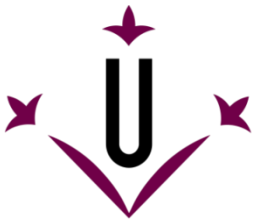

An overview of mycotoxin biomarker application in exposome-health studies

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Highlights

- Monitoring of biomarkers reveal the exposure to AF, OTA and DON, mainly
- Biomarkers results in blood and urine give complementary exposure information.
- Improved instrumental techniques enable multimycotoxin detection
- Few studies deal with simultaneous exposure to mycotoxins and other chemical hazards.
- There is a need to include the mycotoxins in exposome approaches.

List of Abbreviations

Aflatoxin (AF)

Aflatoxin B_1 (AFB1)

Aflatoxin M_1 (AFM1)

Aflatoxin P_1 (AFP1)

Aflatoxin Q_1 (AFQ1)

Alternariol (AOH)

Citrinin (CIT)

Deoxynivalenol (DON)

De-epoxy-deoxynivalenol (DOM-1)

Environmental-wide associations studies (EWAS)

Fumonisins B (FB)

Fumonisin B_1 (FB1)

Glucoside (Glc)

Glucuronide (GlcA)

Hydrolysed Fumonisin (HFB)

High resolution mass spectrometry (HRMS)

Liquid chromatography (LC)

Mass spectrometry (MS)

Nivalenol (NIV)

Ochratoxin A (OTA)

Ochratoxin alpha (OTα)

Patulin (PAT)

Probable daily intake (PDI)

Tolerable daily intake (TDI)

Zearalenone (ZEN)

Zearalanone (ZAN)

Zearalenol (ZEL)

Abstract

Exposure assessment in epidemiological studies remains as a key bias domain, prompting for reliable and accurate methods reflecting the true individual exposure. For that reason, the use of exposure biomarkers has become the gold-standard method for environmental chemicals and food contaminants in epidemiology. In the last few years, a growing list of biomonitoring studies has revealed the widespread exposure of population to mycotoxins, mainly aflatoxins, ochratoxins or trichothecenes, subject to geographical localisation. Despite the advances in mass-spectrometry, mycotoxins remain largely overlooked by mainstream epidemiological research. To date, the scarce epidemiological evidence has elucidated the associations between exposure to aflatoxins and hepatocellular carcinoma, cirrhosis or impairment of infant growth. The novel exposome paradigm offers a unique opportunity to boost the epidemiological research of mycotoxins. Nonetheless, there is an urgent need that mycotoxins catch the attention of mainstream epidemiological researchers, especially those intending to develop chemical-agnostic approaches in pathologies and populations where mycotoxins may represent a concern.

Keywords: mycotoxins, exposome, environmental health, biomonitoring, biomarkers

Biomarkers were defined by Vidal et al. (2018) as characteristics that are objectively measured and evaluated as an indicator of normal biological or pathogenic processes, pharmacologic responses to a therapeutic intervention or toxic responses to a toxic agent. Mycotoxns are fungal secondary metabolites that are capable of causing disease and death in humans and other animals. They are usually present in plant-derived food products; thus human exposure is related to vegetable products intake.

Biomonitoring of mycotoxins

Mycotoxin biomarkers have been defined as the compounds (e.g., parent toxins and/or a metabolite) or the products of their interaction with target molecules (e.g., protein or DNA adducts and glucuronide conjugates) that can be measured in body fluids or tissues and can be correlated with ingested mycotoxins. Very often urine is the matrix of choice, as it is easily collected. Urine biomarkers mainly represent recent mycotoxin intake, whereas measurements in plasma/serum are more likely to represent long-term exposure. Biomarkers analysed both in 24h and first-morning urine paired samples have shown that exposure assessment of mycotoxins with fast excretion rates, such as ZEN, DON and AOH, is influenced by the type of urine sample chosen, with lower concentrations in the first-morning urines than in 24h urines, suggesting that morning urines mainly reflect the exposure from some previous hours and is not representative of the overall daily exposure [1].

Different biomarkers have been proposed for the main mycotoxins in urine. Free DON, DON-15-GlcA, and DON-3-GlcA are considered as the best DON-biomarkers of exposure, whereas HT-2 toxin is the prevailing biomarker of T-2 toxin, and OTA, OTα, and their glucuronides are the main OTA metabolites. Besides, free FB and HFB are the potential FB-biomarkers, while a cascade of ZEN metabolites (α-ZEL, β-ZEL, 8-OH-ZEN, 15-OH-ZEN, and ZEN-14-GlcA) are considered as ZEN biomarkers in urine. To evaluate acute AF exposure, AFM1, AFQ1, AFP1, and AFB1-N7-guanine should be considered in urine.

Few studies have reported the evaluation of human exposure by coupling the analysis of mycotoxins in human urine to that in blood. A recent study in China, showed that in the plasma samples, OTA was the most prevalent one (incidence of 27.7%), followed by AFB1-lysine

(19.6%). On the other hand, in the urine samples, DON-15-GlcA (incidence of 43.8%) was the most abundant mycotoxin, followed by DON-3-GlcA (15.8%), AFM1 (10.4%) and DON (10.0%). In the plasma samples, the mean concentrations of OTA (1.21 mg/L) and ZEN (0.157 mg/L) were higher than those in urine. Conversely, the mean concentrations of FB1 (0.697 mg/L), DON (2.60 mg/L) and ZAN (0.260 mg/L) in plasma were lower than those in urine [2]. Similarly, mycotoxin prevalence was compared in serum and 24h urine by De Ruyck et al. [3], however, they found higher prevalence and smaller differences between these two fluids. Significant correlations have been observed across almost all mycotoxins, when comparing serum to the urinary measurements.

The use of breast milk in biomonitoring studies is gaining interest due to its easy collection, and because it shows not only the mother's internal exposure levels but also the external exposure of infants during critical windows of development. Most studies exploring AFM1 showed percentages of positive samples exceeding the 25% of analysed samples, and mean concentrations of positive samples ranged from 0.56 to 44000 ng/L [4;X,X]. Recent efforts have been devoted to develop highly sensitive multi-biomarker methods for this matrix [5]. Interestingly, wastewater-based epidemiology has recently proved to be a complementary approach to human biomonitoring. It is based on the chemical analysis of biomarkers in urban wastewater to measure the collective consumption or exposure to chemicals [6].

Methods of analysis

Human biomonitoring of mycotoxins relies on suitable methods of analysis, availability of mycotoxin standards and, due to methods improvement, includes an increasing number of co-occurring mycotoxins, although studies rarely include other chemical contaminants. In the last decade, the latest generation of high performance LC-MS/MS instruments, and rapid ‘dilute and shoot’ methods have allowed for convenient simultaneous analysis of a range of parent mycotoxins in urine, usually including their modified forms. However, multidetection of biomarker’s studies in blood plasma or serum are scarce. In the last few years, efforts have been done to develop accurate and sensitive UHPLC-MS/MS methods for multibiomarker’s detection in urine [7, 8]. Recently, Vidal et al. [9] highlighted the capability of HRMS to record

full-scan spectra results of a theoretically unlimited number of compounds that can be detected simultaneously at low concentration levels, often belonging to different classes, at the same time, and consequently, its potential for mycotoxin-biomarker research.

Recent studies on biosurveillance of mycotoxins

In 2018, Marín et al. [10] reviewed the existing surveys on mycotoxins biomonitoring. DON, OTA, and AF were the most often searched and detected mycotoxins in urine, and they co-occurred in most samples. DON and its metabolites were the most frequent mycotoxins' biomarkers detected. As a result, risk characterization showed that between 6 and 29% of the considered populations were exposed to DON at levels over the TDI, suggesting a medium but worrying risk for the population, and, at the same time, they could be co-exposed to OTA or AFB1 at levels of concern.

Recent studies across Europe showed almost 100% occurrence of mycotoxins in urine samples. The most prevalent groups were aflatoxins (51%), ergot alkaloids (56%), fumonisins (40%), ochratoxins (48%), and type B trichothecenes (52%) [3]. In Portugal, DON and its metabolites (DOM-1, DON-15-GlcA and DON-3-GlcA) were the most frequently detected biomarkers in 24h urine samples with 63% (DON), 41% (DOM-1), 52% (DON-15-GlcA), 44% (DON-3-GlcA) of positive samples. If considering DON and its metabolites, 78% of participants were exposed to DON, and 20% of samples were positive also to DON-3Glc. ZEN was the second most frequent detected mycotoxin with 48% of positive samples. Regarding ZEN metabolites, ZEN-14-GlcA was detected in the same proportion. OTA was detected in 18% of urine samples, whereas AOH was detected in 29% of the urine samples for the first time. Regarding FB1, 7% of urine samples were positive for FB1 [1].

In China, 2.3%, 0.4%, 1.2% of the population exhibited PDI exceeding the TDI values for FB1, DON and OTA, respectively [2]. In particular, children are at risk of high-level exposure because of their high cereal intake relative to body weight. Gratz et al. [11] reported mean levels in UK children urine samples of DON (13.10 ± 12.69 ng/mL), NIV (0.36 ± 0.16 ng/mL), OTA (0.05 ± 0.02 ng/mL), and ZEN (0.09 ± 0.07 ng/mL). Some samples contained T-2, HT-2, α-ZEL, and β-

ZEL, but not aflatoxins. Dietary mycotoxin estimation showed that children were frequently exposed to levels exceeding the TDI (52 and 95% of cases for DON and OTA).

Overall, data on the occurrence of mycotoxin biomarkers in human urine indicate high rates of dietary exposure to AF, FB, ZEN, and DON, especially in African and Asian countries. Among the studies that performed the calculation of probable daily intake based on the urinary levels, African countries show worrying levels for FB_1 and DON. In America, only Guatemala presented a level of concern with total fumonisin. From Asia, the worrying level was presented in China, with more than half of the samples above the tolerable level for DON, and in European countries, the DON also shows levels of concern [].

Regarding biomarkers co-occurrence, improved methods have led to up to 13 individual targets co-detected, with a mode of 5 co-detections in 18% of samples, and only 4% returning a single detection [3]. *Fusarium* toxins and OTA have been shown to co-occur [12], for example, the combinations DON-ZEN-OTA and DON-ZEN-FB1-OTA co-occurred in 38 and 52% of urine analysed samples [13].

Application of mycotoxin biomarkers in epidemiological studies

Exposure assessment in epidemiological studies remains as a key bias domain, prompting for reliable and accurate methods reflecting the true individual exposure during the window of interest. For that reason, the use of exposure biomarkers has become the gold-standard method for environmental chemicals and food contaminants in epidemiology.
Epidemiological research on specific mycotoxins have already provided a list of notorious studies encouraging the application of biomarkers in observational settings, especially for aflatoxins, the most studied group. For instance, the body of epidemiological evidence gathered by the International Agency for Research on Cancer (IARC) to evaluate the associations between AFB1 and hepatocellular carcinoma, included 7 cohort studies tracing AFB1 exposures with their metabolite AFM1 in urine, albumin adducts or AF-lysine (AF-lys) in plasma or urinary AF 8-oxodeoxyguanosine (AF 8-oxodG) [14]. Some population-based studies have also

successfully implemented the mutation of codon 249 of the TP53 gene (249ser TP53) measured in plasma DNA as biomarker resulting from AFB1 exposure, supporting the IARC evaluation [14]. The biomarkers AF-albumin adduct or 249ser TP53 mutation have been also implemented in 5 epidemiological studies from China, Taiwan, India and Gambia on cirrhosis risk, which meta-analysis has revealed positive consistent associations with absence of heterogeneity [15].

Child growth may be impaired by AF or FBs, as revealed by observational studies that applied established biomarkers (AF-albumin and AFB-lys in blood or urinary FB) conducted in sub-Saharan African countries such as Tanzania, Benin, Togo or Gambia. On this basis, a novel study design based on a community-based cluster randomized trial has been recently proposed to assess the causal associations between AF and growth of infants from central Tanzania [16].

The exposure of mycotoxins has also been associated with the impairment of pregnancy outcomes in humans, despite the limited number and quality of published studies recently appraised elsewhere [17]. Interestingly, most studies included in the mentioned systematic review used biomarker-based approaches, including AF in blood or the postpartum sphinganine:sphingosine ratio in maternal serum as biomarker of FB exposure. Biomarkers of OTA and CIT in blood and urine, respectively, have been used to explore whether carcinogenic and nephrotoxic mycotoxins may contribute to kidney diseases in a small Czech cohort of patients. Despite the lack of control population, the authors did not notice major differences with published data in the general healthy population [18]. A recent pilot case-control study conducted in Tunisia implemented a multi-mycotoxin detection LC-MS/MS approach for the characterization of PAT and CIT in plasma and urine from 50 patients with colorectal cancer and 50 respective controls [19]. Despite CIT was found in most urine samples, no statistical differences were found between cases and controls.

Mycotoxin biomarkers in exposome-health studies

The ‘exposome’ concept has evolved during the last 15 years as a novel paradigm to better characterize the role of environment in disease risk during the lifetime, complementing the genomic influences [20]. In practice, the new paradigm translates into more comprehensive and

exposure-agnostic approaches to identify environmental exposures and their endogenous metabolic fingerprint. In other words, the traditional hypothesis-driven approaches, targeting few exposures or chemicals, are replaced by large panels of exposure candidates. To tackle the exposome challenge and analogously to the genomic field, environment-wide association studies (EWAS) have been appearing during the last decade applied to metabolic diseases, preterm birth, reproductive function, or cancer, among others [21]. This conception realigns the epidemiological design around the individual profiling and its exposure-disease continuum of biomarkers, boosting the hypothesis generation from the order of some few exposures to large panels of hundreds or thousands. The novel generation of (ultra) high resolution mass spectrometers (U/HRMS) are favouring this transformative process in biomonitoring, enhancing consolidated targeted approaches with suspect or non-targeted workflows [22]. Some inspiring proof-of-concept studies have demonstrated the capabilities of HRMS approaches to screen chemical hazards in biological samples (serum and breast milk), with sufficient sensitivity and selectivity to identify low abundant xenoestrogens, including mycotoxins [23]. However, the published EWAS approaches still favour the conventional targeted approaches, establishing a panel of priority chemicals to screen based on prior knowledge, biospecimens availability, or cost, which in all cases has neglected the role of mycotoxins [24]. Since the exposome concept was first coined in 2005 by the visionary mycotoxin researcher Christopher Wild, the scientific community has delivered an exponentially growing number of publications translating the concept and its applicability to different scientific disciplines. For instance, up to date (13 October 2020) the keyword ‘exposome’ retrieved a total of 806 hits in Pubmed. Nonetheless, if we refine the search adding the keyword ‘mycotoxin’, the list of references dramatically falls to 17 with no applied observational studies, highlighting the mycotoxin research gap. Geographic area of residence, air pollution, dietary habits or lifestyle, account for relevant external variables shaping the lifetime exposome of individuals. Nonetheless, considering the high toxicity and relevant exposure of mycotoxins, especially in low-income countries, it is likely that a relevant number of fungal toxins may have a relevant role in the human exposome [10].

The examples mentioned in the previous section illustrate the successful applicability of validated mycotoxin biomarkers in observational research using conventional methods targeting specific toxins. Nonetheless, there is an urgent need that mycotoxins catch the attention of

mainstream epidemiological researchers, especially those intending to develop exposome-based approaches in pathologies and populations where mycotoxins may represent a concern. Mycotoxins have been identified as a health risk priority in developing countries, where food control policies are scarce and traditional cereal-based diets dominate the nutritional intake for the majority of the population [24]. A high level of concern was concluded for the main mycotoxins consumed in Benin, Cameroon, Mali, and Nigeria, included in the unprecedented total diet study conducted in sub-Saharan Africa [25]. For risk assessment purposes, a final list of 24 chemicals with reliable toxicological data to derive health-based guidance values were retained, of which 7 were mycotoxins. The risk characterization highlighted the high health concern derived from exposure to AFB1, FB, OTA and CIT, especially in Benin, Cameroon and Nigeria. Limited resources for public health and epidemiological research in these low income areas may justify the lack of observational research on mycotoxins [26]. Nonetheless, epidemiological research has also been overlooked in developed countries despite the fact that dietary exposure to mycotoxins such as OTA, DON or T-2 toxins is widespread and may even pose health risks among certain population clusters. The French ANSES expert panel showed in the infant French Total Diet Study that 5 to 10 % of infants (5-12months) may exceed the health-based guidance values for T-2 and HT-2 toxins and 7.5-27% of 5-month or older children would exceed the safety levels for DON [27]. It is noteworthy that in Western countries low-levels of mycotoxins are likely to occur in mixtures with other environmental chemicals such as bisphenol A or polycyclic aromatic hydrocarbons, whose combined effects are unknown [10]

Conclusion

In the last few years, risk characterisation studies based on internal exposure have been preferred over those based on external exposure assessment. In general, there is a discrepancy between internal and external exposure assessments [3], with more accurate estimates derived from internal biomarkers. Mycotoxins vary widely in rates of intestinal absorption which may be further affected by co-exposures with other mycotoxins, or even varying dietary composition [28]. Hence, there is need to accurately predict the intestinally

absorbed fraction of oral mycotoxin intake for suitable assessments of risk associated with mycotoxin contamination [29].

Ten years later since Christopher Wild raised attention on mycotoxins as 'a largely ignored health issue' [26], fungal toxins remains largely ignored. Nonetheless, the current technological HRMS scene and emerging consortiums on exposome-health research represent a unique opportunity to bring mycotoxins into more mainstream areas. To move forward, it is fundamental to establish solid bridges between concerned disciplines such as epidemiology, statistics, mycotoxicology and biomonitoring, especially for those diseases and populations where mycotoxins may be relevant.

Declaration of interest

None

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