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Biomarkers of Mycotoxin Exposure in Humans

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Mycotoxins are secondary metabolites of certain species of fungi and they are frequent contaminants of agricultural products. Contamination of humans by dietary intake may result in various pathophysiological effects, such as nephrotoxicity, gastro-enteric distress, hyperestrogenic conditions, cancer etc.. Therefore, exposure of human population to these compounds needs to be controlled and assessed.

Risk assessment of a toxicant is evaluated with the help of biomarkers which provide information on biological responses following contamination (biomarkers of effect) or allow the quantification of the toxicant or its biotransformation products in body fluids (biomarkers of exposure). The biomarkers are needed to establish the oral dose (intake from food), the internal dose (biologically active) and the dose-response relationship.

Here we describe a series of biomarkers of exposure and effect for aflatoxins, fumonisins, ochratoxin A, zearalenone and deoxynivalenol. For aflatoxins, except the possibility of quantifying the original chemicals per se in biological fluids, the hydroxylation products AFM1 and AFM2 are good indicators of internal exposure, while the albumin adducts and DNA adducts are markers of effect. Ochratoxin A is easily detectable in blood, urine and milk, but its considerably long half-life makes it difficult to correlate with the level of exposure. The main biomarker for fumonisins is the sphinganine:sphingosine ratio, while for deoxynivalenol exposure is evaluated by its presence in urine. For zearalenone there is no reliable biomarker.

Introduction

Mycotoxins are currently a major health threat in human population. The mycotoxins of most importance worldwide, generally contaminating cereals and derived products are the aflatoxins, fumonisins, ochratoxins, deoxynivalenol and zearalenone. Considering the numerous pathophysiological effects of mycotoxins in humans, there is a stringent need of risk assessment (the etiologic relation between the contaminant and a certain condition or illness) [3] and of control for exposure to these compounds. Animal exposure to mycotoxins is also important to observe, due to the possibility of human

contamination from animal product consumption (among which it is notable to mention the milk).

The assessment of exposure to any dietary contaminant is presently evaluated based on its intake from food (or feed), also known as 'external exposure' or 'oral dose' [41]. The most common method of evaluating the dietary intake is based on the occurrence of the contaminant in food products and on consumption data. However, only a certain fraction of the compound reaches the blood stream and is able to exert toxic effects, which represents the 'internal exposure'. The internal exposure is obviously lower than the external one, this leading to confounders in the risk assessment. The effective biological dose is quantifiable with the help of pharmacokinetic studies, in vitro studies or animal models, while risk assessment is described by epidemiologic studies in humans [64].

There are two terms used to describe the uptake of the contaminant within the body: bioaccesibility and bioavailability, both parameters being extremely variable and dependent on a number of factors further discussed in this paragraph. Bioaccesibility is defined as the amount of contaminant that is released from the food and can be absorbed. The bioavailability represents the fraction of this amount which is most likely to reach the systemic circulation. One of the factors that influence these parameters is the extent of contamination of foods; in order to evaluate it, cost-efficient analytical methods and standard operating procedures need to be yet imposed and optimized worldwide. Another issue is that the nature of the food matrix determines the amount of contaminant that is released in the digestive tract; as a result, the same concentration of toxin may or may not exert certain effects *in vivo*, depending on the source of contamination. Moreover, the interaction of the toxin with other dietary components should be taken into consideration, meaning that the individual life-style interferes with the risk assessment of any contaminant [78]. Following uptake, the behavior and the end-point of the assessed compound is a matter of metabolism or biotransformation. It is notable to mention that the two terms are not equivalent, considering that some toxins are (also) chemically modified by the microbionta in the gut, which suggests that 'biotransformation' would be the most suitable term. As a consequence, bioactivation or bioinactivation of the parent compound can occur, depending on the nature of the enzymes existent in the gut, the local microorganisms and the mode of transport across the intestinal epithelium, in other words they are species-specific [9]. Many recent articles suggest that biotransformation is to a considerable extent also individual-specific, seeing that different isoforms of certain

enzymes involved in the metabolism of the original contaminant have selective or variable affinities for their substrates. This leads to the question whether the genetic background of the individual is responsible for the susceptibility to a toxin and if so, to what extent.

The link between the observed disease occurrence and a certain dietary contaminant is made by biomarkers, which provide information on factors that are causative or explanatory towards the respective condition. Biomarkers are distinguishable in two categories: direct (biomarkers of exposure) and indirect (biomarkers of effect). A biomarker of exposure refers to the quantification of the specific compound, its metabolite(s) or interaction products in a body compartment or fluid, which indicates the presence and magnitude of exposure to the agent. Ideally, such a marker should reflect the toxicokinetics, transformation and fate of the assessed contaminant in the body. A biomarker of effect indicates the presence and magnitude of a certain biological response following exposure to the agent [28]. These are generally non-specific of a certain compound, but provide more information on its cumulative effects in relation to other possible environmental factors that should also be taken into consideration. The biomarkers of susceptibility are also to be mentioned; they have been defined as inherited or acquired individual markers that predispose to an increased risk of developing a certain disease. Regardless of the type, all biomarkers should be specific, sensitive and as less invasive as possible. The value of a biomarker is mostly determined by two aspects: sensitivity and specificity [9]. Sensitivity is related to the possibility of detection of the assessed contaminant or its metabolites when found in low amounts either in food or biological samples; the limit of detection is an issue, considering that significant biological effects may occur in humans following long term exposure to such low levels [3]. Specificity is the certainty of associating the marker only to the assessed outcome and not to other unrelated effects.

The most common parameters in quantifying exposure or effect are available from urine, serum and milk, although for some of the toxins there are a few other biological matrixes that can provide us with important information, like feces or hair. So far, aflatoxins have been most studied and therefore a number of biomarkers are available for them, in contrast to Zearalenone which literature has failed to provide us yet with a suitable biomarker for. Nevertheless, the sole correlation of these markers with the oral dose of ingested mycotoxins is not sufficient for the correct assessment of exposure and risk.

Aflatoxins

Aflatoxins are secondary metabolites of *Aspergillus flavus* and *A. parasiticus* and are frequently found in nuts, soya beans, maize and others, especially in areas with appropriate conditions of moisture and heat where these fungi are ubiquitous. The naturally occurring aflatoxins are AFB1, AFB2 (with their hydroxylation products AFM1 and AFM2 found in milk), AFG1 and AFG2.

<u>Aflatoxin metabolism:</u>

AFB1 is metabolized by cytochrome P450 (CYP), more precisely CYP1A2 in humans, to AFB1-8,9-exo-epoxide that binds to DNA to form adducts (AFB1-N7-Gua and the more stable AFB1-FAPY) that lead to G to T transversions in the third nucleotide of codon 249 (p53 gene). This compound can also react with RNA and proteins. Another isoform of P450 (CYP3A4) metabolizes AFB1 to AFB1-endo-epoxide which is less toxic considering that it cannot bind nucleic acids and can be excreted under different forms. Both the exo- and endo-epoxides can undergo rapid non-enzymatic hydrolysis to AFB1-8,9 dihydrodiol, reacting with the -amino group of lysine in serum albumin [81]. Literature data has shown that AFB1 can be transferred through human placenta and metabolized by local enzymes. The placenta contains low levels of P450 enzymes, with variations depending on the stage of placental development. However, the tissue-specific lipoxygenase is capable of epoxidating AFB1 to ALF [36]. Detoxification of the epoxide can also occur enzymatically via conjugation to glutathione, mediated by glutathione S-transferase [31].

Contamination of human milk with AFM1 is of considerable concern, seeing that infant exposure to these compounds can start during prenatal life, due to their ability to cross the human placenta and accumulate in it [17]. However, a recent epidemiological study in Egyptian females also revealed AFB1, AFB2, AFG1 and AFG2 in breast milk [51].

AFB1 has been linked to hepatocellular carcinoma (HCC) in humans, growth impairment, immunosuppression and neural tube defects in animal models. AFB2, AFG1 and AFG2 are generally considered to be a lot less biologically active due to the absence of an 8,9 double bond [81].

Biomarkers of exposure:

Up to the present time, a number of biomarkers of exposure have been developed and applied:

Urinary aflatoxins:

Human studies have shown that AFM1 and AFB1-mercapturic acid (metabolites on the detoxification pathway) are indicators of recent exposure to aflatoxins. The excretion of AFB1-N7-Gua in urine is an indicator of actual genetic damage due to AFB1 exposure. Other urinary biomarkers are AFP1 and AFQ1, less toxic than the parent compound [43], [53], [81].

1-hydroxypirene (1-OHP) was recently accepted as urinary biomarker of polycyclic aromatic hydrocarbon (PAH) exposure, which associated with aflatoxin exposure significantly increases the risk of HCC, as do infections with hepatitis B virus [25].

Biomarkers of effect:

1. DNA mutations:

The direct consequence of DNA binding of aflatoxins is the introduction of point mutations. The G to T transverse mutation in the third nucleotide of codon 249 of the P53 gene on human chromosome 17 in tumoral hepatocytes is direct evidence that aflatoxins are hepatocarcinogenic in both humans and animals [8], [23], [81].

Chronic HBV infection is a risk factor for HCC and is sufficient to induce mutations in the 246 codon, according to studies conducted in Europe, North America and Japan [82]. However, the highest risk of HCC was later on found among individuals both exposed to aflatoxins and chronically infected with HBV [25].

2. DNA adducts:

AFB1-DNA adduct formation is one of the key events in human aflatoxicosis and their levels are proportional to the levels of exposure to AFB1, having been described in rat liver [66] and human colon and cord blood [10]. The formation of such structures in rat liver, kidney, lung and spleen occurs in a linear dose-response relation. In humans, there is less tendency for the formation of adducts as in rats, but they were detected in the colon, both the ring-opened FAPY1 and FAPY2 and the non-ring opened AFB1-N7-Gua [10]. The mutagenic properties of the DNA adducts are chemically explained by their

ability to intercalate between base pairs in the 5' position, where the exo-epoxide enters a nucleophilic reaction with the N7 of the guanine base [82].

As biomarkers of exposure, AFB1-RNA adducts are more sensitive, but show more variability [66].

3. Aflatoxin-albumin adducts

Considering that the half-life of albumin is relatively long (21 days), AFB1-albumin adducts provide information on cumulative exposure over 2 to 3 months [84]. The only protein adduct described of AFB1 is the Lys product. The formation of this compound may contribute to the acute toxicity of AFB1 and increase its carcinogenic properties [19]. Adducts have been found in peripheral blood [10], [69] and cord-blood [71].

4. Salivary IgA levels:

Secreted IgA (sIgA) in saliva, breast milk and mucus of the digestive, genitourinary and bronchial tract has an opsonizing role for bacterial and viral surface antigens. Aflatoxin exposure was significantly associated with decreased levels of salivary sIgA in Gambian children, even with non-detectable aflatoxin-albumin adducts. Considering the local high risk of exposure to aflatoxins and high prevalence of infections, these results suggest that aflatoxins may increase susceptibility to infectious diseases [74].

Fumonisins

The main fumonisin-producing fungus is *Fusarium verticillioides*, but these mycotoxins can also be produced by other species, including *F. proliferatum*, *F. anthophilum*, *Alternaria alternata* etc.. Fumonisin is commonly found in corn and in addition in some other agricultural products, however the Fusarium species are ubiquitous in moisture-damaged buildings, so exposure of human population to fumonisins may also occur by air contamination [26].

So far there have been identified 15 types of fumonisins, classified as A, B, C or P. The most common of them are Fumonisin B1 (FB1), FB2 and FB3, with FB1 being the

most prevalent and apparently the most toxic. Therefore most studies have been focused on FB1, also considering that the other derived are usually found in very low concentrations. FB1 was classified as a possible human carcinogen, seeing that the occurrence of F. verticiloides in corn has been linked to human esophageal cancer. In animal models, FB1 was shown to be neurotoxic, carcinogenic and immunotoxic, the most affected organs being the kidneys and the liver [26]. Also, neural tube defects (NTD) in human embryos have been linked to maternal consumption of fumonisin contaminated corn products during pregnancy, along the Texas-Mexico border in 1991. It has been proposed that the toxins indirectly induce folate deficiency, which is a major factor for NTD [26], [81].

Mechanism of action of FB1:

Structurally, fumonisins resemble sphingolipids, which allows them to interfere biochemically with the sphingolipid metabolism. In the normal metabolic pathway of ceramide synthesis, sphinganine is acylated to dihydroceramide and ceramide by the ceramide synthase enzyme (sphinganine N-acyltransferase) [19]. FB1 was proven to inhibit the ceramide synthase in rats [26], [60], [5] and mice [30], which leads to elevated sphinganine and sphingosine levels in serum and urine.

FB1 metabolism:

The gastric absorption rate of FB1 is very low and the toxin does not undergo major metabolic changes, being found unaltered in urine and biliary excretion in animals [60]. Its serum half-life in humans is of approximately 128 minutes [11], followed by excretion in urine and feces.

Biomarkers of exposure:

Free FB1:

FB1 is in principle detectable in urine, serum and feces [60] and in absence of any metabolite it has been proposed as a biomarker of exposure, despite the fact that the detectable concentrations in all physiological fluids are quite low, approaching the limit of detection. Moreover, the detection window of the toxin is very narrow, so only recent exposures can be quantified. Nevertheless, the correlation of this marker to fumonisin exposure is still to be investigated in humans.

Fumonisins in human hair:

Sewram et al. showed for the first time in 2003 that FB1, FB2 and FB3 can accumulate in human hair as a result of contaminated maize consumption [59]. Hair analysis is relevant in measuring exposure to fumonisins regardless of source or intake profile, in terms of low, medium or high exposure. However, there is a wide degree of interindividual variability of results, due to ethnicity, age, gender and other factors.

<u>Biomarkers of effect:</u>

Sphinganine and Sphinganine:sphingosine ratio

The disruption of sfingolipid metabolism results in an increase of sphinganine levels and of sphinganine to sphingosine ratio (Sa:So).

Although older studies promote usage of Sa as biomarker of effect, recently it has been suggested not to be reliable in relation to the fumonisin exposure levels [80]. The SaSo ratio is a useful marker in animals, but it failed to correlate human exposure to fumonisins in a satisfactory way, probably because the exposure in human population is generally low. Moreover, the normal variation of Sa:So ratio is considerably large and fluctuates over time regardless of the levels of exposure to the toxin. Several studies conducted by the same author in Europe, Africa and South America also show that the Sa:So ratio in urine and plasma may not be a relevant marker [60], [80].

Despite this, considering that most of the studies in literature promote these two parameters as valid in evaluating fumonisins effect, they cannot yet be excluded as biomarkers.

Sphinganine 1-phosphate and Sa-1-P/So-1-P ratio

Sphinganine 1-phosphate (Sa-1-P) has been shown to be in relation with exposure to fumonisins. The cytological role of this compound is not fully understood and it is yet to be clarified how cells convert Sa into Sa-1-P. Remarkably, Sa-1-P/So-1-P in serum is more elevated than the Sa/So ratio, following FB1 exposure [30].

Ochratoxins

Ochratoxins are a group of secondary metabolites of the *Aspergillus* and *Penicillium* genera and contaminate cereals, coffee, dried fruit and other products. This group consists of Ochratoxin A, its methyl ester, its ethyl ester (Ochratoxin C), 4-hydroxyochratoxin A, Ochratoxin B with its methyl and ethyl esters and Ochratoxin . OTA is the most toxic of the ochratoxins.

Ochratoxin B (OTB) often co-occurs with ochratoxin A (OTA) in cereals and it was identified as being a metabolite of OTA within *in vitro* studies. It is 10 times less toxic than OTA.

Ochratoxin C (OTC) also co-occurs with OTA. *In vivo*, it can be converted into OTA, but at the same time, it was found as a metabolite of OTA in ruminants [55].

Ochratoxin A

OTA exerts many toxic effects in human and animals, especially nephrotoxicity, being considered the main etiologic agent of the Balkan Endemic Nephropathy (BEN) and closely associated to chronic interstitial nephropathy (CIN) and urinary tract tumors (UTT). In humans, OTA was proven to impair kidney function by inducing collagen secretion in damaged epithelial cells of the proximal tubules, inflammation [57] and apoptosis [55].

<u>OTA metabolism:</u>

In human serum, it has a half-life of about 35 days, which is due to the ability of this mycotoxin to bind more than 99% of the serum proteins: albumin [77] and lower molecular weight plasma proteins [67]. It is thought to interact with Lys, Arg and His residues within the proteins [55]. The long-time persistence of OTA is also a result of recycling via biliary excretion and of reabsorbtion in the renal tubules [56]. The mycotoxin is slowly eliminated in urine and feces.

Biotransformation of OTA is dependent on the cytochrome P450 enzymes, such as Cyp3A4, Cyp3A5 and Cyp2B6. OTA can undergo hydroxylation, glucurono- and sulphate-conjugation and glutathione conjugation [55]. However, the major metabolic pathway of OTA is represented by its hydrolysis to Ochratoxin , by cleavage of the

peptide bond, which can occur enzymatically (by carboxipeptidases, trypsin, -chemotripsin and cathepsin C) in the presence of the large intestine microbionta.

The role of biotransformation in OTA-related toxicity is yet to be elucidated; the only metabolite found so far in humans was Ochratoxin , which is actually a transformation product of the gut microbionta.

<u>Biomarkers of exposure:</u>

OTA was detected in several human fluids: serum, urine, breast milk and cord blood.

1. Blood OTA:

OTA is ubiquitous in human blood (serum and plasma), being an indicator of continuous exposure to the mycotoxin [42], [86]. Its levels are significantly higher in the blood of patients having certain kidney disorders than in healthy people. However, significant intra-person, regional and seasonal variation of this parameter was observed and further investigation is needed to better correlate OTA concentration to exposure [58]. In regions where BEN is endemic, the majority of studies revealed that OTA amounts in blood are a lot higher than in individuals outside the endemic areas, but it is not possible to directly connect OTA to this condition, seeing that it is also found in blood samples from countries where BEN does not occur [15].

2. Urinary OTA, OTA and 2-Microglobuline:

Despite the fact that urinary levels of OTA are considerably lower than in blood, following a study in the U.K. urine was fund to be a better marker of OTA intake (Gilbert et al. 2001). Around 50% of the ingested OTA is excreted in urine, the rest being metabolites or conjugates of the toxin [58].

Both OTA and OTA were found to be ubiquitous in the urine of healthy individuals in Germany, with OTA in considerable higher amounts than the original compound [42].

2-Microglobuline, a plasmatic protein with low molecular weight, was found to be a sensitive marker of tubular dysfunction, in correlation to exposure to the toxin. It also correlates positively with the clinical diagnosis of BEN and is useful in evaluating proteinuria in CIN affected individuals [21]. However, some authors object that this is a non-specific indicator, considering that all nephropathy patients show increased levels of 2-microglobuline regardless of the cause [13].

Recently, P. Mantle and J. Nagy have shown that OTA also binds to an 2-microglobulin in adult male rats [37].

In rats, mice and pigs 4R-4-hydroxy OTA (the oxidation product of OTA by certain P450s) was shown to be a specific metabolite, frequently detected in the urine of these animals [79]. The existence of this compound in human samples is, however, controversial, as it is not always detectable [7].

3. OTA in human milk:

OTA has been detected in various concentrations in the milk of mothers in Europe, Australia and Sierra Leone [58]. Another recent study conducted in Turkey also revealed that OTA is ubiquitous in breast milk. Nevertheless, up to the present time there is no correlation between levels of the toxin in milk compared to serum, suggesting that the transfer from blood to milk is not yet a fully understood process [4].

Biomarkers of effect:

DNA adducts:

Data regarding the ability of OTA or its metabolites to form adducts with nucleic acids is controversial. Certain studies have shown formation of DNA adducts in mice [24] and humans [50] corresponding to C-C8dG OTA, but it was not obvious that they are covalently bound to OTA. Only recently, Mantle et al. brought mass spectrometry proof of an adduct isolated from mouse kidney [36].

Until recently, studies suggested that OTA is poorly metabolized and does not form reactive intermediates capable of interacting with DNA [33]. However, there is evidence that a quinine/hydroquinone redox couple derived of OTA is involved in the generation of DNA adducts [70].

Zearalenone

Zearalenone (ZEA) is a non-steroidal estrogenic mycotoxin produced by fungi of the *Fusarium* genus, reported to be involved in reproductive disorders in animals and hyperestrogenic conditions in humans. The major dietary sources of ZEA in Europe are wheat, oats and rye, respectively corn in North America. The dietary intake may also occur through consumption of meat, milk and eggs, but this is considered to be less significant, due to the rapid excretion of the mycotoxin in animals [8]. ZEA and its metabolites are considered a danger to human population, provided exposure occurs in high concentrations and over a long period of time.

<u>Toxicity of ZEA:</u>

The estrogenic effects on humans and animals are a result of interaction of the toxin or its metabolites with the estrogen receptor. They are structurally similar to 17 -estradiol, therefore act as estrogenic agonists. Human studies have shown that ZEA may be involved in the etiology of breast cancer [85] and disregulate the hypothalamic-pituitary-gonadal balance, affecting humans in the pre-pubertal stage [38].

Besides its endocrine disrupting and anabolic effects, ZEA was reported to induce hepatocellular lesions in animal models, which are likely to lead to the development of local adenomas.

<u>Biotransformation of ZEA:</u>

Malekinejad et al. showed that the biotransformation of the toxin is species-dependent and there is a small amount of data related to human metabolism of this compound. It is generally known that there are two main metabolites occurring in animals and humans: -zearalenol (-ZEA) and -zearalenol (-ZEA). -ZEA has the highest affinity to the estrogen receptor, so conversion to this compound can be regarded as bioactivation of ZEA, in contrast to -ZEA which has low affinity for the receptor, leading to bioinactivation [32]. The extent to which one of these derivates forms in vivo in detriment to the other depends on the species (-ZEA is predominant in pigs, -ZEA in chicken) [32].

Both metabolites undergo two further biotransformation pathways: hydroxylation by 3 - or 3 -hydroxysteroid dehydrogenase (HSD), and conjugation with glucuronic acid

by uridine diphospate glucuronyl transferase (UDPGT). ZEA itself can undergo conjugation with glucuronic acid [48].

Glucuronation is an important reaction in detoxification and elimination of ZEA, and in some species (e.g. pigs) it undergoes total glucuronation[32]. In vitro studies showed that ZEA and all its metabolites in humans can be conjugated [48].

Other less frequent metabolites of ZEA are zearalanone (ZAN), -zearalanol (-ZOL) and -zearalanol (-ZOL). However, these same compounds have also been found *per se* in wheat and associated products [38].

<u>Biomarkers of exposure to ZEA:</u>

Most data regarding the presence of ZEA in physiological fluids and compartments are available as pharmacokinetic studies in animal models. In rats and pigs, it was isolated from serum, urine, bile and feces following oral and intravenous administration [61]. In humans, ZEA and all its metabolites have been isolated from urine [2], but there is no data correlating their presence in urine with the extent of exposure to the toxin. Very often, ZEA derivates in urine were found to be conjugated with glucuronic acid, so it would be viable to evaluate glucuronides in humans as possible biomarkers for zearalenone.

<u>Biomarkers of effect of ZEA:</u>

A study searching potential biomarkers for Zeranol [54] found four genes having modified expression in cows: IGF and IR are upregulated in the liver, Fas-L is upregulated in the uterus, while bcl-xl is downregulated in the same anatomical site.
At the same time, several papers state that ZEA is responsible for the *in vitro* activation of Erk1/2 and therefore for cell proliferation, which explains the estrogen-receptor-dependent growth induction in animals treated with this toxin or its derived compunds [46], [83].

So far, it can be concluded that there is no biomarker of exposure or effect for zearalenone that could be used in practice.

Deoxynivalenol

Deoxynivalenol (DON) is produced by the fungi *Fusarium*, *Stachybotrys* and *Myrothecium*. It belongs to the group B tricothecenes, a family of low molecular weight toxins with sesquiterpenoid structure. Their toxic properties reside in the common 9,10 double bond and 12,13 epoxide groups that allow the tricothecenes to bind eukaryotic ribosomes, interfere with translation, gene regulation, apoptosis and neuroendocrine signaling [47].

DON is also known as 'vomitoxin', due to its emetic effects in pigs. The acute toxicity of this compound results in nausea, vomiting, diarrhea and gastro-enteric distress. Pathophysiologically, DON has been linked to anorexia, growth retardation and immunotoxicity in animal models. It is yet to be established whether DON is related to certain chronic conditions in humans, such as colitis or inflammatory bowel syndrome. However, so far it has been shown that DON has no carcinogenic effects, neither in animals nor humans [8], [47].

The toxin occurs frequently in corn, wheat and barley, alone or in combination with its acetylated forms: 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON). The latter two compounds have comparable toxic properties to DON [47].

DON metabolism:

The toxicokinetics of DON has been established in rats and swine, but it is not yet fully understood for humans. Generally, DON is rapidly metabolized and does not accumulate in tissues [47]. The mycotoxin is detectable in serum in high amounts immediately after ingestion, but is rapidly cleared from the blood stream. The main fraction is excreted by urine (animal studies suggest that this is more than 30% of the total DON) and a smaller part is eliminated in feces (2-10%) [76].

So far, it is certain that there are two major metabolites of this toxin in mammals: DON glucuronide (DON-G) and de-epoxy deoxynivalenol, also known as DOM-1 [72]-[76]. The mechanistics of DON glucuronation is not yet known; it is unclear at which anatomical site(s) and at which time point between ingestion and excretion this conversion occurs.

DOM-1 is most likely generated by the intestinal microbiota of mammals and in particular in cattle where more than 95% of DON is metabolized to DOM-1 (less toxic than the

parent compound, which explains a certain resistance of this species to the effects of the toxin). Moreover, the glucuronated form of DOM-1 (DOM-G) is also ubiquitous in cows, being found in higher amounts than the parent compound. Literature data suggests that DOM-1 is not a major metabolite in humans [73], considering that it is not found in all individuals. Remarkably, a study conducted in a population of French farmers revealed that 34% of the urine samples collected were positive for this compound, but it was undetectable in UK adults [72]. This may be due to exposure of the farmers by means of cow milk, which sometimes contains DOM-1.

DON biomarkers of exposure:

1. Urinary free DON:

Turner at al. established that there is a quantitative correlation between urinary DON and the exposure to this mycotoxin [76]. In rats, around 37% [39] of the administrated DON can be recovered in urine, out of which 8% as original compound and the rest as metabolites [75].

In human studies, the same authors identified urinary DON as being a reliable biomarker of exposure in U.K. and France populations. The toxin was detectable in approx. 99% of the samples; moreover, the levels of DON in urine significantly correlated to the amount of contaminated cereals ingested one day prior. However, the broad individual variation in amounts of excreted DON is yet to be explained [72].

2. DON-glucuronide (DON-G) and DOM-1 in urine:

The major metabolite of DON found in urine, both for animals and humans, is DON glucuronide. DON-G had been previously found in urine and serum of swine and recent data [73] showed that in humans, about 91% of the total urinary DON is represented by its glucuronide metabolite.

It is yet to be proven that DOM-1 is a relevant biomarker of exposure for deoxynivalenol in humans. Although it was detected in the urine of French farmers, it is unclear whether this is a result of DON exposure or of direct ingestion of DOM-1 from milk. It can also be the case that although DOM-1 is formed in humans, it is not excreted in urine in significant amounts. The authors also state that the samples positive for DOM-1 belonged to individuals with higher urinary free DON, which suggests that DOM-1 could be a biomarker of acute exposure to high levels of mycotoxin.

DON biomarkers of effect:

There are a few potential parameters which could be used as markers of effect for deoxynivalenol, however none of these has been validated yet.

1. Serotonin (5HT), 5-hydroxyindoleacetic acid (5HIAA) and 5HT:5HIAA ratio:

Experiments conducted in swine and poultry fed DON-contaminated grains revealed alterations in the chemistry of neurotransmitters, which were linked to feed refusal. An increase in 5HT and 5HIAA was observed in both animal models, together with modifications of the 5HT:5HIAA ratio, strongly depending on the anatomical site (cortex, pons or hypothalamus) [68].

2. Insuline-like growth factor acid-liable subunit (IGFALS)

Mouse studies revealed that deoxynivalenol leads to downregulation of IGFALS mRNA in a dose-dependent manner, following oral intake. [47]

3. Insuline-like growth factor (IGF1) and liver SOCS3 mRNA:

Considering that DON is known to have immunosuppressive effects in animal models, it is not surprising that this is due to its ability to suppress certain pro-inflammatory cytokines. Mice exposed to DON have lower plasmatic levels of IGF1 and lower expression of SOCS mRNA in the liver. These two parameters might be potential biomarkers of effect in humans, but there is no proof so far that they correlate with the level of exposure to DON. [47]

4. Urinary hippurate:

Hopton et al. published in 2010 a study [22] stating that humans with low, respectively high exposure to DON can be discriminated by the levels of hippurate excreted in urine. The concentration of this compound was found to be up to 1.5 fold higher in individuals with high DON exposure.

Hippurate is normally present in urine as a result of conjugation of glycine and benzoic acid, an important step in detoxification and elimination of aromatic carboxylic acids. This might be a useful biomarker of exposure for human populations, however further data is needed to prove that it is a reliable parameter, considering the fact that its concentration in urine is highly related to general dietary factors.

Discussion:

Risk assessment of different food contaminants in human population is impaired by the inability of correctly quantifying and correlating the level of external exposure to the internal, biologically effective dose. An important confounder in the etiologic studies evaluating the relation between these compounds and different conditions or biological effects is the relation between oral dose, internal dose and biotransformation within the contaminated organism. In order to avoid this, biomarkers of exposure (and especially internal exposure) need to be established and standardized.

Mycotoxins are frequently occurring compounds that contaminate cereals, coffee, nuts and derived products. The main research questions regarding them are whether they are etiologic agents of certain disorders and how we can assess and prevent exposure to them in human population. During the past 50 years, they have been described as being able to interfere with many cellular functions, but a better exposure assessment and finally risk assessment is still needed.

We have indicated two categories of biomarkers (of exposure and of effect) that have been used so far in epidemiologic studies on mycotoxins. The biomarkers of exposure refer to the presence of the contaminant or its metabolites in a certain matrix in humans, offering information on time-average exposure and turnover of the respective chemicals. The biomarkers of effect are associated to any biological outcome which the presence of the contaminant might trigger in the body. They are often more relevant in risk assessment, considering that the sole presence of the toxin in biological matrixes is not necessarily related to a risk.

The aflatoxins are doubtless the best represented of the mycotoxins in terms of biomarkers, both of exposure and effect. Except the possibility of quantifying the original chemicals *per se* in biological fluids, the hydroxylation products AFM1 and AFM2 are good indicators of internal exposure, while the albumin adducts and DNA adducts are reliable markers of effect.

For Zearalenone we have at the moment no reliable biomarker of exposure, due to the lack of information valid for human population. Regarding the few possible biomarkers, the nature of the matrix that these markers are to be found in (e.g. tissue biopsies) makes quantification difficult in humans. Even in the case of easily collectable biological fluids, ZEA and its metabolites have been detected in animals, but there are no

studies in the literature to assess their validity in humans. The effects of Zea, especially on the uterus, are thought to be a result of modulation in the expression levels of certain genes as fas-L, bcl-xl, IGF and IR , but this is yet to be proven in humans.

Studies of Ochratoxin A have been tightly connected to BEN and urinary tract tumor occurence. The toxin and its metabolites are quantifiable in blood, urine and milk. However, the extent to which they correlate to the level of exposure to OTA is not yet certain, possibly because OTA is ubiquitous in body fluids of most individuals. It has a considerably long half-life and therefore can be detected in fluids months after exposure. Moreover, the renal damage induced by the toxin cannot be considered specific, seeing that non-steroid anti-inflammatory drugs have very similar pathophysiological effects. The high incidence of nephropathies in the Balkans might be related to OTA, but their occurrence is also increasing in Western Europe, probably due to the extensive use of medication. As a biomarker of effect, the ability of OTA to form DNA-adducts is still controversial and no such compounds have been isolated from humans.

Fumonisins were so far the first toxins to be detected in human hair which is easy to collect as biological sample and useful in evaluating exposure as low, medium and high. Therefore it is viable to check whether it is a source of biomarkers for the other toxins; so far no data has been published on this issue. The sphinganine:sphingosine ratio, respectively the sphinganin 1-phosphate: sphingosin 1-phosphate remain so far the only biomarkers of effect that we have.

In the case of deoxynivalenol, the main biomarkers of exposure remain urinary DON and DON-G, both appearing to be reliable. The urinary hippurate is interesting to further evaluate as biomarker of effect, even though it is yet to be correlated to the extent of exposure to the toxin.

It can be concluded that there are a number of biomarkers of exposure which are correlated to the intake of mycotoxin contaminated products. In some cases, the markers also correlate with the dietary dose of the contaminants, meaning that a dose-response relationship can be established. The internal dose and the biotransformation have however mostly been assessed in animals or in *in vitro* models, therefore there is a need for validation in human longitudinal epidemiological studies over long periods of time in both high risk and low risk populations. Especially considering the fact that these two phenomena are species-specific, further research should consider the pharmacokinetics of the assessed compound primarily in humans. It is necessary that these studies take into consideration to integrate the markers within environmental and person-specific

information that can act as confounders, such as dietary preferences, smoking behavior, individual susceptibility etc.

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