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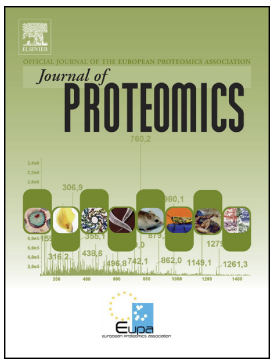

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Title: The importance of accounting for sex in the search of proteomic signatures of mycotoxin exposure

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Abstract

Mycotoxins are natural food and feed contaminants that are toxic to human and animals. Proteomics is an adequate toolbox to investigate the mode of action and the effects of mycotoxins, as these toxicants often alter protein synthesis and degradation, as well as induce changes of important post-translational modifications. For instance, the contaminant deoxynivalenol induces a severe ribosomal stress that affects protein production, whereas the toxin fumonisin B1 can alter the phosphorylation of a large number of proteins, and patulin is a potent proteotoxic molecule. The response to most mycotoxins is sex-dependent, males being generally more sensitive than females. In addition, for some toxins, the toxic effects

observed were different for each sex. Nevertheless, the importance of accounting for a sex-dependent response is often overlooked in toxicology studies involving mycotoxins. Here we review the information that proteomics has provided in pre-clinical studies of mycotoxin exposure as well as the differential response of males and females to these molecules to highlight the need of including male and female individuals when evaluating the impact of mycotoxins in the cell proteome.

Significance

The current trend in mycotoxicology is the combination of several -omics techniques in order to understand the mechanism of action and effects of these toxic natural food contaminants. One of the goals of these experiments is to determine "potential biomarkers" of mycotoxicoses. Nevertheless, the strategy followed in biomarker research must take into account as many possible factors as possible in order to find robust biomarkers for differential diagnosis. Among the factors that can have an influence in the response to mycotoxins, one of the most important is sex. Traditionally, males are preferentially used in research, as they are more sensitive to mycotoxins and their response is not dependent on hormonal levels, thus less variable. However the intrinsic and hormonal differences between sexes makes that results obtained in males are often not directly transferrable to females. In this review, we want to highlight (1) that proteomics has a great potential on mycotoxin research, and (2) the need in taking into account sex differences in proteomic studies, mostly when the discovery of robust biomarkers of mycotoxins response is desired.

Keywords

Mycotoxins; proteomics; sexual-dimorphism; immunotoxicity; genotoxicity; endocrine disruptor

1. What are mycotoxins and why are they so important?

Many fungal species from the *Alternaria*, *Aspergillus*, *Claviceps, Penicillium* and *Fusarium* genera frequently colonize agricultural products such as grains, cereals, fruits, spices and animal forages. In certain conditions of temperature and humidity, the growth of these filamentous fungi results in the synthesis of secondary metabolites known as mycotoxins that will accumulate in the substrate where the fungi grow [1–3]. Mycotoxins are small molecules of diverse chemical nature that are toxic to human and animals at low doses. The production of these metabolites can take place before crops harvest, and/or during storage. Mycotoxins are, therefore, natural food and feed contaminants and so their presence in the food chain is unavoidable nowadays [1–3]. Actually, in spite of the growing efforts in improving pre-and post-harvesting agricultural practices, recent surveys indicate that 70% of raw materials are contaminated with these toxins [4].

Mycotoxins represent a major issue in food safety and public health, as they are related with the development, exacerbation and/or aggravation of very distinct syndromes, diseases and dysfunctions in mammals [5,6]. In order to reduce dietary exposure, many countries have developed regulations and recommendations for the presence of some mycotoxins. For instance, the EU has set maximum levels for zearalenone, deoxynivalenol, aflatoxins, fumonisins, ochratoxin A and patulin in human food [7]. Generalities on the structure, major producing fungi, adverse effects and mode of action of these toxins are summarized in table 1.

2. What are the main challenges we face in mycotoxicology research nowadays?

Outlining the real danger associated with the presence of mycotoxins in food is not a simple task, as this danger is defined by three main factors that can interplay, and that are not all well understood nowadays [8]. The first factor is the presence of the so-known “emerging

mycotoxins" for which not much information on their toxicity is available. In fact, the advancement of new screening and more sensitive detection techniques keeps identifying new candidates to enter the mycotoxin family [9], as well as is giving precise information of their occurrence. However, the real danger associated with the presence of these "emerging mycotoxins" is yet to be defined though toxicological studies [10]. A second factor is that mycotoxins can also be present in different modified forms from the "original" or "parent" mycotoxin [11,12]. The modified forms can be more toxic than the parent compound, and are produced either in the plant or in the human/animal organism in an effort in reducing biologically active fungal metabolites or during food/feed processing [13]. The third factor is the combination of mycotoxins and their interaction. Actually, different mycotoxins are simultaneously present in variable proportions in food and feed. This is because fungi infecting crops can produce more than one mycotoxin, and/or more than one fungal species can contaminate the same commodity [14,15]. Furthermore, different commodities containing different contaminants are associated in the same meal. As simultaneous exposure to different toxins can result in antagonistic, additive or synergistic effects, the presence of mycotoxin mixtures can lead to toxic effects unpredicted by the simple combination of the effect of each toxin alone [14,15]. This means that even if two or more toxins are present in concentrations below those considered safe, the exposure to the mixture can result in a toxic effect [1,16]. The pathology associated with the exposure to mycotoxins is called mycotoxicosis [2]. The symptoms of a mycotoxicosis can be very variable and depend mainly on the type of mycotoxin (some of them summarized in Table 1), but also on its concentration and the duration of the exposure [3]. The combination of concentration and time of exposure defines if the mycotoxicosis is acute (short-term exposure to a high dose of toxin) or chronic (long-term exposure to low doses). Whereas acute toxicity has a rapid onset and a typical symptomatology, the chronic exposure to low doses of mycotoxins has a more insidious

effect, often resulting in unspecific syndromes such as reduced feed intake and slow growth in farm animals, or in cancer or other severe syndromes in humans [2,17]. The presence of mycotoxins can also exacerbate pre-existing inflammatory processes like chronic intestinal inflammatory diseases [18,19] or increase susceptibility to certain infections such as coccidiosis in poultry, colibacillosis, salmonellosis in pig and mice, swine respiratory disease, and aspergillosis in poultry and rabbits, among others [5,20]. Additionally, some host-related factors can influence the in-vivo effect of mycotoxins. The sensitivity to mycotoxins depends in general on differences in the digestive physiology and anatomy, metabolism and excretion capabilities. For each mycotoxin, additional factors related with their specific mode of action/toxicity are important. As a result, the onset of an exposure to a given mycotoxin depends on factors like species, sex, age, nutritional status, pre-existing diseases and microbiota [17,21–26]. Sex has a great influence in the response to xenobiotics including mycotoxins. This is mainly due to differences in hormonal levels as well as differences in pharmacokinetics and pharmacodynamics, although other factors seem to be also involved, such as the influence of hormones in the expression of hepatic detoxifying enzymes or the intrinsic differences in cell composition and structure [27–31]. A sexual dimorphism has been described for the toxicity of many mycotoxins, which will be reviewed below.

3. The proteomic toolbox in mycotoxicology

The result of the consumption of food contaminated with mycotoxins depends on many elements such as the presence of modified forms, the combined presence of different toxins, their interaction, and several factors related with the host. To analyze all the possible effects of mycotoxin exposure in this complex context we need to privilege unsupervised, -omics techniques like proteomics instead of the traditional reductionist, supervised technical approaches that often explore only one specific question. However, if we perform a quick

bibliographic search, we can observe that the use of proteomics in mycotoxicology has been mainly restricted to the study of the pathways involved in mycotoxin synthesis in the corresponding fungal species, and it is still giving its first steps in studying the host response to the presence of mycotoxins. Actually, PubMed searches using the words “proteomics” and “mycotoxin” retrieved 112 studies, from which only 19 were oriented at evaluating the toxic effects of mycotoxins in the organism. Although the use of proteomics approaches to analyze the effect of mycotoxins is increasing, its use is still not as popular as transcriptomics [32–34]. However, we can find some examples in the literature where, for a given gene product, the mRNA and protein response towards a given mycotoxin are different. For instance, in intestinal cells exposed to the mycotoxin deoxynivalenol (DON), the mRNA levels of inducible nitric oxide synthase proteins are elevated in a dose-dependent manner, whereas this effect was not observed at the protein level, possibly due to an enhanced protein degradation though the ubiquitine-proteasome system induced by the toxin [35]. For the same toxin, the time dependent inhibition of the expression of the intestinal mucins MUC1, MUC2 and MUC3 differed from the time-dependent decrease in the number of goblet cells, showing that mRNA levels do not always represent the current functional state of a cell due to transcriptional regulatory/compensatory mechanisms [36]. Same was observed during the DON-dependent disintegration of the intestinal epithelial barrier, where it was shown that claudin protein levels were decreased and correlated with a displacement within the cells, which was accompanied by a compensatory up-regulation of mRNA levels of claudins and their binding partner ZO-1 [37]. Additionally, most mycotoxins show a pleiotropic action [7]. This means that a given toxin has several molecular targets at the genomic, transcriptomic, proteomic and metabolomic level. The best example of a mycotoxin showing a pleiotropic effect is DON. DON is part of a family of mycotoxins called trichothecenes, is frequently found in grains and cereals and is considered the most prevalent food-associated mycotoxin. It

is responsible for gastro-intestinal, immune and cardiotoxic effects among others [38,39]. The mechanisms that trigger DON's toxicity derive from its action at the gene level (it is able to provoke oxidative DNA damage) [40], the transcriptional level (DON binds to the ribosome, inhibits protein and nucleic acid synthesis and triggers ribosomal stress) [41,42], the proteomic level (provokes changes in the phospho- and glycoproteome) [43,44] and the metabolomic level (affects lipids peroxidation) [45]. In this context, it seems clear that following a proteomics approach can present several advantages in exploring the mechanistic aspects of mycotoxin-induced pathologies.

4. Sex determines the toxic effect of many mycotoxins and should be taken into account for data interpretation in proteomics analysis

Several reports describe differences in the response and sensibility to different mycotoxins between males and females. A recent meta-analytical study on the effect of mycotoxins in the pig including 85 articles published between 1968 and 2010 concluded that, in general, mycotoxin exposure had a greater effect in performance in males [46]. Differences were found for different parameters such as feed intake (reduced by 6% in females and by 10% in males), weight gain (reduced by 15% in females and by 19% in males) and feed conversion ratio (8% worse in females versus 10% worse in males) [46]. In spite of this, sex is a factor that has been largely overlooked in mycotoxicological research, where in general, females are under-represented. A review published in 2015 gathered all the in-vivo toxicity studies performed in the last decade on fusarium mycotoxins [47]. Of these, 54% employed only males, 15% used only females, 15% used a mix of both sexes and in 15% of these studies sex was not specified. There is a risk of drawing erroneous conclusions when extrapolating outcome data from one sex to another across different disciplines including toxicology [48], and in fact several clinical biomarkers have different discriminatory power in men and women

[49–52]. Males are more frequently investigated over females in research because they have more constant hormone levels and are thus less variable. Sex hormones interplay with metabolism and immune response [48], and can induce epigenetic changes, thereby predisposing males and females differently to non-Mendelian complex diseases [28]. Furthermore, some differential responses of males and females are due to intrinsic differences in cells that are unrelated with hormonal exposure. Examples of the latter are the differences in male and female cardiac tissue, which include variable ion channel expression as well as diverse responses to sex hormonal regulations via long-term genomic and acute non-genomic pathways [53,54]. Intrinsic differences in male and female liver cells are also very high. Microarray analysis of transcripts from murine liver showed an approximate sexual dimorphism of 70% [55]. This accounts for the higher sensitivity to several toxicants observed in female-derived primary hepatocytes [27], which can be also explained by the fact that female liver cells have more cytochrome CYP3A compared to male cells, a pivotal protein for drug metabolism [56]. Proteomics has proven useful to discovering and/or better describing the toxic effect of mycotoxins, especially for those having an effect in protein synthesis and degradation and/or those inducing post-transcriptional changes such as phosphorylation or glycosylation. However, in none of these publications were sex differences taken into account nor the influence of sex in the chosen model discussed or justified. From these studies (reviewed below), only two employed in-vivo models, using males. Other studies used samples derived from human patients or pig/chicken primary liver cells culture without specifying the sex of donors. The rest of the investigations employed immortalized cell lines cultured in-vitro. From these, most cell lines were originally derived from male individuals (RPMI 1788, Jurkat E6.1, Raw 264.7, HepG2 and MLTC1 cells) and two studies employed cell lines derived from females (H295R and GH3 cells). This is important, since while the importance of stating the sex when performing research in whole

animals is widely known, much less attention is payed to the sex of cells in culture and/or the impact of media composition (usually containing sex hormones), as recently reviewed [31]. In the following section of this review we will critically review how of proteomic analysis has been applied to the study of mycotoxins effect and how taking into account the sex of the model could have changed the results interpretation. Here we have grouped mycotoxins in three main toxicity groups according to their most studied effect, and have discussed if sex dimorphisms have been found for the effect of these toxins.

Genotoxic and carcinogenic mycotoxins: Aflatoxin B1, Ochratoxin A and Fumonisin B1

The incidence of many types of cancer is higher in males than females because of occupational and/or behavioral factors, but also because of other constitutive determinants that are important for cancer initiation, such as the influence of sex hormones, the expression of genes present in sex chromosomes or other intrinsic differences [57]. The exposure to several mycotoxins such as Aflatoxin B1, Ochratoxin A and Fumonisin B1 is linked with the development of cancer in different non-reproductive organs. For these toxins, sex differences in the incidence of cancer development as a consequence of their toxic effect have been described.

Aflatoxin B1 (AFB1) is primarily produced by *Aspergillus flavus* and *Aspergillus parasiticus*, and is the most commonly occurring of all the aflatoxins. This toxin has been classified as a group I human carcinogen by the International Agency for Research on Cancer (IARC) [58,59]. The predominant site of AFB1 metabolism is the liver [60], where AFB1 is metabolized into an aflatoxin-8,9-exo-epoxide by P450 enzymes. This highly reactive exo-epoxide can react with DNA, RNA and proteins to form derivatives, especially the p53 tumor suppressor gene [61], abrogating its function and contributing to carcinogenesis [59].

Carcinogenicity of AFB1 in liver is higher in males than in females in mice, chicken and humans [62–65], which seems to be due to a complex interaction of differences in metabolism, hormonal status influencing inflammation and cancer promotion in the liver [65–69]. In fact, the androgen receptor and the estrogen receptor activation have opposing effects on hepatocyte proliferation and nucleic acid metabolism, through acting on gene transcription [57]. Indeed, the cytochrome P450 is responsible for the activation of AFB1 into the highly reactive exo-epoxide form, and recent studies indicate the existence of sex-specific cytochrome P450, such as P450-male (2C11) and P450-female (2C12) and P450(6) beta (3A2) in rat livers, and also show that their expression levels are markedly different between male and female rats [70]. Growth hormone, thyroid hormone, sex hormones and other chemicals regulate the expressions of sex-specific P450s [70]. Differences in the liver proteome from hepatocarcinoma patients showed that the most common biological processes affected by AFB1 in tumorigenesis were detoxification and drug metabolism pathways, antigen processing and anti-apoptosis pathways [71]. Furthermore, results suggested that AKR1B10 may play a role in AFB1-related hepatocarcinogenesis [71]. However, in this study, the sex of the patients was not taken into account and so no correlation between the higher abundance of AKR1B10 and sex could be established. This could have been interesting, since the mRNA expression of AKR1B10 is known to be significantly higher in men than in women (mean ± SD, males 3.3 ± 7.5 versus females 0.2 ± 0.6, $P < 0.0001$) in non-small-cell lung cancer [72]. Proteomics was also employed to explore the effect of AFB1 exposure during type-1 diabetes mellitus, using exclusively male mice. This study concluded that AFB1 exposure disordered all the pathways typically altered in type-1 diabetes mellitus, and that this toxin could worsen diabetes as it reduced the abundance of the protein MUP1, a marker of increased insulin sensitivity [73]. MUP1 is a known liver male-predominant gene,

that respond differently to hormones in males and females [74]. The decrease of MUP1 protein levels in this male disease model remain therefore to be confirmed in females.

Ochratoxin-A (OTA) is produced by several species of *Aspergillus* and *Penicillium*, and can induce a wide range of toxicological effects, including mutagenicity, carcinogenicity, immunotoxicity, teratogenicity and most notably, nephrotoxicity [75,76]. Most studies have focused on the effect of OTA on the kidney, considered its target organ. A clear sexual dimorphism on the effect of this mycotoxin has been found. OTA can induce renal tumors much more frequently in males than females [77]. The reason for this has been investigated, and the higher male sensibility seems to be related with differences in metabolism [78], bioavailability, body weight [79] and a different regulation of renal transporters expression and binding to renal proteins [80–82]. Unfortunately, most studies employing proteomics used the most popular kidney cell line HEK293, a human embryonic line of unknown sex. This means that results cannot be related with the known differences in sensitivity. The first studies employing proteomics to investigate the molecular mechanisms of OTA cytotoxicity in the HEK293 cells provided evidence of mitochondrial damage [83]. Changes in the mitoproteome of cells exposed to OTA showed changes in abundance in some components of the mitochondrial electron complex II (SHDA and SHDB) as well as complex III (UQCRC2 and UQCRFS1) and complex V (ATP5B), which were all depleted. These changes implied a decrease in ATP levels and a subsequent promotion of apoptosis. Some anti-oxidant proteins were also depleted, which, together with the perturbation of the mitochondrial electron transport chain, promoted an increased state of oxidative stress. Other mitoproteome changes induced by OTA suggested an inhibition of protein synthesis and induction of cell death. It was additionally observed that pre-treatment of cells with *N*-actetyl-L-cysteine was able to reverse of the abovementioned adverse effects almost completely [83]. Results from this study allowed the identification of two proteins whose function could be important for OTA

toxicity, namely apoptosis signal-regulating kinase 1 (ASK1) and Lon Protease 1 (Lonp1). The role of these proteins was investigated by knocking them down in HEK293 kidney cells using RNA interference technology combined with quantitative proteomics to study the impact of silencing the molecule of interest on OTA-exposed cells. ASK1 is an important mediator in the oxidative stress-induced cell apoptosis induced by OTA in the kidney, and it was actually observed that ASK1 knockdown desensitized HEK293 kidney cells to OTA-induced apoptosis [84]. More specifically, evidence of an essential role of ASK1 in the cytotoxic effects of OTA, such as the inhibition of mRNA splicing, nucleotide metabolism and DNA repair, as well as the activation of lipid metabolism was provided. It is difficult to relate these results with the sex-dependent effect of OTA, as the sex of HEK293 is unknown. It is known, however, that in females some tissues like the brain show a constitutive higher expression of enzymes involved in cellular redox maintenance, and that these protect from the activation of ASK-mediated apoptosis, offering neuroprotection in diseases like Parkinson's disease [85]. The protective role of ASK1 could therefore also be different in males and females exposed to OTA. The protective role of mitochondrial Lon Protease 1 (Lonp1) in HEK293 kidney cells exposed to OTA was also confirmed [86]. Proteomic analysis suggested that this function was realized by assisting in mtDNA maintenance, regulation of protein synthesis, modification and repair, protecting against mitochondrial oxidative stress and maintaining the balance of carbohydrate metabolism. There are not data in the literature, to our knowledge, that state id Lonp1 could be more active or being more expressed in females. Other study investigated the effect of OTA in the composition of the hepatoblastoma cell line HepG-2 plasma membrane [87]. This showed that OTA induced a loss of more than 85% of the protein content, being in this way capable of inducing damage in membrane structure and integrity, cell adhesion and vesicle-mediated transport. Membrane transport proteins were also depleted from treated plasma membranes, which could be related with enhanced

hepatotoxicity [87]. HepG2 cells were originally derived from a male patient's liver, and so the same results in liver cells derived from a female patient remain to be confirmed.

Fumonisin B1 (FB1) is produced by a number of *Fusarium* species and affect animals in different ways by interfering with sphingolipid metabolism, causing different syndromes depending on the species [2]. Although different –omics techniques have been applied to evaluate the effect of FB1 in different tissues [88], the changes in the proteome induced by FB1 remain to be explored. The sex-specific effects of fumonisin B1 (FB1) are widely known and have been reviewed [89]. Males and females developed different types of tumors depending on the species: hepatocellular adenomas and carcinomas were induced by FB1 in female mice but not in males, whereas male Fischer F344 rats developed renal tumors that were not seen in females [90]. Female mice seemed more sensitive to FB1 subcutaneous injection than males regarding their immune response, which included changes in cellular and humoral responses [91,92]. In contrast, male pigs were more sensitive to the toxic effects of FB1 than females, having a more marked negative effect on average daily weight gain, serum biochemical parameters, pancreas and adrenal weight, and immune responses such as expression of T-helper (Th) 2 cytokines and the production of specific antibodies upon vaccination [93,94]. It seems clear that, for a given species, male and female subjects should be employed in order to understand the toxicity and the effect of FB1 though proteomics in the future.

Endocrine disrupting mycotoxins: Zearalenone, Alternariol and Patulin

There is a group of mycotoxins including zearalenone, alternariol and patulin that can function as endocrine disruptors. Their effect is hence differential in males and in females, depending on the hormonal unbalance induced by their mode of action.

Zearalenone (ZEN) is a mycotoxin produced by several *Fusarium* species whose main feature is its strong estrogenic activity [2]. Following exposure, ZEN can cause endocrine-disrupting effects, and so in this case the sex-dependent effects of this toxin are expected. Most mechanistic studies regarding this mycotoxin are interested in the effect of ZEN in one specific sex, and hormone differences are usually discussed. ZEN and its metabolites are known to regulate hormone production, cell metabolism, proliferation and apoptosis. As a result, it negatively affects the reproductive system, causing low fertility in both males and females, abnormal fetal development, and precocious puberty (in females) among other effects [95]. In women, ZEN exposure has been associated with the development of breast cancer, and although it seems reasonable that it could also be associated with prostate cancer in men, this link has not been confirmed [96]. Several studies have analyzed the proteomic changes associated with ZEN (or its metabolites) exposure in different models, but the sex of the model employed or the study conditions regarding the presence of sexual hormones was often disregarded. For instance, the effect of a non-cytotoxic dose of ZEN was explored through proteomic analysis in a steroidogenesis model (the angiotensin-II-responsive steroid-producing adrenocortical cell line H295R). The 21 proteins showing abundance changes were mainly grouped into two groups centered on the oncogene ERBB2 and the signaling cascade NF-kB, together with proteins like CCND1, CCNB1, CDK2 and CDKN1B, indeed involved in estrogenicity and endocrine disruption [97]. A later study compared these results to those obtained in the same cellular model when exposing them to low, non-cytotoxic doses of two ZEN metabolites, alpha and beta zearalenol [98]. A total of 14 and 5 proteins showed differential abundance, respectively, in response to the exposure to the mentioned toxins. The lack of common altered functions, as explored by functional analysis, indicated that each compound might have unique biological activities. In fact, whereas alpha- zearalenol altered cellular functions related with movement, cell-to-cell signaling, and proliferation, beta-

zearalenol impacted mainly the hematological system development, tissular morphology and growth [98]. Authors of these two studies did not consider the sex of their model as a possible determining factor to discuss their results. The H295R is a female cell line, and in both studies the cell culture media contained a substitute of fetal calf serum that includes hormones such as progesterone 17β-estradiol and testosterone. Bearing in mind that the compounds being tested are estrogenic, these circumstances should have been considered for data interpretation. The proteome of Leydig cells' (MLTC-1 cell line) mitochondrion also changed following an exposure of this cell line to a low dose of ZEN [99]. Results indicated a strong alteration in the lipid metabolism, which was characterized by an increase of fatty acid uptake and beta-oxidation, as well as an inhibition of steroidogenesis and steroid esterification. Proteomic analysis helped, hence understanding the mechanisms promoting the ZEN-induced reduced hormone secretion and excessive oxidative stress in the male reproductive system [99].

The effect of ZEN exposure in HepG2 hepatic cells has also been explored by proteomics [100]. Several chaperones (HSP90AA1 and HSP90AB1) and anti-apoptotic proteins (NPM, YWHAZ) were significantly more abundant after the treatment [100]. The proteomic analysis also revealed some ZEN early-induced changes related with cellular structure, growth and metabolism. As mentioned early in this review, the liver is one of the organs showing the highest sexual dimorphism and is highly influenced by the presence of steroid hormones. HepG2 are male cells and in this study they were cultured in the presence of serum, which contains steroid hormones. Additional studies using female cells and in absence of sexual hormones would be thus necessary to confirm these results.

Alternariol (AOH) is an emerging mycotoxin produced by *Alternaria* species, for which toxicological knowledge is relatively limited. AOH can be found on many foodstuffs mainly of plant origin [101]. Data suggest that AOH is an endocrine disruptor due to its structural similarity to estrogen. AOH modulates the expression of important genes in steroidogenesis in

H295R cells, with up-regulation of the expression of mRNA for CYP1A1, MC2R, HSD3B2, CYP17, CYP21, CYP11B2 and CYP19 and down-regulation of the expression of the NR0B1 gene being observed [102]. Proteomics later confirmed these results in the same experimental setup [103]. Among other findings, results showed that the early stages of steroid biosynthesis and C21-steroid hormone metabolism were altered upon the exposure to this toxin, as suggested by changes in proteins such as SOAT1, NPC1, ABCD5, CYP21A2 and HSD3B1 [103]. As for some studies using ZEN, here the experiments were performed on a female cell line H295R cultured using a medium containing hormones, so results should be compared to a male model to evaluate differences, mostly because most proteome changes found were centered in cytochrome proteins, which often show a sexual dimorphism depending on the hormone levels [30,104].

Patulin (PAT) is mostly produced by *Penicillium expansum*, the mold that causes soft rot of apples, pears, cherries, and other fruits [2,105]. PAT was initially studied as a potential antibiotic, but subsequent research demonstrated human toxicities, including nausea, vomiting, ulceration and hemorrhage [105]. This toxin is also genotoxic to mammalian cells and can impair DNA synthesis, which might be related to its ability to react with sulfhydryl groups and to induce oxidative damage [105]. Some evidence exist indicating that PAT has the ability to act as a potential endocrine disrupting compound. Male rats exposed to a high dose of PAT for 60 days showed increased plasma levels of testosterone and decreased T4 hormone while there was no change in T3, TSH, LH and GH. When this severe exposure lasted for 90 days, there was an increase in testosterone and in LH [106]. PAT exposure was also associated with low sperm counts and testicular alterations such as edema, fibrosis, local Leydig cell hyperplasia and disorganization of the seminiferous tubule epithelium [107]. An additional in-vitro investigation using the female cell line H295R demonstrated the potential of PAT to modulate the endocrine system, including the alteration of the production of

various hormones as well as interfering with nuclear receptor transcriptional activity [108]. The molecular effect of PAT is however largely unknown, and no proteomic studies have been performed to date. Recent results indicate that proteotoxicity through the regulation of the expression of the proteasome-controlling transcription factor Rpn4 represents a major aspect of patulin toxicity [109]. Proteomic analysis could give more information on the mechanisms of toxicity of PAT, yet given the endocrine disrupting activity of this molecule, both male and female models must be employed for a correct interpretation of results. In fact, even if the available information on the sexual dimorphism of the toxic effect of PAT is scarce, there is some evidence of the influence of sexual hormones in its effect. For instance, exposure to PAT in male rabbits drove to a rise in the levels of testosterone associated with changes associated with intensive bone remodeling, whereas in females the observed changes in bone microstructure induced by exposure were associated with bone turnover [77].

Immunotoxic mycotoxins: Deoxynivalenol and Toxin T-2

Mortality due to infectious diseases is more common in males, whereas autoimmune diseases are more prevalent in females, which already indicates that there must be some differences in the immune response between sexes [31,110]. Sexual hormones are largely responsible for this, as it has been shown that females express a higher number of adaptive immune response genes after puberty, whereas men show a higher expression of innate immunity genes [110]. Moreover, it has been revealed that the cytokine profile produced after lipopolysaccharide-induced inflammation is different in males and females, with females producing more TNF-α and IL-1β [111]. These results have been related with a differential activity of mitogen-activated protein kinases (MAPK) in males and females [112,113]. There are two mycotoxins that exert their immunotoxic effect by altering the MAPK activity: DON [114] and T-2 toxin

[115]. It is therefore not surprising that sex-dimorphisms have been found in the outcome of exposure to these toxins.

DON is the most prevalent food-associated mycotoxin. This type-B tricothecene is produced by several *Fusarium* species and shows a wide range of consequences, with the intestine and the immune system being the main targets, but also showing reproductive and teratological effects [23,116]. A higher sensitivity of males to the toxic effects of DON has been reported in several species including mice and pig [26,117–119]. DON provokes a lower feed intake and weight gain male mice and pigs [26,46]. Levels of IgG, IgA and CCK were higher in female mice exposed to DON, whereas males showed higher levels of IL-6 and higher concentrations of DON in organs and plasma [117,119]. This dimorphism has been attributed to a slower excretion rate in males, since no differences in metabolism were found [118,119]. These differences in excretion have not been confirmed in humans, since the levels of DON present in urines in males and females have been measured in different studies with opposite results [120–124]. Indeed, the small sample sizes employed in those studies limit the interpretation, and larger sample sizes will be needed in future investigations.

Proteomics was used to investigate the effect of DON, as its mechanisms of action. In order to unravel the molecular effect of DON in the immune cells, the proteome of thymoma cells (of unknown sex) upon exposure to DON was investigated [125]. Proteome alterations were characterized by an increase in key metabolic enzymes (FASN, GPI), proteins associated with protein turnover (PDI, ERO1-ialpha), and some immune-related proteins (IgE binding-protein). Such changes were linked with the transcription factor MYBBP1A, which was identified as a possible key molecule in the mechanism of DON toxicity [125]. The activation of the catalytic core of the proteasome was also identified after proteomic analysis of B (RPMI 1788 cell line) and T (JurkatE6.1 cell line) cells exposed to low doses of DON [126]. This study evidenced that the proteomic changes brought about by this toxin were different

for each cell line, suggesting a specific mechanism of action. Proteomic analysis also revealed some metabolic changes induced by DON related with an alteration of nucleotide biosynthesis [126]. The same authors later employed the same experimental setup, this time paying more attention to changes in the phosphoproteome [127]. Indeed, DON-induced ribosomic stress involves the activation of several protein kinases such as PKR, HCK and MAPKs, so phosphoproteomics can give valuable information on the signaling network associated with DON ribotoxicity. Several proteins showed an altered phosphorylation state following DON exposure in B and T lymphocytes, including MTHFD1L, EEF2, NME1, HSP71, GRB2 and EIF3I. In all, phosphoproteomics changes indicated an alteration in purine metabolism and protein synthesis [127]. These studies employed exclusively male cell lines (RPMI 1788 and JurkatE6.1), and so results should be compared to those obtained from female cells, mostly because authors claim that one of their objectives is providing mechanism-based biomarkers for DON exposure. Changes of global phosphorylation dynamics linked with the exposure to DON have been further investigated in the literature. For instance, the early phosphoproteome changes occurring in murine macrophages upon DON exposure revealed that translational regulation and modulation of ribosome biogenesis were the main target during DON-induced ribosomal stress [43]. Moreover, most processes associated with changes in the phosphoproteome were related with signaling networks involving MAPK-, NFkB-, AKT- and MAPK-linked pathways. Those were created with a delicate balance between cellular quiescence and stress response [43]. These changes were further investigated only in the ribosome using the same model and experimental setup [44]. An overall decrease in the levels of translation-related proteins interacting with the ribosome was observed. In parallel, a compensatory response characterized by an increase of proteins mediating cellular organization as well as protein folding and biosynthesis was identified. This phospoproteomic analysis described the molecular mechanisms that enable cells to respond and adapt to the

presence of ribotoxins, such as the phosphorylation of key proteins involved in the early inhibition of translation and the recruitment/maintenance of stress-related proteins [44]. The early phosphoproteomic changes related with DON-induced ribosomal stress in mouse spleen has also been investigated [128]. Several aspects that contribute of the immune dysregulation induced by DON ribosome stress were identified, including changes associated with cytoskeleton organization, apoptosis regulation as well as development and activation of lymphocytes. Interestingly, such alterations were associated not only with the well-known activation of MAPK signaling, but also of the phosphatidylinositol 3-kinase/AKT pathways [128]. Again, all these in-vivo studies were performed exclusively in male subjects, and so the proteome changes induced by DON in females remains unexplored. Differences are expected in females, since MAPK activation is different, and so are the responses associated [111,113].

The T-2 toxin belongs to the group of Type A tricothecenes, and is produced by different *Fusarium* species. It is known to be immunotoxic and also promotes cytotoxic effects at the gastrointestinal level and in fetal tissues [129]. No comparative studies on the effect of T-2 toxin in males and females have been performed, to our knowledge, although the dissimilarities in the immune system between sexes allow us to expect some differences. Proteomics was first employed to study the effect of T-2 toxin combined with transcriptomics in primary cultured porcine hepatocytes [130]. Results evidenced the complementarity of both approaches and showed alterations in lipid metabolism, oxidative stress and apoptosis that were related with the toxic effect. Moreover, several phase I enzymes such as different CYP3As, CESs, and EPHX were observed to be induced by T-2 treatment. From these enzymes, CYP3A46 (a cytochrome P450 isoform) was proven to be directly implicated in T-2 metabolism. Interestingly, the expression of CYP3A46 is male-dominant in several pig breeds, and its expression seems to be dependent of the levels of androgen [131]. This would mean that the metabolism of the T-2 toxin could depend on the sex and the hormonal status.

Unfortunately, the sex from the animal(s) from which hepatocytes were derived was not specified in this study.

In a similar way, the effect of T-2 toxin on primary chicken hepatocytes was also investigated using proteomics [129]. Results confirmed the oxidative stress and protein synthesis inhibition promoted by T-2 toxin that had been already described. In addition, the proteomic analysis also identified that two subunits of mitochondrial complex I (NDUFB10 and NDUFS8) were 20 times more abundant in T-2 toxin- exposed hepatocytes, along with other mitochondrion proteins, thereby suggesting a role of mitochondrion in T-2 exposed liver cells. This hypothesis was verified, since T-2 treated hepatocytes were richer in mitochondrial mass and ATP, probably in compensative response to oxidative stress. Again, sex of the animals employed to derive the primary hepatocytes was not specified in this study. Another approach, this time employing proteomics and transcriptomics, was employed to investigate the molecular basis of the growth retardation induced by T-2 toxin [132]. Rat pituitary adenoma GH3 cells were exposed to T-2 toxin and it was observed that this treatment suppressed the synthesis of growth hormone 1 as well as gene transcription and mRNA translation initiation, together with dysregulation of protein processing and folding. The suppression of growth hormone synthesis was directly linked with the T-2 toxin activation of Hck and EIF2AK2 kinases, which activated MAPK signaling, resulting in inflammatory stress [132]. This study was conducted in the female cell line GH3, and so we must yet establish if the same effect is observed in males. This is of particular importance, since the levels of GH can differentially regulate the expression of different proteins in liver in males and females [30,70], which can be important to define the metabolism of T-2 toxin in each sex.

5. Conclusions and perspectives

There is a growing interest in applying proteomics to investigate the mode of action and effects of mycotoxins. Several examples can be found in the literature, where proteomics has helped understanding the effect of different compounds. However, an appropriate interpretation and future successful utilization of proteomic-derived results should take into account all the factors that might have an impact in the response towards these substances. Sex-differences in the response to mycotoxins have been described in the literature, and so the inclusion of sex as a factor to account for in future proteomic studies is largely justified. Many studies using proteomics to evaluate mycotoxin effects mention the possibility/objective of using results for the discovery of biomarkers of mycotoxins effect. These can be defined as indicators of the molecular changes that result from the exposure to toxins [133]. However, success in discovering and validating good biomarkers depends on following the adequate strategy [134–136]. First, a discovery phase must be established, whose design must be carefully planned; the research question well defined, mostly given the desired future use of the researched biomarker and any important factor to account for [134–136]. Once a list of candidate biomarkers is produced in the basis of the discovery phase results, the validation phase starts. In this phase, we test the actual diagnostic/prognostic performance of each biomarker in a clinical setting. Absolute quantification of candidate biomarkers must ideally then be performed in a representative cohort of samples, taking into account a panel of differential diagnosis to the mycotoxicosis of interest to choose appropriate controls and all the possible confounding factors, including sex [135]. If proteomics is to be employed to discover biomarkers of mycotoxin effect, subjects from both sexes must be included both in the pre-clinical and the clinical stage.

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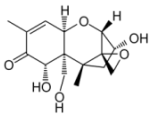
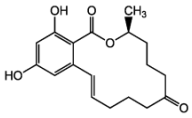
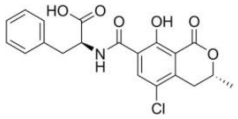
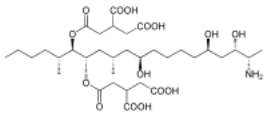
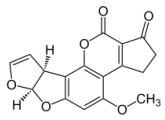
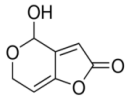
Table legend:

Table 1. Major mycotoxins: formula, producing species, affected crops and adverse effects. Adapted from [137].

Mycotoxin	Chemical structure	Major producing fungi	Main contaminated crops	Main adverse effects & mode of action
Deoxynivalenol (DON)		*Fusarium graminearum, F. culmorum*	Wheat, maize, barley, oats, rye	Feed refusal, emesis Reduction of growth Immunotoxicity Not classifiable as to carcinogenic to humans Binds to ribosomes and activates MAP Kinases
Zearalenone (ZEN)		*F. graminearum, F. culmorum*	Wheat, maize, barley, oats, rye	Endocrine disruptor (interaction with estrogen-receptors)
Ochratoxin A (OTA)		*Aspergillus* section *Circumdati* or section *Nigri*, *Penicillium verrucosum, P. nordicum*	Cereals, nuts, dried fruits, coffee, cocoa	Nephrotoxic (renal tumors) Carcinogenic to animals and possibly to humans
Fumonisin B1 (FB1)		*Fusarium* section *Liseola*, *Aspergillus niger*	Maize (*Fusarium* spp.) Grapes *(A. niger)*	Inhibition of sphingolipid biosynthesis Induction of apoptosis in liver Tumorigenic in rodents Possibly carcinogenic to humans
Aflatoxin B1 (AFB1)		*Aspergillus* section *Flavi*	Maize, peanuts, nuts, pistachious, other dried fruits	Genotoxic carcinogen Carcinogenic to humans
Patulin (PAT)		*Byssochlamys nivea, Penicillium expansum, Aspergillus* section *Clavati*	Fruits especially apples, silage	Gastrointestinal ulceration Immunotoxicity Neurotoxicity

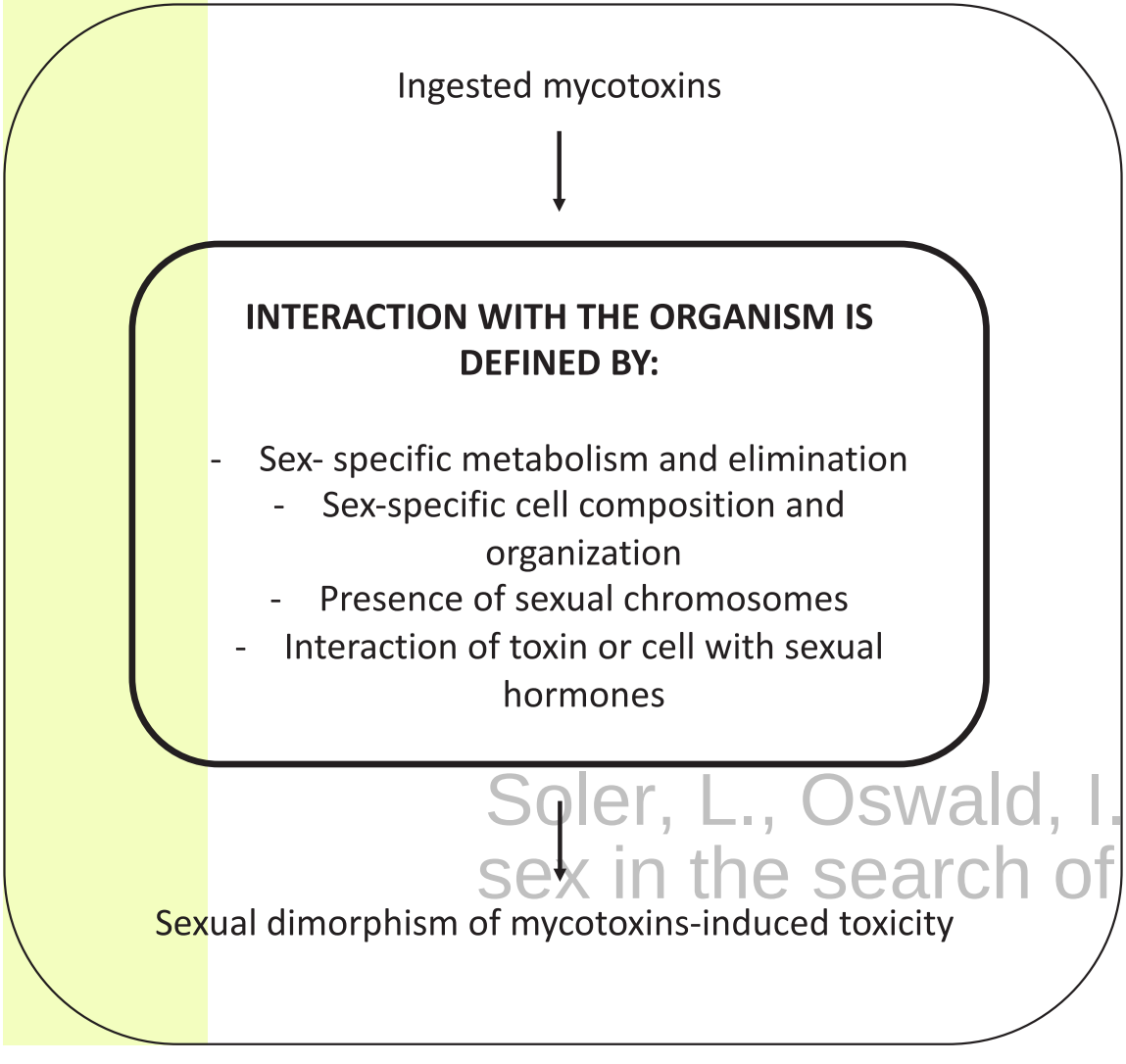
Highlights

- Mycotoxins are a group of more than 300 natural toxic food and feed contaminants.

-The toxic effect of most mycotoxins alter the cell proteome.

-The effect of mycotoxins is known to be sex-dependent.

-The proteome changes induced by mycotoxins are likely influenced by sex.



Graphics Abstract