Title: The role of mycotoxins in the Human Exposome: application of mycotoxin biomarkers in exposome-health studies

Authors:

Marín, Sonia[a,‡*]; Cano-Sancho, German[b,‡]; Sanchis, Vicente[a,§]; Ramos, Antonio J[a, §].

Institutions:

[a]Applied Mycology Unit, Food Technology Department. University of Lleida. UTPV-XaRTA, Agrotecnio Center. Av. Rovira Roure 191. 25198 Lleida. Spain.

[b]LABERCA, Oniris, INRA, Université Bretagne-Loire, 44307, Nantes, France

‡, §: These authors have contributed equally to the present study.

*Corresponding Author : Applied Mycology Unit, Food Technology Department. University of Lleida. UTPV-XaRTA, Agrotecnio Center. Av. Rovira Roure 191. 25198 Lleida. Spain.

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

Highlights

- Humans are exposed to complex chemical mixtures including mycotoxins worldwide
- The intake of mycotoxins in food leads as a consequence to the presence of chemical compounds in biological fluids
- Exposome projects should include mycotoxins within the panel of targeted biomarkers
- The knowledge about mycotoxin exposure and effects on humans may be largely improved through Exposome projects.

Abstract

Mycotoxins are secondary metabolites produced by fungi that may contaminate different foods intended for human consumption, resulting in a widespread exposure worldwide. The novel exposome paradigm has the ambition to decipher the different environmental insults threating human health throughout the entire lifespan. Given the large potential impact of mycotoxins in terms of human exposure and related health effects, the ambition of this review is to present this group of chemical compounds and the high interest to be included in exposome projects. Furthermore, we also attempt to approach the novel exposome paradigm to more traditional disciplines such as mycotoxin exposure assessment and mycotoxicology, introducing the new methodological challenges and translational needs. Hence, we provide an overview of major biomarkers currently developed, biological matrices where these may be found, an overview of internal exposure levels and potential co-occurrence with environmental chemicals and finally an overview of major health effects with the illustrative example of the potent xenoestrogen zearalenol. Conversely, these new approaches may be an excellent opportunity to fill many research gaps on mycotoxins research as the identification of associations with human health, elucidation of join effect with other environmental exposures or the decipher of underlying molecular mechanisms by using advanced OMICs technologies.

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List of Abbreviations

Aflatoxin (AF)

AFB1-albumin (AFB1-Ab)

Citrinin (CIT)

Diacetoxyscirpenol (DAS)

Deoxynivalenol (DON)

De-epoxy-deoxynivalenol (DOM-1)

Diacetoxyscirpenol (DAS)

DH-CIT dihydrocitrinone (DH-CIT)

Enzyme-linked immunosorbent assays (ELISA)

Fumonisin B1 (FB1)

Fumonisin B2 (FB2)

Fusarenon X (FusX)

Gliotoxin (GLIO)

Glucoside (Glc)

Glucuronide (GlcA)

Hydroxy Fumonisin B1 (HFB1)

High resolution mass spectrometry (HRMS)

Immunoaffinity chromatography (IAC)

Liquid chromatography (LC)

Mass spectrometry (MS)

Nivalenol (NIV)

Ochratoxin A (OTA)

Ochratoxin alpha (OTα)

4-hydroxyochratoxin A (4-OH OTA)

45 Sphinganine (Sa)

46 Sphingosine (So)

47 Solid-phase extraction (SPE)

48 Polycyclic aromatic hydrocarbons (PAHs)

49 Patulin (PAT)

50 Zearalenone (ZEA)

51 Zearalanone (ZAN)

52 Zearalenol (ZOL)

53

54

Abstract

Mycotoxins are secondary metabolites produced by fungi that may contaminate different foods intended for human consumption, resulting in a widespread exposure worldwide. The novel exposome paradigm has the ambition to decipher the different environmental insults threating human health throughout the entire lifespan. Given the large potential impact of mycotoxins in terms of human exposure and related health effects, the ambition of this review is to present this group of chemical compounds and the high interest to be included in exposome projects. Furthermore, we also attempt to approach the novel exposome paradigm to more traditional disciplines such as mycotoxin exposure assessment and mycotoxicology, introducing the new methodological challenges and translational needs. Hence, we provide an overview of major biomarkers currently developed, biological matrices where these may be found, an overview of internal exposure levels and potential co-occurrence with environmental chemicals and finally an overview of major health effects with the illustrative example of the potent xenoestrogen zearalenol. Conversely, these new approaches may be an excellent opportunity to fill many research gaps on mycotoxins research as the identification of associations with human health, elucidation of join effect with other environmental exposures or the decipher of underlying molecular mechanisms by using advanced OMICs technologies.

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1. The novel 'exposome' paradigm

The exposome concept refers to 'the totality of environmental exposures from conception onwards', proposed to complement the genome with all those factors that may be related with the human phenotypes and responsible of perturbation of biological processes (Wild et al., 2005). This novel vision involves different levels of complexity and dimensionality, providing an integrative overview of the relationship between the internal biological processes and the environment. Environmental external factors include radiation, infectious agents, chemical contaminants and environmental pollutants, diet, lifestyle factors (e.g. tobacco, alcohol), occupation and medical interventions. The exposome includes also the wider social, economic and psychological dimension for instance the education, the psychological and mental stress, or climate (Rappaport et al., 2011; Wild et al., 2012, 2013). As a major difference to the genome, the exposome has an extremely dynamic nature, entailing complex approaches to accurate characterizations and decipher the interplay of external factors with the biological processes and human health. The limitations and challenges have been already identified and discussed elsewhere, proposing approaches more adapted to the readily accessible technologies and financial budgets, transitioning towards more integrative settings (Siroux et al., 2016).

The exposome concept has been shaped in parallel to the fast development and implementation of advanced mass spectrometry (MS) and high resolution mass spectrometry (HRMS) methodologies to biomonitoring. These approaches, within a "targeted" context, have allowed the accurate screening and characterization of a wide range of known external chemical exposures or related metabolites at individual level. Furthermore, the implementation of HRMS to non-targeted metabolomic approaches has also allowed the development of novel screening framework to massively identify new environmental exposures but also internal molecules generated by the intermediary metabolism. The

chemical spectra of molecules in the organism is believed to account for about 40 nutrients, 2,000 intermediary metabolites, 200,000 peptides and 500000 lipids, whereas about 400,000 chemicals are believed to be part of the exposome (Jones, 2016). The diet is a major pathway of intake of environmental chemicals, including non-nutritive molecules with potentially harmful properties like pesticides, environmental pollutants or chemicals often underscored, like mycotoxins. Currently, more than 300 mycotoxins are known, but scientific and regulatory attention is focused only on a reduced number of major toxins, in terms of known occurrence and toxicity.

Given the large potential impact of mycotoxins in terms of human exposure and related health effects, the ambition of this review is to present this group of chemical compounds and the high interest to be included in exposome projects. Furthermore, we also attempt to approach the novel exposome paradigm to more traditional disciplines such as mycotoxin exposure assessment and mycotoxicology, introducing the new methodological challenges and translational needs. Hence, we provide an overview of main mycotoxins, major biomarkers currently developed and biological matrices where these may be found. The present review is an introductory overview about available methods of detection for mycotoxins biomarkers, hence we refer readers to more comprehensive reviews if interested in a deeper insight (Escrivà et al., 2017a; Vidal et al., 2018; Warth et al., 2016). We also provide an overview of internal exposure levels and potential co-occurrence with environmental chemicals which highlight the interest of studying the effect of complex mixtures including mycotoxins. Finally, a general overview about major health effects of mycotoxins is provided with an example of the potent xenoestrogen zearalenol (ZOL) to illustrate the high interest of considering fungal toxins in exposome-health studies.

2. Mycotoxins, chemical contaminants produced by fungi

Mycotoxins are natural toxicants produced by a high number of species of different fungal genera. The main mycotoxigenic species belong to the genera *Fusarium*, *Claviceps*, *Alternaria*, *Aspergillus* and *Penicillium* (Marin et al., 2013). These species need particular eco-physiological conditions, like temperature and humidity (water activity), to grow and synthesize these secondary metabolites which have adverse effects in animal and human health. The main substrates or crops with capacity to support the growth and accumulation of these toxins are cereals, nuts, oilseeds, dried fruits, coffee and spices, and their by-products. The contamination can occur throughout the food chain, both in the field and in the post-harvest stage, depending on the species involved. It must be borne in mind that the same species can produce more than one mycotoxin, such is the case of *Fusarium graminearum*, which produces deoxynivalenol (DON) and zearalenone (ZEA), and also that the same mycotoxin can be produced by several fungal species, such as ochratoxin A (OTA), which is produced by *Penicillium verrucosum*, *Aspergillus ochraceus* and *Aspergillus carbonarius*.

Mycotoxins are a structurally diverse group of relatively low molecular mass compounds that can occur in three possible forms: as free or unmodified, as matrix associated and as modified forms (Rychlik et al., 2014). The free or unmodified mycotoxins describe the basic mycotoxin structures formed by a high number of fungi in well-known biosynthetic pathways. Some examples of these mycotoxins are aflatoxin B_1 (AFB1), OTA, patulin (PAT), DON, fumonisin B_1 (FB1), and ZEA. Their chemical structures are very diverse. So, we find microcyclic lactones like ZEA, small lactones condensed with hetero- or alicycles like PAT, furan derivatives like aflatoxins (AFs), alicyclic compounds like T-2 toxin, among others. The matrix associated forms are either complexes with matrix compounds or are physically dissolved or trapped or are covalently bound to matrix components or a combination of both effects. Examples of this group are the fumonisins (FBs) bound to

carbohydrates or proteins. The third group known as modified mycotoxins describes any modification of the basic chemical structure of the molecules. One of these modifications are produced by plants through conjugation reactions such as the formation of DON-3-glucoside (DON-3-Glc). Other conjugation reactions are produced by animals such as the formation of DON-3-glucuronide (DON-3-GlcA) or by fungi as for example the formation of ZEA-14-sulfate. Among the chemically modified mycotoxins it is possible to distinguish between the thermally formed and non-thermally formed ones. The first group describes the modifications produced during the thermal process of foods and feeds with the example of norDON A-C. The second group is formed by degradation products of the mycotoxins produced under alkaline conditions like DON-sulfonate.

It is necessary to highlight that the routine analysis of foods and feeds usually determines the free or parent mycotoxins. In order to identify and quantify the modified forms, validated methods using highly sensitive equipment like LC-MS/MS is necessary. The industries don't usually dispose of these facilities mostly because of economic issues. However, there is a danger related to the ingestion of these modified mycotoxins, particularly because of their release into the digestive system and the formation of free forms, which toxic action has been proved already. Thus, the exposure assessment might not be accurate enough due to the presence of the modified mycotoxins. Another possible scenario is the exceptional case when the modified form is more toxic than its parent molecule (e.g. α-ZOL possesses a stronger oestrogenic potency than ZEA) (Frizzell et al., 2011).

3. Biomarkers of mycotoxin exposure

Biomarkers are measurable biochemical or molecular indicators of either exposure (exposure biomarker) or biological response (effect biomarker) to a mycotoxin that can be specifically linked to the proximate cause (Baldwin et al., 2011). More specifically, a biomarker is a biological measure (parent toxins, protein or DNA adducts, glucuronide conjugates…

measured in urine or plasma/serum) which is correlated with the quantity of xenobiotic ingested (Table 1). Validation of a biomarker requires demonstration of assay robustness, intake versus biomarker level, and stability of stored samples.

Biomarkers have contributed largely to understanding the causative role of AFB1 in human hepatocellular carcinoma (Kensler et al., 2011). These have included both biomarkers of exposure, based on urinary aflatoxin M_1 (AFM1) and serum AFB1-albumin adduct as markers of internal dose, and a biomarker of effect, based on urinary AFB1-N7-guanine as a measure of biological effective dose, since DNA adduct formation is the biochemical mechanism whereby AFB1 exerts its carcinogenic potency.

Traditional biomonitoring studies of internal exposure through urine or plasma analysis of target chemicals, metabolites, or reaction products are useful to link exposures to health outcomes. Biologically persistent chemicals are well-characterized with traditional methods, whereas short-lived chemicals are effectively measured only if the individual is undergoing continuous or continual exposures or if the timing of exposures is known. In particular, urinary excretion mainly represents recent mycotoxin intake, whereas measurements in plasma/serum are more likely to represent long-term exposure. Very often urine is the matrix of choice, as it is easily collected, however, its limitation is the differing urine excretion owing to different fluid intakes. This can be addressed partially by normalization for the creatinine concentration of a urine sample. In exposure studies it is always recommended to collect 24-h urine. Stability studies revealed that a wide range of target analytes were stable for 12 h at 25 °C post-collection (Njumbe Ediage et al., 2012), but to avoid fermentation problems that can alter any of the components of the sample, conservation at 2-4ºC is recommended.

Table 1. Biomarkers of mycotoxin exposure.

Mycotoxin	Biomarker	Validated	Reference
Aflatoxin B1	AFM1 in urine	Yes (1.2-2.2% of ingested AFB1))	Zhu et al (1987)
	Aflatoxin –N7-guanine adduct in urine	Yes (0.2% of ingested AFB1)	Groopman and Kensler (1993)
	AFB1 in urine		
	AF-Alb in plasma	No	
		No specified	Chapot and Wild, 1991
Fumonisin B1	FB1 in urine	Yes (0.08-0.5 of ingested FB1)	Van der Westhuizen et al. (2011)
	HFB1 in urine	No	Riley et al. (2012)
	Sa/So in plasma	Yes	Shephard and Van der Westhuizen (1998)
Deoxynivalenol	'total DON' (free DON+DON released by b-glucuronidase) in urine	Yes	Turner et al. (2008)
	DON in urine	No	
	DOM in urine	No	
Ochratoxin A	OTA in urine	Yes	Gilbert et al. (2001)
	OTα in urine	No	
	4-OH OTA in urine	No	
	OTA in plasma	Yes	Breitholtz et al. (1991)
Zearalenone	ZEA+α-zearalenol+ß-zearalenol in urine	No	
	ZEA-14-GlcA in urine		
	ZEA–Glu in urine		
	ZEA+α-ZOL+ß-ZOL in plasma	Yes	Prelusky et al. (1989)
Fumonisin B1	FB2 in urine	No	
HT-2 toxin	HT-2 toxin in urine	No	
Citrinin	CIT in urine	No	
	DH-CIT in urine	No	
	CIT in plasma	Yes	Blaszkewicz et al. (2013)
T-2 toxin	T-2 toxin in urine	No	

4. Analytical methods to identify mycotoxins biomarkers in urine

Traditional biomonitoring implies developing analysis protocols for each mycotoxin. This multiplies the volume of sample required, and may be slower and more expensive. Most mycotoxin exposure assessments in developing countries have focused on the AFs and FB1, while DON and its modified forms were usually monitored in developed countries. Recently, an increasing number of studies include biomonitoring of a range of mycotoxins, as a result

217 of the advent of the latest generation of high performance LC-MS/MS instruments, however,
218 they rarely include simultaneously contaminants other than mycotoxins.

219 Traditional biomonitoring of aflatoxins, has been mainly carried out through AFM1
220 determination in urine. The method of choice has been usually competitive enzyme-linked
221 immunosorbent assays (ELISA) kits, or IAC clean-up followed by HPLC-FD detection.
222 UPLC-MS/MS methods are nowadays preferred. In these cases, urine samples are
223 centrifuged, diluted in IAC column compatible buffers, cleaned up, evaporated to dryness and
224 suspended before injection in LC-MS/MS (Jager et al., 2014).

225 Total urinary DON (free DON plus DON-GlcA) has been usually analysed using
226 immunoaffinity enrichment and liquid chromatography mass spectrometry (LC-MS)
227 quantification according to Turner et al. (2008). This method includes a β-glucuronidase
228 treatment of the centrifuged and pH- adjusted sample, and a subsequent clean-up using a
229 DON IAC column, with final LC-MS detection (Wallin et al., 2013). Based on samples
230 analysed with or without enzymatic treatment, it has been observed that free DON is 22% of
231 the total urinary DON (Srey et al., 2014). Gratz et al. (2013b) developed a similar method of
232 extraction and clean-up, for urine samples analysis for DON+DON-glucuronide and de-
233 epoxy-deoxynivalenol (DOM-1) using an LC-MS/MS for detection.

234 Exposure to OTA has been traditionally analysed through urine dilution and clean-up using
235 IAC prior HPLC-FD quantification of OTA and OTα aglycones (Manique et al., 2008;
236 Duarte et al., 2015). However, the increasing evidence of the presence of OTA glucuronides
237 has led to the search for an indirect method, i.e. by comparing levels of OTA aglycone in
238 urines without and after enzymatic hydrolysis. Considerable increases in OTA levels are
239 found after enzymatic hydrolysis in some urine samples which provides evidence for the
240 excretion of OTA-conjugates. Thus enzymatic treatment of urine samples with ß-
241 glucuronidase/arylsulfatase is recommended before samples clean-up with IAC (Muñoz et al.,

2017). Analysis of OTA in enzymatically hydrolized urine samples have demonstrated considerably higher detection frequencies for OTA than when those samples were directly analysed by LC-MS/MS, even when OTA-8-β-glucuronide is used as standard, as it shows very low sensitivity for the metabolite compared to detection of OTA (aglycone) due to a far lower ionization efficiency (Muñoz et al., 2017). This suggests that OTA conjugates may escape detection when direct ('dilute and shoot') methods are applied for urinary biomarker analysis (Ali et al., 2018).

Regarding fumonisins, IAC or SPE (Oasis® MAX cartridge, Waters, UK, Gong et al., 2008) clean-up prior detection by HPLC/MS system is preferred. A highly sensitive method has been optimized for FB1 and FB2 determination in urine using IAC followed by liquid chromatography with tandem mass detection (Silva et al., 2009a). Urine has been also analyzed to identify the surrogates of fumonisin exposure sphinganine (Sa) and sphingosine (So). The most common method of choice is HPLC–FD after prior derivatization with o-phthaldahyde or naphthalene-2,3-dicarboxaldehyde. An optimized method for urine also included the isolation of exfoliated cells followed b extraction with ethyl acetate prior derivatization (Silva et al., 2009b).

Nowadays multibiomarker studies are often undergone by using separation by LC and detection using triple-quadrupole analyzers coupled via an electrospray ionization (ESI) interface. However, the degree of sample purification greatly differs from one study to another. An excellent review on the main analytical issues related to multibiomarkers determination was published by Warth et al. (2013) and also covered by Vidal et al. (2018).

The first multibiomarker studies in human urine involved separated IAC clean-up for each toxin of interest and pooling of the purified extracts, or using multi-IAC containing a range of antibodies against the more relevant mycotoxins, AFM1, OTA, FB1 and FB2 (Ahn et al., 2010). Similarly, using IAC concentration, DON, T-2 toxin, HT-2 toxin, ZEA, OTA, AFB1,

aflatoxin B_2 (AFB2), aflatoxin G_1 (AFG1), aflatoxin G_2 (AFG2), as well as FB1 and fumonisin B_2 (FB2) were analysed in urine by LC-MS/MS (Rubert et al., 2011). AFM1, OTA, DON, DOM-1, α-zearalenol/β-zearalenol (α-ZOL/β-ZOL) and FB1 were simultaneously analysed by LC-MS/MS (plus other SPE after IAC for subsequent sample preparation, Solfrizzo et al., 2011). Another urinary biomarker study applying an LC-MS/MS method for the simultaneous determination of DON, OTA, FB1, AFB1, ZEA, T-2 toxin and citrinin (CIT), as well as their main metabolites in human urine, was developed and validated (Ediage et al., 2012). The urine samples required solvent extraction and SPE clean-up prior to analysis by LC-MS/MS. Later, a method developed by Njumbe Ediage et al. (2013) covered seven mycotoxins and several important conjugation and breakdown products (in total 18 analytes). Sample cleanup was optimized in a progressive procedure where urine samples were extracted with ethyl acetate/formic acid (99:1, v/v) followed by strong anion exchange (SAX) SPE cleanup of the acidified aqueous fraction. The combined extracts of the evaporated organic phase and the SAX eluate were injected into the LC-MS/MS system.

Simultaneously, a multi-biomarker method was developed based on the LC-MS 'dilute-and-shoot' approach for the direct detection of the 15 most relevant key mycotoxin metabolites in human urine without extract purification (Warth et al., 2012). This rapid method utilized an UHPLC system in tandem with a QTrap 5500 LC-MS/MS system equipped with a Turbo electrospray ionisation source. The urine sample was simply diluted 1:10 with acetonitrile/water (10:90) and injected directly into the LC-MS/MS system. The resulting LODs defined as the signal-to-noise ratio of 3:1 were for each analyte as follows: AFM1 (0.05 µg/L), FB1 (0.5 µg/L), FB2 (0.5 µg/L), OTA (0.05 µg/L), DON (4.0 µg/L), DON-3-GlcA (6.0 µg/L), DOM-1 (10 µg/L), NIV (3.0 µg/L), T-2 toxin (2.0 µg/L), HT-2 toxin (20 µg/L), ZEA (0.4 µg/L), zearalenone-14-glucuronide (ZEA-14-GlcA) (1.0 µg/L), α-ZOL (0.5 µg/L), and β-ZOL (0.5 µg/L). Besides the simplification, the advantage of this workflow is

292 the full recovery of the polar conjugates such as glucuronides which are frequently lost
293 during sample cleanup. The disadvantage of the dilute and shoot approach is the need for the
294 latest state-of-the art triple-quadrupole mass analyzer to achieve the very low LODs required.
295 Even when these highly advanced instruments are used, only moderate to high exposure is
296 detectable, rather than very low background levels. Some other authors have used such
297 'dilute and shoot' approach (Abia et al., 2013), using H_2O/ACN/FA as dilution solvent
298 (Gerding et al., 2014), 1% ammonium acetate (Vidal el al., 2016), or just injection without
299 dilution (Huybrechts et al., 2015).

300 Interestingly, Shephard et al. (2013) compared results of urine analysis both with sample
301 clean-up (single and multi-biomarker) and by a 'dilute-and-shoot' multibiomarker method.
302 Firstly, urinary FB1 was separately determined using a tailor-made single target method as
303 previously described (Gong et al., 2008) (SPE+LC-MS/MS), secondly, DON, AFM1, FB1, α-
304 ZOL, β-ZOL, ZEA and OTA) were determined as previously described (Solfrizzo et al.,
305 2011) (enzymatic treatment +IAC+SPE+UPLC-MS/MS), finally, urine samples were
306 analysed for the biomarkers FB1, FB2, AFM1, OTA, DON, DON-3-GlcA, DON-15
307 glucuronide (DON-15-GlcA), DOM-1, nivalenol (NIV), T-2 toxin, HT-2 toxin, ZEA, ZEA-
308 14-GlcA, and α- and β-ZOL using a 'dilute-and-shoot' method without prior β-glucuronidase
309 treatment as previously described (Warth et al., 2012). The single biomarker method detected
310 FB1 (87% incidence; mean ± standard deviation 0.342 ± 0.466 ng/mg creatinine) and DON
311 (100% incidence; mean 20.4 ± 49.4 ng/mg creatinine) after hydrolysis with β-glucuronidase.
312 The multi-biomarker 'dilute-and-shoot' method showed only 51% of FB1 positive samples,
313 with a maximum value of 2.59 ng/mg, and indicated that DON-15-GlcA was predominantly
314 present. The multi-biomarker method with β-glucuronidase and immunoaffinity clean-up
315 determined ZEA (100%; 0.529 ± 1.60 ng/mg creatinine), FB1 (96%; 1.52 ± 2.17 ng/mg
316 creatinine), α-ZOL (92%; 0.614 ± 1.91 ng/mg creatinine), DON (87%; 11.3 ± 27.1 ng/mg

creatinine), β-ZOL (75%; 0.702 ± 2.95 ng/mg creatinine) and OTA (98%; 0.041 ± 0.086 ng/mg creatinine). Given its higher LOD, lower incidence was reported for the 'dilute-and-shoot' method. On the other hand, the tandem clean-up procedure led to higher mean and medium values than using SPE clean-up only. Low correlation was observed among the different methods for FB1 detection. Better correlation was found among DON biomarkers of exposure (either DON or DON glucuronides). Similarly, Solfrizzo et al. (2103) compared single biomarker methods for DON and FB1 to multibiomarker methods (dilute and shoot and tandem IAC), and showed good performance of the three methods for DON, but questionable for FB1. Between the multibiomarker methods, acceptable performance was observed for DOM-1, AFM1, ZEA, α-ZOL and β-ZOL, but not for OTA.

Recently, Turner et al. (2017) compared Wallin et al. (2013) single method (SM) to Solfrizzo et al. (2014) multidetection method (MM) for DON and its modified forms. Both methods measure free DON plus the β-glucuronidase digest of DON glucuronides. A higher number of samples were <LOQ by using the MM method probably due to increased LOD as a results of an increase of matrix effect, that is, higher ion suppression. The higher matrix effect could be due to the use of an SPE column for urine purification. However, mean DON concentrations were not statistically different ($p > 0.05$). Although the analytical approaches used in the two methods are similar, including immunoaffinity enrichment and LC-MS/MS in both, several important details are different. First, the pH of urine before enzymatic digestion was adjusted for the SM method but not the MM method. Second, the enzyme used for urine digestion was different. Third, the volume of urine analyzed was 1 mL for SM and 6 mL for MM. Fourth, the enrichment for the mycotoxin included a single-antibody column for the SM method and several distinct antibodies in the columns plus an SPE-OASIS HLB column for the MM method. Finally, the SM approach included an internal individual standard spiked at the outset of extraction, whereas the MM used a matrix-assisted calibration adjusting all

342 samples as the mean recovery for the method. As DON-glucuronides can represent a
343 significant portion of the total DON in urine, it is plausible that these analytical differences
344 may have affected the efficacy of deconjugation of DON-glucuronides.

345 In the later years, salting-out assisted liquid/liquid extraction methods and dispersive solid
346 phase extraction methods have also been developed for multiple mycotoxins and metabolites
347 analysis in urine (Song et al., 2013), linked to either LC-MS/MS or GC-MS/MS analysis
348 (Rodríguez-Carrasco et al. 2014). That latter GC-MS/MS method has been successfully
349 applied to a 24 h pilot diet study revealing that DON was the main mycotoxin in diet and
350 urine among the 15 *Fusarium* toxins analyzed (Rodríguez-Carrasco et al., 2015).

351 Recently, some studies have specifically compared different extraction and micro-extraction
352 techniques for *Fusarium* mycotoxins applied to human urine, showing that dispersive liquid–
353 liquid microextraction (DLLME) was the most performant compared to salting-out liquid–
354 liquid extraction (SALLE), miniQuEChERS (quick, easy, cheap, effective, rugged, and safe)
355 methods (Escrivà et al., 2017b). Conversely, SALLE showed better accuracy and precision
356 than DLLME in combination with GC-MS/MS for the determination of 10 *Fusarium*
357 mycotoxins (Rodríguez-Carrasco et al., 2017).

358 A comparison of relevant multidetection biomarker methods for analysis of mycotoxins in
359 urine is presented in Table 2. Warth et al. (2012) reviewed the main limitations encountered
360 in multibiomarker monitoring. A range of analytical challenges were listed.

361 a) First is the extremely low analyte concentrations present in biological fluids following
362 dietary exposure, thus sample preparation is crucial to obtain acceptable LODs. However, the
363 great chemical diversity of analytes (including polar conjugates such as glucuronides which
364 are frequently lost during common cleanup approaches such as SPE or IAC procedures)
365 makes it difficult. IAC cleanup allows for high enrichment, however, no conjugates or other

366 biomarkers/analytes of interest can be included in a method, and enzymatic hydrolysis should
367 be performed to include conjugates. On the other hand, the dilute and shoot approach does
368 not need further pretreatment; however, to overcome matrix effects and interfering matrix
369 peaks, eluents, the chromatographic gradient, and the dilution factor need to be carefully
370 optimized.

371 b) Second, co-eluting matrix components can negatively influence the accuracy of
372 quantitative methods through ion suppression or enhancement in the ion source. Ion
373 suppression can be reduced efficiently by careful optimization of the eluents and gradient.
374 Using internal standards and matrix-matched calibration is critical.

375 c) Third, there is a lack of authentic reference standards and certified reference materials.

376 **Table 2**. Comparison of the number of detected biomarkers and LODs of relevant multidetection biomarker methods for analysis of mycotoxins
377 in urine.

Reference	AFM1	FB1+FB2	DON	OTA	ZEA	T-2	HT-2	Others	Clean-up	Total
Rubert et al. 2011		9	10	0.5	3	2	3	AFB1, AFB2, AFG1, AFG2	IAC	11
Warth et al. 2012	0.05	0.4	4	0.05	0.4	1	12	DON-3-GlcA, DOM1, NIV, ZEA-14-GlcA, α-ZOL, β-ZOL, DON-15-GlcA	No	15
Ediage et al. 2012	0.15	2.7	4	0.15	0.6	1	40	AFB1, CIT	SPE	7
Ediage et al. 2013	0.01	0.01	0.04	0.03	0.1	0.05	0.42	AFB1, DOM1, CIT, α-ZOL, β-ZOL, ochratoxin α (OTα), 4-hydroxyochratoxin A (4-OH-OTA), DON–3-GlcA, HFB1, ZEA–GlcA	SPE	18
Abia et al. 2013	0.05	1	4	0.05	0.4	2	20	DON-3-GlcA, ZEA-14-GlcA, DON-15-GlcA, DOM-1, NIV, α-ZOL β-ZOL	No	15
Gerding et al. 2014	0.025	0.25	0.5	0.1	0.025	0.25	2	Zearalanone (ZAN), α-ZOL, β-ZOL, AFB1, AFB2, AFG1, AFG2, dihydrocitrinone (DH-CIT), enniatin B, OTα, DON-3-GlcA, ZEA-14-GlcA, ZAN-14-GlcA, α-ZOL-14-GlcA, β-ZOL-14-GlcA, and HT-2-4-GlcA	No	24
Rodriguez-Carrasco et al. 2014			0.12		3	0.5	1	DOM1, 3-acetyl-DON, fusarenone-X (FusX), diacetoxyscirpenol (DAS), NIV, neosolaniol , ZAN, α-zearalanol, ß-zearalanol, α-ZOL, ß-ZOL	Dispersive SPE	15
Huybrechts et al.	0.002	0.1	0.2	0.001	0.02	0.01	0.2	AFB1, AFB2, AFG1, AFG2, CIT, OH-CIT, DAS , DON-3-GlcA, DON-15-GlcA, 3-ADON, 3-ADON-15-	No, except for	32

2015			GlcA, 15-ADON, 15-ADON-3-GlcA, DOM1, DOM1-GlcA, FusX, OTA, ZEA-14-GlcA, α-ZOL, α-ZOL-GlcA, α-ZOL-14-GlcA, β-ZOL, β-ZOL-14-GlcA	OTA, CIT, AFM1 (IAC)	
Vidal et al. 2016	0.5	0.003	OTα DON-3-glucoside, 3-ADON, DOM-1 DON-3-GlcA	No	8

5. Analytical methods to identify mycotoxins biomarkers in blood

Biomonitoring of aflatoxins in blood was initially carried out by directly analyzing this group of toxins (including AFB1, AFB2, AFG1, AFG2, AFM1, aflatoxin B_{2a} (AFB2a), aflatoxin BG_{2a} (AFG2a), aflatoxin P (AFP) and aflatoxicol) in serum samples by 2D-TLC, previous extraction with dichloromethane and purification on a silica gel column (Hatem et al., 2005), or by RP-HPLC with fluorescence detection, previous extraction of samples with chloroform and hexane (Lopez et al., 2002).

However, nowadays the determination of the AFB1-albumin (AFB1-Ab) adduct in serum (adduct formed with the lysine amino acid of albumin) is more frequently used, as it has been demonstrated that the concentration of this adduct in serum is strongly correlated with aflatoxin intake, which makes it a very useful exposure biomarker (Wild et al., 1992). Adducts could be formed not only with AFB1, but also with the other main aflatoxin (AFB2, AFG1 and AFG2). Besides, the AFB1-Ab adducts presents a half-life in the organism of around 2-3 months, what makes this compound a good biomarker to reflect exposures over long periods of time, in contrast to what happens with the AFB1-N7-guanine adduct biomarker in urine, that better reflects day-to-day variations in aflatoxin intake. Moreover, AFB1-Ab adducts are stable in serum samples stored at -80 °C for over 25 years, allowing for re-analysis of archived samples years later (Scholl and Groopman, 2008).

For the analysis of the AFB1-Ab adducts in serum, ELISA seems to be the routine analysis method; samples are previously digested with pronase, extracted and purified and measured using a competitive ELISA (Chapot and Wild, 1991; Turner et al., 2007, 2008; Gong et al., 2012; Piekkola et al., 2012; Shirima et al., 2013, 2015; Asiki et al, 2014; Chen et al., 2018). However, other techniques as RIA (Jiang et al., 2005; Tang et al., 2009), HPLC-FD (Mizrak et al., 2009; Shuaib et al., 2012) and HPLC with isotope dilution mass spectrometry (IDMS) (McCoy et al., 2005, 2008) have been also used.

In relation to FBs, direct detection of FB1 in blood samples has not been considered an appropriate biomarker, due to its rapid elimination and low oral bioavailability. Taking into account the effect of FBs on the metabolism of sphingolipids (inhibition of the ceramide synthase enzyme and increase of intracellular Sa concentration), the ratio between Sa and So (or between Sa-1-phosphate and So-1-phosphate) in plasma has been considered an indirect indicator of human FBs exposition, and therefore it has been frequently used. However, this ratio has been considered useful in studies with animals, but in human exposure studies, when the level of food contamination is relatively low, wide ranges of Sa:So ratios and bad correlation coefficients have been observed when linear regression was fitted, which suggests that this ratio present low sensitivity and imprecision in humans (Cano-Sancho et al., 2010). The analytical method used to determinate these metabolites frequently included plasma deproteinization, liquid-liquid extraction, hydrolysis and purification, and HPLC-FD analysis prior derivatisation with o-phthaldialdehyde (Riley, 1994; Shephard and Van der Westhuizen, 1998; Castegnaro et al., 1998), although use of blood spots, LC-MS determination has also been used (Riley et al., 2015).

Direct OTA detection in human plasma has been widely used, as OTA binds rapidly and with high affinity to plasma proteins, constituting therefore a good biomarker of exposure (Coronel et al., 2010; Lino et al., 2008). The method of choice for detection is the HPLC-FD, and usual methods of analysis comprise liquid-liquid extraction of plasma samples, for example with acidified ethyl acetate or other solvents, and analysis by HPLC-FD with postcolumn confirmation through the formation of OTA-methyl ester (Palli et al., 1999), purification of acidified plasma samples with a C18 Sep-Pak cartridge and analysis by HPLC-FD with confirmation through the formation of OTα after carboxypeptidase treatment of samples (Creppy et al., 2005), and other similar methods (Ali et al., 2018), many of them using immunoaffinity columns (Ghali et al., 2008). Other methods used included detection of

ochratoxins in plasma by ELISA (Ueno et al., 1998) or LC/MS/MS (Lau et al., 2000; Medina et al., 2010; Cramer et al., 2015).

Regarding DON, to date, DON, DON-GlcA and DOM-1 in urine are the preferred biomarkers for the study of DON exposure. However, several attempts have been made to find DON biomarkers linked to blood samples. Thus, from studies with rodents, Kim et al. (2008) have proposed the use of plasma haptoglobin, measured using SELDI-TOF/MS as a diagnostic biomarker for DON intoxication when this is combined with examining the serum immunoglobulins. These findings have led to a patent application in Korea (patent reference number KR100809952B1) protecting a diagnostic kit for the evaluation of toxicity and exposure for DON using haptoglobin-specific protein. However, different results have been found in experiments with lactating dairy cows (Kinoshita et al., 2015), and, to date, no data are available in the case of human blood.

With respect to other mycotoxins few studies have been conducted to assess the presence of other fungal toxins in human blood. Thus, in the case of ZEA, the presence of this metabolite or its congeners (α-ZOL, ß-ZOL) has been studied in plasma of patients with breast and cervical cancer by HPLC and GC (Pillay et al., 2002), whereas Massart et al. (2008) studied the presence of ZEA and derivatives in the serum of healthy girls and affected by central precocious puberty, performing an enzymatic treatment of samples with glucuronidase, followed by purification through a immunoaffinity column and analysis by HPLC-FD. On the other hand, Fleck et al. (2016) have studied the presence of total ZEA (ZEA plus conjugated metabolites) and total α-ZOL in serum of pregnant women by UPLC-MS/MS and electrospray ionization (ESI).

Finally, for CIT, studies on human plasma have been developed by means of an acetonitrile protein precipitation followed by centrifugation and analysis by HPLC-FD and LC-MS/MS (Blaszkewicz et al., 2013; Ali et al., 2018).

453 Regarding plasma or serum, few studies have carried out on multi-detection analysis of
454 mycotoxins including multiple mycotoxin biomarkers of different mycotoxins groups in one
455 sample at the same time, mainly due to the high matrix complexity. Thus, in plasma most
456 methods have only focused on the analysis of structurally-related mycotoxins belonging to a
457 single family.
458 Osteresch et al. (2017) have developed a rapid multi-mycotoxin method, using dried whole
459 blood spots and dried serum spots, which allows for the simultaneous detection and
460 quantification of a great variety of fungal toxins by HPLC-MS/MS in less than 10 minutes.
461 This method is able to detect till 27 mycotoxins, of the following groups (data of LOD in
462 ng/mL is given): aflatoxins (AFB1: 0.012; AFB2: 0.013; AFG1: 0.021; AFG2:0.037; AFM1:
463 0.017), *Alternaria* toxins (alternariol: 0.142; alternariol monomethyl ether: 0.146; altenuene:
464 0.147), enniatins (A: 0.0016,: A1: 0.0055; B: 0.0012; B1: 0.0044), ochratoxins (OTA/2'R-
465 ochratoxin A: 0.012; OTα: 0.014; 10-hydroxyochratoxin A: 0.015), T-2/HT-2 group (T-2
466 toxin: 0.227; HT-2 toxin: 1.344; HT-2-4-glucuronide: 0.709), ZEA (0.294) and ZAN (0.273),
467 CIT (0.066) and DH-CIT (0.268), FB1 (0.521) and beauvericin (0.014), with average
468 recoveries above 90% in most of the cases.
469 De Santis et al. (2017) have described a method for the analysis of 8 mycotoxins (AFB1,
470 AFM1, FB1, OTA, ZEA, DON, DOM-1, and gliotoxin –GLIO–) that combine pronase
471 treatment of serum samples followed by QuEChERS purification and LC-MS detection.
472 Limits of quantification were low for AFB1 (0.01 ng/mL), AFM1 (0.22 ng/mL) and OTA
473 (0.16 ng/mL), but in other mycotoxins were above 5 ng/mL (DON, DOM-1) or 11 ng/mL
474 (GLIO). The absolute recoveries of the method were not too high, since, with the exception
475 of AFB1 (82%), all the toxins had recoveries below 63%.
476 Cao et al. (2018) have also described a method for the quantitative determination of
477 mycotoxins in human plasma, as well as in other biological matrices (like urine) and animal

derived foods, by HPLC-MS/MS. This method is valid for the determination of aflatoxins in human plasma (data of LOD in ng/mL is given) (AFB1: 0.07; AFB2: 0.05; AFG1: 0.13; AFG2:0.15; AFM1: 0.16), as well as of FBs (FB1: 0.41; FB2: 0.39), sterigmatocystin (0.05), PAT (0.35), CIT (0.18) and OTA (0.15). Sample preparation consisted in a treatment of plasma with ß-glucuronidase, deproteinization with acetonitrile/acetic acid and evaporation.

Recently, Slobodchikova and Vuckovic (2018) have described a LC-MS method for the simultaneous detection of 17 mycotoxins in human plasma. Studied mycotoxins are NIV, DON, 3-ADON, 15-ADON, T-2 toxin, HT-2 toxin, AFB1, AFB2, AFG1, AFG2, ZEA, ZAN, α-ZOL, ß-ZOL, α-zeranol, ß-zeranol and fusarenon X (FusX). The method avoids the use of immunoaffinity columns thanks to a three-step liquid-liquid extraction procedure with ethyl acetate. LOQ of all mycotoxins ranged from 0.1 to 0.5 ng/mL, except NIV (3 ng/mL). This method is not suitable for OTA, FB1 and FB2

Covering a smaller number of mycotoxins, Serrano et al. (2015) have developed a method for the simultaneous determination of enniatins (A, A1, B and B1) and beauvericin in human plasma by HPLC-MS/MS. The method consisted in the deproteinization of samples with MeOH/H2O (40/60, v/v) followed by solid phase extraction, using in-house prepared Carbograph-4 SPE column, and detection by HPLC-tandem mass spectrometry with an electrospray ion source. Experimental LOD obtained were 10 ng/L for enniatins A1 and B, 20 ng/L for enniatin B1 and beauvericin, and 40 ng/L for enniatin A, and recoveries ranged between 90 to 120%.

6. Biomarkers of mycotoxins in breast milk

The use of breast milk in biomonitoring studies and epidemiological birth cohort studies is gaining interest due to the large chemical information contained and the easy collection methods resulting in a non-invasive and valuable biological matrix. By default, breastfeeding is considered the “gold-standard” diet for infants, however it has been questioned the

potential health risk associated when mothers are subjected to contaminated diets. The tight relationship between blood and breast milk compartments results in high and rapid transference of lipophilic chemicals, however the transference of mycotoxins from blood to human breast milk and overall occurrence, has been scarcely explored (Reviewed by Warth et al., 2016). The high interest of studying the concentrations of harmful chemicals in breast milk is justified not only by the exploration of mother's internal exposure levels but also the external exposure of infants during critical windows of development. The vulnerability is reflected by the low maximum tolerable levels established in baby foods and infant formulas by regulatory agencies, which enforce those products to rigorous inspections. Surprisingly, little effort has been addressed to evaluate the levels of mycotoxins in breast milk and risk-benefits derived from breastfeeding. The preparation of samples commonly involves immunoaffinity columns, liquid-liquid or solid-phase extraction, and the major methods of detection are based on ELISA kits, liquid HPLC-FD and LC-MS/MS. Maternal determinants of AFM1 in breast milk determined by ELISA included the season of collection, education level, lactation stage or consumption of rice and chocolate (Bogalho et al., 2018). The implementation of multi-mycotoxin detection methods in breast-milk remains as a major challenge nowadays, yet few studies have published screening exploratory studies (Andrade et al., 2013; Rubert et al., 2014). As recently summarized by Warth et al. (2016), most studies have evaluated the occurrence of AFM1 (Brazil, Cameroon, Columbia, Egypt, Iran, Italy, Jordan, Kuwait, Nigeria, Serbia, Sudan, Tanzania and Turkey) or OTA and related metabolites (Chile, Egypt, German, Iran, Italy, Poland, Slovakia, Turkey, Brazil and Chile). Conversely, few studies have explored the levels of AFB1 (Turkey and Egypt), FB1 (Tanzania) or ZEA (Italy). 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 and 44000 ng/L (Warth et al., 2016). These values appear specially

528 concerning if we consider the maximum concentration levels set up by the European
529 Commission in infant formula was 0.025 ng/mL (European Commission, 2006).

530 **7. Internal exposure of general population to mycotoxins.**

531 In the last few years an increasing number of studies have been published on assessment of
532 exposure to mycotoxins in different countries using biomarkers. Most single biomarker
533 studies dealt with exposure to AFB1 through AFM1 determination in urine. Moderate to high
534 frequencies were reported all over the world, depending on the LOD of the methods used. In
535 general, mean and median values under 0.1 ng/mL were detected in different countries in
536 Asia, America and Europe. Higher absolute concentrations were reported in some countries
537 in Africa (up to 3.7 ng/mL) (Smith et al., 2017). Using direct detection of AFB1 in blood,
538 values from different studies ranged from 0.08 to 7.4 ng/mL, whereas when the AFB1-
539 albumin biomarker was used the values ranged from not detected to values as high as 268
540 pg/mg. A good review about these data can be found in Waseem et al. (2014).
541 Secondly, DON exposure through urine analysis was assessed mainly in European countries,
542 where frequencies in the range 90-100% were usually reported in urine samples, with 12%
543 found as free DON and 88% as DON glucuronides (Wells et al., 2016). Mean levels of DON
544 were around 10 ng/mL, while when total DON was assessed higher levels were reported, and
545 higher total levels could be over 400 ng/mL (Wells et al., 2016). Several studies confirmed
546 that a significant percentage of the populations were exposed to levels over the TDI. Lower
547 frequencies of occurrence were observed in other countries like Bangladesh or Tanzania,
548 where the different dietary habits may be determinant.
549 Finally, those studies devoted to OTA in Europe reported widely variable frequencies, but
550 low general levels (mean under 0.3 ng/mL) (Ali et al., 2018; Wallin et al., 2013; Duarte el al.,
551 2015). In blood, OTA has been detected in a great number of studies, with OTA occurrence
552 frequently over 74% and usually over 90% (Coronel et al., 2010; Waseem et al., 2014), and

553 with a global estimation (derived from a big number of published studies) of minimum,
554 maximum and mean levels of 0.15, 9.15 and 0.45 ng OTA/mL plasma, respectively (Coronel
555 et al, 2010).
556 Differences in nutritional habits and quality of consumed foodstuffs are likely the reason for
557 interregional variations in mycotoxin excretion.
558 Regarding multibiomarker studies, as shown in Table 3, DON, OTA and AF were the more
559 often searched and detected mycotoxins, and they co-occurred in most samples. The
560 frequencies for DON and OTA were high, but low for AFM1 (in contrast to what observed
561 using single analysis). Only one study reported frequent exposure to ZEA (Solfrizzo et al.,
562 2014). The detected levels, in general, paralleled those observed in the single biomarker
563 studies, with high concentration for total DON, followed by FB1, DH-CIT, OTA, total ZEA
564 and AFM1. Nevertheless, differences in analytical methodology and diversity in available
565 biomarkers limit comparison of the results.
566 In contrast to what happens with urine, to date there are not many multimycotoxin studies
567 conducted in blood (Table 3).
568 De Santis et al. (2017) studied 8 different mycotoxins (AFB1, AFM1, ZEA, OTA, FB1,
569 DON, DOM-1, GLIO) in the serum of autistic patients and two control groups (one of
570 siblings and the other of non-parental persons). In all groups OTA was the prevalent
571 mycotoxin, with mean prevalence of 82.9% of samples in the whole group and 85.1% in the
572 autistic. For the rest of mycotoxins, all mean values found were below LOQ. Few samples
573 showed co-occurrence of different mycotoxins (AFB1, AFM1 and OTA in 4% samples, and
574 AFB1, AFM1, OTA and GLIO in 2% samples).
575 The most complete is a recent study by Cao et al. (2018), developed in the People's Republic
576 of China, in which the plasma of 30 healthy individuals has been analyzed and compared to
577 that of 30 hepatocellular carcinoma patients. Eleven mycotoxins were simultaneously

analyzed by HPLC-MS/MS. In the plasma of control patients the most frequently mycotoxin found was AFB2 (1.37-3.89 ng/mL; 16.6% samples), followed by AFB1 and sterigmatocystin (13.3%), and AFG1, AFG2, AFM1, FB1 and FB2 (3.3%). No PAT nor CIT were found in these samples. Higher percentage of positive samples was found in plasma from hepatocellular carcinoma patients, with sterigmatocystin being the more frequently found mycotoxin (1.06-3.23 ng/mL; 40%), followed by AFB1 (33.3%) and AFB2 (23.3%). However, in plasma AFG1, AFG2, AFM1, OTA and CIT were detected just at the LOD of the method in both kinds of samples, authors not excluding that these results could be false positives.

587 Table 3. Mycotoxins detected in urine and blood/plasma samples through multidetection methods (%positives/mean (ng/mL))

Urine biomarkers

Reference Country (Samples)	DON	DON-GlcA	OTA	AFM1	ZEA	∝-ZOL	β-ZOL	FB1-FB2	DH-CIT
Gerding et al. 2015 Haiti (142)	17/3.2	21/17.0	33/0.109	8/0.06		3/1.46		3/0.44	14/0.49
Germany (50)	16/2.0	54/11.2	30/0.040	n.d.		n.d.		n.d.	28/0.12
Bangladesh (95)	n.d.	n.d.	76/0.203	8/0.06		n.d.		1/-	75/2.75
Solfrizzo et al. 2014 Italy (52)	96/11.89		100/0.144	6/0.068	100/0.057	100/0.077	98/0.090	56/0.055	
Wallin et al. 2015 Sweden (252)	63/5.38		51/0.90			21/0.13	18/0.10	6/0.07	
Abia et al. 2013 Cameroon (175)	6/-	41/5.49	16/0.09	9/0.05	2/0.22	1/-		3/0.63	
Gerding et al. 2014 Germany (101)	29/3.38	82/12.21							12/-
Heyndrickx et al. 2015 Belgium (239)	37/3.9	100/61.3	35/0.278			0.4/0.005			12/0.752
Ezekiel et al. 2014 Nigeria (120)	0.8/2	5/3.5	28/0.2	14/0.3	0.8/0.3			13/4.6	
Warth et al. 2014 Thailand (60)		12/12.4	2/-	5/0.33					

Blood/serum biomarkers

Reference Country (Samples)	DON	DOM-1	OTA	AFB1-AFB2	AFG1-AFG2	AFM1	CIT	ST	PAT	ZEA	FB1-FB2	GLIO

De Santis *et al.* 2017 Italy												
Control group 1 (35)	22.9/0.5 12.5/0.8	17.1/0.3 6.3/0.1	77.1/0.27 75/0.28	25.7/0.002 (AFB1) 6.3/0.00 (AFB1)		45.7/0.07 31.3/0.06				8.6/0.1 0/0	2.9/0.04 0/0	14.3/06 18.8/10.3
Control group 2 (18)												
Cao *et al.* 2018 PR of China (30)			traces	13/0.95-1.78 (AFB1) 16.6/1.37-3.89 (AFB2)	3.3/0.61 (AFG1)-0.43(AFG2)	3.3/0.57	traces	13/0.88-2.05	n.d.		3.3/1.92 (FB1) 3.3/2.03 (FB2)	

588

589

590 Table 4 summarizes the calculated daily intakes from the mycotoxin concentration in urine in
591 some multibiomarker studies. In summary, between 6 and 29% of the populations considered
592 were exposed to DON at levels over the TDI, suggesting a medium but worrying risk for the
593 population, and at the same time they could be exposed to OTA or AFB1 levels of concern.
594 Since AFB1 is a potent mutagenic carcinogen, no TDI values are established. The presence of
595 AFM1 in urine samples is of concern. The TDI of 2 µg/kg b.w. for FB1 was never exceeded
596 by healthy population.

597

598 Table 4. Calculated daily intake from some exposure studies (mean/max/% exceeding TDI).

Reference (n)	**DON** TDI 1 µg/kg bw	**OTA** TDI 0.017 µg/kg bw	**AFB1** - -	**FB1** TDI 2 µg/kg bw
Gerding *et al.* 2015 Haiti (142)	0.27/4.38/6		0.03/0.23/-	0.05/1.74/0
Germany (50)	0.3/2.15/6		-	-
Bangladesh (95)	-		0.03/0.195/-	0.03/1.362/0
Solfrizzo *et al.* 2014 Italy (52)	0.59/3.37/6	0.139/2.07/94	0.668/0.142/-	0.274/1.759/0
Abia *et al.* 2013 Cameroon (175) HIV sub-populations	0.21/2.59/-	0.004/0.094/-	0.0425/1.15/-	5.25/123.3/-
Gerding *et al.* 2014 Germany (101)	0.52/5.67/12			
Heyndrickx *et al.* 2015 Belgium (239)	1.24/10.08/29	0.001/0.021/1		

599

Multi-detection methods have allowed assessing the levels of co-exposure to different mycotoxins by an individual through urine analysis. Consequently, it has been confirmed that co-occurrence of two toxins in a urine sample is usually common (more than single contamination), however results depend highly on the analysed toxins, if only parent mycotoxins were analysed, 1-2 toxins are usually reported, while if both parent and modified mycotoxins are analysed 2 to 4 toxins are usually found in a sample. Moreover, lower LOD of the methods led to higher reported co-occurrence. For example, Gerding et al. (2015) reported that between 16-54% samples contained two detectable toxins, between 6 and 20 samples contained 3 toxins and 1-2% contained 4 toxins. DH-CIT and OTA usually co-occurred as well as DON and DON-GlcA, and also 3 of them. *Fusarium* toxins and OTA have been also shown to occur (Wallin et al., 2015), for example, DON, ZEA, OTA and DON, ZEA, FB1, OTA, co-occurred in 38 and 52% of urine analysed samples (Solfrizzo et al., 2014). Studies on exposure in Cameroon reported 4% co-exposure to AFM1 and DON, 3% to OTA and DON and 5% to DON and NIV (Abia et al., 2013).

8. Co-exposure of mycotoxins with other environmental chemicals

A major research gap is the potential concurrent exposure of mycotoxins with other environmental chemicals that may exhibit some interactive activity and/or exert some biological function converging in the same molecular pathways. As far as we know, there are not biomonitoring studies exploring the simultaneous presence of a panel of environmental chemicals including some type mycotoxin. However, the estimates relying on dietary exposure modelling suggest that multiple patterns of co-exposure are likely within general population. The research on mixture identification from the second French Total Diet Study revealed the extended exposure to mycotoxins in complex mixtures with other environmental chemicals in most of French diet clusters. For instance, a first cluster containing 18% of the whole population, was expected to have a significantly higher exposure to mycotoxins (HT-2

toxin, DON, ZEA and NIV), polycyclic aromatic hydrocarbons (PAHs) (pyrene and phenanthrene) and bisphenol A, than the whole population. The estrogenic ZEA was also identified in another cluster with many PAHs, acrylamide, trace elements, pesticides and the sum of eight polybrominated diphenyl ethers in a cluster representing the 21% of the population with dietary habits related to junk food and identified as "Snacking" (Traore et al., 2016). Using a similar approach based on the identification of consumption systems integrated with exposure data, different clusters of pregnant mothers from the two large French cohorts "Étude Longitudinale Française depuis l'Enfance" (ELFE) and "L'étude des déterminants pré et post natals du développement et de la santé des enfants" (EDEN), were identified to be more exposed to mycotoxins simultaneously with other environmental chemicals. The model was comprehensive including 210 chemicals of which 18 were major mycotoxins or parent compounds. The "Myco-Pest-PAH" mixture identified from EDEN before pregnancy was also found in EDEN during pregnancy. It contained eight mycotoxins (α-ZAL, α-ZOL, diacetoxyscirpenol (DAS), DON-3-GlcA, FusX, OTB, OTA and HT-2 toxin), three pesticides (chlorpyrifos-methyl, cyproconazole and pirimiphosmethyl) and four PAHs (benzo[g,h,i]perylene, benzo[e]pyrene, cyclopenta(c,d)pyrene and indeno[1,2,3-cd]pyrene). In EDEN before pregnancy, these substances were associated with nine other pesticides (pyriproxyfen, tetradifon, sulphur, chlorothalonil, diethofencarb, flutriafol, iprodione, ethion and bifenthrin) and an additive (sulphites). In EDEN during pregnancy, these substances were associated with three other mycotoxins (DON, DON-15-GlcA and ZEA), a PAH (pyrene), two phytoestrogens (daidzein and genistein), a trace element (gallium), a pesticide (sulphur) and two perfluoroalkyl acids, perfluorobutane sulfonate and perfluorohexanesulfonic acid (Traore et al., 2018). Despite the uncertainties related to the dietary modelling methodologies, these results provide strong evidence concerning the potential co-exposure of highly bioactive mycotoxins like α-ZEA with many environmental

chemicals during highly sensitive developmental windows. These modelling studies provides also light on the potential weight of mycotoxins in the human chemical exposome, as suspected by the extensive occurrence of mycotoxins in diets.

These profiles extracted from a European diet only represent a region where strict mycotoxin control regulation is enforced, underscoring the weight of mycotoxins in the chemical exposome of population in developing countries. The "traditional" fungal contamination of cereals with the mycoestrogen ZEA, has been identified as a major public health challenge co-existing with emerging chemical exposures resulting of unstructured industrial development resulting on high exposures to lead, air pollution, pesticides or e-waste by-products (Bornman et al., 2017).

We have failed to find in the literature examples of targeted approaches that use liquid or gas MS methods for the simultaneous detection of mycotoxins and environmental contaminants in biological specimens. An inspiring example is the method developed by LC-MS with previous SPE for the simultaneous determination of mycotoxins (AFB1, OTA and PAT) and bisphenol A in food matrices that could be adapted and applied for urine samples (Song et al., 2013). Novel analytical workflows based on HRMS untargeted metabolomic approaches may become efficient solutions to overcome existing analytical challenges for the screening of large panel of chemicals including well-known chemicals. A proof-of-concept study has recently presented a novel workflow for analysis of blood and urine based on HPLC coupled to Bruker Impact II quadrupole time-of-flight (Q-TOF) mass spectrometer with a previous simple sample preparation (Warth et al., 2017). The panel of detected features are further explored using machine-learning algorithms combined to the XCMS/METLIN platform to elucidate the pathways related to the annotated signature. Through the pilot study the authors

demonstrated to efficiently identify low concentrations of common xenoestrogens such as genistein, ZEA and triclosan at in commercial biological matrices.

9. Mycotoxins and human health effects

The disease caused by mycotoxins is called mycotoxicosis. Mycotoxins can be a threat to both animal and human health. Oral ingestion of contaminated food is the most frequently exposure way, however dermal contact and inhalation can also occur (Marin et al., 2013). Their toxic effect depends on the toxicity of each mycotoxin, the extent of exposure, age and nutritional status of the individual and possible synergistic effects with other chemicals that the individual is exposed. Infants is considered the most vulnerable population group due to the relative inefficiency of detoxification pathways and high relationship of between internal doses per body weight.

There are more than 300 known mycotoxins which are suspected of widely differing modes of action, however formal toxicological evaluation and comprehensive risk assessment have been conducted only for environ 10 of most known or major mycotoxins. Consequently, very little is known about the potential toxicological and biological effects of secondary mycotoxins, metabolites or emerging mycotoxins.

To date, most toxicological evaluation is based on experimental studies, including *in vitro* and *in vivo* studies, conversely the body of evidence from human studies is limited to few epidemiological studies or case studies conducted shortly after human mycotoxicosis outbreaks. An overview of major health effects of mycotoxins at different toxicological levels is summarized at Table 5, nonetheless readers may find more detailed reviews published in the literature (Bui-Klimke et al., 2015; EFSA 2017; Kensler et al., 2011; Marin et al., 2013; Peraica et al., 1999; Puel et al., 2010).

Table 5. Overview of major health effects of mycotoxins at different toxicological levels.

Mycotoxin group	Interaction Nuclear Receptor	Cellular responses	Health effects Animals	Health effects Humans

AFB1 AFM1	Pregnane X receptor Constitutive androstane receptor Aryl hydrocarbon receptor Vitamin D receptor	Formation of DNA adducts Lipid peroxidation Bioactivation by cytochromes P450 Conjugation to GS-transferases	Hepatotoxicity Genotoxicity Carcinogenicity Immunomodulation	Cancer Impaired child growth
FB1	-	Inhibition of ceramide synthesis Adverse effect on the sphinganine/sphingosine ratio Adverse effects on the cell cycle.	Central nervous system damage Hepatotoxicity Genotoxicity Immunomodulation	Oesophageal cancer Liver cancer Neural tube defects Impaired child growth
OTA	-	Effect on protein synthesis. Inhibition of ATP production Detoxification by peptidases	Nephrotoxicity Genotoxicity Immunomodulation	Nephritic syndrome BEN
PAT	Ø	Indirect enzyme inhibition *In vitro* mutagenesis	Neurotoxicity Immunotoxicty Disruption of barrier function	-
DON NIV T-2 toxin HT-2 toxin	Peroxisome proliferator-activated receptor Liver X receptor Retinol X receptor G-protein coupled receptor	Apoptosis Oxidative stress Inhibition protein synthesis	Hematotoxicity Immunomodulation Skin toxicity Anorexia and vomiting Reduced weight gain Disruption of barrier function	Hormone-dependent cancer Acute mycotoxicosis
ZEA ZOL	Estrogen Receptor-α and -β	Binding to oestrogen receptors Bioactivation by reductases Conjugation to glucuronyltransferases	Reproductive adverse effects	Thelarche Precocious puberty Breast cancer

Among the toxicological initiating events, the interaction of most mycotoxins with nuclear receptors has been scarcely explored (reviewed by Dall'Asta, 2016). The exception is the case of ZEA and ZOL whose potent actions to activate the oestrogen receptor pathway and trigger endocrine perturbations merits an entire section presented hereafter. AFB1 has been found to modify the expression of nuclear receptors such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (AhR) at

705 transcriptional level, and also causing the downregulation of vitamin D receptor. Hormone
706 exocytosis caused by DON was found to be triggered by G-protein coupled receptor (GPCR)-
707 mediated $Ca2^{+}$ signaling, using the murine neuroendocrine tumor STC-1 cell line (Zhou and
708 Pestka, 2015). PAT has been evaluated against different endocrine disrupting models and no
709 studies have revealed effects on reporter gene assays at the receptor level.
710 A broad range of adverse health effects have been identified for mycotoxins in animals and
711 humans, including hepatotoxicity, estrogenicity, immuno/haematotoxicity, nephrotoxicity or
712 neurotoxicity. And some of them are recognized as genotoxic and/or carcinogenic, including
713 AFB1, one of the most carcinogenic food contaminants in human diets and classified as
714 carcinogenic to humans by the International Agency for Research on Cancer (Group 1), or
715 OTA and FBs classified as possible carcinogens (Group 2B). AFB1 is a primary cause of
716 human hepatocarcinoma, and in developing countries it acts synergistically with the hepatitis
717 B virus infection.
718 Mycotoxins also affects the intestinal barrier function, impairing the permeability and
719 integrity of epithelial cells. Most prominent effects have been document for the trichothecene
720 DON that may strongly impair the expression, localization and function of tight junction
721 proteins which seal the epithelial monolayer and prevents the para-cellular diffusion of
722 luminal antigens and microorganisms. Other trichotecenes including T-2 and HT-2 toxin and
723 mycotoxins such as PAT or FB1 have been found to impair some of the physiological
724 parameters that characterize the intestinal barrier function (Akbari et al., 2017). On this basis,
725 it has been hypothesized the role of mycotoxins in the pathophysiology of chronic intestinal
726 inflammatory diseases, such as inflammatory bowel disease, and in the prevalence of food
727 allergies.
728 The potential effect of mycotoxins in infant growth parameters has been recognised as a
729 priority research gap, especially in developing countries, where the high occurrence of

mycotoxins comes together with the high prevalence of intrauterine growth restriction, infant and young stunting, underweight wasting. Whereas no epidemiological studies have been conducted to evaluate the associations between exposure to DON or ZEA and infant growth parameters, some studies that analysed exposure to AFs and FBs consistently found negative associations (Lombard et al., 2014). For instance, in African countries, strong dose response relationships were found between exposure in utero and/or early infancy to AFs and growth retardation, identified by reduced birth weight and/or low weight-for-age or height-for-age Z scores (Turner et al., 2013).

Another important aspect to be considered is that many foods can present a simultaneous presence of different food contaminants, like mycotoxins, pesticides, heavy metals or radioactive particles (Kosalec et al., 2009). This multi-contamination can strongly modify the toxic effects of some of them resulting in a range of interactive effect as demonstrated by the simultaneous exposure of Caco-2 cells and HEK-293 kidney cells to cadmium and DON (Le et al., 2017). The toxicological evaluation of combinations of mycotoxins for the characterization of potential interactions is an emerging and very active field of research (Alassane-Kpembi et al., 2017).

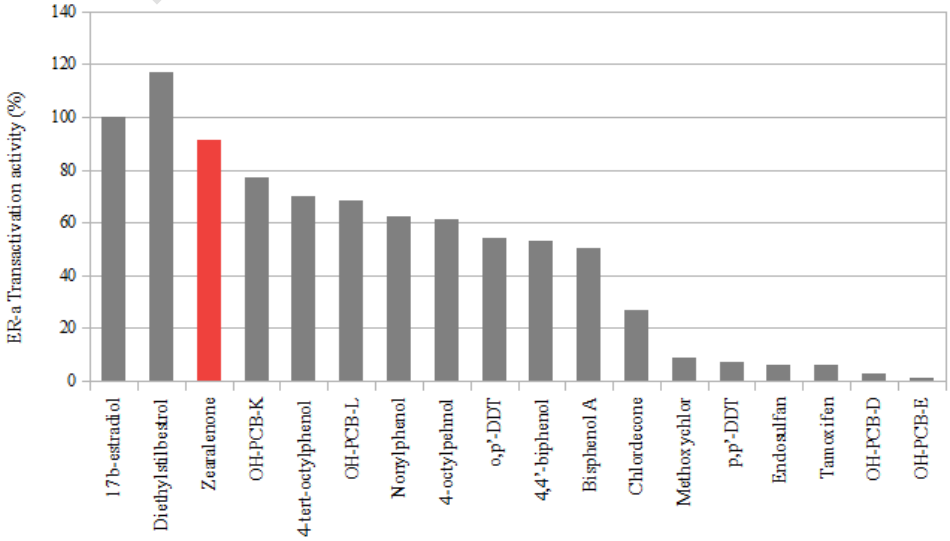
10. Mycotoxins as endocrine disruptors: case of ZEA and its derivatives

The Endocrine Society's Scientific Statement on Endocrine-Disrupting Chemicals (EDCs) says that an EDCs is "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action" (Gore et al., 2015). The particular mechanism of actions of EDCs represent a novel paradigm in chemical risk assessment introducing new challenges related to ability of inducing biological effects at very low concentrations or the presence non-monotonic dose-responses. Hence, the emerging concern with EDCs emphasize the high interest of including mycotoxins, specially ZEA and its metabolites within the panel of

targeted analytes in exposome projects addressing hormone-dependent diseases. Despite the knowledge about the endocrine disrupting action of ZEA and its strong oestrogenic effects was identified decades ago (recently reviewed by Kowalska et al., 2016 and Metzler et al., 2010), little attention has been paid by most researchers on EDCs who has extensively focused on industrial compounds such as bisphenol A or phthalates. Occurrence studies have demonstrated the pervasive presence of ZEA in cereal-based foods and the extended exposure among general population supporting that ZEA and specially the metabolite ZOL may be a relevant contributor on the total body burden of xenoestrogenic activity.

In silico and *in vitro* studies have demonstrated the high affinity of ZEA and ZOL to bind and activate estrogen receptor, exhibiting potencies similar to 17-β–estradiol, and substantially higher than many industrial xenoestrogens such as bisphenol A (See Figure 1, based on Kuiper et al., 1998). Metabolite ZOL has non-estrogenic chemical structure but resembles that of 17-β–estradiol, exhibiting similar key contacts in the binding pocket of ER, resulting on high bioactivity (Delfosse et al., 2014; Balaguer et al., 2017). For that reason, a-ZON is recognised as one the most active xenoestrogens that can modulate ER activity at concentrations as low as 0.1 nM. (Balaguer et al., 2017).

Figure 1. Relative transactivation activity of environmental endocrine disruptors for estrogenic receptor alpha created from data published by Kuiper et al., (1998). ZEA appears highlighted in red.



The related effects of ZEA and its derivatives in cells include the stimulation of growth of estrogen receptor-positive human breast cells, stimulation of cell cycle progression of MCF-7 cells (Metzler et al., 2010). It has been also shown that ZEA may affect the metabolism of rat adipocytes, including the stimulation of basal lipolysis and reduced epinephrine stimulated lipolysis (Kandulska et al., 1999), suggesting ZEA also as a metabolic disruptor candidate.

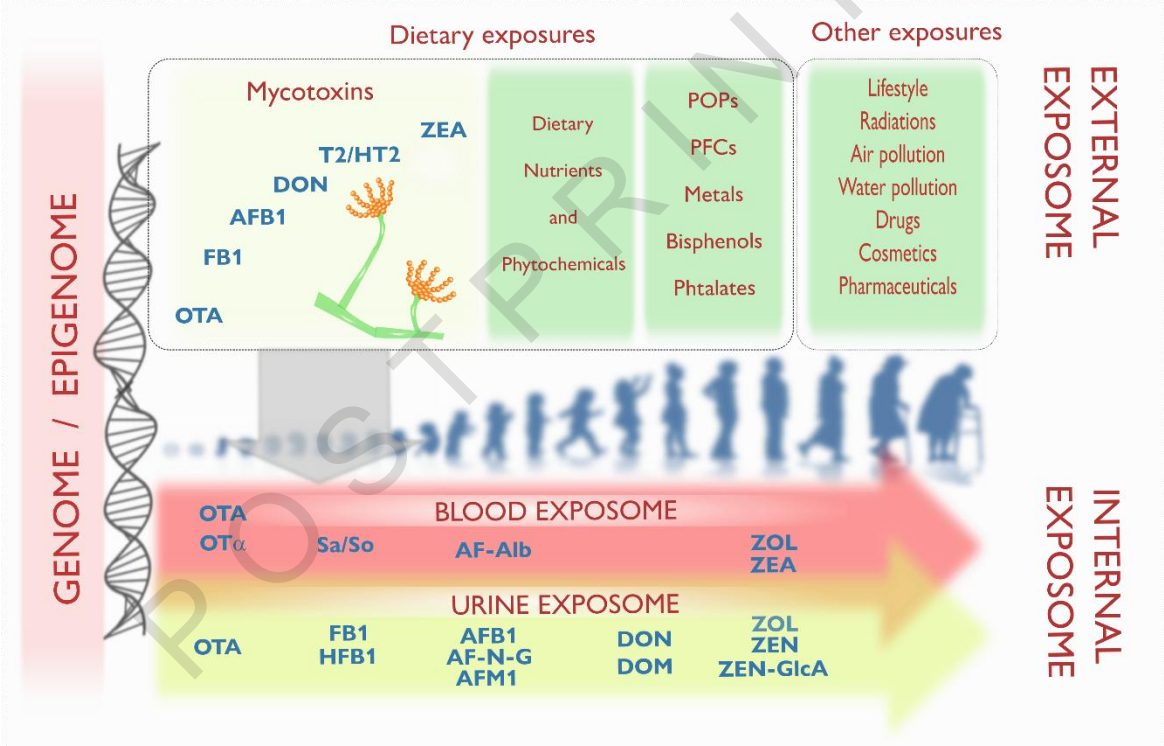
The endocrine disrupting effects of ZEA in animals includes the impairment of reproduction, uterotropic activity, hyperoestrogenism and inflammation of the vagina, endocrine-disruptive effects during gestation and neonatal life vaginal cornification, persistent estrus, reduced fertility, anovulation and decreased gonadotropic hormone output by the hypophysis among others (Kowalska et al., 2016; Metzler et al., 2010). Despite the large evidence suggesting the potential hormone disrupting effects of ZEA, few epidemiological studies have been conducted in humans to explore associations with estrogen-dependent diseases. Food contaminated with ZEA and its natural metabolites was associated with the development of precocious puberty, a risk factor for breast cancer (Gray et al., 2017). Furthermore, higher urinary ZEA levels, resulting from recent intake of beef or popcorn, were inversely associated with the onset of breast development (Bandera et al., 2011).

11. Mycotoxins within the human exposome: challenges and opportunities

The mycotoxins constitute a large group of chemicals that can be found regularly in foodstuff worldwide often resulting in the chronic exposures of low doses of complex mixtures of mycotoxins concurrently with industrial chemicals, phytochemicals and nutrients as represented in the Figure 2. The fast excretion and the low concentration levels of mycotoxins challenge their detection in biological samples, however current evidence demonstrate that major mycotoxins can be commonly found within the urine and blood chemical spectra (Marin et al., 2013). Dietary exposure assessment studies support that

infants and children are the most exposed population groups and the mycotoxin exposures continue through the entire life. Despite the relevance in terms of exposure and health effects, mycotoxins are often underscored and/or overlooked in epidemiological research as acknowledged by the visionary Christopher Wild (Wild and Gong, 2009), and no attention have received by major exposome projects launched in Europe, such as the impressive HELIX or EXPOsOMICS projects.

Figure 2. Conceptual representation of the mycotoxin compartment within the human exposome framework including the sum of the most important mycotoxins that may found in the diet and respective forms or metabolites that are used as biomarkers in blood and urine.



The novel exposome paradigm proposes a chemical-agnostic approach, that appears as an excellent opportunity to evaluate the role of mycotoxins in human health through more integrative approaches. This approach contrasts with the historical expertise of scientist that have been focusing on specific chemicals or group of chemicals. Hence, it looks like a big communicative and cross-talk effort will be required to efficiently optimize the already

available knowledge across disciplines. For instance, the mycotoxin compartment of human exposome, has been pretty well characterized for the main mycotoxins in terms of exposure and health effects, despite little is known about the rest of mycotoxins, modified forms and more emerging toxins.

To date, most mycotoxin exposure assessment studies have been based on dietary modelling approaches although many limitations exist on these indirect exposure assessment methods, especially if we consider the uncertainties related to these modelling methods, and also the intra-individual or seasonal variability. Hence biomonitoring methods are considered the 'gold-standard' to evaluate the individual exposures, however the field is still on its emergence and few biomarkers have been fully validated. The biomonitoring studies applied to mycotoxins have been mainly focused on surveillance of general population with regulatory or risk assessment purposes and few epidemiological studies have considered the evaluation of mycotoxins. Methodological approaches used to detect the mycotoxin biomarkers will strongly determine the performance (e.g. sensitivity), resulting on dramatically different results depending on the detection/quantification thresholds achieved (p.e. direct vs indirect methods). The development of reliable, accurate and sensitive multibiomarker methods to simultaneously characterize a large panel of mycotoxins, but also industrial pollutants, will strongly help to understand the potential associations between environment and health. On this sense, the application of non-targeted or semi-targeted HRMS metabolomic methods appears as a promising screening approach to identify exposure risk factors, and related biomarkers of biological perturbation (Warth et al., 2017). It appears also as a great opportunity to explore the underlying toxicological effects of mycotoxins in humans. Coupling other OMICs platforms for the identification of endogenous chemical signatures we may gain access to early biomarkers of health effects and biological perturbation triggered by mycotoxins. In any case, a list of challenges associated with the

accurate determination of biomarkers of non-persistent pollutants exposure applies directly to the mycotoxins, including the high intra- and inter-day individual variability, requiring repeated sampling protocols to avoid the exposure misclassification (Perrier et al., 2016). Additionally, specific issues related to mycotoxins such as the variability related to seasonal or weather influences on mycotoxin productions will challenge the estimations of individual trajectories.

The simultaneous determination of mixtures of mycotoxins within more complex cocktails of environmental pollutants will allow address major research gaps related to mixture effects. A growing interest on the effect of mycotoxin mixtures have led toxicologist dosing binary and tertiary combinations of mycotoxins, sometimes with little similarities on mechanism of action, whereas few or non-studies have evaluated the simultaneous effect of mycotoxins with other environmental pollutants with similar biological actions (e.g. the xenoestrogens bisphenol A and ZEA).

12. Concluding remarks

In the present review we have shown that mycotoxins maybe commonly found at high concentrations in blood and urine from individuals from developing countries, but also frequently found at moderate or low concentrations in developed regions. For instance, it has been estimated that 500 million of the poorest people in sub-Saharan Africa, Latin America, and Asia are exposed to mycotoxins at levels that substantially increase mortality and severe diseases (Wild and Gong, 2010). The health effects of mycotoxins are very wide, targeting different toxicological endpoints, biological functions and have been related with multiple diseases. To date, few studies have been able to demonstrate consistent associations of health effects in humans relying most of studies on animal or *in vitro* settings.

We strongly believe that mycotoxins represent a relevant component of the human exposome and that exposome-based projects aiming to explore the role of chemical exposome on human health, should strongly consider the mycotoxins within the panel chemical candidates.

Whereas industrial chemicals may be banned and set out of the market, mycotoxins will not be completely removed of raw foods and food items intended for human consumption. Even with very stringent regulations, humans will continuously be exposed to low level of mycotoxins whose combined effect and their combined effect with other environmental exposures whose effects are completely unknown. As we have shown in this document, currently there are available robust and accurate analytical methods that allow the identification and characterization of multiple mycotoxins and/or their metabolites in most common biological samples, allowing the direct implementation in epidemiological research.

Overall, we acknowledge that the exposome projects will be a great opportunity to better translate the knowledge generated on mycotoxicology during the past decades in environmental health. Conversely, these new approaches may be an excellent opportunity to fill many research gaps on mycotoxins research as the identification of associations mycotoxins with human health, elucidation of join effect with other environmental exposures or the decipher of underlying molecular mechanisms by using advanced OMICs technologies.

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1315

Title:

The role of mycotoxins in the Human Exposome: application of mycotoxin biomarkers in exposome-health studies

Authors:

Marín, Sonia[a,‡*]; Cano-Sancho, German[b,‡]; Sanchis, Vicente[a,§]; Ramos, Antonio J[a, §].

Institutions:

[a]Applied Mycology Unit, Food Technology Department. University of Lleida. UTPV-XaRTA, Agrotecnio Center. Av. Rovira Roure 191. 25198 Lleida. Spain.

[b]LABERCA, Oniris, INRA, Université Bretagne-Loire, 44307, Nantes, France

‡, §: These authors have contributed equally to the present study.

*Corresponding Author : Applied Mycology Unit, Food Technology Department. University of Lleida. UTPV-XaRTA, Agrotecnio Center. Av. Rovira Roure 191. 25198 Lleida. Spain.

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List of Abbreviations

Aflatoxin (AF)

AFB1-albumin (AFB1-Ab)

Citrinin (CIT)

Diacetoxyscirpenol (DAS)

Deoxynivalenol (DON)

De-epoxy-deoxynivalenol (DOM-1)

Diacetoxyscirpenol (DAS)

DH-CIT dihydrocitrinone (DH-CIT)

Enzyme-linked immunosorbent assays (ELISA)

Fumonisin B1 (FB1)

Fumonisin B2 (FB2)

Fusarenon X (FusX)

Gliotoxin (GLIO)

Glucoside (Glc)

Glucuronide (GlcA)

Hydroxy Fumonisin B1 (HFB1)

High resolution mass spectrometry (HRMS)

Immunoaffinity chromatography (IAC)

Liquid chromatography (LC)

Mass spectrometry (MS)

Nivalenol (NIV)

Ochratoxin A (OTA)

Ochratoxin alpha (OTα)

4-hydroxyochratoxin A (4-OH OTA)

45 Sphinganine (Sa)

46 Sphingosine (So)

47 Solid-phase extraction (SPE)

48 Polycyclic aromatic hydrocarbons (PAHs)

49 Patulin (PAT)

50 Zearalenone (ZEA)

51 Zearalanone (ZAN)

52 Zearalenol (ZOL)

53

54

Abstract

Mycotoxins are secondary metabolites produced by fungi that may contaminate different foods intended for human consumption, resulting in a widespread exposure worldwide. The novel exposome paradigm has the ambition to decipher the different environmental insults threating human health throughout the entire lifespan. Given the large potential impact of mycotoxins in terms of human exposure and related health effects, the ambition of this review is to present this group of chemical compounds and the high interest to be included in exposome projects. Furthermore, we also attempt to approach the novel exposome paradigm to more traditional disciplines such as mycotoxin exposure assessment and mycotoxicology, introducing the new methodological challenges and translational needs. Hence, we provide an overview of major biomarkers currently developed, biological matrices where these may be found, an overview of internal exposure levels and potential co-occurrence with environmental chemicals and finally an overview of major health effects with the illustrative example of the potent xenoestrogen zearalenol. Conversely, these new approaches may be an excellent opportunity to fill many research gaps on mycotoxins research as the identification of associations with human health, elucidation of join effect with other environmental exposures or the decipher of underlying molecular mechanisms by using advanced OMICs technologies.

Highlights

- Humans are exposed to complex chemical mixtures including mycotoxins worldwide
- The intake of mycotoxins in food leads as a consequence to the presence of chemical compounds in biological fluids
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- Exposome projects should include mycotoxins within the panel of targeted biomarkers
- The knowledge about mycotoxin exposure and effects on humans may be largely improved through Exposome projects.

1. The novel 'exposome' paradigm

The exposome concept refers to 'the totality of environmental exposures from conception onwards', proposed to complement the genome with all those factors that may be related with the human phenotypes and responsible of perturbation of biological processes (Wild et al., 2005). This novel vision involves different levels of complexity and dimensionality, providing an integrative overview of the relationship between the internal biological processes and the environment. Environmental external factors include radiation, infectious agents, chemical contaminants and environmental pollutants, diet, lifestyle factors (e.g. tobacco, alcohol), occupation and medical interventions. The exposome includes also the wider social, economic and psychological dimension for instance the education, the psychological and mental stress, or climate (Rappaport et al., 2011; Wild et al., 2012, 2013). As a major difference to the genome, the exposome has an extremely dynamic nature, entailing complex approaches to accurate characterizations and decipher the interplay of external factors with the biological processes and human health. The limitations and challenges have been already identified and discussed elsewhere, proposing approaches more adapted to the readily accessible technologies and financial budgets, transitioning towards more integrative settings (Siroux et al., 2016).

The exposome concept has been shaped in parallel to the fast development and implementation of advanced mass spectrometry (MS) and high resolution mass spectrometry (HRMS) methodologies to biomonitoring. These approaches, within a "targeted" context, have allowed the accurate screening and characterization of a wide range of known external chemical exposures or related metabolites at individual level. Furthermore, the implementation of HRMS to non-targeted metabolomic approaches has also allowed the development of novel screening framework to massively identify new environmental exposures but also internal molecules generated by the intermediary metabolism. The

chemical spectra of molecules in the organism is believed to account for about 40 nutrients, 2,000 intermediary metabolites, 200,000 peptides and 500000 lipids, whereas about 400,000 chemicals are believed to be part of the exposome (Jones, 2016). The diet is a major pathway of intake of environmental chemicals, including non-nutritive molecules with potentially harmful properties like pesticides, environmental pollutants or chemicals often underscored, like mycotoxins. Currently, more than 300 mycotoxins are known, but scientific and regulatory attention is focused only on a reduced number of major toxins, in terms of known occurrence and toxicity.

Given the large potential impact of mycotoxins in terms of human exposure and related health effects, the ambition of this review is to present this group of chemical compounds and the high interest to be included in exposome projects. Furthermore, we also attempt to approach the novel exposome paradigm to more traditional disciplines such as mycotoxin exposure assessment and mycotoxicology, introducing the new methodological challenges and translational needs. Hence, we provide an overview of main mycotoxins, major biomarkers currently developed and biological matrices where these may be found. The present review is an introductory overview about available methods of detection for mycotoxins biomarkers, hence we refer readers to more comprehensive reviews if interested in a deeper insight (Escrivà et al., 2017a; Vidal et al., 2018; Warth et al., 2016). We also provide an overview of internal exposure levels and potential co-occurrence with environmental chemicals which highlight the interest of studying the effect of complex mixtures including mycotoxins. Finally, a general overview about major health effects of mycotoxins is provided with an example of the potent xenoestrogen zearalenol (ZOL) to illustrate the high interest of considering fungal toxins in exposome-health studies.

2. Mycotoxins, chemical contaminants produced by fungi

Mycotoxins are natural toxicants produced by a high number of species of different fungal genera. The main mycotoxigenic species belong to the genera *Fusarium*, *Claviceps*, *Alternaria*, *Aspergillus* and *Penicillium* (Marin et al., 2013). These species need particular eco-physiological conditions, like temperature and humidity (water activity), to grow and synthesize these secondary metabolites which have adverse effects in animal and human health. The main substrates or crops with capacity to support the growth and accumulation of these toxins are cereals, nuts, oilseeds, dried fruits, coffee and spices, and their by-products. The contamination can occur throughout the food chain, both in the field and in the post-harvest stage, depending on the species involved. It must be borne in mind that the same species can produce more than one mycotoxin, such is the case of *Fusarium graminearum*, which produces deoxynivalenol (DON) and zearalenone (ZEA), and also that the same mycotoxin can be produced by several fungal species, such as ochratoxin A (OTA), which is produced by *Penicillium verrucosum*, *Aspergillus ochraceus* and *Aspergillus carbonarius*.

Mycotoxins are a structurally diverse group of relatively low molecular mass compounds that can occur in three possible forms: as free or unmodified, as matrix associated and as modified forms (Rychlik et al., 2014). The free or unmodified mycotoxins describe the basic mycotoxin structures formed by a high number of fungi in well-known biosynthetic pathways. Some examples of these mycotoxins are aflatoxin B_1 (AFB1), OTA, patulin (PAT), DON, fumonisin B_1 (FB1), and ZEA. Their chemical structures are very diverse. So, we find microcyclic lactones like ZEA, small lactones condensed with hetero- or alicycles like PAT, furan derivatives like aflatoxins (AFs), alicyclic compounds like T-2 toxin, among others. The matrix associated forms are either complexes with matrix compounds or are physically dissolved or trapped or are covalently bound to matrix components or a combination of both effects. Examples of this group are the fumonisins (FBs) bound to

carbohydrates or proteins. The third group known as modified mycotoxins describes any modification of the basic chemical structure of the molecules. One of these modifications are produced by plants through conjugation reactions such as the formation of DON-3-glucoside (DON-3-Glc). Other conjugation reactions are produced by animals such as the formation of DON-3-glucuronide (DON-3-GlcA) or by fungi as for example the formation of ZEA-14-sulfate. Among the chemically modified mycotoxins it is possible to distinguish between the thermally formed and non-thermally formed ones. The first group describes the modifications produced during the thermal process of foods and feeds with the example of norDON A-C. The second group is formed by degradation products of the mycotoxins produced under alkaline conditions like DON-sulfonate.

It is necessary to highlight that the routine analysis of foods and feeds usually determines the free or parent mycotoxins. In order to identify and quantify the modified forms, validated methods using highly sensitive equipment like LC-MS/MS is necessary. The industries don't usually dispose of these facilities mostly because of economic issues. However, there is a danger related to the ingestion of these modified mycotoxins, particularly because of their release into the digestive system and the formation of free forms, which toxic action has been proved already. Thus, the exposure assessment might not be accurate enough due to the presence of the modified mycotoxins. Another possible scenario is the exceptional case when the modified form is more toxic than its parent molecule (e.g. α-ZOL possesses a stronger oestrogenic potency than ZEA) (Frizzell et al., 2011).

3. Biomarkers of mycotoxin exposure

Biomarkers are measurable biochemical or molecular indicators of either exposure (exposure biomarker) or biological response (effect biomarker) to a mycotoxin that can be specifically linked to the proximate cause (Baldwin et al., 2011). More specifically, a biomarker is a biological measure (parent toxins, protein or DNA adducts, glucuronide conjugates…

measured in urine or plasma/serum) which is correlated with the quantity of xenobiotic ingested (Table 1). Validation of a biomarker requires demonstration of assay robustness, intake versus biomarker level, and stability of stored samples.

Biomarkers have contributed largely to understanding the causative role of AFB1 in human hepatocellular carcinoma (Kensler et al., 2011). These have included both biomarkers of exposure, based on urinary aflatoxin M_1 (AFM1) and serum AFB1-albumin adduct as markers of internal dose, and a biomarker of effect, based on urinary AFB1-N7-guanine as a measure of biological effective dose, since DNA adduct formation is the biochemical mechanism whereby AFB1 exerts its carcinogenic potency.

Traditional biomonitoring studies of internal exposure through urine or plasma analysis of target chemicals, metabolites, or reaction products are useful to link exposures to health outcomes. Biologically persistent chemicals are well-characterized with traditional methods, whereas short-lived chemicals are effectively measured only if the individual is undergoing continuous or continual exposures or if the timing of exposures is known. In particular, urinary excretion mainly represents recent mycotoxin intake, whereas measurements in plasma/serum are more likely to represent long-term exposure. Very often urine is the matrix of choice, as it is easily collected, however, its limitation is the differing urine excretion owing to different fluid intakes. This can be addressed partially by normalization for the creatinine concentration of a urine sample. In exposure studies it is always recommended to collect 24-h urine. Stability studies revealed that a wide range of target analytes were stable for 12 h at 25 °C post-collection (Njumbe Ediage et al., 2012), but to avoid fermentation problems that can alter any of the components of the sample, conservation at 2-4ºC is recommended.

Table 1. Biomarkers of mycotoxin exposure.

Mycotoxin	Biomarker	Validated	Reference
Aflatoxin B1	AFM1 in urine	Yes (1.2-2.2% of ingested AFB1))	Zhu et al (1987)
	Aflatoxin –N7-guanine adduct in urine	Yes (0.2% of ingested AFB1)	Groopman and Kensler (1993)
	AFB1 in urine		
	AF-Alb in plasma	No	
		No specified	Chapot and Wild, 1991
Fumonisin B1	FB1 in urine	Yes (0.08-0.5 of ingested FB1)	Van der Westhuizen et al. (2011)
	HFB1 in urine	No	Riley et al. (2012)
	Sa/So in plasma	Yes	Shephard and Van der Westhuizen (1998)
Deoxynivalenol	'total DON' (free DON+DON released by b-glucuronidase) in urine	Yes	Turner et al. (2008)
	DON in urine	No	
	DOM in urine	No	
Ochratoxin A	OTA in urine	Yes	Gilbert et al. (2001)
	OTα in urine	No	
	4-OH OTA in urine	No	
	OTA in plasma	Yes	Breitholtz et al. (1991)
Zearalenone	ZEA+α-zearalenol+ß-zearalenol in urine	No	
	ZEA-14-GlcA in urine		
	ZEA–Glu in urine		
	ZEA+α-ZOL+ß-ZOL in plasma	Yes	Prelusky et al. (1989)
Fumonisin B1	FB2 in urine	No	
HT-2 toxin	HT-2 toxin in urine	No	
Citrinin	CIT in urine	No	
	DH-CIT in urine	No	
	CIT in plasma	Yes	Blaszkewicz et al. (2013)
T-2 toxin	T-2 toxin in urine	No	

4. Analytical methods to identify mycotoxins biomarkers in urine

Traditional biomonitoring implies developing analysis protocols for each mycotoxin. This multiplies the volume of sample required, and may be slower and more expensive. Most mycotoxin exposure assessments in developing countries have focused on the AFs and FB1, while DON and its modified forms were usually monitored in developed countries. Recently, an increasing number of studies include biomonitoring of a range of mycotoxins, as a result

217 of the advent of the latest generation of high performance LC-MS/MS instruments, however,
218 they rarely include simultaneously contaminants other than mycotoxins.

219 Traditional biomonitoring of aflatoxins, has been mainly carried out through AFM1
220 determination in urine. The method of choice has been usually competitive enzyme-linked
221 immunosorbent assays (ELISA) kits, or IAC clean-up followed by HPLC-FD detection.
222 UPLC-MS/MS methods are nowadays preferred. In these cases, urine samples are
223 centrifuged, diluted in IAC column compatible buffers, cleaned up, evaporated to dryness and
224 suspended before injection in LC-MS/MS (Jager et al., 2014).

225 Total urinary DON (free DON plus DON-GlcA) has been usually analysed using
226 immunoaffinity enrichment and liquid chromatography mass spectrometry (LC-MS)
227 quantification according to Turner et al. (2008). This method includes a β-glucuronidase
228 treatment of the centrifuged and pH- adjusted sample, and a subsequent clean-up using a
229 DON IAC column, with final LC-MS detection (Wallin et al., 2013). Based on samples
230 analysed with or without enzymatic treatment, it has been observed that free DON is 22% of
231 the total urinary DON (Srey et al., 2014). Gratz et al. (2013b) developed a similar method of
232 extraction and clean-up, for urine samples analysis for DON+DON-glucuronide and de-
233 epoxy-deoxynivalenol (DOM-1) using an LC-MS/MS for detection.

234 Exposure to OTA has been traditionally analysed through urine dilution and clean-up using
235 IAC prior HPLC-FD quantification of OTA and OTα aglycones (Manique et al., 2008;
236 Duarte et al., 2015). However, the increasing evidence of the presence of OTA glucuronides
237 has led to the search for an indirect method, i.e. by comparing levels of OTA aglycone in
238 urines without and after enzymatic hydrolysis. Considerable increases in OTA levels are
239 found after enzymatic hydrolysis in some urine samples which provides evidence for the
240 excretion of OTA-conjugates. Thus enzymatic treatment of urine samples with ß-
241 glucuronidase/arylsulfatase is recommended before samples clean-up with IAC (Muñoz et al.,

2017). Analysis of OTA in enzymatically hydrolized urine samples have demonstrated considerably higher detection frequencies for OTA than when those samples were directly analysed by LC-MS/MS, even when OTA-8-β-glucuronide is used as standard, as it shows very low sensitivity for the metabolite compared to detection of OTA (aglycone) due to a far lower ionization efficiency (Muñoz et al., 2017). This suggests that OTA conjugates may escape detection when direct ('dilute and shoot') methods are applied for urinary biomarker analysis (Ali et al., 2018).

Regarding fumonisins, IAC or SPE (Oasis® MAX cartridge, Waters, UK, Gong et al., 2008) clean-up prior detection by HPLC/MS system is preferred. A highly sensitive method has been optimized for FB1 and FB2 determination in urine using IAC followed by liquid chromatography with tandem mass detection (Silva et al., 2009a). Urine has been also analyzed to identify the surrogates of fumonisin exposure sphinganine (Sa) and sphingosine (So). The most common method of choice is HPLC–FD after prior derivatization with o-phthaldahyde or naphthalene-2,3-dicarboxaldehyde. An optimized method for urine also included the isolation of exfoliated cells followed b extraction with ethyl acetate prior derivatization (Silva et al., 2009b).

Nowadays multibiomarker studies are often undergone by using separation by LC and detection using triple-quadrupole analyzers coupled via an electrospray ionization (ESI) interface. However, the degree of sample purification greatly differs from one study to another. An excellent review on the main analytical issues related to multibiomarkers determination was published by Warth et al. (2013) and also covered by Vidal et al. (2018).

The first multibiomarker studies in human urine involved separated IAC clean-up for each toxin of interest and pooling of the purified extracts, or using multi-IAC containing a range of antibodies against the more relevant mycotoxins, AFM1, OTA, FB1 and FB2 (Ahn et al., 2010). Similarly, using IAC concentration, DON, T-2 toxin, HT-2 toxin, ZEA, OTA, AFB1,

aflatoxin B_2 (AFB2), aflatoxin G_1 (AFG1), aflatoxin G_2 (AFG2), as well as FB1 and fumonisin B_2 (FB2) were analysed in urine by LC-MS/MS (Rubert et al., 2011). AFM1, OTA, DON, DOM-1, α-zearalenol/β-zearalenol (α-ZOL/β-ZOL) and FB1 were simultaneously analysed by LC-MS/MS (plus other SPE after IAC for subsequent sample preparation, Solfrizzo et al., 2011). Another urinary biomarker study applying an LC-MS/MS method for the simultaneous determination of DON, OTA, FB1, AFB1, ZEA, T-2 toxin and citrinin (CIT), as well as their main metabolites in human urine, was developed and validated (Ediage et al., 2012). The urine samples required solvent extraction and SPE clean-up prior to analysis by LC-MS/MS. Later, a method developed by Njumbe Ediage et al. (2013) covered seven mycotoxins and several important conjugation and breakdown products (in total 18 analytes). Sample cleanup was optimized in a progressive procedure where urine samples were extracted with ethyl acetate/formic acid (99:1, v/v) followed by strong anion exchange (SAX) SPE cleanup of the acidified aqueous fraction. The combined extracts of the evaporated organic phase and the SAX eluate were injected into the LC-MS/MS system.

Simultaneously, a multi-biomarker method was developed based on the LC-MS 'dilute-and-shoot' approach for the direct detection of the 15 most relevant key mycotoxin metabolites in human urine without extract purification (Warth et al., 2012). This rapid method utilized an UHPLC system in tandem with a QTrap 5500 LC-MS/MS system equipped with a Turbo electrospray ionisation source. The urine sample was simply diluted 1:10 with acetonitrile/water (10:90) and injected directly into the LC-MS/MS system. The resulting LODs defined as the signal-to-noise ratio of 3:1 were for each analyte as follows: AFM1 (0.05 µg/L), FB1 (0.5 µg/L), FB2 (0.5 µg/L), OTA (0.05 µg/L), DON (4.0 µg/L), DON-3-GlcA (6.0 µg/L), DOM-1 (10 µg/L), NIV (3.0 µg/L), T-2 toxin (2.0 µg/L), HT-2 toxin (20 µg/L), ZEA (0.4 µg/L), zearalenone-14-glucuronide (ZEA-14-GlcA) (1.0 µg/L), α-ZOL (0.5 µg/L), and β-ZOL (0.5 µg/L). Besides the simplification, the advantage of this workflow is

292 the full recovery of the polar conjugates such as glucuronides which are frequently lost
293 during sample cleanup. The disadvantage of the dilute and shoot approach is the need for the
294 latest state-of-the art triple-quadrupole mass analyzer to achieve the very low LODs required.
295 Even when these highly advanced instruments are used, only moderate to high exposure is
296 detectable, rather than very low background levels. Some other authors have used such
297 'dilute and shoot' approach (Abia et al., 2013), using H_2O/ACN/FA as dilution solvent
298 (Gerding et al., 2014), 1% ammonium acetate (Vidal el al., 2016), or just injection without
299 dilution (Huybrechts et al., 2015).

300 Interestingly, Shephard et al. (2013) compared results of urine analysis both with sample
301 clean-up (single and multi-biomarker) and by a 'dilute-and-shoot' multibiomarker method.
302 Firstly, urinary FB1 was separately determined using a tailor-made single target method as
303 previously described (Gong et al., 2008) (SPE+LC-MS/MS), secondly, DON, AFM1, FB1, α-
304 ZOL, β-ZOL, ZEA and OTA) were determined as previously described (Solfrizzo et al.,
305 2011) (enzymatic treatment +IAC+SPE+UPLC-MS/MS), finally, urine samples were
306 analysed for the biomarkers FB1, FB2, AFM1, OTA, DON, DON-3-GlcA, DON-15
307 glucuronide (DON-15-GlcA), DOM-1, nivalenol (NIV), T-2 toxin, HT-2 toxin, ZEA, ZEA-
308 14-GlcA, and α- and β-ZOL using a 'dilute-and-shoot' method without prior β-glucuronidase
309 treatment as previously described (Warth et al., 2012). The single biomarker method detected
310 FB1 (87% incidence; mean ± standard deviation 0.342 ± 0.466 ng/mg creatinine) and DON
311 (100% incidence; mean 20.4 ± 49.4 ng/mg creatinine) after hydrolysis with β-glucuronidase.
312 The multi-biomarker 'dilute-and-shoot' method showed only 51% of FB1 positive samples,
313 with a maximum value of 2.59 ng/mg, and indicated that DON-15-GlcA was predominantly
314 present. The multi-biomarker method with β-glucuronidase and immunoaffinity clean-up
315 determined ZEA (100%; 0.529 ± 1.60 ng/mg creatinine), FB1 (96%; 1.52 ± 2.17 ng/mg
316 creatinine), α-ZOL (92%; 0.614 ± 1.91 ng/mg creatinine), DON (87%; 11.3 ± 27.1 ng/mg

creatinine), β-ZOL (75%; 0.702 ± 2.95 ng/mg creatinine) and OTA (98%; 0.041 ± 0.086 ng/mg creatinine). Given its higher LOD, lower incidence was reported for the 'dilute-and-shoot' method. On the other hand, the tandem clean-up procedure led to higher mean and medium values than using SPE clean-up only. Low correlation was observed among the different methods for FB1 detection. Better correlation was found among DON biomarkers of exposure (either DON or DON glucuronides). Similarly, Solfrizzo et al. (2103) compared single biomarker methods for DON and FB1 to multibiomarker methods (dilute and shoot and tandem IAC), and showed good performance of the three methods for DON, but questionable for FB1. Between the multibiomarker methods, acceptable performance was observed for DOM-1, AFM1, ZEA, α-ZOL and β-ZOL, but not for OTA.

Recently, Turner et al. (2017) compared Wallin et al. (2013) single method (SM) to Solfrizzo et al. (2014) multidetection method (MM) for DON and its modified forms. Both methods measure free DON plus the β-glucuronidase digest of DON glucuronides. A higher number of samples were <LOQ by using the MM method probably due to increased LOD as a results of an increase of matrix effect, that is, higher ion suppression. The higher matrix effect could be due to the use of an SPE column for urine purification. However, mean DON concentrations were not statistically different ($p > 0.05$). Although the analytical approaches used in the two methods are similar, including immunoaffinity enrichment and LC-MS/MS in both, several important details are different. First, the pH of urine before enzymatic digestion was adjusted for the SM method but not the MM method. Second, the enzyme used for urine digestion was different. Third, the volume of urine analyzed was 1 mL for SM and 6 mL for MM. Fourth, the enrichment for the mycotoxin included a single-antibody column for the SM method and several distinct antibodies in the columns plus an SPE-OASIS HLB column for the MM method. Finally, the SM approach included an internal individual standard spiked at the outset of extraction, whereas the MM used a matrix-assisted calibration adjusting all

samples as the mean recovery for the method. As DON-glucuronides can represent a significant portion of the total DON in urine, it is plausible that these analytical differences may have affected the efficacy of deconjugation of DON-glucuronides.

In the later years, salting-out assisted liquid/liquid extraction methods and dispersive solid phase extraction methods have also been developed for multiple mycotoxins and metabolites analysis in urine (Song et al., 2013), linked to either LC-MS/MS or GC-MS/MS analysis (Rodríguez-Carrasco et al. 2014). That latter GC-MS/MS method has been successfully applied to a 24 h pilot diet study revealing that DON was the main mycotoxin in diet and urine among the 15 *Fusarium* toxins analyzed (Rodríguez-Carrasco et al., 2015).

Recently, some studies have specifically compared different extraction and micro-extraction techniques for *Fusarium* mycotoxins applied to human urine, showing that dispersive liquid–liquid microextraction (DLLME) was the most performant compared to salting-out liquid–liquid extraction (SALLE), miniQuEChERS (quick, easy, cheap, effective, rugged, and safe) methods (Escrivà et al., 2017b). Conversely, SALLE showed better accuracy and precision than DLLME in combination with GC-MS/MS for the determination of 10 *Fusarium* mycotoxins (Rodríguez-Carrasco et al., 2017).

A comparison of relevant multidetection biomarker methods for analysis of mycotoxins in urine is presented in Table 2. Warth et al. (2012) reviewed the main limitations encountered in multibiomarker monitoring. A range of analytical challenges were listed.

a) First is the extremely low analyte concentrations present in biological fluids following dietary exposure, thus sample preparation is crucial to obtain acceptable LODs. However, the great chemical diversity of analytes (including polar conjugates such as glucuronides which are frequently lost during common cleanup approaches such as SPE or IAC procedures) makes it difficult. IAC cleanup allows for high enrichment, however, no conjugates or other

366 biomarkers/analytes of interest can be included in a method, and enzymatic hydrolysis should
367 be performed to include conjugates. On the other hand, the dilute and shoot approach does
368 not need further pretreatment; however, to overcome matrix effects and interfering matrix
369 peaks, eluents, the chromatographic gradient, and the dilution factor need to be carefully
370 optimized.

371 b) Second, co-eluting matrix components can negatively influence the accuracy of
372 quantitative methods through ion suppression or enhancement in the ion source. Ion
373 suppression can be reduced efficiently by careful optimization of the eluents and gradient.
374 Using internal standards and matrix-matched calibration is critical.

375 c) Third, there is a lack of authentic reference standards and certified reference materials.

376 **Table 2**. Comparison of the number of detected biomarkers and LODs of relevant multidetection biomarker methods for analysis of mycotoxins
377 in urine.

Reference	AFM1	FB1+FB2	DON	OTA	ZEA	T-2	HT-2	Others	Clean-up	Total
Rubert et al. 2011		9	10	0.5	3	2	3	AFB1, AFB2, AFG1, AFG2	IAC	11
Warth et al. 2012	0.05	0.4	4	0.05	0.4	1	12	DON-3-GlcA, DOM1, NIV, ZEA-14-GlcA, α-ZOL, β-ZOL, DON-15-GlcA	No	15
Ediage et al. 2012	0.15	2.7	4	0.15	0.6	1	40	AFB1, CIT	SPE	7
Ediage et al. 2013	0.01	0.01	0.04	0.03	0.1	0.05	0.42	AFB1, DOM1, CIT, α-ZOL, β-ZOL, ochratoxin α (OTα), 4-hydroxyochratoxin A (4-OH-OTA), DON–3-GlcA, HFB1, ZEA–GlcA	SPE	18
Abia et al. 2013	0.05	1	4	0.05	0.4	2	20	DON-3-GlcA, ZEA-14-GlcA, DON-15-GlcA, DOM-1, NIV, α-ZOL β-ZOL	No	15
Gerding et al. 2014	0.025	0.25	0.5	0.1	0.025	0.25	2	Zearalanone (ZAN), α-ZOL, β-ZOL, AFB1, AFB2, AFG1, AFG2, dihydrocitrinone (DH-CIT), enniatin B, OTα, DON-3-GlcA, ZEA-14-GlcA, ZAN-14-GlcA, α-ZOL-14-GlcA, β-ZOL-14-GlcA, and HT-2-4-GlcA	No	24
Rodriguez-Carrasco et al. 2014			0.12		3	0.5	1	DOM1, 3-acetyl-DON, fusarenone-X (FusX), diacetoxyscirpenol (DAS), NIV, neosolaniol , ZAN, α-zearalanol, ß-zearalanol, α-ZOL, ß-ZOL	Dispersive SPE	15
Huybrechts et al.	0.002	0.1	0.2	0.001	0.02	0.01	0.2	AFB1, AFB2, AFG1, AFG2, CIT, OH-CIT, DAS , DON-3-GlcA, DON-15-GlcA, 3-ADON, 3-ADON-15-	No, except for	32

2015			GlcA, 15-ADON, 15-ADON-3-GlcA, DOM1, DOM1-GlcA, FusX, OTA, ZEA-14-GlcA, α-ZOL, α-ZOL-GlcA, α-ZOL-14-GlcA, β-ZOL, β-ZOL-14-GlcA	OTA, CIT, AFM1 (IAC)	
Vidal et al. 2016	0.5	0.003	OTα DON-3-glucoside, 3-ADON, DOM-1 DON-3-GlcA	No	8

5. Analytical methods to identify mycotoxins biomarkers in blood

Biomonitoring of aflatoxins in blood was initially carried out by directly analyzing this group of toxins (including AFB1, AFB2, AFG1, AFG2, AFM1, aflatoxin B_{2a} (AFB2a), aflatoxin BG_{2a} (AFG2a), aflatoxin P (AFP) and aflatoxicol) in serum samples by 2D-TLC, previous extraction with dichloromethane and purification on a silica gel column (Hatem et al., 2005), or by RP-HPLC with fluorescence detection, previous extraction of samples with chloroform and hexane (Lopez et al., 2002).

However, nowadays the determination of the AFB1-albumin (AFB1-Ab) adduct in serum (adduct formed with the lysine amino acid of albumin) is more frequently used, as it has been demonstrated that the concentration of this adduct in serum is strongly correlated with aflatoxin intake, which makes it a very useful exposure biomarker (Wild et al., 1992). Adducts could be formed not only with AFB1, but also with the other main aflatoxin (AFB2, AFG1 and AFG2). Besides, the AFB1-Ab adducts presents a half-life in the organism of around 2-3 months, what makes this compound a good biomarker to reflect exposures over long periods of time, in contrast to what happens with the AFB1-N7-guanine adduct biomarker in urine, that better reflects day-to-day variations in aflatoxin intake. Moreover, AFB1-Ab adducts are stable in serum samples stored at -80 °C for over 25 years, allowing for re-analysis of archived samples years later (Scholl and Groopman, 2008).

For the analysis of the AFB1-Ab adducts in serum, ELISA seems to be the routine analysis method; samples are previously digested with pronase, extracted and purified and measured using a competitive ELISA (Chapot and Wild, 1991; Turner et al., 2007, 2008; Gong et al., 2012; Piekkola et al., 2012; Shirima et al., 2013, 2015; Asiki et al, 2014; Chen et al., 2018). However, other techniques as RIA (Jiang et al., 2005; Tang et al., 2009), HPLC-FD (Mizrak et al., 2009; Shuaib et al., 2012) and HPLC with isotope dilution mass spectrometry (IDMS) (McCoy et al., 2005, 2008) have been also used.

In relation to FBs, direct detection of FB1 in blood samples has not been considered an appropriate biomarker, due to its rapid elimination and low oral bioavailability. Taking into account the effect of FBs on the metabolism of sphingolipids (inhibition of the ceramide synthase enzyme and increase of intracellular Sa concentration), the ratio between Sa and So (or between Sa-1-phosphate and So-1-phosphate) in plasma has been considered an indirect indicator of human FBs exposition, and therefore it has been frequently used. However, this ratio has been considered useful in studies with animals, but in human exposure studies, when the level of food contamination is relatively low, wide ranges of Sa:So ratios and bad correlation coefficients have been observed when linear regression was fitted, which suggests that this ratio present low sensitivity and imprecision in humans (Cano-Sancho et al., 2010). The analytical method used to determinate these metabolites frequently included plasma deproteinization, liquid-liquid extraction, hydrolysis and purification, and HPLC-FD analysis prior derivatisation with o-phthaldialdehyde (Riley, 1994; Shephard and Van der Westhuizen, 1998; Castegnaro et al., 1998), although use of blood spots, LC-MS determination has also been used (Riley et al., 2015).

Direct OTA detection in human plasma has been widely used, as OTA binds rapidly and with high affinity to plasma proteins, constituting therefore a good biomarker of exposure (Coronel et al., 2010; Lino et al., 2008). The method of choice for detection is the HPLC-FD, and usual methods of analysis comprise liquid-liquid extraction of plasma samples, for example with acidified ethyl acetate or other solvents, and analysis by HPLC-FD with postcolumn confirmation through the formation of OTA-methyl ester (Palli et al., 1999), purification of acidified plasma samples with a C18 Sep-Pak cartridge and analysis by HPLC-FD with confirmation through the formation of OTα after carboxypeptidase treatment of samples (Creppy et al., 2005), and other similar methods (Ali et al., 2018), many of them using immunoaffinity columns (Ghali et al., 2008). Other methods used included detection of

ochratoxins in plasma by ELISA (Ueno et al., 1998) or LC/MS/MS (Lau et al., 2000; Medina et al., 2010; Cramer et al., 2015).

Regarding DON, to date, DON, DON-GlcA and DOM-1 in urine are the preferred biomarkers for the study of DON exposure. However, several attempts have been made to find DON biomarkers linked to blood samples. Thus, from studies with rodents, Kim et al. (2008) have proposed the use of plasma haptoglobin, measured using SELDI-TOF/MS as a diagnostic biomarker for DON intoxication when this is combined with examining the serum immunoglobulins. These findings have led to a patent application in Korea (patent reference number KR100809952B1) protecting a diagnostic kit for the evaluation of toxicity and exposure for DON using haptoglobin-specific protein. However, different results have been found in experiments with lactating dairy cows (Kinoshita et al., 2015), and, to date, no data are available in the case of human blood.

With respect to other mycotoxins few studies have been conducted to assess the presence of other fungal toxins in human blood. Thus, in the case of ZEA, the presence of this metabolite or its congeners (α-ZOL, ß-ZOL) has been studied in plasma of patients with breast and cervical cancer by HPLC and GC (Pillay et al., 2002), whereas Massart et al. (2008) studied the presence of ZEA and derivatives in the serum of healthy girls and affected by central precocious puberty, performing an enzymatic treatment of samples with glucuronidase, followed by purification through a immunoaffinity column and analysis by HPLC-FD. On the other hand, Fleck et al. (2016) have studied the presence of total ZEA (ZEA plus conjugated metabolites) and total α-ZOL in serum of pregnant women by UPLC-MS/MS and electrospray ionization (ESI).

Finally, for CIT, studies on human plasma have been developed by means of an acetonitrile protein precipitation followed by centrifugation and analysis by HPLC-FD and LC-MS/MS (Blaszkewicz et al., 2013; Ali et al., 2018).

Regarding plasma or serum, few studies have carried out on multi-detection analysis of mycotoxins including multiple mycotoxin biomarkers of different mycotoxins groups in one sample at the same time, mainly due to the high matrix complexity. Thus, in plasma most methods have only focused on the analysis of structurally-related mycotoxins belonging to a single family.

Osteresch et al. (2017) have developed a rapid multi-mycotoxin method, using dried whole blood spots and dried serum spots, which allows for the simultaneous detection and quantification of a great variety of fungal toxins by HPLC-MS/MS in less than 10 minutes. This method is able to detect till 27 mycotoxins, of the following groups (data of LOD in ng/mL is given): aflatoxins (AFB1: 0.012; AFB2: 0.013; AFG1: 0.021; AFG2:0.037; AFM1: 0.017), *Alternaria* toxins (alternariol: 0.142; alternariol monomethyl ether: 0.146; altenuene: 0.147), enniatins (A: 0.0016,: A1: 0.0055; B: 0.0012; B1: 0.0044), ochratoxins (OTA/2'R-ochratoxin A: 0.012; OTα: 0.014; 10-hydroxyochratoxin A: 0.015), T-2/HT-2 group (T-2 toxin: 0.227; HT-2 toxin: 1.344; HT-2-4-glucuronide: 0.709), ZEA (0.294) and ZAN (0.273), CIT (0.066) and DH-CIT (0.268), FB1 (0.521) and beauvericin (0.014), with average recoveries above 90% in most of the cases.

De Santis et al. (2017) have described a method for the analysis of 8 mycotoxins (AFB1, AFM1, FB1, OTA, ZEA, DON, DOM-1, and gliotoxin –GLIO–) that combine pronase treatment of serum samples followed by QuEChERS purification and LC-MS detection. Limits of quantification were low for AFB1 (0.01 ng/mL), AFM1 (0.22 ng/mL) and OTA (0.16 ng/mL), but in other mycotoxins were above 5 ng/mL (DON, DOM-1) or 11 ng/mL (GLIO). The absolute recoveries of the method were not too high, since, with the exception of AFB1 (82%), all the toxins had recoveries below 63%.

Cao et al. (2018) have also described a method for the quantitative determination of mycotoxins in human plasma, as well as in other biological matrices (like urine) and animal

derived foods, by HPLC-MS/MS. This method is valid for the determination of aflatoxins in human plasma (data of LOD in ng/mL is given) (AFB1: 0.07; AFB2: 0.05; AFG1: 0.13; AFG2:0.15; AFM1: 0.16), as well as of FBs (FB1: 0.41; FB2: 0.39), sterigmatocystin (0.05), PAT (0.35), CIT (0.18) and OTA (0.15). Sample preparation consisted in a treatment of plasma with ß-glucuronidase, deproteinization with acetonitrile/acetic acid and evaporation.

Recently, Slobodchikova and Vuckovic (2018) have described a LC-MS method for the simultaneous detection of 17 mycotoxins in human plasma. Studied mycotoxins are NIV, DON, 3-ADON, 15-ADON, T-2 toxin, HT-2 toxin, AFB1, AFB2, AFG1, AFG2, ZEA, ZAN, α-ZOL, ß-ZOL, α-zeranol, ß-zeranol and fusarenon X (FusX). The method avoids the use of immunoaffinity columns thanks to a three-step liquid-liquid extraction procedure with ethyl acetate. LOQ of all mycotoxins ranged from 0.1 to 0.5 ng/mL, except NIV (3 ng/mL). This method is not suitable for OTA, FB1 and FB2

Covering a smaller number of mycotoxins, Serrano et al. (2015) have developed a method for the simultaneous determination of enniatins (A, A1, B and B1) and beauvericin in human plasma by HPLC-MS/MS. The method consisted in the deproteinization of samples with MeOH/H2O (40/60, v/v) followed by solid phase extraction, using in-house prepared Carbograph-4 SPE column, and detection by HPLC-tandem mass spectrometry with an electrospray ion source. Experimental LOD obtained were 10 ng/L for enniatins A1 and B, 20 ng/L for enniatin B1 and beauvericin, and 40 ng/L for enniatin A, and recoveries ranged between 90 to 120%.

6. Biomarkers of mycotoxins in breast milk

The use of breast milk in biomonitoring studies and epidemiological birth cohort studies is gaining interest due to the large chemical information contained and the easy collection methods resulting in a non-invasive and valuable biological matrix. By default, breastfeeding is considered the “gold-standard” diet for infants, however it has been questioned the

potential health risk associated when mothers are subjected to contaminated diets. The tight relationship between blood and breast milk compartments results in high and rapid transference of lipophilic chemicals, however the transference of mycotoxins from blood to human breast milk and overall occurrence, has been scarcely explored (Reviewed by Warth et al., 2016). The high interest of studying the concentrations of harmful chemicals in breast milk is justified not only by the exploration of mother's internal exposure levels but also the external exposure of infants during critical windows of development. The vulnerability is reflected by the low maximum tolerable levels established in baby foods and infant formulas by regulatory agencies, which enforce those products to rigorous inspections. Surprisingly, little effort has been addressed to evaluate the levels of mycotoxins in breast milk and risk-benefits derived from breastfeeding. The preparation of samples commonly involves immunoaffinity columns, liquid-liquid or solid-phase extraction, and the major methods of detection are based on ELISA kits, liquid HPLC-FD and LC-MS/MS. Maternal determinants of AFM1 in breast milk determined by ELISA included the season of collection, education level, lactation stage or consumption of rice and chocolate (Bogalho et al., 2018). The implementation of multi-mycotoxin detection methods in breast-milk remains as a major challenge nowadays, yet few studies have published screening exploratory studies (Andrade et al., 2013; Rubert et al., 2014). As recently summarized by Warth et al. (2016), most studies have evaluated the occurrence of AFM1 (Brazil, Cameroon, Columbia, Egypt, Iran, Italy, Jordan, Kuwait, Nigeria, Serbia, Sudan, Tanzania and Turkey) or OTA and related metabolites (Chile, Egypt, German, Iran, Italy, Poland, Slovakia, Turkey, Brazil and Chile). Conversely, few studies have explored the levels of AFB1 (Turkey and Egypt), FB1 (Tanzania) or ZEA (Italy). 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 and 44000 ng/L (Warth et al., 2016). These values appear specially

528 concerning if we consider the maximum concentration levels set up by the European
529 Commission in infant formula was 0.025 ng/mL (European Commission, 2006).

530 **7. Internal exposure of general population to mycotoxins.**

531 In the last few years an increasing number of studies have been published on assessment of
532 exposure to mycotoxins in different countries using biomarkers. Most single biomarker
533 studies dealt with exposure to AFB1 through AFM1 determination in urine. Moderate to high
534 frequencies were reported all over the world, depending on the LOD of the methods used. In
535 general, mean and median values under 0.1 ng/mL were detected in different countries in
536 Asia, America and Europe. Higher absolute concentrations were reported in some countries
537 in Africa (up to 3.7 ng/mL) (Smith et al., 2017). Using direct detection of AFB1 in blood,
538 values from different studies ranged from 0.08 to 7.4 ng/mL, whereas when the AFB1-
539 albumin biomarker was used the values ranged from not detected to values as high as 268
540 pg/mg. A good review about these data can be found in Waseem et al. (2014).
541 Secondly, DON exposure through urine analysis was assessed mainly in European countries,
542 where frequencies in the range 90-100% were usually reported in urine samples, with 12%
543 found as free DON and 88% as DON glucuronides (Wells et al., 2016). Mean levels of DON
544 were around 10 ng/mL, while when total DON was assessed higher levels were reported, and
545 higher total levels could be over 400 ng/mL (Wells et al., 2016). Several studies confirmed
546 that a significant percentage of the populations were exposed to levels over the TDI. Lower
547 frequencies of occurrence were observed in other countries like Bangladesh or Tanzania,
548 where the different dietary habits may be determinant.
549 Finally, those studies devoted to OTA in Europe reported widely variable frequencies, but
550 low general levels (mean under 0.3 ng/mL) (Ali et al., 2018; Wallin et al., 2013; Duarte el al.,
551 2015). In blood, OTA has been detected in a great number of studies, with OTA occurrence
552 frequently over 74% and usually over 90% (Coronel et al., 2010; Waseem et al., 2014), and

553 with a global estimation (derived from a big number of published studies) of minimum,
554 maximum and mean levels of 0.15, 9.15 and 0.45 ng OTA/mL plasma, respectively (Coronel
555 et al, 2010).
556 Differences in nutritional habits and quality of consumed foodstuffs are likely the reason for
557 interregional variations in mycotoxin excretion.
558 Regarding multibiomarker studies, as shown in Table 3, DON, OTA and AF were the more
559 often searched and detected mycotoxins, and they co-occurred in most samples. The
560 frequencies for DON and OTA were high, but low for AFM1 (in contrast to what observed
561 using single analysis). Only one study reported frequent exposure to ZEA (Solfrizzo et al.,
562 2014). The detected levels, in general, paralleled those observed in the single biomarker
563 studies, with high concentration for total DON, followed by FB1, DH-CIT, OTA, total ZEA
564 and AFM1. Nevertheless, differences in analytical methodology and diversity in available
565 biomarkers limit comparison of the results.
566 In contrast to what happens with urine, to date there are not many multimycotoxin studies
567 conducted in blood (Table 3).
568 De Santis et al. (2017) studied 8 different mycotoxins (AFB1, AFM1, ZEA, OTA, FB1,
569 DON, DOM-1, GLIO) in the serum of autistic patients and two control groups (one of
570 siblings and the other of non-parental persons). In all groups OTA was the prevalent
571 mycotoxin, with mean prevalence of 82.9% of samples in the whole group and 85.1% in the
572 autistic. For the rest of mycotoxins, all mean values found were below LOQ. Few samples
573 showed co-occurrence of different mycotoxins (AFB1, AFM1 and OTA in 4% samples, and
574 AFB1, AFM1, OTA and GLIO in 2% samples).
575 The most complete is a recent study by Cao et al. (2018), developed in the People's Republic
576 of China, in which the plasma of 30 healthy individuals has been analyzed and compared to
577 that of 30 hepatocellular carcinoma patients. Eleven mycotoxins were simultaneously

analyzed by HPLC-MS/MS. In the plasma of control patients the most frequently mycotoxin found was AFB2 (1.37-3.89 ng/mL; 16.6% samples), followed by AFB1 and sterigmatocystin (13.3%), and AFG1, AFG2, AFM1, FB1 and FB2 (3.3%). No PAT nor CIT were found in these samples. Higher percentage of positive samples was found in plasma from hepatocellular carcinoma patients, with sterigmatocystin being the more frequently found mycotoxin (1.06-3.23 ng/mL; 40%), followed by AFB1 (33.3%) and AFB2 (23.3%). However, in plasma AFG1, AFG2, AFM1, OTA and CIT were detected just at the LOD of the method in both kinds of samples, authors not excluding that these results could be false positives.

587 Table 3. Mycotoxins detected in urine and blood/plasma samples through multidetection methods (%positives/mean (ng/mL))

Urine biomarkers

Reference Country (Samples)	DON	DON-GlcA	OTA	AFM1	ZEA	∝-ZOL	β-ZOL	FB1-FB2	DH-CIT
Gerding et al. 2015 Haiti (142)	17/3.2	21/17.0	33/0.109	8/0.06		3/1.46		3/0.44	14/0.49
Germany (50)	16/2.0	54/11.2	30/0.040	n.d.		n.d.		n.d.	28/0.12
Bangladesh (95)	n.d.	n.d.	76/0.203	8/0.06		n.d.		1/-	75/2.75
Solfrizzo et al. 2014 Italy (52)	96/11.89		100/0.144	6/0.068	100/0.057	100/0.077	98/0.090	56/0.055	
Wallin et al. 2015 Sweden (252)	63/5.38		51/0.90			21/0.13	18/0.10	6/0.07	
Abia et al. 2013 Cameroon (175)	6/-	41/5.49	16/0.09	9/0.05	2/0.22	1/-		3/0.63	
Gerding et al. 2014 Germany (101)	29/3.38	82/12.21							12/-
Heyndrickx et al. 2015 Belgium (239)	37/3.9	100/61.3	35/0.278			0.4/0.005			12/0.752
Ezekiel et al. 2014 Nigeria (120)	0.8/2	5/3.5	28/0.2	14/0.3	0.8/0.3			13/4.6	
Warth et al. 2014 Thailand (60)		12/12.4	2/-	5/0.33					

Blood/serum biomarkers

Reference Country (Samples)	DON	DOM-1	OTA	AFB1-AFB2	AFG1-AFG2	AFM1	CIT	ST	PAT	ZEA	FB1-FB2	GLIO

De Santis *et al.* 2017 Italy												
Control group 1 (35)	22.9/0.5 12.5/0.8	17.1/0.3 6.3/0.1	77.1/0.27 75/0.28	25.7/0.002 (AFB1) 6.3/0.00 (AFB1)		45.7/0.07 31.3/0.06				8.6/0.1 0/0	2.9/0.04 0/0	14.3/06 18.8/10.3
Control group 2 (18)												
Cao *et al.* 2018 PR of China (30)			traces	13/0.95-1.78 (AFB1) 16.6/1.37-3.89 (AFB2)	3.3/0.61 (AFG1)-0.43(AFG2)	3.3/0.57	traces	13/0.88-2.05	n.d.		3.3/1.92 (FB1) 3.3/2.03 (FB2)	

588

589

590 Table 4 summarizes the calculated daily intakes from the mycotoxin concentration in urine in
591 some multibiomarker studies. In summary, between 6 and 29% of the populations considered
592 were exposed to DON at levels over the TDI, suggesting a medium but worrying risk for the
593 population, and at the same time they could be exposed to OTA or AFB1 levels of concern.
594 Since AFB1 is a potent mutagenic carcinogen, no TDI values are established. The presence of
595 AFM1 in urine samples is of concern. The TDI of 2 µg/kg b.w. for FB1 was never exceeded
596 by healthy population.

597

598 Table 4. Calculated daily intake from some exposure studies (mean/max/% exceeding TDI).

Reference (n)	**DON** TDI 1 µg/kg bw	**OTA** TDI 0.017 µg/kg bw	**AFB1** - -	**FB1** TDI 2 µg/kg bw
Gerding *et al.* 2015 Haiti (142)	0.27/4.38/6		0.03/0.23/-	0.05/1.74/0
Germany (50)	0.3/2.15/6		-	-
Bangladesh (95)	-		0.03/0.195/-	0.03/1.362/0
Solfrizzo *et al.* 2014 Italy (52)	0.59/3.37/6	0.139/2.07/94	0.668/0.142/-	0.274/1.759/0
Abia *et al.* 2013 Cameroon (175) HIV sub-populations	0.21/2.59/-	0.004/0.094/-	0.0425/1.15/-	5.25/123.3/-
Gerding *et al.* 2014 Germany (101)	0.52/5.67/12			
Heyndrickx *et al.* 2015 Belgium (239)	1.24/10.08/29	0.001/0.021/1		

599

600 Multi-detection methods have allowed assessing the levels of co-exposure to different
601 mycotoxins by an individual through urine analysis. Consequently, it has been confirmed that
602 co-occurrence of two toxins in a urine sample is usually common (more than single
603 contamination), however results depend highly on the analysed toxins, if only parent
604 mycotoxins were analysed, 1-2 toxins are usually reported, while if both parent and modified
605 mycotoxins are analysed 2 to 4 toxins are usually found in a sample. Moreover, lower LOD
606 of the methods led to higher reported co-occurrence. For example, Gerding et al. (2015)
607 reported that between 16-54% samples contained two detectable toxins, between 6 and 20
608 samples contained 3 toxins and 1-2% contained 4 toxins. DH-CIT and OTA usually co-
609 occurred as well as DON and DON-GlcA, and also 3 of them. *Fusarium* toxins and OTA
610 have been also shown to occur (Wallin et al., 2015), for example, DON, ZEA, OTA and
611 DON, ZEA, FB1, OTA, co-occurred in 38 and 52% of urine analysed samples (Solfrizzo et
612 al., 2014). Studies on exposure in Cameroon reported 4% co-exposure to AFM1 and DON,
613 3% to OTA and DON and 5% to DON and NIV (Abia et al., 2013).

614 **8. Co-exposure of mycotoxins with other environmental chemicals**

615 A major research gap is the potential concurrent exposure of mycotoxins with other
616 environmental chemicals that may exhibit some interactive activity and/or exert some
617 biological function converging in the same molecular pathways. As far as we know, there are
618 not biomonitoring studies exploring the simultaneous presence of a panel of environmental
619 chemicals including some type mycotoxin. However, the estimates relying on dietary
620 exposure modelling suggest that multiple patterns of co-exposure are likely within general
621 population. The research on mixture identification from the second French Total Diet Study
622 revealed the extended exposure to mycotoxins in complex mixtures with other environmental
623 chemicals in most of French diet clusters. For instance, a first cluster containing 18% of the
624 whole population, was expected to have a significantly higher exposure to mycotoxins (HT-2

toxin, DON, ZEA and NIV), polycyclic aromatic hydrocarbons (PAHs) (pyrene and phenanthrene) and bisphenol A, than the whole population. The estrogenic ZEA was also identified in another cluster with many PAHs, acrylamide, trace elements, pesticides and the sum of eight polybrominated diphenyl ethers in a cluster representing the 21% of the population with dietary habits related to junk food and identified as "Snacking" (Traore et al., 2016). Using a similar approach based on the identification of consumption systems integrated with exposure data, different clusters of pregnant mothers from the two large French cohorts "Étude Longitudinale Française depuis l'Enfance" (ELFE) and "L'étude des déterminants pré et post natals du développement et de la santé des enfants" (EDEN), were identified to be more exposed to mycotoxins simultaneously with other environmental chemicals. The model was comprehensive including 210 chemicals of which 18 were major mycotoxins or parent compounds. The "Myco-Pest-PAH" mixture identified from EDEN before pregnancy was also found in EDEN during pregnancy. It contained eight mycotoxins (α-ZAL, α-ZOL, diacetoxyscirpenol (DAS), DON-3-GlcA, FusX, OTB, OTA and HT-2 toxin), three pesticides (chlorpyrifos-methyl, cyproconazole and pirimiphosmethyl) and four PAHs (benzo[g,h,i]perylene, benzo[e]pyrene, cyclopenta(c,d)pyrene and indeno[1,2,3-cd]pyrene). In EDEN before pregnancy, these substances were associated with nine other pesticides (pyriproxyfen, tetradifon, sulphur, chlorothalonil, diethofencarb, flutriafol, iprodione, ethion and bifenthrin) and an additive (sulphites). In EDEN during pregnancy, these substances were associated with three other mycotoxins (DON, DON-15-GlcA and ZEA), a PAH (pyrene), two phytoestrogens (daidzein and genistein), a trace element (gallium), a pesticide (sulphur) and two perfluoroalkyl acids, perfluorobutane sulfonate and perfluorohexanesulfonic acid (Traore et al., 2018). Despite the uncertainties related to the dietary modelling methodologies, these results provide strong evidence concerning the potential co-exposure of highly bioactive mycotoxins like α-ZEA with many environmental

chemicals during highly sensitive developmental windows. These modelling studies provides also light on the potential weight of mycotoxins in the human chemical exposome, as suspected by the extensive occurrence of mycotoxins in diets.

These profiles extracted from a European diet only represent a region where strict mycotoxin control regulation is enforced, underscoring the weight of mycotoxins in the chemical exposome of population in developing countries. The "traditional" fungal contamination of cereals with the mycoestrogen ZEA, has been identified as a major public health challenge co-existing with emerging chemical exposures resulting of unstructured industrial development resulting on high exposures to lead, air pollution, pesticides or e-waste by-products (Bornman et al., 2017).

We have failed to find in the literature examples of targeted approaches that use liquid or gas MS methods for the simultaneous detection of mycotoxins and environmental contaminants in biological specimens. An inspiring example is the method developed by LC-MS with previous SPE for the simultaneous determination of mycotoxins (AFB1, OTA and PAT) and bisphenol A in food matrices that could be adapted and applied for urine samples (Song et al., 2013). Novel analytical workflows based on HRMS untargeted metabolomic approaches may become efficient solutions to overcome existing analytical challenges for the screening of large panel of chemicals including well-known chemicals. A proof-of-concept study has recently presented a novel workflow for analysis of blood and urine based on HPLC coupled to Bruker Impact II quadrupole time-of-flight (Q-TOF) mass spectrometer with a previous simple sample preparation (Warth et al., 2017). The panel of detected features are further explored using machine-learning algorithms combined to the XCMS/METLIN platform to elucidate the pathways related to the annotated signature. Through the pilot study the authors

demonstrated to efficiently identify low concentrations of common xenoestrogens such as genistein, ZEA and triclosan at in commercial biological matrices.

9. Mycotoxins and human health effects

The disease caused by mycotoxins is called mycotoxicosis. Mycotoxins can be a threat to both animal and human health. Oral ingestion of contaminated food is the most frequently exposure way, however dermal contact and inhalation can also occur (Marin et al., 2013). Their toxic effect depends on the toxicity of each mycotoxin, the extent of exposure, age and nutritional status of the individual and possible synergistic effects with other chemicals that the individual is exposed. Infants is considered the most vulnerable population group due to the relative inefficiency of detoxification pathways and high relationship of between internal doses per body weight.

There are more than 300 known mycotoxins which are suspected of widely differing modes of action, however formal toxicological evaluation and comprehensive risk assessment have been conducted only for environ 10 of most known or major mycotoxins. Consequently, very little is known about the potential toxicological and biological effects of secondary mycotoxins, metabolites or emerging mycotoxins.

To date, most toxicological evaluation is based on experimental studies, including *in vitro* and *in vivo* studies, conversely the body of evidence from human studies is limited to few epidemiological studies or case studies conducted shortly after human mycotoxicosis outbreaks. An overview of major health effects of mycotoxins at different toxicological levels is summarized at Table 5, nonetheless readers may find more detailed reviews published in the literature (Bui-Klimke et al., 2015; EFSA 2017; Kensler et al., 2011; Marin et al., 2013; Peraica et al., 1999; Puel et al., 2010).

Table 5. Overview of major health effects of mycotoxins at different toxicological levels.

Mycotoxin group	Interaction Nuclear Receptor	Cellular responses	Health effects Animals	Health effects Humans

AFB1 AFM1	Pregnane X receptor Constitutive androstane receptor Aryl hydrocarbon receptor Vitamin D receptor	Formation of DNA adducts Lipid peroxidation Bioactivation by cytochromes P450 Conjugation to GS-transferases	Hepatotoxicity Genotoxicity Carcinogenicity Immunomodulation	Cancer Impaired child growth
FB1	-	Inhibition of ceramide synthesis Adverse effect on the sphinganine/sphingosine ratio Adverse effects on the cell cycle.	Central nervous system damage Hepatotoxicity Genotoxicity Immunomodulation	Oesophageal cancer Liver cancer Neural tube defects Impaired child growth
OTA	-	Effect on protein synthesis. Inhibition of ATP production Detoxification by peptidases	Nephrotoxicity Genotoxicity Immunomodulation	Nephritic syndrome BEN
PAT	Ø	Indirect enzyme inhibition *In vitro* mutagenesis	Neurotoxicity Immunotoxicty Disruption of barrier function	-
DON NIV T-2 toxin HT-2 toxin	Peroxisome proliferator-activated receptor Liver X receptor Retinol X receptor G-protein coupled receptor	Apoptosis Oxidative stress Inhibition protein synthesis	Hematotoxicity Immunomodulation Skin toxicity Anorexia and vomiting Reduced weight gain Disruption of barrier function	Hormone-dependent cancer Acute mycotoxicosis
ZEA ZOL	Estrogen Receptor-α and -β	Binding to oestrogen receptors Bioactivation by reductases Conjugation to glucuronyltransferases	Reproductive adverse effects	Thelarche Precocious puberty Breast cancer

Among the toxicological initiating events, the interaction of most mycotoxins with nuclear receptors has been scarcely explored (reviewed by Dall'Asta, 2016). The exception is the case of ZEA and ZOL whose potent actions to activate the oestrogen receptor pathway and trigger endocrine perturbations merits an entire section presented hereafter. AFB1 has been found to modify the expression of nuclear receptors such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (AhR) at

705 transcriptional level, and also causing the downregulation of vitamin D receptor. Hormone
706 exocytosis caused by DON was found to be triggered by G-protein coupled receptor (GPCR)-
707 mediated $Ca2^{+}$ signaling, using the murine neuroendocrine tumor STC-1 cell line (Zhou and
708 Pestka, 2015). PAT has been evaluated against different endocrine disrupting models and no
709 studies have revealed effects on reporter gene assays at the receptor level.
710 A broad range of adverse health effects have been identified for mycotoxins in animals and
711 humans, including hepatotoxicity, estrogenicity, immuno/haematotoxicity, nephrotoxicity or
712 neurotoxicity. And some of them are recognized as genotoxic and/or carcinogenic, including
713 AFB1, one of the most carcinogenic food contaminants in human diets and classified as
714 carcinogenic to humans by the International Agency for Research on Cancer (Group 1), or
715 OTA and FBs classified as possible carcinogens (Group 2B). AFB1 is a primary cause of
716 human hepatocarcinoma, and in developing countries it acts synergistically with the hepatitis
717 B virus infection.
718 Mycotoxins also affects the intestinal barrier function, impairing the permeability and
719 integrity of epithelial cells. Most prominent effects have been document for the trichothecene
720 DON that may strongly impair the expression, localization and function of tight junction
721 proteins which seal the epithelial monolayer and prevents the para-cellular diffusion of
722 luminal antigens and microorganisms. Other trichotecenes including T-2 and HT-2 toxin and
723 mycotoxins such as PAT or FB1 have been found to impair some of the physiological
724 parameters that characterize the intestinal barrier function (Akbari et al., 2017). On this basis,
725 it has been hypothesized the role of mycotoxins in the pathophysiology of chronic intestinal
726 inflammatory diseases, such as inflammatory bowel disease, and in the prevalence of food
727 allergies.
728 The potential effect of mycotoxins in infant growth parameters has been recognised as a
729 priority research gap, especially in developing countries, where the high occurrence of

mycotoxins comes together with the high prevalence of intrauterine growth restriction, infant and young stunting, underweight wasting. Whereas no epidemiological studies have been conducted to evaluate the associations between exposure to DON or ZEA and infant growth parameters, some studies that analysed exposure to AFs and FBs consistently found negative associations (Lombard et al., 2014). For instance, in African countries, strong dose response relationships were found between exposure in utero and/or early infancy to AFs and growth retardation, identified by reduced birth weight and/or low weight-for-age or height-for-age Z scores (Turner et al., 2013).

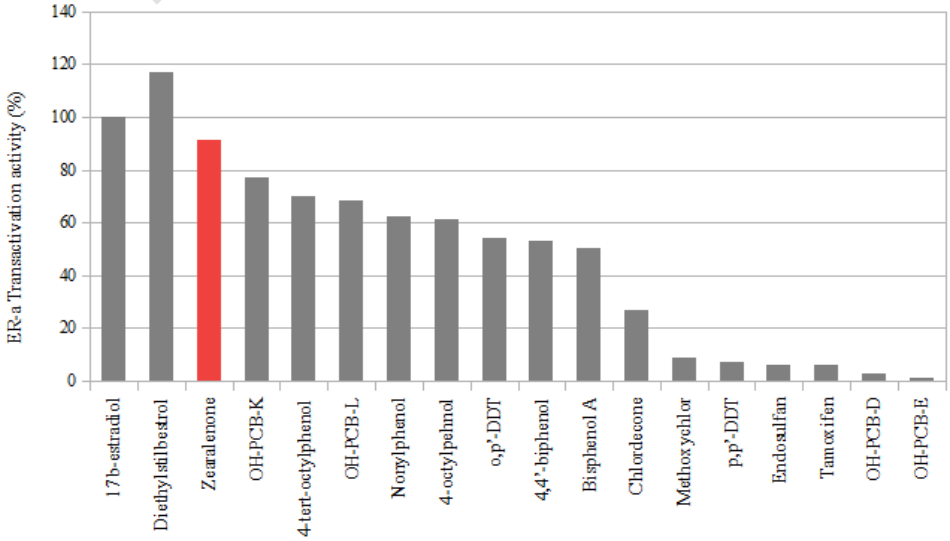
Another important aspect to be considered is that many foods can present a simultaneous presence of different food contaminants, like mycotoxins, pesticides, heavy metals or radioactive particles (Kosalec et al., 2009). This multi-contamination can strongly modify the toxic effects of some of them resulting in a range of interactive effect as demonstrated by the simultaneous exposure of Caco-2 cells and HEK-293 kidney cells to cadmium and DON (Le et al., 2017). The toxicological evaluation of combinations of mycotoxins for the characterization of potential interactions is an emerging and very active field of research (Alassane-Kpembi et al., 2017).

10. Mycotoxins as endocrine disruptors: case of ZEA and its derivatives

The Endocrine Society's Scientific Statement on Endocrine-Disrupting Chemicals (EDCs) says that an EDCs is "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action" (Gore et al., 2015). The particular mechanism of actions of EDCs represent a novel paradigm in chemical risk assessment introducing new challenges related to ability of inducing biological effects at very low concentrations or the presence non-monotonic dose-responses. Hence, the emerging concern with EDCs emphasize the high interest of including mycotoxins, specially ZEA and its metabolites within the panel of

755 targeted analytes in exposome projects addressing hormone-dependent diseases. Despite the
756 knowledge about the endocrine disrupting action of ZEA and its strong oestrogenic effects
757 was identified decades ago (recently reviewed by Kowalska et al., 2016 and Metzler et al.,
758 2010), little attention has been paid by most researchers on EDCs who has extensively
759 focused on industrial compounds such as bisphenol A or phthalates. Occurrence studies have
760 demonstrated the pervasive presence of ZEA in cereal-based foods and the extended exposure
761 among general population supporting that ZEA and specially the metabolite ZOL may be a
762 relevant contributor on the total body burden of xenoestrogenic activity.
763 *In silico* and *in vitro* studies have demonstrated the high affinity of ZEA and ZOL to bind and
764 activate estrogen receptor, exhibiting potencies similar to 17-β–estradiol, and substantially
765 higher than many industrial xenoestrogens such as bisphenol A (See Figure 1, based on
766 Kuiper et al., 1998). Metabolite ZOL has non-estrogenic chemical structure but resembles
767 that of 17-β–estradiol, exhibiting similar key contacts in the binding pocket of ER, resulting
768 on high bioactivity (Delfosse et al., 2014; Balaguer et al., 2017). For that reason, a-ZON is
769 recognised as one the most active xenoestrogens that can modulate ER activity at
770 concentrations as low as 0.1 nM. (Balaguer et al., 2017).

771 Figure 1. Relative transactivation activity of environmental endocrine disruptors for
772 estrogenic receptor alpha created from data published by Kuiper et al., (1998). ZEA appears
773 highlighted in red.

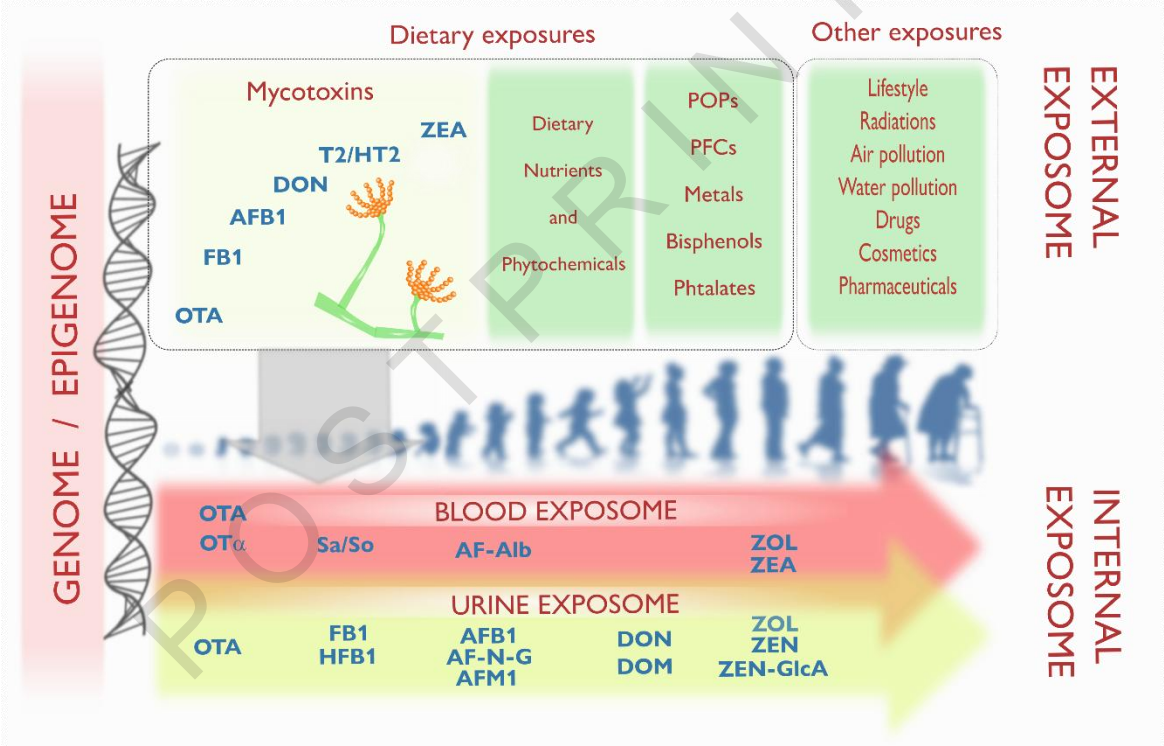


774

775 The related effects of ZEA and its derivatives in cells include the stimulation of growth of
776 estrogen receptor-positive human breast cells, stimulation of cell cycle progression of MCF-7
777 cells (Metzler et al., 2010). It has been also shown that ZEA may affect the metabolism of rat
778 adipocytes, including the stimulation of basal lipolysis and reduced epinephrine stimulated
779 lipolysis (Kandulska et al., 1999), suggesting ZEA also as a metabolic disruptor candidate.
780 The endocrine disrupting effects of ZEA in animals includes the impairment of reproduction,
781 uterotropic activity, hyperoestrogenism and inflammation of the vagina, endocrine-disruptive
782 effects during gestation and neonatal life vaginal cornification, persistent estrus, reduced
783 fertility, anovulation and decreased gonadotropic hormone output by the hypophysis among
784 others (Kowalska et al., 2016; Metzler et al., 2010). Despite the large evidence suggesting the
785 potential hormone disrupting effects of ZEA, few epidemiological studies have been
786 conducted in humans to explore associations with estrogen-dependent diseases. Food
787 contaminated with ZEA and its natural metabolites was associated with the development of
788 precocious puberty, a risk factor for breast cancer (Gray et al., 2017). Furthermore, higher
789 urinary ZEA levels, resulting from recent intake of beef or popcorn, were inversely
790 associated with the onset of breast development (Bandera et al., 2011).
791
792 **11. Mycotoxins within the human exposome: challenges and opportunities**
793 The mycotoxins constitute a large group of chemicals that can be found regularly in foodstuff
794 worldwide often resulting in the chronic exposures of low doses of complex mixtures of
795 mycotoxins concurrently with industrial chemicals, phytochemicals and nutrients as
796 represented in the Figure 2. The fast excretion and the low concentration levels of
797 mycotoxins challenge their detection in biological samples, however current evidence
798 demonstrate that major mycotoxins can be commonly found within the urine and blood
799 chemical spectra (Marin et al., 2013). Dietary exposure assessment studies support that

infants and children are the most exposed population groups and the mycotoxin exposures continue through the entire life. Despite the relevance in terms of exposure and health effects, mycotoxins are often underscored and/or overlooked in epidemiological research as acknowledged by the visionary Christopher Wild (Wild and Gong, 2009), and no attention have received by major exposome projects launched in Europe, such as the impressive HELIX or EXPOsOMICS projects.

Figure 2. Conceptual representation of the mycotoxin compartment within the human exposome framework including the sum of the most important mycotoxins that may found in the diet and respective forms or metabolites that are used as biomarkers in blood and urine.



The novel exposome paradigm proposes a chemical-agnostic approach, that appears as an excellent opportunity to evaluate the role of mycotoxins in human health through more integrative approaches. This approach contrasts with the historical expertise of scientist that have been focusing on specific chemicals or group of chemicals. Hence, it looks like a big communicative and cross-talk effort will be required to efficiently optimize the already

available knowledge across disciplines. For instance, the mycotoxin compartment of human exposome, has been pretty well characterized for the main mycotoxins in terms of exposure and health effects, despite little is known about the rest of mycotoxins, modified forms and more emerging toxins.

To date, most mycotoxin exposure assessment studies have been based on dietary modelling approaches although many limitations exist on these indirect exposure assessment methods, especially if we consider the uncertainties related to these modelling methods, and also the intra-individual or seasonal variability. Hence biomonitoring methods are considered the 'gold-standard' to evaluate the individual exposures, however the field is still on its emergence and few biomarkers have been fully validated. The biomonitoring studies applied to mycotoxins have been mainly focused on surveillance of general population with regulatory or risk assessment purposes and few epidemiological studies have considered the evaluation of mycotoxins. Methodological approaches used to detect the mycotoxin biomarkers will strongly determine the performance (e.g. sensitivity), resulting on dramatically different results depending on the detection/quantification thresholds achieved (p.e. direct vs indirect methods). The development of reliable, accurate and sensitive multibiomarker methods to simultaneously characterize a large panel of mycotoxins, but also industrial pollutants, will strongly help to understand the potential associations between environment and health. On this sense, the application of non-targeted or semi-targeted HRMS metabolomic methods appears as a promising screening approach to identify exposure risk factors, and related biomarkers of biological perturbation (Warth et al., 2017). It appears also as a great opportunity to explore the underlying toxicological effects of mycotoxins in humans. Coupling other OMICs platforms for the identification of endogenous chemical signatures we may gain access to early biomarkers of health effects and biological perturbation triggered by mycotoxins. In any case, a list of challenges associated with the

accurate determination of biomarkers of non-persistent pollutants exposure applies directly to the mycotoxins, including the high intra- and inter-day individual variability, requiring repeated sampling protocols to avoid the exposure misclassification (Perrier et al., 2016). Additionally, specific issues related to mycotoxins such as the variability related to seasonal or weather influences on mycotoxin productions will challenge the estimations of individual trajectories.

The simultaneous determination of mixtures of mycotoxins within more complex cocktails of environmental pollutants will allow address major research gaps related to mixture effects. A growing interest on the effect of mycotoxin mixtures have led toxicologist dosing binary and tertiary combinations of mycotoxins, sometimes with little similarities on mechanism of action, whereas few or non-studies have evaluated the simultaneous effect of mycotoxins with other environmental pollutants with similar biological actions (e.g. the xenoestrogens bisphenol A and ZEA).

12. Concluding remarks

In the present review we have shown that mycotoxins maybe commonly found at high concentrations in blood and urine from individuals from developing countries, but also frequently found at moderate or low concentrations in developed regions. For instance, it has been estimated that 500 million of the poorest people in sub-Saharan Africa, Latin America, and Asia are exposed to mycotoxins at levels that substantially increase mortality and severe diseases (Wild and Gong, 2010). The health effects of mycotoxins are very wide, targeting different toxicological endpoints, biological functions and have been related with multiple diseases. To date, few studies have been able to demonstrate consistent associations of health effects in humans relying most of studies on animal or *in vitro* settings.

We strongly believe that mycotoxins represent a relevant component of the human exposome and that exposome-based projects aiming to explore the role of chemical exposome on human health, should strongly consider the mycotoxins within the panel chemical candidates.

Whereas industrial chemicals may be banned and set out of the market, mycotoxins will not be completely removed of raw foods and food items intended for human consumption. Even with very stringent regulations, humans will continuously be exposed to low level of mycotoxins whose combined effect and their combined effect with other environmental exposures whose effects are completely unknown. As we have shown in this document, currently there are available robust and accurate analytical methods that allow the identification and characterization of multiple mycotoxins and/or their metabolites in most common biological samples, allowing the direct implementation in epidemiological research.

Overall, we acknowledge that the exposome projects will be a great opportunity to better translate the knowledge generated on mycotoxicology during the past decades in environmental health. Conversely, these new approaches may be an excellent opportunity to fill many research gaps on mycotoxins research as the identification of associations mycotoxins with human health, elucidation of join effect with other environmental exposures or the decipher of underlying molecular mechanisms by using advanced OMICs technologies.

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Figure

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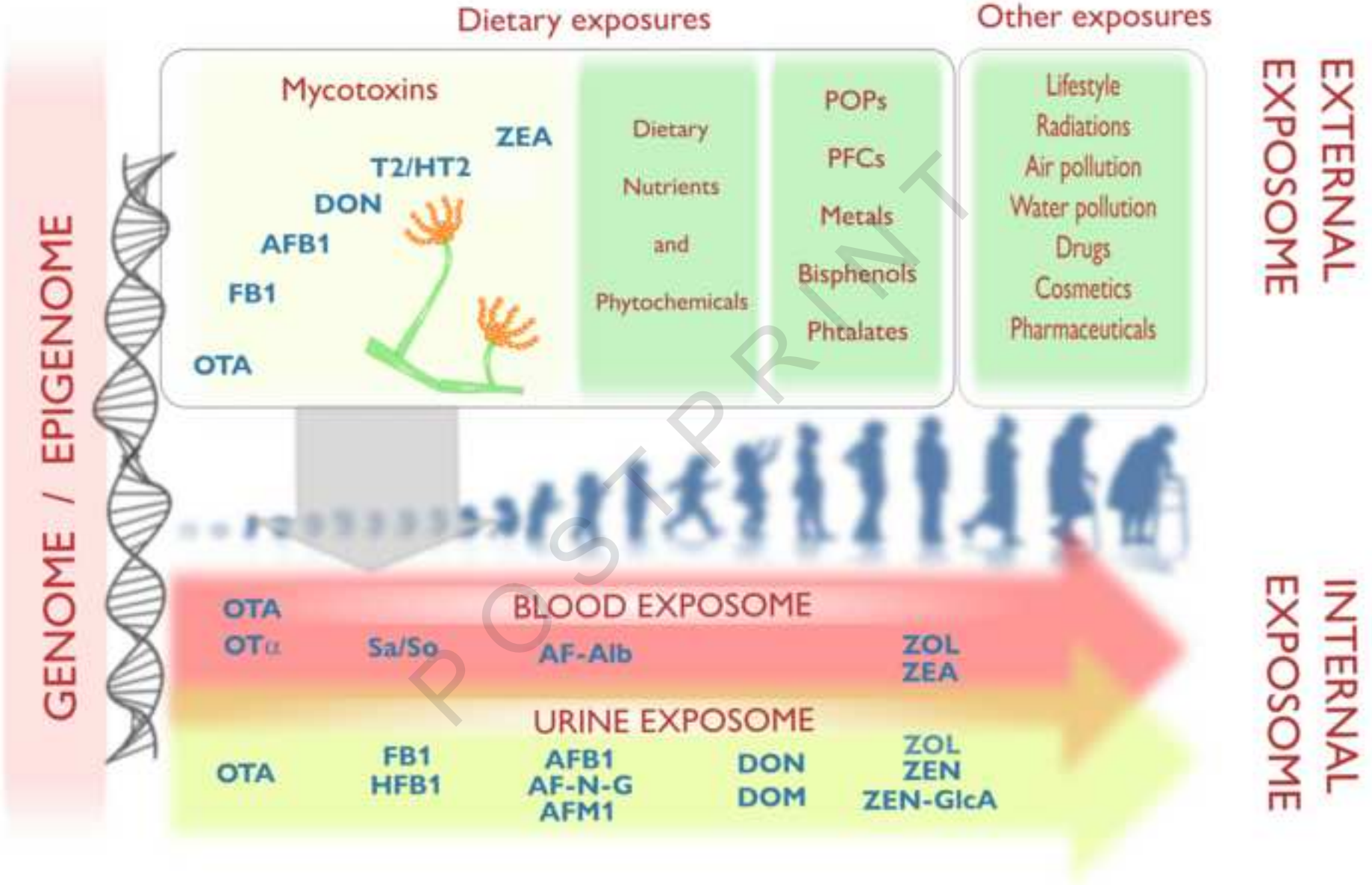

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