Rahman M, Alim A, Hossain K, Degen GH (2015d). Mycotoxin Res 31: 9–16

Contribution statement

NA contributed to the concept and designed study, co-arranged the sample collection, performed the biomarker analysis, statistical analysis of data and interpretation and drafted the manuscript. MB provided support in instrumental analysis and contributed to final editing of the manuscript. NCM, MR, AA and KH supported in study area selection and in sampling. GHD contributed to the study concept, data interpretation, and final editing of the manuscript.

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ORIGINAL PAPER

First results on citrinin biomarkers in urines from rural and urban cohorts in Bangladesh

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Abstract Citrinin (CIT) is a mycotoxin contaminant in food commodities and can co-occur with ochratoxin A (OTA), another nephrotoxic contaminant in food and feed. Presence of OTA in maize from Bangladesh has been reported, but no data exist on CIT occurrence in food or feed in Bangladesh. Since biomonitoring provides the best approach to assess human exposure to contaminants from various sources and by all routes, a validated method for biomarker analysis has been used to investigate the presence of CIT and its metabolite dihydrocitrinone (HO-CIT) in urines from two Bangladeshi cohorts: Both analytes were determined in urine samples collected from inhabitants of a rural (n=32) and an urban (n=37) area in the Rajshahi district of Bangladesh. After cleanup by immunoaffinity columns, extracts were analyzed by LC-MS/MS; the limits of detection for CIT and HO-CIT in urine were 0.02 and 0.05 ng/mL, respectively. CIT and HO-CIT were detectable in 94 and 71 % of all urine samples. Urinary biomarker levels did not show significant correlations with age, gender, and body mass index of the donors. However, excretion of CIT together with its metabolite HO-CIT was significantly higher (p<0.01) in the rural cohort (mean 1.1±1.9 ng/mL) than in the urban cohort (mean 0.14±0.14 ng/mL). This clearly indicates differences in mycotoxin exposure. As food habits differ between rural and urban people and also their main areas of occupation, further research is needed with regard to the major contributors of CIT exposure in the two cohorts. In conclusion, this first biomarker analysis indicates widespread and variable exposure to CIT in Bangladeshi adults.

Keywords Citrinin · Dihydrocitrinone · Exposure · Mycotoxins · Urine

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Introduction

Citrinin (CIT) is a toxic fungal metabolite produced by several fungi of the genera *Penicillium* and *Aspergillus* (Frisvad et al. 2006). CIT contamination occurs mainly in grains and grain-based products (European Food Safety Authority EFSA 2012). CIT is also known to occur as undesirable contaminant in *Monascus* fermentation products, so-called "red mold rice" (Li et al. 2012; Liao et al. 2014) that is used in Asia for meat preservation and for food coloring but now is also marketed as a food supplement (DFG Permanent Senate Commission on Food Safety 2013).

In repeated dose studies in animals, the kidney was identified as the principal target organ for CIT toxicity. Thus, CIT shows similar toxic properties as ochratoxin A (OTA), although CIT is a less potent nephrotoxin compared to OTA (European Food Safety Authority EFSA 2006, 2012; Frank 1992). Co-occurrence of CIT and OTA in various foodstuffs and in animal feeds has raised concerns on possible risks for human and animal health (European Food Safety Authority EFSA 2006, 2012; Flajs and Peraica 2009; Ostry et al. 2013). Both mycotoxins have been found in cereals from Bulgarian villages with a history of Balkan endemic nephropathy (Petkova-Bocharova et al. 1991; Vrabcheva et al. 2000a, b). CIT and OTA were detected in grains in Germany (Meister 2003; Schmidt et al. 2003), in breakfast cereals in France (Molinie et al. 2005), and in rice in Vietnam and India

(Nguyen et al. 2007; Reddy et al. 1983). Further reports on CIT occurrence in wheat in Tunisia (Zaied et al. 2012) and in various feed ingredients in Russia (Kononenko and Burkin 2008) support the view that CIT, like other mycotoxins, is a contaminant of various food commodities in several parts of the world.

While many surveys have been conducted on OTA contamination of human foods (EC/SCOOP 2002), data on CIT occurrence are still scarce. This may be in part related to the fact that no legal requirements for food surveillance exist as yet, except for a recent Commission regulation (Commission Regulation EU No 212/2014 of 6 March 2014) with regard to maximum levels of CIT in food supplements based on rice fermented with *Monascus purpureus*. Moreover, CIT analysis in food or feed is not trivial (Meister 2003; Xu et al. 2006). The available data, recently reviewed by EFSA, are therefore insufficient to reliably estimate CIT exposure in the human population (European Food Safety Authority EFSA 2012).

In situations where food analysis data is scarce or insufficient, as in the case of CIT, analysis of mycotoxin biomarkers in human body fluids (biomonitoring) can provide useful insights into human exposure. As exemplified for OTA, biomarker analysis in blood, urine, or breast milk serves to assess the occurrence and levels in certain population groups (e.g., Castegnaro et al. 2006; Duarte et al. 2011; Muñoz et al. 2014). Biomonitoring is thus a complementary approach to classical exposure assessments that are based on analysis of mycotoxin concentrations in food commodities combined with food intake estimates for the general population or population subgroups (EC/SCOOP 2002). Biomonitoring covers human exposure from various sources of dietary intake, but also by all other potential routes; it is thus a useful tool to assess additional workplace-related exposure by inhalation of mycotoxins (Degen 2011; Turner et al. 2010) or by dermal contact (Boonen et al. 2012).

We have recently developed a sensitive method for targeted analysis of CIT and its metabolite dihydrocitrinone (HO-CIT) in human blood plasma and urine: When applied in a small pilot study, the presence of measurable biomarker levels in these matrices was detected (Blaszkewicz et al. 2013). This method now proved to be useful for analysis of CIT and HO-CIT (Fig. 1) in urine samples from 50 healthy German adults (Ali et al. 2014a) and for the present study. In Bangladesh, the occurrence of OTA in plant-derived foods (maize, cereals, and ground-nuts) and in feeds has been reported (Dawlatana et al. 2002, 2008), and a recent human biomonitoring study documented the presence of OTA in blood plasma of adult students in Bangladesh (Ali et al. 2014b). However, no study has been carried out on CIT contamination in foods or feed in Bangladesh. Therefore, biomarkers of CIT exposure were analyzed in urine samples from two cohorts in the Rajshahi district of Bangladesh. The first results of this larger project on mycotoxin biomonitoring in this developing country are reported here.

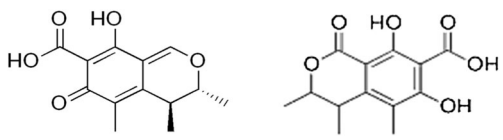

Fig. 1 Structures of analytes: citrinin (*left*) and its metabolite dihydrocitrinone (*right*)

Materials and methods

Chemicals and materials

Citrinin standard material (5 mg, purity >98 %) was purchased from Sigma-Aldrich (Taufkirchen, Germany); CIT solutions in methanol were calibrated at 321 nm using the molar absorptivity $\varepsilon = 5490\ M^{-1}\ cm^{-1}$ (Molinie et al. 2005). The CIT metabolite dihydrocitrinone [$C_{13}H_{14}O_6$; (3R,4S)-6,8-Dihydroxy-3,4,5-trimethyl-1-oxo-3,4-dihydro-1H-isochromene-7-carboxilic acid; purity 98.9 %] was from AnalytiCon Discovery GmbH (Potsdam, Germany). Working standard solutions of CIT and HO-CIT were prepared weekly from a stock solution of CIT (200 μg/mL acetonitrile) and HO-CIT (500 μg/mL acetonitrile), by dilution in methanol in a range of 1–20 ng/mL. Acetonitrile, methanol (LC-MS grade), and acetic acid (96 %) were purchased from Merck (Darmstadt, Germany).

Cohorts and collection of urines

Ethical permission for conducting this study was granted by the Institute of Biological Sciences, Rajshahi University, Bangladesh. Sixty-nine volunteers in total participated in this study of which 32 (15 females, 17 males) were residents of a rural area (Mongol Para, Puthia) and 37 (19 females, 18 males) of an urban area (Rajshahi University region) in the Rajshahi district of Bangladesh. All participants were of good health and gave written consent before inclusion in the study. Volunteers were asked to fill a short questionnaire for anthropometric information (age, gender, height, and weight), occupation, and food habits. First morning urine samples were collected into a non-sterile disposable container (approximately 50 mL) in May 2013. Urine samples were first stored at −20 °C at Rajshahi University and then mailed to Dortmund for subsequent biomarker analysis. Also, creatinine was measured by a modified Jaffe method (Blaszkewicz and Liesenhoff-Henze 2012) to control for differences in urine dilution between individual spot urines.

Extraction of urine samples

The immunoaffinity CitriTest® columns (Vicam®, purchased from Ruttmann, Hamburg, Germany) were used for cleanup and enrichment of analytes as described by Blaszkewicz et al. (2013): 5 mL of urine was diluted with 5 mL of 1 mM acetic acid in water and mixed for 15 min on a rotary shaker. The sample was then loaded on a CitriTest® column at a flow rate of 1 drop/s. The column was washed twice with 5 mL distilled water; then, CIT and its metabolite HO-CIT were eluted (flow rate 1 drop/s) using 4 mL of methanol. The eluate was evaporated to dryness under a stream of nitrogen at 40 °C, and the residue was dissolved in 500 μL methanol, vortexed, and filtered through a 0.45-μm pore size Teflon syringe filter (WICOM GmbH, Germany) prior to LC-MS/MS analysis.

LC-MS/MS analysis

CIT and its metabolite HO-CIT were measured in urine sample extracts by liquid chromatography with tandem mass spectrometry according to a validated method (Blaszkewicz et al. 2013). Detection was performed with a Varian 1200-L Quadrupole MS/MS equipped with an electrospray ionization (ESI) source and a Prostar® Varian HPLC system and Varian MS Workstation version 6.9.1 data system (Agilent Technologies, Germany). The following settings were used: nitrogen as drying gas (19 psi), gas temperature 200 °C, and argon used as collision gas (2.0 mTorr). Chromatographic separation was carried out at 21 °C on a Nucleosil® 100-5 C18 HD 125× 3 mm column with ammonium formate 1 mM in water (mobile phase A) and ammonium formate 1 mM in methanol (mobile phase B) as eluents in the following gradient: 0–1 min 20 % B, 1–3 min 20–75 % B, 3–11 min 75 % B, 11–12 min 75–100 % B, 12–14 min 100 % B (column wash), 14–14.5 min 100–20 % B, and from 14.5 to 20 min 20 % B (re-equilibration). The flow rate was 0.2 mL/min. The retention times for CIT and its metabolite HO-CIT were 9.3 and 8.7 min, respectively. ESI–MS/MS was executed by multiple reaction monitoring (MRM) in negative ion mode. The specific transitions of precursor ion and product ion were as follows: 249.0→204.7 *m*/*z* (loss of the carboxyl group) and 249.0→176.7 for CIT. The optimized collision energies (CE) were 15 and 19 eV, respectively. For HO-CIT, the transitions of precursor and product ions were 265.0→221.0 and 265.0→176.7 *m*/*z* with optimized CE of 16 and 20 eV, in that order.

Statistical analysis

The results are presented as means±standard deviation or medians and interquartile ranges. Those samples containing CIT and HO-CIT below the limit of detection (LOD) were assigned a value of one-half the detection limit for calculation of mean and median values, since this is considered a better approach to estimate average concentrations for left censored data rather than assigning a value of zero to measurements below a detection limit (Hornung and Reed 1990). Differences in urinary CIT and HO-CIT levels between the rural and the urban cohort were analyzed by independent sample *t* test. Correlation between CIT and HO-CIT with age and body mass index (BMI) was assessed by Spearman correlation coefficient (two-tailed). Box plots were used to describe analytes by regions. The box represents the central data of distribution where upper and lower limits of the box indicate 25th and 75th percentiles (first quartile or Q1 and third quartile or Q3, respectively) and the median value is presented as a line inside the box. A level of alpha 0.05 was assigned for statistical significance. Analysis of data was performed using the software IBM SPSS Statistics version 22.

Results

Validation parameters

The calibration curve with pure standards in mobile phase showed linearity in the range of 0.1–20 ng/mL for both analytes. Analysis of CIT and HO-CIT spiked urine extracts yielded similar data in the relevant concentration range. The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the lowest quantity of analyte that can be clearly distinguished (LOD) from the background (S/N=3) or quantified (LOQ; S/N≥6). For CIT, the LOD was 0.02 and LOQ was 0.05 ng/mL. For HO-CIT, the LOD and LOQ were 0.05 and 0.10 ng/mL, respectively. Recovery assays were performed in a urine with no measurable background of CIT and HO-CIT: Recovery of analytes were assessed at three concentration levels in triplicate spiked samples with mean values of 78 and 86 % for CIT and HO-CIT, respectively (Table 1). The intra-day and inter-day repeatability (Table 2) at a spike concentration of 0.25 ng/mL (n=5) were determined using also the mycotoxin-free urine and showed acceptable precisions for these low concentrations.

Characteristics of the study cohorts

The basic characteristics of the cohorts are summarized in Table 3. Of the 69 subjects, 49 % are females and 51 % are males. The mean age of the participants was 38.3±10.6 years (range=21–60 years), with no significant difference between females and males. The average BMI for all subjects was 22.7 ±3.2 kg/m^2. People of the urban cohort had significantly higher BMI (24.1±3.1 kg/m^2) than those in the rural cohort (21.2±2.6 kg/m^2). There were no significant differences in urinary creatinine levels (data not shown) between the rural

Table 1 Recovery and RSD of analytes CIT and HO-CIT in human urine

Spike level (ng/mL)	CIT		HO-CIT	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
0.10 (n=3)	82	11.2	80	2.5
0.25 (n=3)	76	12.4	84	8.4
1.00 (n=3)	77	13.4	94	2.2

and urban cohort. The rural people are mainly farmers or farm workers involved in grain production; housewives in rural areas often also take part in farm work. The urban residents are university employees or work at home (housewives). All participants consumed the typical Bangladeshi staple food rice and, to a lesser extent, roti (whole wheat flatbread); these are generally consumed with vegetables, lentils, fish, poultry, and beef. For some differences in eating habits between the two cohorts, see the "Discussion" section.

Levels of CIT and HO-CIT in the rural and the urban cohort

Urines containing analyte concentration at or above the limit of detection (LOD) were considered as positive samples: Overall, CIT was detected in 65 (94 %) and HO-CIT in 49 (71 %) of the urines from both cohorts (Table 4). The distribution of urinary levels of citrinin biomarkers (parent compound and metabolite) in the rural and urban cohort is shown in Fig. 2. Samples with CIT or HO-CIT below the LOD were assigned a value of one half the detection limit for the distribution graph and for the calculation of mean and median values. CIT and HO-CIT urinary levels of the two cohorts and of subgroups are given in Table 4. Statistical analysis revealed no significant correlation of urinary biomarkers with age, gender, and BMI of the study subjects. However, a significant difference was found for the CIT and HO-CIT concentration between the rural and the urban cohort (p<0.05 and p<0.01).

In urines of most Bangladeshi subjects the metabolite was present at about fivefold higher levels than the parent compound, indicating that the metabolite HO-CIT is a useful second biomarker of citrinin exposure. On the one hand, the ratio of CIT to OH-CIT in the male and female subgroups of both cohorts can vary considerably (data not shown). On the other hand, the rural subgroups with higher urinary levels of the parent compound apparently excrete also more of the non-toxic metabolite (Table 4). As depicted in Fig. 3, excretion of total CIT (sum of CIT and HO-CIT) is significantly higher in rural than in urban Bangladeshi adults, indicative of different exposure. Whether this may be related to differences in their food habits and/or additional occupational mycotoxin exposure in the rural cohort is unclear at present, as no data on CIT contamination of grains or food commodities are available.

Discussion

This is the first study that examined the occurrence of CIT and its metabolite in urines from two cohorts of Bangladeshi adults. The method with IAC cleanup and analysis by LC-MS/MS (Blaszkewicz et al. 2013) allowed frequent detection of CIT and HO-CIT (94 and 71 %, respectively) in the 69 urine samples (Table 4). Detection frequencies in the present study with Bangladeshi urines are similar to those (82 and 84 % for CIT and HO-CIT, respectively) of a recent analysis for urines from 50 German adults (Ali et al. 2014a). In German urine samples, the mean analyte levels were 30± 20 pg/mL for CIT and 100±100 pg/mL for HO-CIT with maximum values of 80 pg/mL CIT and 510 pg/mL HO-CIT (Ali et al. 2014a). The "German" data resemble results from a study with urine samples from n=29 Belgian adults, which reported average urinary levels for CIT of 26 pg/mL (range 2–117 pg/mL) and for HO-CIT of 35 pg/mL (range 13–210 pg/mL) upon analysis by a direct multi-mycotoxin method (Huybrechts et al. 2014).

The biomarker concentration data available now for adults in two Western European countries are somewhat lower than those measured in the present study for Bangladeshi adults. Our first findings on the occurrence of CIT and its metabolite in urine indicate a frequent exposure in the Bangladeshi population. Of further interest are the significantly higher

Table 2 Precision of intra-day and inter-day assay repeatability for CIT and HO-CIT in urine

Spike level (ng/mL)	CIT			HO-CIT		
	Mean±SD (ng/mL)	Recovery (%)	RSD (%)	Mean±SD (ng/mL)	Recovery (%)	RSD (%)
Intra-day (n=5) 0.25	0.20±0.02	80	10.4	0.21±0.01	84	6.6
Inter-day (n=5) 0.25	0.19±0.02	76	11.9	0.20±0.02	80	9.8

Table 3 Basic characteristics of the study participants in the rural and the urban area

Characteristics	All subjects	Rural	Urban
Subjects (*n*)	69	32	37
Gender (*n*)			
Female	34	15	19
Male	35	17	18
Age (years)			
Mean±SD	38.3±10.6	40±12.5	36.8±8.5
Range	21–60	21–60	24–57
Occupation [*n*, (%)]			
Female			
Housewives	27 (79.4)	15 (100)	12 (63.2)
Office workers	4 (11.8)	0	4 (21.1)
Student	3 (8.8)	0	3 (15.8)
Male			
Farmers	11 (31.4)	11 (64.7)	0
Office worker	17 (48.6)	0	17 (94.4)
Student	3 (8.6)	2 (11.8)	1 (5.6)
Others	4 (11.4)	4 (23.5)	0
BMI (kg/m^2)[a]			
Mean±SD	22.7±3.2	21.2±2.6	24.1±3.1
Range	16.2–32.9	16.2–27.3	18.9–32.9

[a] BMI: significantly different ($p<0.001$) between rural and urban people

levels of CIT and its metabolite in the rural cohort compared to the urban cohort (Table 4 and Fig. 3). At present it is unclear, whether this biomarker result reflects a difference in food habits and/or an additional occupational exposure: Most of the people in the rural cohort of Bangladesh are grain farmers or housewives taking part in field work while urban residents mainly work in offices or at home. There are also some differences in eating habits between the rural and the urban population in Bangladesh. Rural people mainly depend on home-cooked food while urban people also take restaurant food occasionally beside the homemade food. Rural people consume rice for breakfast, lunch, and dinner with one or two curries everyday. For breakfast, they eat much rice and sometimes roti (whole wheat flatbread) with some vegetables before they work in the fields. The urban people eat less rice than the rural people: For breakfast, normally they take bread, parata (handmade fried bread), vegetables, eggs, and some take banana. For daily food, the rural population cannot bear the expenses of so many different food items as the urban population. In contrast to other Asian countries, red mold rice is not popular in Bangladesh.

To further assess the variability in both cohorts, a follow-up biomarker analysis for urines collected in another season will be conducted. Bangladesh has a subtropical monsoon climate, characterized by seasonal variations in rainfall, temperature, and humidity (http://en.wikipedia.org/wiki/Geography_of_

Table 4 Levels of CIT and HO-CIT in urines according to region

Analyte cohort subgroup	*n*	Positive samples (%)	Mean±SD (ng/mL)	Median (ng/mL)	75th percentile	Range (ng/mL)
CIT						
Rural						
Female	15	14 (93)	0.17±0.31	0.07	0.12	nd–1.22
Male	17	17 (100)	0.11±0.11	0.08	0.15	0.02–0.41
All	32	31 (97)	0.14±0.22*	0.08	0.13	nd–1.22
Urban						
Female	19	17 (89)	0.04±0.03	0.03	0.06	nd–0.13
Male	18	17 (94)	0.07±0.10	0.03	0.07	nd–0.45
All	37	34 (92)	0.06±0.07	0.03	0.07	nd–0.45
HO-CIT						
Rural						
Female	15	13 (87)	0.80±1.30	0.19	1.12	nd–4.39
Male	17	16 (94)	1.11±2.10	0.22	0.87	nd–7.47
All	32	29 (91)	0.97±1.75**	0.20	0.99	nd–7.47
Urban						
Female	19	9 (47)	0.06±0.05	0.03	0.07	nd–0.22
Male	18	11 (61)	0.11±0.11	0.05	0.17	nd–0.36
All	37	20 (54)	0.08±0.09	0.05	0.11	nd–0.36

Positive sample refer to urines containing the analyte ≥LOD. For calculation of mean and median values, those samples that contained CIT and HO-CIT below LOD were assigned a value of one half the detection limit

nd level below LOD

*$p<0.05$ and **$p<0.01$ when compared to urban, p value obtained from independent sample t test

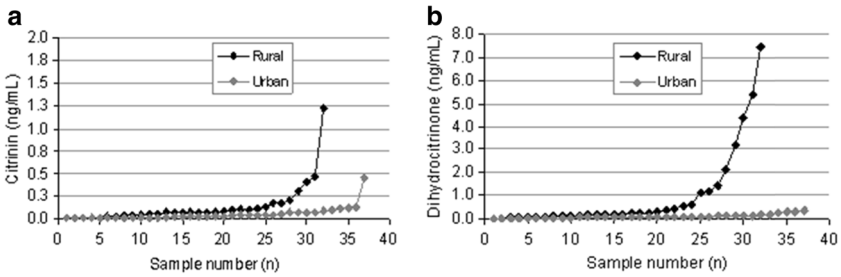

Fig. 2 Distribution of individual levels of biomarkers **a** citrinin and **b** dihydrocitrinone in the rural and urban cohorts, plotted by increasing concentration

Bangladesh). Three seasons are usually recognized: a hot, humid summer (32–40 °C) from March to June; a humid rainy monsoon season (28–35 °C) from July to October; and a warm, dry winter (15–25 °C) from November to February. In Bangladesh, crops are mainly produced in summer and in winter time. Food stored in one season (e.g., summer) is largely consumed in the next season until new seasonal (e.g., winter) crops become available. In the absence of fungal analysis or data on CIT level in food commodities from Bangladesh, one can only speculate on possible sources of CIT intake in the two cohorts. We assume that *Aspergillus* and *Penicillium* species rather than *Monascus* are responsible for producing of citrinin in food consumed by our cohorts, because "red mold rice" is not common in their food. Rice is staple food in Bangladesh and may be one important source of CIT, as occurrence of this mycotoxin has been reported in rice from other Asian countries (Reddy et al. 1983; Nguyen et al. 2007). Spices might be another source of CIT: One study (Saxena and Mehrotra 1989) reported on the occurrence of CIT in different spices commonly marketed in India. Spices such as turmeric, coriander, fennel, black pepper, cardamom, and cumin are also used to make curries in Bangladesh.

At present, urine biomarker levels cannot be converted to amounts of CIT intake since kinetic studies in humans have not been conducted which allow to estimate bioavailability and renal clearance, as in the case of OTA (Studer-Rohr et al. 2000). This strengthens the view that additional research on CIT is needed (European Food Safety Authority EFSA 2012).

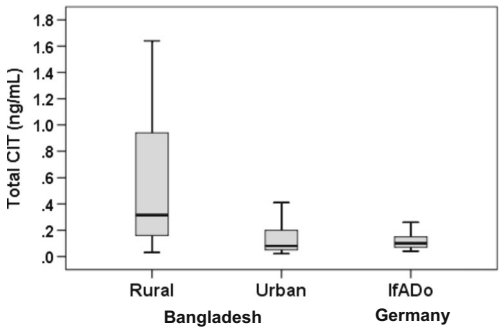

Fig. 3 Boxplot of urinary biomarker levels in the rural and urban cohorts of Bangladesh. The concentration for total CIT (sum of CIT and HO-CIT) in the rural cohort is significantly different ($p<0.01$) compared to that for the urban cohort that presents similar values as those determined in urines of a German cohort (Ali et al. 2014a); see text for details

In rats dosed with radiolabeled mycotoxin (^{14}C-CIT), either by i.v. or s.c. injection, about 75 % of the dose was recovered in urine within 24 h and a biphasic elimination from blood was observed (Phillips et al. 1979; Reddy et al. 1982). In the only study with per os administration of ^{14}C-CIT, HO-CIT has been first identified in urine of rats, albeit only as minor metabolite of CIT and only unchanged CIT was found in blood collected 24 h after administration (Dunn et al. 1983). As HO-CIT is much less toxic than CIT, formation of this metabolite can be considered as detoxication reaction (Föllmann et al. 2014). This is of interest for a toxicological risk assessment since the kinetics of CIT can differ between rodents and humans, at least in quantitative terms.

In this context, it is worth noting that the novel data point to HO-CIT as major metabolite in humans, in contrast to the rat where it is a minor metabolite in urine. In urines of most Bangladeshi subjects, HO-CIT is present at clearly (up to fivefold) higher levels than the parent compound CIT, although the ratios for urinary CIT/HO-CIT levels are variable among individuals (1.3–6.6). Also, biomarker analysis in German urines revealed on average about threefold higher levels of the metabolite than the parent compound and a variable ratio of urinary CIT/HO-CIT concentrations among individuals (Ali et al. 2014a).

In recent studies, where blood plasma and first morning urines were repeatedly collected from two male volunteers over a period of several weeks, the analyte concentrations showed small fluctuations over time but distinct differences in biomarker levels (and the CIT/HO-CIT ratio) for the two persons (Degen et al. 2014). Interestingly, the data showed also consistently higher levels of CIT and HO-CIT in blood plasma than in urine, although at lower plasma concentrations than those measured for OTA. Nonetheless, further studies are needed to validate CIT and HO-CIT as biomarkers of exposure in humans. Presently, such studies are underway to gain further insights in the kinetics of CIT in humans and to determine the fraction of ingested CIT that is eliminated with urine within a day.

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Conflict of interest None

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Publication 6

Deoxynivalenol exposure assessment for pregnant women in Bangladesh

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Contribution statement

NA and GHD co-conceived the study idea, its design and funding. NA conducted biomarker analysis, statistical data analysis and he drafted the manuscript. MB supervised LC-MS/MS analysis and its optimization, contributed to final editing of the manuscript. AAN and MR contributed in collection of urine samples and questionnaires; aided in FFQ data interpretation. GHD supervised data collection and interpretation; co-wrote manuscript with NA.

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Article

Deoxynivalenol Exposure Assessment for Pregnant Women in Bangladesh

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Abstract: The trichothecene mycotoxin deoxynivalenol (DON) is a contaminant of crops worldwide and known to cause adverse health effects in exposed animals and humans. A small survey reported the presence of DON in maize samples in Bangladesh, but these data are insufficient to assess human exposure, and also, biomonitoring data are still scarce. The present study applied biomarker analysis to investigate the DON exposure of pregnant women in Bangladesh. Urine samples were collected from pregnant women living in a rural (n = 32) and in a suburban (n = 22) area of the country. Urines were subjected to enzymatic hydrolysis of glucuronic acid conjugates and to immunoaffinity column clean-up prior to LC-MS/MS analysis of DON and its de-epoxy metabolite DOM-1. The limits of detection (LOD) for DON and DOM-1 in urine were 0.16 ng/mL and 0.10 ng/mL, respectively. DOM-1 was not detected in any of the urines, whilst DON was detectable in 52% of the samples at levels ranging from 0.18–7.16 ng/mL and a mean DON concentration of 0.86 ± 1.57 ng/mL or 2.14 ± 4.74 ng/mg creatinine. A significant difference in mean urinary

DON levels was found between the rural (0.47 ± 0.73 ng/mL) and suburban (1.44 ± 2.20 ng/mL) cohort, which may be related to different food habits in the two cohorts. Analysis of food consumption data for the participants did not show significant correlations between their intake of typical staple foods and DON levels in urine. The biomarker concentrations found and published urinary excretion rates for DON were used to estimate daily mycotoxin intake in the cohort: the mean DON intake was 0.05 µg/kg b.w., and the maximum intake was 0.46 µg/kg b.w., values lower than the tolerable daily intake of 1 µg/kg b.w. These first results indicate a low dietary exposure of pregnant women in Bangladesh to DON. Nonetheless, further biomonitoring studies in children and in adult cohorts from other parts of the country are of interest to gain more insight into DON exposure in the population of Bangladesh.

Keywords: deoxynivalenol; deepoxy-deoxynivalenol; exposure; mycotoxins; urine

1. Introduction

Deoxynivalenol (DON) is a trichothecene mycotoxin produced by *Fusarium* species, mainly *F. graminearum* and *F. culmorum*, and the most frequently-detected mycotoxin contaminant of maize, wheat and barley in temperate regions of the world [1,2]. Consumption of DON-contaminated feed has been associated with a number of adverse effects in animals, including feed refusal, vomiting, diarrhea, dizziness and fever, and chronic exposure to DON can lead to growth faltering, immunological and neurological dysfunction [3,4]. The acute effects of DON (or "vomitoxin") in humans are similar to those seen in animals, and chronic dietary exposure of animals to DON causes altered nutritional efficiency [2,5], whilst long-term effects in humans have not been established so far. The primary toxic effect of DON is inhibition of protein synthesis, and it activates a signaling pathway known as ribotoxic stress response in cells and induces apoptosis [4,6]. Moreover, the effects include altered neuroendocrine signaling, impaired gut integrity and immune function [7,8].

DON is thus a concern for human health, and exposure early in life is of special interest in light of the following: DON transfer via the placenta to the fetus occurs in sows [9], and maternal exposure has been linked to growth retardation [10] and immunosuppression in the offspring [11]. Since DON has been shown to cross also the human placenta, dietary exposure during pregnancy will lead to DON exposure of the fetus, whose detoxification mechanisms are not yet well developed [12].

Along with conventional mycotoxin analysis in food, biomonitoring has greatly advanced an assessment of human DON exposure. As reviewed by Turner *et al.* (2012) [13], sensitive analytical methods for the analysis of DON and its metabolites in urine have been developed and validated as biomarkers of exposure: unmetabolized or "free" DON together with DON-glucuronides in urine reflect rather strong dietary mycotoxin exposure, and the mean estimated amount of biomarkers excreted within a day are rather high, with about 68% [14] and 72% of the DON intake [15]. As DON is largely present as a glucuronide conjugate in human urine [14,15], enzymatic hydrolysis of samples (deconjugation) is used in most single- or multi-analyte methods for biomarker determination to increase the detectability of total DON [16]. Analysis should also include DOM-1, the detoxification product of DON formed by

gut microbiota in animals and humans: a recent study found relatively high levels of DOM-1, indicating that a substantial proportion of DON can be detoxified in humans [17], whilst the DOM-1 metabolite has been rarely detected or at only very low levels in urines from other cohorts [15,18–20].

DON biomarker occurrence has been analyzed in the urines of pregnant women from the U.K., Egypt and Croatia [21–23]. More studies have been conducted in the general population of some Asian [19,24], European [15,18,20,25–30] and African countries [31,32]. Overall, the results from these biomonitoring studies (in terms of frequency of detection and urinary analyte levels) indicate quite variable human exposure to DON, a finding in accord with reported differences in DON contamination of food commodities in various regions of the world [1,6].

When food contaminant data are scarce, as often is the case in developing countries, analysis of biomarkers in human body fluids provides useful insights, since biomonitoring covers mycotoxin intake from all dietary sources and exposure by various routes [14,33]. In Bangladesh, a small survey detected the presence of DON in 10 maize samples (17% positive) with the highest level of 337 μg/kg in maize from the northern part of the country [34]. The reported DON levels did not exceed the U.S. or EU regulatory limits, but this survey focused on maize alone and did not include analysis of other possibly contaminated food commodities. In short, these data are insufficient to assess human DON exposure in Bangladesh.

Therefore, to gain more insight into maternal DON exposure during pregnancy, urine samples were collected from inhabitants of a rural and a suburban area of the Savar region in the Dhaka district of Bangladesh. The present study is the first biomarker-based DON exposure assessment for pregnant women in Bangladesh.

2. Results

2.1. Validation Parameters for Biomarker Analysis

The calibration curve with pure standards in the mobile phase showed linearity in the range of 0.5–20 ng/mL for both analytes with a coefficient of determination (R^2) of 0.998 for DON and 0.997 for DOM-1, respectively. Analysis of DON and DOM-1 spiked urine yielded similar data for linearity in the range of LOD, 10 ng/mL. The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the lowest quantity of analyte that can be clearly distinguished from the background (LOD; signal to noise ratio, S/N = 3) or quantified (LOQ; S/N ≥ 6). For DON, the LOD was 0.16, and the LOQ was 0.30 ng/mL. For DOM-1, the LOD and LOQ were 0.10 and 0.20 ng/mL, respectively.

Recovery assays were performed in a urine with no measurable background of DON and DOM-1. Isotope-labeled internal standard ([$^{13}C_{15}$] DON) was used to correct for recovery. Recovery of analytes was assessed at three concentration levels in triplicates of spiked samples with mean values of 93% and 86% for DON and DOM-1, respectively (Table 1). The intra-day and inter-day repeatability (Table 2) at a spike concentration of 1.0 ng/mL (n = 6) were determined using also the mycotoxin-free urine and showed acceptable precision for the measurements.

Table 1. Recovery of DON and DOM-1 in human urine.

Spike Level (ng/mL)	DON		DOM-1	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
0.5 (n = 3)	102	12.2	88	8.5
1.0 (n = 3)	90	8.4	92	4.8
2.0 (n = 3)	94	10.4	78	4.2

Table 2. Intra-day and inter-day assay repeatability for DON and DOM-1 in urine.

Spike Level (ng/mL)	DON			DOM-1		
	Mean ± SD (ng/mL)	Recovery (%)	RSD (%)	Mean ± SD (ng/mL)	Recovery (%)	RSD (%)
Intra-day (1.0) n = 6	0.92 ± 0.12	92	13.0	0.82 ± 0.06	82	7.3
Inter-day (1.0) n = 6	0.88 ± 0.08	88	9.1	0.80 ± 0.08	80	10.0

2.2. Demographic Characteristics of the Participants

The baseline anthropometric data of the participants are summarized in Table 3. The mean age of the participants was 25 ± 5 years, ranging from 18–36 years with no significant difference between the rural and suburban cohort. The average body mass index (BMI) for all subjects was 20.1 ± 3.0 kg/m^2. Volunteers of the rural cohort had significantly higher BMI (21.2 ± 2.3 kg/m^2) than those in the suburban cohort (18.6 ± 3.2 kg/m^2). There were no significant differences ($p < 0.01$) in urinary creatinine levels between the rural (652 ± 509 mg/L) and suburban (710 ± 472 mg/L) cohort. Regarding occupation, 83% of the participants were housewives, and 17% were office workers. All participants consumed the typical Bangladeshi staple food rice and, to a lesser extent, roti (whole wheat flatbread); these are generally consumed with vegetables, lentils, fish, poultry and beef.

Table 3. Demographic characteristics of the participant.

Characteristics	Rural	Suburban	Total
n	31	22	54
Age (years)			
Mean ± SD	25 ± 5	26 ± 5	25 ± 5
Range	18–36	18–36	18–36
Occupation (n, %)			
Housewives	29 (91)	16 (73)	45 (83)
Office workers	3 (9)	6 (27)	9 (17)
BMI (kg/m^2)			
Mean ± SD	21.2 ± 2.3 *	18.6 ± 3.2	20.1 ± 3.0
Range	14.3–28.2	12.0–24.4	12.0–28.2
Creatinine (mg/L)			
Mean ± SD	652 ± 509	710 ± 472	676 ± 490

* $p < 0.01$ when compared to suburban; p-value obtained from independent sample t-test.

2.3. Urinary Level of DON in the Cohorts

Urines with analyte concentrations at or above the limit of detection (LOD) were considered as positive samples. DOM-1 was not detected in any of the urines, whilst DON was detectable in 52% of the urines, at concentrations ranging from 0.18–7.16 ng/mL (Table 4). The distribution of urinary DON levels according to age of both cohorts is presented in Figure 1. The mean concentration of urinary DON was 0.86 ± 1.57 ng/mL or 2.14 ± 4.74 ng/mg creatinine for all study participants. A significant difference ($p < 0.05$) in mean urinary DON levels was found between the rural (0.47 ± 0.73 ng/mL) and the suburban (1.44 ± 2.20 ng/mL) cohort (Table 4), which may be related to different food habits in the two cohorts. Statistical analysis revealed no significant correlation of urinary biomarkers with age, gender and BMI of the study subjects.

Table 4. Occurrence and contamination levels of DON in urine.

Cohort	*n*	Positive Samples *n* (%)	Mean ± SD (ng/mL)	Median (Range) (ng/mL)	75th Percentile (ng/mL)	Mean ± SD (ng/mg Creatinine)
Rural	32	13 (41)	0.47 ± 0.73	nd (nd–3.09)	0.65	1.14 ± 2.47
Suburban	22	15 (68)	1.44 ± 2.20 *	0.51 (nd–7.16)	1.94	3.60 ± 6.63
Total	54	28 (52)	0.86 ± 1.57	0.19 (nd–7.16)	0.85	2.14 ± 4.74

A positive sample refers to urines containing the analyte ≥LOD; nd refers to levels below the LOD. For the calculation of mean and median values, biomarker concentration was set to half of the LOD if the DON urine concentration was below the LOD. * $p < 0.05$ when compared to rural; *p*-value obtained from independent sample *t*-test.

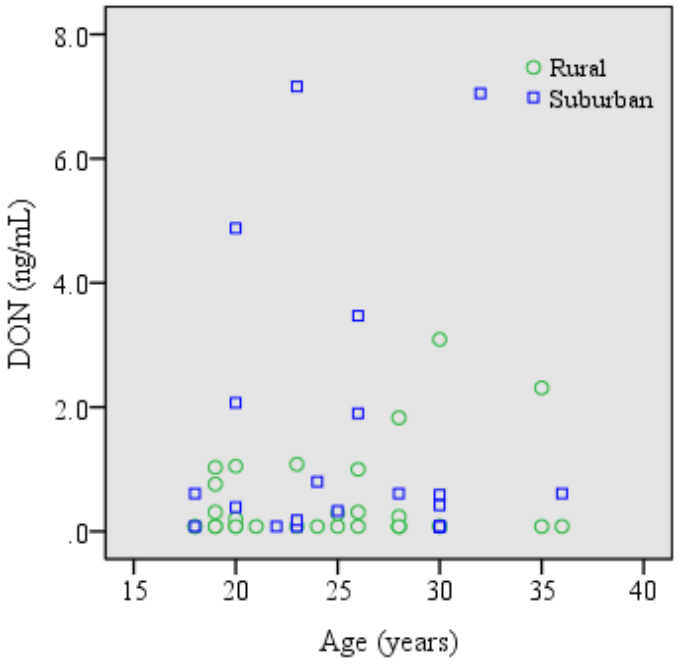

Figure 1. Distribution of urinary DON in the rural and suburban cohort. Biomarker concentration was set to half of the LOD for samples with DON below the LOD.

2.4. Estimation of DON Intake

Using the urinary biomarker analysis data, the provisional daily DON intake among all participants was on average 52.5 ± 94.4 ng/kg body weight (b.w.), and the highest DON intake was 461 ng/kg b.w. (Table 5). A significant difference ($p < 0.05$) was found between the rural (27 ng/kg b.w.) and suburban (90 ng/kg b.w.) cohort. No individual had an estimated daily DON intake above the tolerable daily intake (TDI) of 1000 ng/kg b.w./day [1].

Table 5. Provisional daily intake (PDI) of DON [a]. TDI, tolerable daily intake.

Cohort	*n*	Mean ± SD ng/kg b.w.	Median ng/kg b.w.	Range ng/kg b.w.	Exceeding TDI *n* (%)
Rural	32	26.8 ± 39.0	5.0	3.1–154.0	-
Suburban	22	89.9 ± 133.2 *	31.1	4.7–460.8	-
Total	54	52.5 ± 94.4	11.2	3.1–460.8	-

[a] Dietary DON intake was calculated based on urinary DON concentrations, adjusted for 24-h urine volume, assuming a 68% clearance rate and individual body weight (see the Experimental Section for details). * $p < 0.05$ when compared to rural; *p*-value obtained from independent sample *t*-test.

2.5. Correlation between Urinary DON and Food Intake

Based on information provided in the food frequency questionnaires (FFQ), possible correlations were analyzed between food consumption (graded 1–4) and urinary DON levels in the pregnant women cohort. The food items rice, wheat/maize, lentils and chicken meat were considered in Spearman correlation analysis (Table 6). Consumption of rice, the main staple food in Bangladesh, did not show a significant correlation ($p = 0.39$) with the urinary DON level of the subjects. No significant correlations were observed between DON in urine and other food items, such as wheat/maize ($p = 0.75$), lentil soup ($p = 0.37$) or chicken meat ($p = 0.46$).

Table 6. Correlation (*r*) between food consumption and DON concentration in urine.

Foodstuff	Correlation (*r*)	*p*-Value
Rice	−0.174	0.395
Wheat/maize	0.067	0.746
Lentil soup	0.182	0.374
Chicken meat	0.151	0.462

Only positive samples (≥LOD) are considered for the calculation. Assessment of food consumption was done using the score 1–4 (1 = do not consume at all, 2 = 1 time daily, 3 = 2 times daily, 4 = 3 times daily. *p*-values are obtained from Spearman's correlation coefficient (two-tailed).

3. Discussion

As briefly outlined in the Introduction, DON is known to cause a range of adverse effects in animals and can also cross the placenta. Thus, exposure of pregnant women to DON is of particular concern, yet only few biomonitoring studies have been conducted so far in this group of the population.

The present study is the first biomarker survey for DON exposure of pregnant women in Bangladesh: frequent detection of DON (52%) at a mean concentration of 0.86 ± 1.57 ng/mL or 2.14 ± 4.74 ng/mg creatinine (Table 4) indicates DON exposure in pregnant women living in a rural and a suburban area of Bangladesh, albeit at comparatively moderate levels. The urinary DON concentrations determined in this study are similar to levels found in the urines of pregnant women from Egypt (geometric mean 1.11 ng/mg creatinine, range 0.5–59.9 ng/mg creatinine [22]). On the other hand, values in our cohort (mean 2.1 ng/mg creatinine, range 0.6–24.3 ng/mg creatinine) are clearly lower than those reported for pregnant women (n = 85) from Bradford, the U.K. (geometric mean 10.3 ng/mg creatinine, range 0.5–116.7 ng/mg creatinine [21]) and for pregnant women (n = 40) from Croatia (DON equivalents: mean 111.8 ng/mL, range 4.8–1238 ng/mg [23]). The authors noted that maximum concentration in the rural sub-cohort of Croatian pregnant women indicated a nine-times higher exposure to DON than in U.K. pregnant women. Moreover, biomarker-based calculation of DON intake revealed mycotoxin exposures that clearly exceeded the tolerable daily intake (TDI) for DON of 1 µg/kg b.w., both in the U.K. and the Croatian cohort [21,23]. In the Bangladeshi cohort of pregnant women, even the highest calculated provisional daily DON intake of 461 ng/kg b.w. (Table 5) did not exceed the TDI.

In our survey, DOM-1 metabolite was not detected in any of the 54 Bangladeshi urines. This is of interest, since human data on the occurrence of this detoxication product are limited. DOM-1 was found at modest levels in urines from Egyptian pregnant women (2/69; [22]) and from French farmers (26/76; [18]), but it was not detected in studies of U.K. adults [20] or U.K. pregnant women [21]. However, in a recent small pilot study with 15 volunteers who provided spot urines in two years, DOM-1 was detected in 37% and 40% of the samples: The prevalence and the proportion of DOM-1 (up to 17.8% of urinary DON in some individuals) indicated that a substantial fraction of DON can be detoxified in humans harboring gut microbiota capable of DON detoxication [17]. Other recent studies from Europe detected DOM-1 less frequently and also at very low levels compared to DON and DON glucuronide [28,35,36]. DON toxicity in humans may be also related to their ability to produce DON-glucuronide [37]. Yet, in light of the limited availability of urines, we did not determine the ratio of free DON (aglycone) to DON-glucuronides in our cohort.

Regarding possible sources of DON intake in Bangladesh: analysis of correlations between urinary biomarker levels and food consumption pattern based on FFQ data did not show significant associations with typical food items (Table 6), and this may be due to the overall rather low DON exposure among the participants. Interestingly, pregnant women from the suburban area had a significantly higher mean level of urinary DON and also provisional daily DON intake than our rural cohort (Tables 4 and 5). The higher DON exposure in the suburban participants may be related to higher consumption of wheat bread by the suburban residents than the rural residents. However, maize cannot be excluded, since our FFQ did not assess wheat and maize consumption separately, and wheat and maize flours are sometimes mixed together for making homemade bread and also in the bakery industry in Bangladesh. A small survey reported DON contamination at a low level in maize samples collected from the northern part of Bangladesh [34], but food analysis data for DON are not available for other staple food, such as rice or wheat.

Finally, the present biomonitoring study revealed only moderate exposure of pregnant women in Bangladesh. Nonetheless, further studies are indicated: urine samples were collected in wintertime (2014), but the country has a subtropical monsoon climate, characterized by clear seasonal variations in

rainfall, temperature and humidity (http://en.wikipedia.org/wiki/Geography_of_Bangladesh). Since the growth and distribution of *Fusarium* fungi depends on climatic factors, such as moisture and temperature, quite variable DON contamination of crops cannot be excluded [17,38].

4. Experimental Section

4.1. Standards and Reagents

Standards for deoxynivalenol (DON), de-epoxy DON (DOM-1) and isotope-labeled internal standard ([$^{13}C_{15}$] DON) were obtained from Romer Labs Diagnostics GmbH (Tulln, Austria). The enzyme β-glucuronidase/arylsulfatase (β-Gluc/ArylS) from *Helix pomatia* (with specific activity 5.5 U/mL β-glucuronidase, 2.6 U/mL arylsulfatase at 37 °C) was from Roche Diagnostics (Mannheim, Germany) and used with 10-fold hydrolysis buffer (13.6 g sodium acetate hydrate, 1.0 g ascorbic acid, 0.1 g EDTA in 100 mL deionized water, adjusted to pH 5.0 with acetic acid 98%) for enzymatic treatment of urine samples. Immunoaffinity DONTest™ columns (Vicam®, purchased from Ruttmann, Hamburg, Germany) were used for clean-up and enrichment of analytes. Methanol (LC-MS grade) was purchased from Merck (Darmstadt, Germany).

4.2. Participants and Sample Collection

During February–March 2014, fifty-four pregnant women (n = 47 in the third, n = 6 in the second and n = 1 in the first trimester) were recruited from a rural (Dhamrai, Dhamsona, Nolam, Paichail) and a suburban area (Baipail, Dhamrai, Kashimpur, Modhupur, Namabazar) of the Savar region in the Dhaka district of Bangladesh. All participants were apparently healthy according to external clinical examination by professional nurses. Written consent was obtained from the women before inclusion in this study. Volunteers were asked to fill out a short questionnaire for anthropometric information (age, height and weight), occupation and food habits, using the same food frequency questionnaire (FFQ) as in our previous studies in Bangladesh [39]. The spot urine samples were collected in the morning (between 8:00 and 11:00 am) into non-sterile disposable containers and then stored at −20 °C. All urine samples were shipped on dry ice to IfADo (Dortmund, Germany) in March 2014, and biomarker analysis was conducted in January 2015. This study was approved by the Institute of Biological Sciences of Rajshahi University, Rajshahi-6205, Bangladesh (Memo no. 40/320/IAMEBBC/IDSC), and by the Institutional Internal Review Board of IfADo.

4.3. Enzymatic Hydrolysis

To cleave DON (and DOM-1) conjugates, 250 µL hydrolysis buffer (pH 5.0) and 40 µL of β-Gluc/ArylS enzyme were added to 3 mL urine aliquots and incubated at 37 °C overnight before sample extraction.

4.4. Sample Preparation

Urine sample clean-up and enrichment of analytes was performed by immunoaffinity column (IAC) extraction with DONTest™ following the protocol provided by the manufacturer (VICAM). Briefly, after rinsing the column with 1 mL of water, the entire hydrolyzed urine sample was loaded on

a DONTest™ column at a flow rate of 1 drop/s. The column was washed with 5 mL distilled water, then DON was eluted (flow rate 1 drop/s) from the column using 2 mL of methanol. The elute was evaporated to dryness under a stream of nitrogen at 45 °C; the residue was dissolved in 500 µL water/methanol (90:10), vortexed and filtered through a 0.45-µm pore size Teflon syringe filter prior to LC-MS/MS analysis. Thus, the enrichment factor was 6.

4.5. LC-MS/MS Analysis

DON and its metabolite DOM-1 were measured in urine extracts by liquid chromatography with tandem mass spectrometry. Analysis was performed with a Varian 1200-L Quadrupole MS/MS equipped with an electrospray ionization (ESI) source, a Prostar® Varian HPLC system and a Varian MS Workstation version 6.9.1 data system (Agilent Technologies, Germany). The following settings were used: nitrogen as the drying gas (21 psi), gas temperature 250 °C, and argon used as the collision gas (2.0 mTorr). Chromatographic separation was carried out at 25 °C on a Nucleosil® 100-5 C_{18} 125 × 3 mm column (Macherey-Nagel, Dŭren, Germany) with water (Mobile Phase A) and methanol (Mobile Phase B) as eluents in the following gradient: 0–1.1 min 45% B, 1.1–5.3 min 60% B, 5.3–7.3 min 95% B (column wash), 8–18 min 45% B (re-equilibration). The flow rate was 0.2 mL/min, and the injection volume was 20 µL. The retention time for DON and its metabolite DOM-1 was 4.5 and 5.6 min and for the internal standard (IS) 4.5 min. ESI-MS/MS was executed by multiple reaction monitoring (MRM) in negative ion mode. The specific transitions of precursor ion and product ion were as follows: 295.1 → 265.1 *m*/*z* and 295.1 → 138.1 for DON. The optimized collision energy (CE) was −10 and −15.5 eV, respectively. For DOM-1, the transitions of precursor and product ions were 279.1 → 248.9 and 279.1 → 231.1 *m*/*z* with an optimized CE of −9 and −13 eV, and for the internal standard (IS), the transition of precursor and product ions was 310.0 → 279.2 *m*/*z* with an optimized CE of −9 in that order.

4.6. Creatinine Analysis

Urinary creatinine was measured by a modified Jaffe method on a 96-well plate reader (Tecan Genios) [40] to account for variability in urine dilution between individual samples. Urinary DON levels determined in ng/mL were adjusted for creatinine in the urine sample and their concentrations expressed as ng/mg creatinine to allow also comparison with other biomarker data.

4.7. Exposure Assessment

The estimation of DON intake among the participants was performed based on the results of urinary DON analysis. The following equation was used to assess the provisional daily intake (PDI) of DON among the participants:

$$PDI\left(\frac{\mu g}{Kg}\,\text{bodyweight}\right)=\frac{C\times V\times 100}{W\times E} \quad (1)$$

with C = biomarker concentration (µg/L), V = daily urine excretion (L), W = body weight (kg) and E = excretion rate (%). In the calculation, urinary output during pregnancy was considered as 2 L per day [41]. Individual body weight was used during the calculation of PDI and a daily urinary DON excretion rate of 68% [14], a value close to estimates in other studies [15,42].

4.8. Food Consumption Data

All participants were asked to fill in a questionnaire with a focus on the last two days of food consumption prior to urine sampling and in addition to record their regular food habits. The food frequency questionnaire (FFQ) asked for the intake of typical food items consumed by Bangladeshi people: staple foods, mainly cereals, such as rice, wheat, maize and lentils, as the major pulses. Consumption frequency was graded 1–4 (see Table 4). Chicken meat, eggs, groundnuts, milk and milk-based products were also included. Among these items, only rice is regularly consumed one to three times in a day and, to a lesser extent, also roti (whole wheat flatbread) for breakfast by the majority of participants.

4.9. Statistical Analysis

Descriptive statistics are presented as means (±SD), medians and interquartile ranges. Those samples containing DON below the limit of detection (LOD) were assigned a value of one-half the detection limit for the calculation of mean and median values, since this is considered a better approach to estimate average concentrations for left censored data, rather than assigning a value of zero to measurements below the detection limit [43]. Differences in urinary DON levels between the rural and the suburban cohort were analyzed by an independent sample *t*-test. The Spearman correlation coefficient (two-tailed) was used to assess the correlation between urinary DON concentration with food consumption, age and body mass index (BMI) of the participants. All analyses were carried out using IBM SPSS Statistics Version 22. A level of alpha 0.05 was assigned for statistical significance.

5. Conclusions

DON exposure in pregnant women in Bangladesh appears to be modest and lower than observed in biomonitoring studies performed in Europe and Africa. However, the present investigation comprises analysis of urine samples collected in one district and season only. Therefore, further studies in children and adult cohorts from other parts of the country and another season are indicated to gain more insights into the DON exposure of the Bangladeshi population.

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Author Contributions

Nurshad Ali and Gisela H. Degen co-conceived of the study idea, its design and funding. Nurshad Ali conducted biomarker analysis, statistical data analysis and drafted the manuscript. Gisela H. Degen supervised data collection and interpretation; and co-wrote the manuscript with the first author. Meinolf Blaszkewicz supervised LC-MS/MS analysis and its optimization; and contributed to the final editing of the manuscript. Abdullah Al Nahid and Mustafizur Rahman were instrumental in the collection of urine samples and questionnaires; and aided in FFQ data interpretation.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Reprint of peer-reviewed original publications

Publication 7

A comparative study of the human urinary mycotoxin excretion patterns in Bangladesh, Germany and Haiti using a rapid and sensitive LC-MS/MS approach

Gerding J, **Ali N**, Schwartzbord J, Cramer B, Brown DL, Degen GH, Humpf HU (2015). Mycotoxin Res 31:127–36

Contribution statement

JG performed biomarker analysis, statistical data analysis and drafted the manuscript. NA provided urine samples from Bangladesh and Germany, supported data analysis and interpretation. JS collected urine samples from Haiti cohorts and provided input in the manuscript draft. BC supervised the LC-MS/MS analysis. All senior co-authors, DLB, GHD and HUH contributed to the concept and final editing of the manuscript. HUH co-conceived study idea and acted as corresponding author for the manuscript.

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ORIGINAL PAPER

A comparative study of the human urinary mycotoxin excretion patterns in Bangladesh, Germany, and Haiti using a rapid and sensitive LC-MS/MS approach

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Abstract An improved "dilute and shoot" LC-MS/MS multibiomarker approach was used to monitor urinary excretion of 23 mycotoxins and their metabolites in human populations from Asia (Bangladesh), Europe (Germany), and the Caribbean region (Haiti). Deoxynivalenol (DON), deoxynivalenol-3-glucuronide (DON-3-GlcA), T-2-toxin (T-2), HT-2-toxin (HT-2), HT-2-toxin-4-glucuronide (HT-2-4-GlcA), fumonisin B_1 (FB_1), aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1), zearalenone (ZEA), zearalanone (ZAN), their urinary metabolites α-zearalanol (α-ZEL) and β-zearalanol (β-ZEL), and corresponding 14-O-glucuronic acid conjugates (ZEA-14-GlcA, ZAN-14-GlcA, β-ZEL, α/β–ZEL-14-GlcA), ochratoxin A (OTA), and ochratoxin alpha (OT_α) as well as enniatin B (EnB) and dihydrocitrinone (DH-CIT) were among these compounds. Eight urinary mycotoxin biomarkers were detected (AFM_1, DH-CIT, DON, DON-GLcA, EnB, FB_1, OTA, and α-ZEL). DON and DON-GlcA were exclusively detected in urines from Germany and Haiti whereas urinary OTA and DH-CIT concentrations were significantly higher in Bangladeshi samples. AFM_1 was present in samples from Bangladesh and Haiti only. Exposure was estimated by the calculation of probable daily intakes (PDI), and estimates suggested occasional instances of toxin intakes that exceed established tolerable daily intakes (TDI). The detection of individual mycotoxin exposure by biomarker-based approaches is a meaningful addition to the classical monitoring of the mycotoxin content of the food supply.

Keywords Urinary biomarker · Exposure assessment · Mass spectrometry · Mycotoxins · Urine

Introduction

Mycotoxins are toxic secondary metabolites of numerous fungal strains infesting a large variety of food and feed commodities. Mycotoxin production can take place at all stages of plant growth, processing, or storage (Bennett and Klich 2003). In order to protect humans against adverse health effects of mycotoxins, maximum levels in foodstuffs were set by regulatory authorities (Egmond et al. 2007). A reliable database concerning the human exposure to mycotoxins is critical to these regulatory attempts (Marin et al. 2013). Biomarker-based approaches to assess human exposure to mycotoxins have gained increased acceptance in recent years (Warth et al. 2013b). State-of-the-art LC-MS/MS methods allow detection and quantitation of mycotoxins and their metabolites in blood or urine at trace levels. Several pilot-scale studies applying multibiomarker

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approaches to investigate exposure of populations in Africa, Asia, or Europe have revealed regional differences in urinary biomarker excretion patterns (Warth et al. 2014; Njumbe Ediage et al. 2013; Gerding et al. 2014; Abia et al. 2013). Differences in nutritional habits and quality of consumed foodstuffs are likely the reason for interregional variations in mycotoxin excretion. Nevertheless, differences in analytical methodology and diversity in available biomarkers limit comparison of the results.

The present study provides comparable data on the urinary mycotoxin excretion patterns of populations from Bangladesh, Germany, and Haiti using an improved "dilute and shoot" LC-MS/MS multibiomarker approach for the simultaneous detection of 23 analytes, parent mycotoxins, and mycotoxin metabolites. Compounds monitored in this study were deoxynivalenol (DON), deoxynivalenol-3-glucuronide (DON-3-GlcA), T-2-toxin (T-2), HT-2-toxin (HT-2), HT-2-toxin-4-glucuronide (HT-2-4-GlcA), fumonisin B_1 (FB_1), aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1), zearalenone (ZEA), zearalanone (ZAN), their urinary metabolites α-zearalanol (α-ZEL), and β-zearalanol (β-ZEL) and corresponding 14-O-glucuronic acid conjugates (ZEA-14-GlcA, ZAN-14-GlcA, α/β–ZEL-14-GlcA), ochratoxin A (OTA), and ochratoxin alpha (OT_α), as well as enniatin B (EnB) and dihydrocitrinone (DH-CIT), a urinary metabolite of citrinin (Dunn et al. 1983; Ali et al. 2015b). Since in previous human or animal studies a good correlation has been observed between dietary mycotoxin intake and urinary excretion, AFM_1, OTA, DON, FB_1, and ZEA can be considered as suitable biomarkers of exposure (Zhu et al. 1987; Gilbert et al. 2001; Šarkanj et al. 2013; Turner et al. 2010; Riley et al. 2012; Gambacorta et al. 2013; Muñoz et al. 2014). When reliable urinary excretion rates were available, these data were used in our study to calculate the PDI to estimate the human exposure to mycotoxins.

Material and methods

Chemicals and reagents

Methanol and acetonitrile were of LC gradient grade purity (VWR, Darmstadt, Germany). Formic acid was purchased from Merck (Darmstadt, Germany). ZAN, α-ZEL, β-ZEL, AFM_1, and $AFB_{1/2}$, $AFG_{1/2}$ were purchased from Sigma Aldrich (Schnelldorf, Germany). DH-CIT was purchased from AnalytiCon Discovery (Potsdam, Germany). EnB was from Enzo Life Sciences (Lörrach, Germany). ZEA, DON, FB_1, OTA, OTα, T-2, and HT-2 were isolated and purified from fungal cultures (Beyer et al. 2009; Cramer et al. 2007, 2010; Bittner et al. 2013; Bretz et al. 2006; Hübner et al. 2012). Glucuronic acid conjugates (DON-3-GlcA, ZEA-14-GlcA, ZAN-14-GlcA, α-ZEL-14-GlcA, β-ZEL-14-GlcA, and HT-2-4-GlcA) were obtained from enzymatic synthesis using rat and pig liver microsomes (Welsch and Humpf 2012). Solid analytes were dissolved in ACN or ACN/H_2O (DON-3-GlcA, FB_1). Stock solutions were prepared at a concentration of 10 μg/mL in ACN or ACN/H_2O and stored at −20 °C. A working solution was freshly prepared at 20-fold concentration of the highest calibration point at each day of measurement in ACN/H_2O (50/50, *v*/*v*).

Sample collection and origin

Samples from Bangladesh and Germany were collected from 2013 to 2014 from healthy adult volunteers with ethical permission granted by the respective Institutional Internal Review Boards (Institute of Biological Sciences, Rajshahi University, Bangladesh; and IfADo, Germany). Samples from Bangladesh (n=95) were collected from residents of rural and urban areas of Rajshahi District (Ali et al. 2015c). Samples from Germany (n=50) were collected in the area of the city of Dortmund. Sample collection in Haiti (n=142) took place in the areas of Port au Prince and Quartier Morin with participation of healthy adult and children volunteers. For sample collection in Port-au-Prince, approval was received from the GHESKIO internal review board, and in Quartier Morin the department director of the North for the Ministry of Health (MSPP, in French). Sample collection in Port-au-Prince took place during July of 2012 and in Quartier Morin during September and October of 2013 and January of 2014. This study was reviewed by and received approval from the Cornell Institutional Review Board for Human Participants (ID no. 0908000519).

Sample preparation

After collection, samples were aliquoted, transported to Germany on dry ice, and stored at −80 °C until further analysis. On the day of analysis, samples were allowed to reach room temperature and centrifuged at 14,000×*g* for 10 min to remove solid residues. One-hundred μL of the supernatant were diluted with 900 μL H_2O/ACN/formic acid (FA) (0.94/0.05/0.01, *v*/*v*/*v*) and subjected to LC-MS/MS analysis. All measurements were performed in duplicate and included the measurement of a blank urine sample pooled from German samples and a blank urine sample spiked with analytes at medium concentration level for quality control. Runs were rejected, if quality control samples failed to be within 15 % of their correct value.

LC-MS/MS conditions

Analysis was carried out on an Agilent 1260 Infinity HPLC system coupled to an AB SCIEX QTRAP®6500 mass spectrometer.

HPLC setup

Separation of analytes was carried out by reversed-phase chromatography on a NUCLEODUR® C_{18} Pyramid column (3 μm, 2.0×150 mm, MACHEREY-NAGEL, Düren, Germany) equipped with a C_{18} EC guard column (2 mm×4 mm, MACHEREY-NAGEL, Düren, Germany). Eluent A was acetonitrile (0.1 % formic acid) and eluent B water (0.1 % formic acid) at a flow rate of 600 μL/min. The total runtime was 13 min. The gradient started at 5 % A for 2 min. Solvent A was increased to 95 % until 9.5 min and kept for 1 min at 95 % A. Afterwards percentage of A was decreased to starting conditions (11 min) and the column was allowed to re-equilibrate until 13 min.

MS/MS setup

Detection of analytes of interest was carried out on an AB SCIEX QTRAP®6500 mass spectrometer with ESI ionization in Scheduled MRM™ (multiple reaction monitoring) detection mode. ESI-source parameters were optimized and preset for all measurements as follows: source temperature, 500 °C; curtain gas, 30 psi; gas 1, 35 psi; gas 2, 45 psi. Ion spray voltage was set to −4,500 V in negative ionization mode and 5,500 V in positive ionization mode. Two characteristic MRM transitions per analyte were monitored to ensure accurate identification. MS/MS parameters were optimized for each analyte in positive and negative ionization mode by direct infusion of pure standard solutions. The final method was built based on the best S/N ratio in positive or negative ionization mode. Scheduled MRM™ parameters were set to a window width of 25 s and a target scan time of 0.15 s in negative and 0.20 s in positive ionization mode. The software used was Analyst 1.6.2. Detailed MS-Parameters are in Table S1 in the supporting information.

Validation of the analytical method

The analytical method was subject to an in-house validation comprising linearity, apparent recovery (R_A), reproducibility, and accuracy along with the determination of LOD and LOQ. Quantitation was carried out with an external matrix-matched calibration with spiked blank urine samples using a freshly prepared working solution. Blank urine was pooled out of samples negative for all analytes in a previous measurement. The calibration curves covered two magnitudes in concentration of each analyte. Coefficient of determination was defined as a measure of linearity of the calibration curves. The calibration range was chosen based on the preliminary determination of LOD and LOQ of the analytes. LOD was defined at a signal to noise (S/N) ratio of S/N=3 and LOQ at S/N=10. All validation experiments were executed in triplicate. R_A was calculated from the slope of the linear calibration curves of spiked urine samples and neat standard solutions. Reproducibility was investigated at low, medium, and high spiking level in blank urine on three different days. Intraday precision was determined by 10-fold injection of a spiked urine sample at second highest spiking level.

Statistical analysis

Means of biomarker concentrations detected in urines of different origins were subjected to a one-way analysis of variance (ANOVA). Differences among means of populations were further characterized using a post hoc test (Scheffe's method). Statistical significance was assumed at a p value$\leq$0.05. IBM SPSS 22 program was used for statistical analysis.

Estimation of probable daily intakes (PDI) for a theoretical reference subject weighing 60 kg

PDI was calculated if samples from an investigated population were tested positive for a specific biomarker and previously published data on human excretion rates were available. In cases in which no data on human excretion were available, estimates from piglet biomarker excretion were used for calculations. Table 4 summarizes underlying excretion rates and references. Concerning the excretion of DON, we assumed an excretion rate of 68 % where 52 % were excreted as DON-GlcA and 16 % as unmetabolized DON (Warth et al. 2013a). PDI was calculated using Formula 1. Since no specific data on individual body weight and the daily excreted amount of urine volume of the investigated populations were available, calculations were based on an average body weight of 60 kg. Results therefore have to be considered as rough estimates rather than accurate exposure assessments, but give valuable insights to the exposure situation of the investigated populations. A mean body weight of 60 kg and a mean daily urine excretion of 1.5 L (EFSA 2010) were assumed.

$$\mathrm{PDI}\left(\frac{\mu g}{\mathrm{kg}}\,\mathrm{body\ weight}\right) = \frac{C \times V \times 100}{W \times E} \quad (1)$$

With:

C Biomarker concentration (in microgram per liter)
V (Mean) daily urine excretion (in liter)
W (Mean) body weight (in kilogram)
E Excretion rate (in percent)

Results

Method development and validation

This improved LC-MS/MS method is capable of the simultaneous separation and sensitive detection of 23 mycotoxin-biomarkers in one single chromatographic run of 13 min (Gerding et al. 2014). DON-3-GlcA and DON-15-GlcA were not chromatographically separated and the resulting signal was assumed to be the sum of both glucuronides. LOD of OTA, FB_1, EnB, DH-CIT, and other analytes could be lowered compared to the previously published method and allowed the frequent detection of OTA throughout the sample pool. Obtained LODs were in the low nanogram per milliliter scale or below. Apparent recovery (R_A) values ranged from 73 to 183 % and indicate an acceptable suppressing or enhancing effect of the matrix on signal intensities. Relative standard deviation (RSD) during intraday reproducibility was lower than 15 % for all analytes. RSD during interday reproducibility was lower than 15 % at high and medium spiking level and below 20 % at low calibration level for the majority of analytes. With the exception of HT-2-4-GlcA, all analytes met the criteria set during in-house validation. A higher RSD of 36 % in the low and 20 % at high spiking level was tolerated for HT-2-4-GlcA since a reliable screening at low concentration levels was the major aim during method development. Retention times were highly reproducible and stable for all analytes, allowing the application of Scheduled MRM™ algorithm. Detailed validation parameters are listed in Table 1.

Mycotoxin and metabolite excretion patterns of urine samples from Germany, Haiti, and Bangladesh

Some mycotoxins and their metabolites could be detected in the samples from all countries of origin. The detailed results are summarized in Tables 2 and 3. Analyte occurrence differs widely among the investigated populations. Eighty-seven percent of the samples from Bangladesh were tested positive for one or more mycotoxin biomarker, and 80 % of German urine samples contained detectable amounts of biomarkers. The presence of mycotoxin biomarkers was confirmed for 68 % of the Haitian samples (Table 3). Eight different biomarkers were detected in the samples from Haiti. OTA, DON-GlcA, DON, and DH-CIT were the most prevalent analytes, followed by the infrequent detection of AFM_1, EnB, FB_1, and α-ZEL. DON-GlcA, DON, OTA, and DH-CIT were detected in urines of the German cohort. Samples from Bangladesh showed an abundant occurrence of OTA and DH-CIT, whereas AFM_1, FB_1, and EnB were only occasionally detected. DON and DON-GlcA were frequently detected in samples from Germany and Haiti at similar concentrations, but were absent in samples from Bangladesh (Table 2). DH-CIT and OTA occurred more frequently and at higher mean concentrations in urine samples from Bangladesh than in those from Germany or Haiti. α-ZEL was the sole metabolite related to zearalenone contamination of ingested foodstuffs and was only identified in Haitian samples. Neither T-2-toxin nor HT-2-toxin or HT-2-4-GlcA were detected in any of the three cohorts.

Estimation of probable daily intake rates (PDI)

The obtained biomarker concentrations of DON, DON-GlcA, FB_1, AFM_1, and α-ZEL were used to estimate probable daily intake (PDI) values for the mycotoxins DON, FB_1, AFB_1, and ZEA based on published urinary excretion rates for these mycotoxins. The OTA data was not considered for estimation of intakes as a robust estimate of human urinary OTA excretion rates is not available (Duarte et al. 2011). Table 4 summarizes the calculated PDI values and relates to the established TDI values. Mean PDI was below the TDI for all toxins in samples from the different geographic origins. PDI calculated for DON exceeded the TDI of 1 μg/kg b.w. in 6 % of the samples at maximum by the factor of two in German and four in Haitian samples. α-ZEL was occasionally detected in urines from Haiti. Assuming a urinary excretion rate of 8.3 %, 1 % of the samples exceed the TDI set for ZEA at 0.2 μg/kg b.w. with a maximum exceedance by the factor of four.

Discussion

Interregional differences in urinary mycotoxin and mycotoxin metabolite excretion patterns exist between German, Haitian, and Bangladeshi populations. DON and its urinary metabolite DON-GlcA were frequently detected in urine samples from Germany and Haiti, whereas all samples from Bangladesh tested negative for these compounds. Presence of OTA and DH-CIT was confirmed in samples from all geographic regions, with samples from Bangladesh showing higher occurrence rates and mean concentrations than samples originating from Germany or Haiti (Tables 2 and 3). AFM_1 and FB_1 were exclusively found in samples from Bangladesh and Haiti. Frequent detection of DON or its urinary metabolites DON-3-GLcA and DON-15-GlcA in Europe was previously reported in populations from Italy, Germany, and Croatia (Solfrizzo et al. 2014; Gerding et al. 2014; Šarkanj et al. 2013). The outcomes of these studies match the present data of mean concentrations below the LOQ (4 ng/mL for DON) and 11.2 ng/mL for DON-GlcA in the German population. Both Solfrizzo et al. (2014) and Gerding et al. (2014) concluded a medium risk for the investigated populations based on PDI calculations ranging close to the established TDI of 1 μg DON/kg b.w. with an occasional exceedance of the TDI. Exposure to DON was low in all of the three investigated locations. Whereas DON-Biomarkers were absent in samples

Table 1 Results of method validation. Limit of detection (LOD) and limit of quantitation (LOQ) are defined at a S/N ratio of 3 (LOD) and 10 (LOQ)

Analyte	Calibration range [ng/mL]	LOD [ng/mL] [a]	LOQ [ng/mL][a]	R_A [%]	Intraday [%][b]	Interday[c]	Retention times [min]
DON-GlcA	0.4–40	0.1 (1)	0.4 (4)	91 %	9	16/9/11	4.48±0.014
ZAN-14-GlcA	0.1–10	0.0125 (0.125)	0.1 (1)	73 %	8	18/13/13	6.97±0.01
ZAN	0.15–15	0.0375 (0.375)	0.15 (1.5)	82 %	10	19/5/6	8.34±0.02
ZEA	0.03–3	0.015 (0.15)	0.03 (0.3)	86 %	8	19/15/15	8.38±0.013
α-ZEL	0.025–2.5	0.0125 (0.125)	0.025 (0.25)	74 %	10	16/12/13	7.86±0.013
α-ZEL-14-GlcA	0.5–50	0.25 (2.5)	0.5 (5)	129 %	7	13/14/15	6.62±0.012
ZEA-14-GlcA	0.75–75	0.1875 (1.875)	0.75 (7.5)	183 %	7	17/12/12	6.98±0.013
OTα	0.1–10	0.05 (0.5)	0.1 (1)	86 %	7	19/11/11	8.32±0.026
β-ZEL	0.025–2.5	0.0125 (0.125)	0.025 (0.25)	79 %	8	14/11/12	7.49±0.013
β-ZEL-14-GlcA	0.5–50	0.25 (2.5)	0.5 (5)	76 %	8	19/14/11	6.29±0.012
AFB_1	0.01–1	0.0025 (0.025)	0.01 (0.1)	124 %	8	20/11/11	7.02±0.015
AFB_2	0.0029–0.289	0.0014 (0.014)	0.0029 (0.029)	109 %	10	16/14/14	6.77±0.027
AFG_1	0.01–1	0.0025 (0.025)	0.01 (0.1)	117 %	11	18/15/15	6.77±0.014
AFG_2	0.0029–0.289	0.0014 (0.014)	0.0029 (0.029)	122 %	6	15/14/14	6.53±0.01
AFM_1	0.01–1	0.0025 (0.025)	0.01 (0.1)	129 %	9	13/9/12	6.26±0.01
T-2	0.02–2	0.01 (0.1)	0.02 (0.2)	86 %	8	15/14/14	8.03±0.01
HT2	0.9–90	0.45 (4.5)	0.9 (9)	63 %	5	17/14/12	7.2±0.01
HT-2-4-GlcA	0.15–15	0.0375 (0.375)	0.15 (1.5)	111 %	14	36/20/14	6.72±0.01
DH-CIT	0.02–2	0.01 (0.1)	0.02 (0.2)	76 %	9	20/15/15	11.31±0.13
FB_1	0.01–1	0.00125 (0.0125)	0.01 (0.1)	170 %	12	7/6/12	6.12±0.02
EnB	0.0005–0.05	0.000125 (0.00125)	0.0005 (0.005)	144 %	15	8/10/15	10.18±0.03
OTA	0.0075–0.75	0.001 (0.01)	0.0075 (0.075)	93 %	11	7/9/9	8.37±0.01
DON	0.4–40	0.1 (1)	0.4 (4)	82 %	8	15/9/9	4.27±0.01

Retention times are means of 58 chromatographic runs on three different days of analysis

[a] Values in parentheses take the 10-fold dilution of urine samples into account

[b] Apparent recovery (R_A) was calculated from the slope of the linear calibration curves of spiked urine samples and neat standard solutions. Intraday reproducibility was determined by 10-fold injection of a blank urine sample spiked at second highest level of the calibration range

[c] Interday precision was determined by 3-fold injection of a spiked urine sample on three subsequent days at second lowest, medium, and second highest calibration point

from Bangladesh, the TDI for DON was only occasionally exceeded in Germany and Haiti. The biomarker data reflect the high occurrence rates of DON in 99 % of raw grains at mean concentrations of 61 μg/kg (wheat) as reported in the annual German harvest report (Bundesministerium für Ernährung und Landwirtschaft 2013). Our data indicate that the exposure of the investigated Haitian population to DON is similar or higher, including peak values, compared to DON exposure in Germany. This finding is rather interesting as no data on the natural occurrence of DON in Haitian foodstuffs are available. High peak concentrations of DON-GlcA of 60 and 104 ng/mL in the German and Haitian population furthermore demonstrate the advantage of biomarker-based approaches to assess the exposure of individuals and sub-populations. Since neither DON nor its metabolites were detected in samples from Bangladesh, we conclude a low rate of exposure among Bangladeshi participants. This result is consistent with low occurrence rates reported for DON in foodstuffs of Bangladesh (Dawlatana et al. 2002) and the outcome of a biomarker based study on DON exposure conducted in Southeast Asia, in the area of Bangkok and Thailand (Dawlatana et al. 2002; Warth et al. 2014).

OTA was frequently detected in samples of all origins, with mean values of 0.040, 0.109, and 0.203 ng/mL in urines from Germany, Haiti, and Bangladesh. Samples from Bangladesh showed significantly higher mean concentrations than samples from Germany and Haiti. The data of the present study resemble ranges of OTA concentrations reported for urine samples from Germany, Italy, and Croatia (Gerding et al. 2014; Solfrizzo et al. 2014; Klapec et al. 2012). From a mean urinary concentration of 0.144 ng/mL in Italian urines, Solfrizzo et al. (2014) calculated a PDI of 0.139 μg/kg b.w., i.e., a significant exceedance of the established TDI of 0.017 μg/kg b.w. for OTA. We did not estimate exposure to OTA by quantitative means, since uncertainties remain concerning urinary excretion rates of OTA due to its complex toxicokinetics (Duarte et al. 2011). Excretion rates applied in previous biomarker studies range from 2.6 % (Solfrizzo et al.

Table 2 Summary of mycotoxin and mycotoxin metabolite content detected in urine samples from Germany, Haiti, and Bangladesh

	Sample origin								
	Haiti			Germany			Bangladesh		
	Cohort size (*n*), 142			Cohort size (*n*), 50			Cohort size (*n*), 95		
Toxin	Positives % (*n*)	Mean, ng/mL±SD (ng/mg creatinine)	Median, ng/mL (range)	Positives % (*n*)	Mean, ng/mL±SD (ng/mg creatinine)	Median, ng/mL (lowest, highest)	Positives % (*n*)	Mean, ng/mL±SD (ng/mg creatinine)	Median, ng/mL (range)
DON	17 (24)	3.2±2.0 (3.6)	2.0 (<LOQ–16.9)	16 (8)	2.0±n.a. (2.0)	2.0 (<LOQ)	n. d.	n. d.	n. d.
DON-GlcA	21 (30)	17.0±21.1 (20.0)	10.8 (<LOQ–104.3)	54 (27)	11.2±13.0 (11.7)	7.5 (<LOQ–60.9)	n. d.	n. d.	n. d.
EnB	3 (4)	0.036±0.020 (0.029)	0.028 (0.021–0.065)	14 (7)	0.012±0.002 (0.017)	0.011 (0.010–0.014)	2 (2)	0.019±0.000 (0.015)	0.019 (0.0119–0.0187)
OTA	33 (47)	0.109±0.044 (0.091)	0.097 (<LOQ–0.225)	30 (15)	0.040±0.011 (0.030)	0.038 (<LOQ–0.082)	76 (72)	0.203[a]±0.282 (0.207)	0.115 (<LOQ–2.010)
AFM_1	8 (11)	0.06±0.03 (0.06)	0.05 (<LOQ–0.14)	n. d.	n. d.	n. d.	8 (8)	0.06±0.02 (0.06)	0.05 (<LOQ–0.12)
DH-CIT	14 (20)	0.49±0.95 (0.28)	0.27 (<LOQ–4.34)	28 (14)	0.12±0.02 (0.09)	0.10 (<LOQ–0.33)	75 (71)	2.75[a]±8.43 (3.15)	0.42 (<LOQ–58.82)
FB_1	3 (4)	0.44±0.21 (0.29)	0.41 (0.23–0.70)	n. d.	n. d.	n. d.	1 (1)	–	–
α-ZEL	3 (4)	1.46±1.02 (0.97)	1.42 (0.52-2.49)	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.

The portion of positive findings for a compound is given as a percentage (in percent) and an absolute number (*n*). Means are stated in nanogram per milliliter and normalized to creatinine (in nanogram per milligram of creatinine). Negative findings are indicated as not detected (n. d.). Toxin concentrations between the limit of detection (LOD) and the limit of quantitation (LOQ) were set to LOQ/2 of the respective compound for the calculation of means. Two samples of the Bangladeshi sample pool exceeded the calibration range for DH-CIT.

[a] Measurement of these samples was repeated with an adjusted calibration. Mean concentrations of DH-CIT and OTA were significantly higher in urine samples from Bangladesh than in Germany and Haiti at a p value≤0.05

Table 3 Occurrence and co-occurrence of investigated mycotoxins and their metabolites in urine samples from Haiti, Germany, and Bangladesh

	Occurrence/co-occurrence % (*n*)						
Toxins	Haiti	Germany	Bangladesh	Toxins	Haiti	Germany	Bangladesh
1 Toxin	43 (61)	42 (21)	23 (22)	3 Toxins	6 (9)	20 (10)	11 (10)
AFM_1	3 (4)	n. d.	n. d.	AFM_1/DH-CIT/DON-GlcA	1 (1)	n. d.	n. d.
α-ZEL	1 (2)	n. d.	n. d.	AFM1/DH-CIT/OTA	1 (1)	n. d.	8 (8)
DH-CIT	5 (7)	8 (4)	11 (10)	DH-CIT/DON/DON-GlcA	1 (2)	4 (2)	n. d.
DON	4 (5)	n. d.	n. d.	DH-CIT/DON-GlcA/OTA	n.d	6 (3)	n. d.
DON-GlcA	6 (9)	22 (11)	n. d.	DH-CIT/FB_1/OTA	n.d	n. d.	1 (1)
EnB	1 (1)	4 (2)	n. d.	DH-CIT/ENB/OTA	n.d	2 (1)	1 (1)
FB_1	1 (2)	n. d.	n. d.	DON/DON-GlcA/EnB	n.d	4 (2)	n. d.
OTA	22 (31)	8 (4)	13 (12)	DON/DON-GlcA/OTA	3 (4)	4 (2)	n. d.
2 Toxins	17 (24)	16 (8)	54 (51)	EnB/DON-GlcA/OTA	1 (1)	n. d.	n. d.
AFM_1/DH-CIT	1 (2)	n. d.	n. d.	4 Toxins	1 (2)	2 (1)	n. d.
AFM_1/DON	1 (1)	n. d.	n. d.	DH-CIT/DON/DON-GlcA/OTA	1 (1)	2 (1)	n. d.
AFM_1/DON-GlcA	1 (2)	n. d.	n. d.	DH-CIT/DON/OTA/FB_1	1 (1)	n. d.	n. d.
α-ZEL/DH-CIT	1 (1)	n. d.	n. d.	Total positives	68 (96)	80 (40)	87 (83)
α-ZEL/DON	1 (1)	n. d.	n. d.	Total negatives	32 (46)	20 (10)	13 (12)
DH-CIT/DON-GlcA	n.d	2 (1)	n. d.				
DH-CIT/EnB	n.d	n. d.	1 (1)				
DH-CIT/FB_1	1 (1)	n. d.	n. d.				
DH-CIT/OTA	2 (3)	4 (2)	53 (50)				
DON/DON-GlcA	6 (8)	2 (1)	n. d.				
DON/OTA	1 (1)	n. d.	n. d.				
DON-GlcA/OTA	1 (2)	4 (2)	n. d.				
EnB/DON-GlcA	n.d	4 (2)	n. d.				
EnB/OTA	1 (2)	n. d.	n. d.				

Values are given as percentage and absolute number of occurrence in each cohort (*n*, in brackets)

2014) to 50 % (Abia et al. 2013), which demonstrates the need for better and robust estimates urinary OTA excretion rates. On the other hand good, correlations of OTA intake or dietary habits with urinary OTA concentrations were previously reported (Gilbert et al. 2001; Coronel et al. 2011; Muñoz et al. 2014). We therefore conclude a higher exposure of the Bangladeshi cohort to OTA compared to Germany and Haiti. Further investigations are warranted in light of the frequent detection of OTA in plasma samples from Bangladeshi students (Ali et al. 2014). Whereas comprehensive data on the occurrence of OTA in food commodities is available for Europe (EFSA 2006), our findings support efforts to identify dietary sources of OTA exposure in Haiti and Bangladesh.

AFM_1, as a urinary biomarker of AFB_1, was detected in 8 % of samples from Bangladesh and 11 % in samples from Haiti. Since AFB_1 is a potent mutagenic carcinogen, no TDI values are established. The presence of AFM_1 in urine samples from Bangladesh and Haiti support concerns about the aflatoxin contamination of foodstuffs in these regions. Aflatoxin exposure in Haiti was recently reviewed, revealing high aflatoxin levels in common staple foods like peanut butter and maize (Schwartzbord et al. 2013; Filbert and Brown 2012; Castor 1987). Recent data from Bangladesh showed evidence for high AFB_1 levels in common foodstuffs above EU regulatory limits (Roy et al. 2013). Elevated aflatoxin concentrations were also reported in chili samples from Sri Lanka, with 67 % of the samples exceeding EU regulatory limits (Yogendrarajah et al. 2014). Future efforts in monitoring aflatoxin contents in Haiti and Bangladeshi foodstuffs should therefore also include spices to investigate their impact on aflatoxin exposure.

Fumonisin FB_1 was detected in four urine samples from Haiti and one sample from Bangladesh at a maximum concentration of 0.7 ng/mL. The TDI of 2 μg/kg b.w. was never exceeded. No data on the occurrence of FB_1 in food are available for Haiti. Biomarker-based studies conducted in regions of endemic FB_1 occurrence reported more frequent detection of FB_1 in urine samples than in our Haitian cohort. Torres et al (2014) investigated urinary mycotoxin levels in nearby Guatemala, reporting the abundant presence of urinary FB_1 (Torres et al. 2014). Shephard et al. (2013) detected FB_1 in 96 % of the investigated South African population at mean

Table 4 Calculated probable daily intake (PDI) of selected mycotoxins

Biomarker	DON+DON-GlcA	FB1	AFM1	α-ZEL
Mycotoxin	DON	FB1	AFB1	ZEA
Excretion rate %	68	1	1.5	8.3
Reference	Warth et al. (2013a)	Riley et al. (2012)	Zhu et al. (1987)	Gambacorta et al. (2013)
Species	Human	Human	Human	Piglet
TDI (μg/kg b.w.)	1	2	–	0.2
Haiti				
Mean PDI (μg/kg b.w.)	0.27	0.05	0.03	0.03
Max. PDI (μg/kg b.w.)	4.38	1.74	0.23	0.75
Samples exceeding TDI % (*n*)	6 (8)	–	–	1 (2)
Germany	–	–	–	–
Mean PDI (μg/kg b.w.)	0.3	–	–	–
Max. PDI (μg/kg b.w.)	2.15	–	–	–
Samples exceeding TDI % (*n*)	6 (3)	–	–	—
Bangladesh	–	–	–	–
Mean PDI (μg/kg b.w.)	–	0.03	0.03	–
Max. PDI (μg/kg b.w.)	–	1.362	0.195	–
Samples exceeding TDI % (*n*)	–	–	–	–

Concentration was set to LOD/2, if a biomarker was not detected and to LOQ/2 when biomarker concentration was below LOQ. Excretion rates were obtained from human excretion data and piglet excretion data, where no data on human excretion was available (α-ZEL) TDI values were obtained from opinions of the Scientific Committee on Food (SCF) and European Food Safety Authority (EFSA) (EFSA 2006; SCF 2000, 2002, 2003). No TDI for AFB_1 is set due to its carcinogenicity

urine concentrations of 1.52 ng/mL. Combining our results with the outcome of the studies from Guatemala and South Africa, we conclude a lower risk of exposure to FB_1 among the Haitian participants of our study. Moreover, the biomarker based approach demonstrates value identifying exposure of individuals, since occasional peak exposures to FB_1 were not detected in the German or Bangladeshi sample cohort.

DH-CIT is a metabolite of citrinin and was frequently detected in samples from all geographic areas covered by this study. Whereas DH-CIT occurrence was frequent but at low concentrations in the German and Haitian sample pool (0.27 and 0.49 ng/mL), mean concentrations in Bangladeshi urines were higher: DH-CIT was detected in 76 % of these samples, with a mean concentration of 2.75 ng/mL. This is in line with differences reported in recent studies using targeted analysis of citrinin biomarkers in urines from Bangladeshi cohorts collected during another season and in German volunteers (Ali et al. 2015b, c). DH-CIT has not been validated as biomarker of exposure to citrinin, but new data indicate that a correlation of citrinin intake and DH-CIT in urine can be anticipated (Ali et al. 2015a). Citrinin occurrence in food and feed has been described in Europe, Asia, and the USA, frequently present with OTA (EFSA 2012). Nguyen et al. (2007) detected citrinin in rice samples from Vietnam and reported co-occurrence with OTA and AFB_1. Rice is a common staple food in Bangladesh, and urinary OTA and DH-CIT concentrations were elevated in urines from Bangladesh. Rice is also the most common grain ingested in Haiti, but DH-CIT and OTA levels there were not as high as those found in Bangladesh. It would therefore be of particular interest to investigate if differences in rice contamination with OTA and citrinin lead to the observed effect.

EnB was most frequently detected in German urines and less so among urines from Haiti and Bangladesh. To date, little attention has been paid to the occurrence of enniatins in foodstuffs by regulatory authorities (Jestoi 2008). Several studies have examined the presence of enniatins in food commodities in Europe, revealing a widespread occurrence in grain and grain-derived products (Juan et al. 2013; Uhlig et al. 2006). The data of this biomarker survey indicate a frequent intake of enniatins in Germany, supporting further studies on the occurrence of enniatins. There is also a need for more toxicological studies to determine the impact of enniatin contaminated food and feed on the health of humans and animals.

Zearalenone-related biomarkers were detected occasionally in samples from Haiti and calculated PDI values exceeded the TDI of 0.2 μg/kg b.w. in 1 % of samples by a factor of six. Solfrizzo et al. (2014) frequently detected ZEA in southern Italy at mean concentrations below 0.1 ng/mL of α-ZEL, β-ZEL, and ZEA after enzymatic cleavage with β-glucuronidase (Solfrizzo et al. 2014). That study concluded a limited risk of exposure to the Italian population since calculated mean PDI values were lower than the established TDI by the factor of 10. The low frequency of positivity for ZEA

and its metabolites in the present study may be attributed to the high LOD of our current approach, which compromised detection at lower ranges but achieved direct assessment of multiple mycotoxins. This was also discussed by Warth et al. (2013a) detecting only ZEA and no other metabolites after enzymatic treatment of the urine samples of a human volunteer consuming a ZEA containing diet. Nevertheless, the present method detects ZEA and its metabolites in concentrations that would result in PDI values exceeding the TDI.

No T-2, HT-2, or the urinary HT-2 biomarker HT-2-4-GlcA was detected in any of the samples. The occurrence of T-2 toxin in Europe was reviewed by EFSA in 2011, finding a low risk of exposure to the European population but underscoring a lack of data on suitable T-2 biomarkers in humans (EFSA 2011). We confirm a low exposure of the investigated populations by means of the urinary presence of T-2 or its metabolites.

A validated and improved multi-biomarker LC-MS/MS approach was applied to samples of three different geographic origins to investigate differences in human urinary mycotoxin excretion, on the basis of directly comparable data. Exposure to DON was higher in the German and Haitian cohorts, whereas exposure to citrinin and OTA was elevated in Bangladeshi persons, based on comparatively high urine concentrations of DH-CIT and OTA. This calls for further biomonitoring studies in Bangladesh with a focus on nephrotoxic mycotoxins. Attention should be paid also to aflatoxin exposure in Bangladesh and Haiti. We report a low exposure of the investigated populations to DON, FB_1, and ZEA with occasional exceedance of tolerable daily intakes. Valuable information is gained through the identification of peak exposures of individuals using the multibiomarker LC-MS/MS approach. The approach of this study furthermore demonstrates its usefulness in estimating human exposure to mycotoxins in geographic areas with little or no data available on mycotoxin occurrence in food commodities.

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Conflict of interest The authors declare no conflict of interests.

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