Urinary Mycotoxins: A Review of Contaminated Buildings and Food in Search of a Biomarker Separating Sick Patients from Controls

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List of acronyms:
CFS: Chronic Fatigue Syndrome
CIRS: chronic inflammatory response syndrome
CIRS-WDB: chronic inflammatory response syndrome acquired from exposure to WDB
CRS: chronic rhinosinusitis
DON: deoxynivalenol
GAO: US Government Accountability Office
GST: glutathione transferase
LPS: lipopolysaccharide
MARCoNS: multiply antibiotic resistant coagulase negative staphylococci
OTA: ochratoxin
SRL: sarcin ricin loop
STC: sterigmatocystin
TGF beta-1: transforming growth factor beta-1
WDB: water-damaged buildings
VCS: visual contrast sensitivity
VIP: vasoactive intestinal polypeptide

Abstract:
Beginning in 2010, there have been an increasing number of patients with a chronic multisystem illness who have been using measurements of mycotoxins in urine to diagnose a putative illness for which antifungals in various forms (oral, IV, sublingual and intranasal) are being used as therapy. Many of these patients and providers believe that the illness is caused by fungi living in the human body, making toxins, or has been acquired by exposure to the interior of water-damaged buildings (WDB). This practice persists despite the absence of (i) an accepted case definition; (ii) any validated control groups; (iii) any rigorous case/control studies; (iv) any prospective, placebo-controlled studies; (v) any confirmation of active fungal infection; (vi) any confirmation that urinary mycotoxins are not simply derived from diet; and presence (vii) of a sharp repudiation from the CDC of this practice and the use of antifungals in 2015; and presence (viii) of a robust literature demonstrating causation of illness acquired from WDB is inflammatory in causation, not infectious.

This review looks at the extensive published materials, including a definition of mycotoxins; a case definition for illness acquired following exposure to WDB from 2008 US GAO, multiple biomarkers, proteomics, transcriptomics, volumetric CNS imaging studies and more supporting the diagnosis of an active chronic inflammatory response syndrome (CIRS), acquired following exposure to the interior environment of WDB as a validated diagnosis that leads to use of published therapies with documented efficacy. The review also looks at (i) published literature of fungal contamination of foods; (ii) multiple world-wide studies showing contamination of urine with mycotoxins and metabolites in healthy controls finding 21 studies of 2756 controls with a range of positive urinary mycotoxins from 60 to 100%; (iii) a tutorial on diagnostic testing for mycotoxins, including mass spectrometry and liquid chromatography; (iv) protective mechanisms to prevent intoxication following ingestion of mycotoxins. The review also includes a critical analysis of two papers cited by the antifungal community as supportive of their diagnosis of mycotoxicosis.

Keywords: CIRS, antifungals, mycotoxins, TGF beta-1, transcriptomics, ochratoxin A, deoxynivalenol, aflatoxin, trichothecenes, metabolites

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Background:
Attempts at definition: form or function?

There are multiple definitions for mycotoxins. Simple approaches such as "secondary metabolites of fungi that can injure humans and other animals," omit some potentially pathogenic compounds or fungal elements, including beta glucans, hemolysins, mannans and spirocyclic drimanes. Defining mycotoxins as "toxic substances made by fungi," would include other secondary metabolites such as antibiotics and immune suppressants. The main role for mycotoxins is to enhance efficiency of predation on plants,[1] not acting as offensive or defensive functions, as is oft claimed.

These attempts at defining mycotoxins fail to address the problem of including a mechanism of injury or toxicity in vivo. Consideration of route of exposure such as ingestion of mycotoxins or skin exposure, versus inhalation brings about additional confounders. Assessing inhalation exposure injury stemming from exposure to mycotoxins in vitro ignores (i) protective host mechanisms, including antigen presentation; (ii) loss of regulatory control of immune responses; and in the case of water-damaged buildings (WDB), (iii) eliminates the role of inflammatory responses, which taken as a whole, has been called chronic inflammatory response syndrome (CIRS) since 2010. This syndrome is marked by innate immune activation following exposure to a diverse series of immunogenic effectors including over 30 published effectors found inside WDB.[2]

As we have seen [3], omitting consideration of differential gene activation following exposure to mycotoxins ignores the main mechanism of mycotoxin injury to people, namely ribotoxin and ribosomal inhibitory protein attack on ribosomal production (including initiation, elongation and termination) of protein [4]. Further, failure to note suppression of nuclear encoded mitochondrial gene transcription is fatal to accurate assessment of adverse health effects.[3]

A more detailed definition of mycotoxins, but yet one that is still incomplete, focuses more on the role of mycotoxins in plants [5].

"Mycotoxins are toxic secondary metabolic products of molds present on almost all agricultural commodities worldwide. Unlike primary metabolites (sugars, amino acids and other substances), secondary metabolites are not essential in the normal metabolic function of the fungus. Other known secondary metabolites are phytotoxins and antibiotics.

"Currently there are around 400 mycotoxins reported. These compounds occur under natural conditions in feed as well as in food. Some of the most common mycotoxins include aflatoxins, trichothecenes, fumonisins, zearalenone, ochratoxin and ergot alkaloids. Mycotoxins are produced by different strains of fungi and each strain can produce more than one mycotoxin.

"Each plant can be affected by more than one fungus and each fungus can produce more than one mycotoxin. Consequently, there is a high probability that many mycotoxins are present in one feed ingredient, thus increasing the chances of interaction between mycotoxins and the occurrence of synergistic effects, which are of great concern in livestock health and productivity. Synergistic effects occur when the combined effects of two mycotoxins are greater than individual effects of each toxin alone."

Given that mycotoxins in feed and food can be metabolized (in stomach, gut and liver) to make degradation daughters, we must expand our consideration of adverse effects of ingestion to include consumption of or endogenous production of metabolites of parent mycotoxins. These compounds can stay

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in blood for variable amounts of time before appearing in urine, as enterohepatic recirculation can greatly reduce fecal excretion.[6] Urinary excretion of mycotoxins and metabolites has become the main source of information regarding dietary exposure and metabolism, with a robust literature on findings of mycotoxins in urine of cases and controls.

The goal of this paper is to take the reader from assessment of a chronically ill patient, one exposed to the interior environment of a WDB before onset of illness, using a published protocol and to examine what benefit measuring mycotoxins in urine brings. We will review pertinent literature regarding mycotoxins in food; and mycotoxins found in urine in control populations to understand the firm stance of the Centers for Disease Control and Prevention (CDC) against use of urinary mycotoxin testing that leads to therapy based on antifungals.[7] Recently published papers advocating treatment with antifungals based on urine findings will be reviewed.[8, 9]

Consider a functional definition of mycotoxins as different from what was presented above. "Mycotoxins are products of fungal metabolism in which secondary metabolites are manufactured in response to environmental stimuli that turn on mycotoxin synthetic gene clusters in the fungi, so they can make products that can be directly injurious to animals and people; and indirectly by adversely affecting protein production by impairing function of the sarcin-ricin loop in ribosomes and mitoribosomes; and mitochondrial function by interfering with nuclear encoded mitochondrial gene function."

We also cannot limit our discussion of naturally occurring mycotoxins as the main source of adverse human health effects. Fungi live in ecosystems in which a plethora of bacteria and actinomycetes invariably co-exist.[10] Actinomycetes ("actinos") are adept at making compounds that are toxic; they can manufacture ribotoxins as well that co-occur with mycotoxins. Actinos are richly endowed with gene sequences to make a host of bio-active compounds, [11] including antibiotics, anti-virals, anti-parasites and immune suppressants, among others.

We need to separate out toxins made by actinos from endotoxins and fungal mycotoxins if we are going to impute adverse human health effects to mycotoxins. Assuming mycotoxins cause illness from simple exposure is untenable in the face of studies showing presence in healthy controls of urinary mycotoxins and their metabolites.

Overview on dietary mycotoxins

For our discussion herein, we will be looking at three main categories of mycotoxins commonly ingested. Trichothecenes include some of the "dreaded" toxins made by some *Stachybotry*s *spp*. but they also are also produced by *Aspergillus* species.[12] Trichothecenes are widely known, with types A and B toxins described. These compounds share unique structures that create a lack of specificity when measured using ELISA. Deoxynivalenol (DON) as a by-product of fungal gene activation, DON will induce production of peroxidases that block generation of hydrogen peroxide by a plant when the plant is being "eaten" by the *Aspergillus* species.[1] The idea that fungi make mycotoxins as a defensive mechanism or as an offensive weapon to kill other fungi is not well supported.

The second category of mycotoxins of concern are ochratoxins. Ochratoxins have notoriety in the medical literature for their ability to cause renal injury, called Balkan nephropathy.

The third group of mycotoxins are aflatoxins. Aflatoxins are made by several species of *Aspergillus*, especially *A. flavus*. Aflatoxins

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have a reputation for causing human health effects including liver damage and possibly cancer but when we discuss mycotoxins and prevention of mycotoxin injury following ingestion, it appears that in pigs, that supplementation of protein in the diet, to include glutamic acid in small amounts can prevent mycotoxin injury.

There are other toxins of importance to human health. When one thinks of trichothecenes one will hear of satratoxins and roridins. When one thinks of *Wallemia sp. (*and *Aspergillus sp.*) one will think of production of sterigmatocystin (STC). When we think of *Chaetomium spp.*, it has its own suite of toxins, including chaetoglobosins.

Inside look: what adverse health effects are caused by exposure to the interior environment of WDB?

Over the last 25 years there have been a series of changes in opinions regarding causation of adverse health effects seen in patients exposed to the interior environment of WDB. Legal decisions mirror some views of society towards so-called "mold" cases.

Before it became known that "mold illness" was caused by inflammation, which was caused by exposure to WDB, followed by genomic response to biological elements found inside the WDB, including toxins, inflammagens and fragments of microbes (fungi, actinomycetes and bacteria, among others), some defense consultants suggested that the illness was allergy. There are people who do have allergies to mold; hypersensitivity pneumonitis can occur following exposure to thermophilic actinomycetes.

Allergy is based on excessive antibody responses to exposure; that finding is not involved in CIRS. Treatment of allergy by removal from exposure will resolve symptoms but not in CIRS. High levels of IgE is typically found in allergy but rarely is high IgE seen in CIRS. Defective antigen presentation is seen in CIRS; excessive antibody response is seen in allergy. Proteomic findings seen in CIRS but not seen in allergy include excessive levels of cytokines, split products of complement activation, TGF beta 1, with increased relative risk for a limited number of HLA haplotypes. CIRS is not allergy

We now know that the genomic injuries in CIRS are commonly initiated by ribotoxins. These are compounds made by one-celled microbes, including fungi, bacteria and actinomycetes that stop or reduce normal protein synthesis by disrupting an evolutionarily conserved structural element on ribosomes called the sarcin-ricin loop (SRL). These are the actual players that initiate the cascades of inflammatory events seen in CIRS. The fact that the differential gene activation seen in CIRS comes from biowarfare among one-celled creatures that began four billion years ago is stunning.[13]

The legal importance of defining allergy as causative of symptoms acquired from damp buildings is straightforward: Negligent maintenance or construction defects don't cause allergy, but they do cause CIRS-WDB. Landlords might be accused causing injury to a tenant if the problem is CIRS but convincing a jury that the problem was allergy usually shields the landlord from paying awards.

Published research expanded our knowledge of harmful abnormal physiology of CIRS-WDB. We found that CIRS was definable. The condition had a variety of defining objective biomarkers. Findings that confirmed CIRS include visual contrast sensitivity (VCS), cluster analysis of symptoms, genetic susceptibility (HLA DR) and prospective repetitive re-exposure trials conducted over a

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course of six days (sequential activation of innate immune elements, SAIIE). [14-16]

While there are more science-based markers found in CIRS, nowhere will we see any that the illness is marked by presence of mycotoxins in urine. Presence of mycotoxins will tell us, however, if the patient has eaten warehoused foods in the last sixty days. The CDC has rejected such testing in no uncertain terms[7], saying that foods have mycotoxins and that mycotoxins will appear in the urine of healthy persons following consumption of contaminated foods.

Additional insight into CIRS-WDB comes from multiple published, peer-reviewed case/control studies involving over 5000 patients. Patients have known exposures to WDB; controls do not. Cases have a large roster of symptoms, controls do not. Cases have lab abnormalities, controls do not. Cases respond to treatment with reduction of symptoms, VCS deficits and lab abnormalities. These elements form the accepted case definition of "mold illness" published by the US GAO in 2008.[17] There is no mention of urinary mycotoxins in that Federal publication.

Prospective, double blinded, placebo-controlled clinical trials have also been published to confirm that causation of CIRS and treatment benefit is not random.[6, 18]

Additional biomarkers come from NeuroQuant; an FDA-cleared software program added to MRI of the brain which shows a distinctive fingerprint in CIRS-WDB patients.[19] Correction of the inflammation that causes the illness results in simultaneous abatement of symptoms and NeuroQuant deficits, demonstrating that the neurologic and cognitive abnormalities in CIRS are not permanent.[20] The neurologic injury is caused by inflammation; it is treated by reduction of inflammation.

A subset of patients with CIRS-WDB have excessive gray matter nuclear atrophy that has been shown to respond to treatment protocols employing vasoactive intestinal polypeptide (VIP).[2] Published in 2017, no other subsequent studies have shown correction of gray matter nuclear atrophy. Clinicians using CIRS treatment protocols see this salutary result on a daily basis.

Sequential mycotoxin testing has never been correlated with improvement of brain volumes.

There are other objective biomarkers in CIRS patients including development of pulmonary hypertension. Here, the velocity of tricuspid regurgitation (TR), measured in meters/per second, is elevated such that four times the square of TR, added to right atrial pressure, will exceed 30 as a cut off separating normal pulmonary artery pressure from acquired pulmonary hypertension. In a stress echo, measured by achievement of pulse rate of greater than 90% of predicted, we will see a rise greater than 8mm of mercury (Hg) in patients with acquired pulmonary hypertension.[22] Sequential mycotoxin testing in urine does not correlate with improvement of pulmonary hypertension.

Measurement of maximal oxygen consumption exercise, VO2 max, can also be demonstrated in patients with CIRS-WDB. Correction of inflammation results in improvement of VO2 max. Sequential mycotoxin testing in urine does not correlate with improvement of VO2 max.

The greatest progress in looking at definable, objective biomarkers for CIRS[23] and CIRS-WDB comes from transcriptomics.[3, 4] Using state of the art molecular platforms, transcriptomics shows differences in gene activity in cases compared to controls, as well as in patients observed prospectively who

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develop specific gene abnormalities with exposure to WDB that resolve with standard treatment. When combined with NeuroQuant studies showing resolution of gray matter nuclear atrophy and/or reduction of the enlarged forebrain parenchyma and/or cortical gray, these transcriptomic studies give us basis for better understanding of neuronal injury and repair. Sequential mycotoxin testing in urine does not correlate with improvement of transcriptomics.

A remarkable discovery of disproportionate increase in activation of coagulation genes in CIRS, together with beta tubulin genes, demonstrates the marked correlation of a subset of CIRS cases with enhanced gray matter nuclear atrophy. The significance of these findings for those with developing dementia supports the vascular hypothesis of neuronal injury,[24-26] as targeted treatment of patients with excessive coagulation gene activation and early dementia is anecdotally associated with improvement of cognitive state.

Unfortunately, mycotoxicosis medical practice moves away from science

Against this deluge of overwhelming confirmation of causation of mold related CIRS illness and correction with published treatment protocols, one cannot forget discredited defense arguments presented in litigation. One of those opinions, called *Geffcken*, demanded that evidence of mycotoxins be found in patient tissues that were identical to mycotoxins found in a given building to which the affected patient was exposed. Unfortunately, the identification of mycotoxins accurately in a room is compromised even if mycotoxins could be found accurately in tissue. Geffcken held sway in medical mold cases until 2006 in the United States. Sequential assessment of urinary mycotoxins could have provided cover for Geffcken-type arguments in court had urinary measurements ever been shown in published literature to be correlated with exposure.

When *Geffcken* and allergy defenses didn't work to win cases, resourceful legal arguments from defense interests became that the illness seen in patients in water-damaged buildings was due to *ingestion* of mycotoxins. The idea of ingestion creating illness was supported largely by a study from Russia in 1947 reporting horses dying after eating hay contaminated by *Stachybotrys sp.* The study had little objective data to support it and yet, because it was a convenient way to "make the jury look the other way," it was attempted without much success.

We still hear that ingestion remains the dominant source of exposure without evidence. Ingestion is the main route for appearance of mycotoxins in urine but remember metabolites (there are at least 18) of ochratoxin A can stay in human blood and tissue for over sixty days.[27]

Beginning in the mid-2000s, a new concept arose as advocated by several physicians from the Mayo Clinic. These physicians were convinced that fungi growing in sinuses were a marker for chronic rhinosinusitis (CRS) and that nasal cultures could be used to demonstrate the fungi.[28] Despite the evidence to the contrary,[29, 30] this idea, while it changed radically over the next five years from fungal causation of CRS in favor of an inflammatory condition with eosinophilic basic protein actually causing the runny nose, the idea has persisted. An important paper from the German literature showed that fungi could indeed be found in nearly every one's nose, with cases of CRS having 2.3 species of fungi, but controls had 3.1 species.[31]

Beginning in 2009, a new approach to explaining adverse health effects found in people exposed to water-damaged buildings

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was proposed. A pathologist published a paper[8] showing that patients exposed to WDB had putative evidence of mycotoxin carriage that could be detected using an ELISA technique in urine. Not with-standing the lack of precision of ELISA analyses, laboratories started selling tests for urinary mycotoxins. The idea that exposure alone equated to illness causation was promoted.

The paper referred to a control group but specific (i) control group demographics and (ii) building testing that confirmed microbial amplification were omitted. Absence of reliable control data remains a problem. If one is maintaining causation of illness, there needs to be prospective studies demonstrating acquisition of illness coincident with appearance of abnormal urine findings. If one relies on only a case/control study, there must be a transparent association in which abnormalities of exposed patients are different from abnormalities in non-exposed patients derived following transparent and thorough differential diagnosis.

The scientific concept is simple: no controls, no conclusions about cases.

Since the theory for finding mycotoxins in urine was that fungi growing in the nose were making toxins, use of antifungal nasal sprays rose in 2014 and 2015. By 2016, extensive acquisition of anti-fungal resistance was seen not just in fungi but in bacteria as well, apparently through the mechanism of horizonal gene transfer (personal communication, MicrobiologyDx, 8/2016).

The alarming feature of the new-found antibiotic resistance in bacteria was resistance to (i) vancomycin, an antibiotic necessary for dangerously ill septic patients, as well as (ii) gentamicin, an aminoglycoside, emerged in a group of organisms called coagulase negative staph. These staphs are multiply antibiotic resistant and are known by their acronym of MARCoNS. Kirby-Bauer resistant biograms in MARCoNS let us trace development of vancomycin and gentamicin resistance which in turn could be traced back to physicians who used antifungals. Now that the antifungal resistance has spread (likely through plasmid exchange as well as free DNA transfer) the genes for fungal resistance are found not just in antifungal users but have spread rapidly in the MARCoNS population. MARCoNS are promiscuous exchangers of DNA and antibiotic resistance factors with other one-celled creatures. We simply need to look at the experience with Staph aureus, a coagulase positive staph, in the 1970's to 1980's to suspect that the reservoir of resistance to penicillin was in MARCoNS.

Dietary sources of mycotoxins

Fungi are ubiquitous in nature. Foods carry fungi. Moist food, especially starches, will support fungal growth in a few days. Dry foods will take longer to spoil, but fungal presence in foods can create problems for health of humans and animals. Predictably, toxigenic fungi are also found routinely in food supplies worldwide. In spite of the massive potential to cause adverse human health effects, i.e., if people eat fungi and mycotoxins at the same time, people will be ill and urinary mycotoxins will be positive, we may ask, "Where are the cases?" Absence of massive numbers of cases of mycotoxicosis suggests that the role of foods in producing chronic, multisystem, multi-symptom human illness has not been confirmed. Yet, when we hear "experts" telling us to avoid coffee, mushrooms, wine, cheeses, breads and more because of fungi, as shown by mycotoxins in urine, we don't see any epidemiologic confirmation of the basis for such advice.

The source of mycotoxins in food can be divided into three categories of pre-harvest;

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post-harvest and warehouse-based growth. The foods involved are diverse, but colonization of foods is primarily due to several genera of fungi. These are *Aspergillus*, *Fusarium* and *Penicillium*, with *Stachybotry*s less common. *Fusarium* species are confined to corn products and will not be discussed.

In food manufacturing, from warehouse to table, attempts to destroy mycotoxins by food processing is an ongoing challenge. Mycotoxins are resistant to most physical methods; detection is complicated by the sheer volume of foodstuffs that must be analyzed.[32]

The health concerns about ingestion of mycotoxin-contaminated food have been heavily weighted to in vitro studies but confirmation of the same risks is rare in vivo. These problems could include autoimmunity, allergy, birth defects, cancers and mutagenesis.[33] While the vast majority of patients seen following exposure to the interior environment of WDB have inhalation as their route of exposure, and not ingestion, in animals, ingestion can clearly cause mycotoxicosis. Species differences are dominant in mycotoxicosis.

There is variation from climate to climate of foods grown, as well as fungi found in those foods that contaminate harvested food. The most important mycotoxins are aflatoxin, ochratoxins, zearalenone, patulin and trichothecenes. This latter group includes deoxynivalenol (DON), metabolites of DON, T2 toxin and satratoxin.

Additional human health concerns include renal dysfunction due to ochratoxin A exposure, together with the largely uninvestigated field of chronic low-level, long-term exposure to multiple mycotoxins.[34]

The appearance of the most common fungi in food is at variance with fungi identified with confirmed adverse human health effects, measured with MSQPCR, called Health Effects Roster of Type Specific (formers) of Mycotoxins, version 2.[35] For example, there are *Aspergillus* species that make ochratoxin found in food and WDB, but none of those fungi are seen in the top ten species list associated with adverse human health effects. Similarly, *Stachybotrys*, one of the top five species most pathogenic for human hosts, is associated with significant appearance of parent trichothecenes and their metabolites in urine of control patients. Interestingly, aflatoxin, which derives its name from *Aspergillus flavus*, a fungus that rarely appears in the top ten most commonly associated with human illness.

Patulin is a toxin found in fruit juices, especially in apple and grape juice, but also with stone fruits including apricots, peaches and plums. Patulin rarely is found in intact fruit but any fruit with a damaged surface is susceptible to fungal infestation. The key to preventing exposure to patulin is to maintain high fruit quality.

We are faced with the near ubiquitous finding of patulin in apple juice. But where is the evidence that when apple juice is ingested, bringing a significant gastric burden of patulin, that illness follows? Perhaps clues to the relative absence of adverse human health effects from ingested mycotoxins come from the findings that patulin, for example, is rapidly destroyed before leaving the stomach, resulting in a residual of less than 3%.[33]

Much of the concern about food contamination with mycotoxins has given rise to odd dietary alterations together with claims of enhanced safety of mycotoxin-free foods. As much as 50% of human daily intake of ochratoxin and its metabolites is due to its direct consumption in cereals or grains, but the remainder will be due to consumption of animals after they ate

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contaminated feed. The list of common food sources of ochratoxins includes foods made from corn, rice, wheat, barley, oats, rye, sorghum and millet. Contaminated foods include cereals or trail mixes; together with bread, bread products and baby food. Any brans from rice, corn, wheat or oats can be contaminated as well as cracked grains, wine and beer.

Ochratoxin A is found in cheese and meat products, as well as dried and smoked fish, soybeans, garbanzo beans, nuts and dried fruits. Additional food sources of ochratoxin are raisins, wine and wine vinegars. Coffee and pork also are known to harbor ochratoxins.[33]

The problem of analysis for ochratoxin is complex, usually requiring high performance liquid chromatography (HPLC) and mass spectrometry (MS) in an attempt to separate an apparent molecule of ochratoxin A from its 18 known congeners that have variable half-life in blood, ranging up to 60 days.[33] With so many degradation daughters of ochratoxin A, where are the billions of people suffering worldwide from ochratoxin A-associated nephropathy? The stated risks of OTA appear to be over-stated.

With regard to cancer causation, aflatoxin (AFB1), especially when associated with hepatitis B virus, is widely reported to be associated with hepatic cell carcinoma. The marker for the breakdown product of aflatoxin is AFB1-N7-guanine adduct.[32] This adduct is secreted into urine; mycotoxins labs could look for this marker of cancer causation from aflatoxins. One might again ask, where are the billions of people with cancer associated with aflatoxin?

Host/farm protective factors

It is well known that detoxification of aflatoxins is accomplished internally by an enzyme, glutathione S-transferase (GST), which will bind to an ingested metabolite of aflatoxin, then combine with glutathione to detoxify the compound. GST is ancient and evolutionarily conserved, with a complex gene family in plants.[12] T2 toxin and DON will induce activation of groups of the GST genome. Of interest is the existence of gene super-families for GST in honeybees and Drosophila;[36] polymorphisms are felt to have a role in loss of protection from sporadic colorectal cancer risk in Caucasians.[37]

Regarding sources of T-2 toxins in the world, the natural occurrence of host genera *Fusarium* or *Sporotrichioides* has been reported in Asia, Africa, South America, Europe and in North America. Predominant genera that make other trichothecenes include *Stachybotrys*, *Trichoderma* and *Trichothecium*. All of these sources of T-2 will be detected when an ELISA assay is used.[33] The main production of mycotoxins is associated with the greatest water content before and immediately after harvest. Once food materials dry out, mycotoxin production declines in step with reduction of A_w.

DON and its metabolites are the most prevalent of trichothecenes found in food; DON is found with its metabolites, together with T-2 toxin and nivalenol. DON in urine is easily separated from other trichothecenes. Only a paucity of commercial labs, however, will perform this standard assay. Given the disparity of known effects of trichothecenes compared to known adverse ribotoxin effects creating the fundamental mechanism of molecular hypometabolism with ribosomal break down in mammalian cells following exposure, more accurate delineation of trichothecene effects is needed.

A problem faced by microbiologists is how to separate direct ribosomal injury from DON versus indirect injury from ribotoxins or

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ribosomal inhibitory proteins. This problem of lack of specificity as part of DON toxicity is partially solved by identifying a ribotoxic stress response that is manifested by immunotoxicity causing enhanced activation of mitogen-activated protein kinases (MAPK), which can be used as a marker for exposure to trichothecenes.

The association of enhanced mycotoxin production with activity of water (A_w) bears consideration. While there are variable activities of water associated with growth of fungal species, production of mycotoxins is most commonly accomplished at a higher A_w of 0.98 for ochratoxins, 0.93 for fumonisins and 0.90 for DON noted.[33] These values of A_w are not found on mucus membranes in humans, especially the nose. These data rule out the possibility of intra-nasal production of mycotoxins.

Given the common finding of mycotoxins in food and the common finding of mycotoxins in urine, what can we decide about the absence of significant health effects of the billions of patients exposed to mycotoxins on a daily basis? Could there be dietary factors associated with protection from mycotoxicosis? Are we looking at enhanced metabolism? Are we looking at the effect of antioxidants? Glutathione? Perhaps we can learn from mammals that are far more sensitive to mycotoxin effects, as shown by reduction in growth rates, than others. Pigs lead the list. As mentioned, pork will have mycotoxins in it and while the diet of a factory raised feedlot swine operation is not typified by feeding animals "slop," mycotoxins, especially DON, in pig food can slow growth causing the grower significant financial loss.

Three papers from the Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South Central China[38-40] bear significant weight in assessment for host factors protecting piglets from ingested mycotoxin injury. Multiple groups of piglets were assessed for DON-damage by measurements of oxidative parameters in the first study[38] including catalase, malic dialdehyde, nitric oxide, peroxide levels in blood; total antioxidant capacity; d-lactate and amino acids. DON decreased catalase but this effect was blocked by feed that was supplemented by 2% glutamic acid. Peroxide too was higher in DON, but glutamic acid blocked that oxidative effect as well. Similar results were noted for malic dialdehyde and nitric acid: glutamic acid prevented harm. DON increased lactate (NB: this is an indirect marker for molecular hypometabolism) but glutamic acid prevented that abnormality.

Villus height was diminished in jejunum and ileum by DON but was increased in glutamic acid-fed piglets. Similarly, glutamic acid blocked increased lymphocytes induced by DON, and protected goblet cells as well. The indicator genes for DON-induced ribotoxic stress response were activated in DON-fed pigs and protein synthesis reduced, but not in pigs fed the combination of DON and 2% glutamic acid.

The second study[39] used the same control and experimental designs. This time, glutamic acid blocked mycotoxin-induced decreased weight gain and blocked reduced feed conversion rate.

The final study[40] used nuclear magnetic resonance to show additional benefits of glutamic acid in DON-challenged piglets. Here the authors showed additional manifestations of protection, namely glutamic acid treatment corrected DON-driven raised levels of LDL cholesterol, lowered levels of HDL; corrected elevated levels of alanine, arginine, acetate, glycoprotein, trimethylamine-N-oxide, glycine, lactate, urea and glutamate/creatinine ratio. Further, glutamic acid increased superoxide dismutase and glutathione peroxidase. The

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authors conclude that glutamic acid can repair injuries associated with oxidative stress as well as disturbances of energy and amino acid metabolism induced by DON.

Remember that glutamate, consists of two enantiomers. One is L-glutamate and the other is D-glutamate. The D-enantiomer of glutamate is better known as MSG. Glutamate is found in proteins and peptides, with virtually every food containing glutamate. If we superimpose the occurrence of aflatoxicosis on areas of protein/calorie malnutrition, we see that if adequate protein in food is ingested, mycotoxicosis from food is dramatically reduced. This is an association suggesting that something found in food has much to do with protection from fungal injury. Protein rich foods including meat, eggs, poultry, milk, cheese and fish are major components of glutamate in the diet.

The 3-D structure of glutamate has an amino group in the middle of the chain of five carbons with a carboxylic acid moiety found on either end. At acid pH, one hydrogen can be lost from a hydroxyl group balanced as a zwitterion with a NH3 replacing an NH2. At gastric pH, there can be production of a single positive charge with the amino group becoming an ammonium group with each hydroxyl group losing an electron. This mini-molecular anion dipole could create a mechanism for binding the anion rings of mycotoxins by the cation found in glutamate at gastric pH. This is the putative mechanism that has been shown for cholestyramine binding to a variety of mycotoxins.[41, 42]

As at least 25% of foods are felt to be contaminated worldwide, we would expect anywhere between 1 ½ to 2 billion individuals sickened by mycotoxins if this were a simple linear expression of causation with exposure resulting in illness. The absence of 2 billion sick people suggests strongly the model used for excessive mycotoxin pathogenicity is flawed. Even though food safety concerns and best farming practices emphasize mechanisms to reduce fungal contamination, we need to look at host factors as the variable controlling acquisition of illness from ingestion.

Host factor analysis itself is potentially flawed as rarely mentioned fungi may be confounders. Such is the case with sterigmatocystin (STC) in foodstuffs. While we worry about production indoors of STC by *Wallemia sebi*, STC is also found in grains, corn, bread, cheese, spices, beans, soybeans, pistachios, animal feed and silage.[43]

In a Turkish population,[44] hydroxydeoxyguanosine and malondialdehyde were correlated with ochratoxin A. The advantage of finding specific urinary markers with mass spectrometry is that there are 18 different metabolites known for ochratoxin A. Trying to pick one of those 18 accurately as a carcinogen by any other method is problematic.

Dietary protection factors

The degradation of patulin showed 94% disappearance from blood within 2 minutes of ingestion.[45] Given the high concentrations of patulin in particular foods, especially fruits, with apples leading the way, these compounds are rapidly degraded before reaching other tissues inside the upper gastrointestinal tract.

Further confirmation of the disintegration of patulin comes from studies in rats.[46] Isolated rat stomachs had luminal application of patulin with rapid emergence of mycotoxin into the stomach. Concentrations of 350 mg per liter and 3.5 mgs per liter were tested, with mycotoxins appearing almost instantly with both the high and the low dose. Residual toxin was 3% and 0.06%, respectively, in gastric tissue. This disintegration of 8400 micrograms and 700 micrograms, respectively, was in part

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due to the role of intracellular glutathione (GSH). The massive dose of patulin did reduce GSH content of tissue by 87% of controls but not the low dose.

Dietary supplementation of animal feed with organic activated bentonite, a clay product, and humic acid polymer, both have shown benefit in vitro,[47] confirmed by binding by both bentonite and humic acids of ochratoxin and zearalenone, with binding exceeding 96% of total burden. These products have not been demonstrated to provide benefit of reduction in inflammatory biomarkers acquired following exposure to WDB. Given the ability of these compounds to adsorb toxins but not to prevent disease suggests that ingestion and gastrointestinal exposure to mycotoxins is not the relevant causative feature of CIRS-WDB.

Other dietary strategies[48] with the ability to prevent toxic effects of ingested mycotoxins include antioxidants (selenium, vitamins, pro-vitamins); food components, including fructose, aspartame, chlorophyll and phenols; together with biological binding agents, hydrated sodium, calcium, aluminosilicate, bentonites, zeolites, activated carbons; bacteria and yeast. While these dietary strategies provide promise, the discrepancy between in vitro studies and in vivo studies are difficult to reconcile. These additional dietary compounds just don't work to prevent CIRS-WDB; the problem is not ingested mycotoxins.

Additional efforts to use microbiologicals for deactivating mycotoxins show initial promise. A *Eubacterium* sp., (BBSH 797), isolated from the rumen of a cow, deactivated trichothecenes. Also, a novel yeast strain, *Trichosporon mycotoxinivorans* was isolated and characterized as being able to degrade ochratoxin A and zearalenone.[49]

In a study that shows great promise,[50] 32 separate strains of *Rhodococcus* were demonstrated to be able to degrade aflatoxin B-1, zearalenone, fumonisins B-1, T-2 toxin and ochratoxin A. In addition, *Rhodococcus* species were able to protect from injury from multi-mycotoxin exposure. While this was a promising study in 2013, no *Rhodococcus* strains are available commercially.

Other microbiologic interventions include mycotoxin-degrading bacteria and fungi isolated from agricultural soils and animal digestive tracts.[51] Biotransformation effects included acetylation, glycosylation, ring cleavage, hydrolysis, deamination and decarboxylation. These promising solutions have not been tested in humans yet.

Similarly, *Trichosporon* shows promise in its ability to detoxify ochratoxin A. Certainly, this organism could be used in clinical trials to deactivate mycotoxins in animal feeds. This yeast, isolated from a hind gut of the termite,[52] *Mastotermes darwiniensis,* shows promise.

Enzymatic inactivation (biological detoxification) of fungal toxins has been accomplished using pure cultures of bacteria and fungi.[53] Following isolation of a complex microbial population, after differential gene activation has been identified, cloned and expressed in other hosts, the ability to detoxify aflatoxins, cercosporin, fumonisins, fusaric acid, ochratoxin A, oxalic acid, patulin, trichothecenes and zearalenone was accomplished.

Regarding glutathione,[54] in tests performed in lambs, there was localization of injected aflatoxin found in liver, nasal olfactory mucosa, nasopharynx, esophagus. larynx, trachea, bronchi, bronchial and conjunctiva. The nasal mucosa was the most active in forming DNA bound aflatoxin metabolites. When incubated in the presence of reduced glutathione, a drastic decrease in active DNA binding was seen without the addition of GST.

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Experiments done on chickens fed ochratoxin A[55] provided yet an additional approach of prevention of mycotoxin-induced injury in animals. When chickens were fed ochratoxin-contaminated diets of up 1.5mg/kg for three weeks, there was marked reduction of relative weight of immune organs (bursa of Fabricius and spleen).

Phagocytic function and lymphoproliferative responses in a follow-up experiment, when chickens were treated with silymarin, vitamin E or antioxidants,[56] the ochratoxin-induced immunotoxicity was prevented. This approach shows promise for chickens but as yet there is no indication of benefit in humans.

The mechanism of DON-induced proinflammatory gene expression[57] appears in humans and animals to involve activation of protein kinases located on the damaged ribosome. DON-induced activation of mitogen-activated protein kinases (MAPKs) is the known ribotoxic stress response. Pathological abnormalities in chronic low dose exposure showed anorexia, impaired weight gain and growth hormone dysregulation together with high dose exposure-evoked gastroenteritis, vomiting and a shock-like syndrome. DON evokes a ribotoxin stress response in mononuclear phagocytes which is an important contribution to acute and chronic toxic effects in vivo.

Early work in this field from Pestka[58] has shown that the mechanism of ribotoxic stress response involves double stranded RNA activated protein kinase (PKR) as well as hematopoietic cell kinases (Hck). Inhibitors in gene silencing studies have revealed PKR plays roles in both DON-induced gene expression and apoptosis.

Pestka has investigated the role of trichothecenes[59] on white blood cells. His lab has found that monocytes, macrophages as well as T- and B-lymphocytes are cellular targets of DON and trichothecenes. Exposure, even to low dose concentrations, reflected upregulation both transcriptionally and post-transcriptionally of cytokines, chemokines and inflammatory genes. High concentrations of exposure bring about apoptosis of leukocytes. Again, Pestka discusses the ribotoxic stress response, with binding to ribosomes and rapidly activating MAPKs. We are seeing that the series of immune events in CIRS-WDB is not related to ingestion but is related to genomic and transcriptomic abnormalities induced by toxin and/or ribotoxin exposure.

Experiments with DON inoculation[60] showed a whole series of gene activation in the MAPK family. For example, tyrosine phosphorylation of the hematopoietic cell kinase, Hck, was detected within 1 to 5 minutes after addition of toxin, with this gene activation suppressed by incubation with inhibitors of the family of tyrosine kinase.

Investigating the source of apoptosis has shown that BAK, a pro-apoptotic Bcl-2 family protein, is expressed in a wide variety of tissues.[61] Bcl-2 proteins regulate apoptosis as well as autophagy. When activation of apoptosis occurred following treatment with nigericin, a ribosomal toxin, both transient and stable overexpression of various forms of BAK exerted a protective role but it did not inhibit the extent of nigericin-mediated activation of caspase-3. This study strengthens the link between an exposure to ribotoxins and induction of apoptosis.

Accurate mycotoxin analysis in urine, feed and food: GC, MS and others
A tutorial

Mycotoxins are toxic fungal secondary metabolites that frequently contaminate food and feed worldwide, and hence represent a

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major hazard for food and feed safety. To estimate human exposure arising from contaminated food, so-called biomarker approaches have been developed as a complementary biomonitoring tool besides traditional food analysis. [62-73, 110] The first methods based on radio-immunoassays and enzyme-linked immunosorbent assays as well as on liquid chromatography were developed in the late 1980s and early 1990s for the carcinogenic aflatoxins. In the last two decades further tailor-made methods for some major mycotoxins have been published.

Since 2010, there has been a clear trend towards the development and application of multianalyte methods based on liquid chromatography/electrospray ionization/tandem mass spectrometry for assessment of mycotoxin exposure made possible by the increased sensitivity and selectivity of modern mass spectrometry instrumentation and sophisticated sample clean-up approaches. With use of these advanced methods, traces of mycotoxins and relevant breakdown and conjugation products can be quantified simultaneously in human urine as so-called biomarkers and can be used to precisely describe the real exposure, toxicokinetics, and bioavailability of the toxins present.

In this article, we present a short overview of the above cited articles and a comparison of published multi-biomarker methods focusing on the determination of mycotoxins and relevant excretion products in human urine is presented. Special attention is paid to the main challenges when analyzing these toxic food contaminants in urine, i.e., very low analyte concentrations, appropriate sample preparation, matrix effects, and a lack of authentic, NMR-confirmed calibrants and reference materials. Finally, the progress in human exposure assessment studies facilitated by these analytical methods is described and an outlook on probable developments and possibilities is presented.

Traditionally, mycotoxin testing used enzyme linked immuno-sorbent analysis (ELISA) technology which relies on antibodies, sometimes monoclonal but more often polyclonal. Among all published immunological based methods, these enzyme-linked immunosorbent assay (ELISA) were the most commonly used for mycotoxin determination. ELISA provides rapid screening, with many kits commercially available for detection and quantification of major mycotoxins including AFs, AFM1, OTA, ZEA, DON, fumonisins, and T-2 toxin.

ELISA methods have been validated in a wide variety of food matrices by only in a few instances for urine. The principle of ELISA is based on the competitive interactions between mycotoxins (acting as an antigen) and assigned antibodies labelled with toxin-enzyme conjugate for many binding sites. The amount of antibody-bound toxin-enzyme conjugate will determine the level of color development. This technique provides a rapid, specific, and relatively simple method for analysis of mycotoxins.

However, ELISA has certain disadvantages including potential cross-reactivity, dependent on antibody specificity. In addition, the kit detects only a single mycotoxin and is designed for one-time use; thus, it can be costly, impracticable even, if one needs to test samples contaminated with multiple mycotoxins. Moreover, each test kit is specified by the manufacturer for a set matrix and while some third-party validations, e.g., by AOAC, have been done for some mycotoxin ELISA kits, the validations are for use with specific toxins under specific contamination levels within specified matrixes; therefore, the kit cannot be used for all food matrices and all contamination levels, let alone human samples like blood and urine. Even when used in their appropriate

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settings, the manufacturers of these kits recommend that positive ELISA results should be confirmed by a suitable chromato-graphic method, especially when used in a matrix not specified by the manufacturer.

Alternatively, lateral flow devices (LFD) has been developed as a single-step test that includes a negative control line along with the sample lines on the same strip. A lateral flow test can provide semi-quantitative results in less than 10 min and requires no specialized equipment. It consists of three parts: a conjugate pad, a porous membrane, and an absorbent pad. The test is based on a competitive immuno-assay, where a labelled antibody is used as a signal reagent. This device has also recently been coupled with spectrometric readers to provide quantitative results. LFDs are commercially available for detection of AFs, DON, T-2 toxin, OTA, and ZEA. However, their applications in the field is limited due to numerous problems associated with the sensitivity and reliability in different matrices in addition to their high cost.

Another simplified system comprises flow-through membranes which utilize the same basic principle as LFD but may not yield accurate results near the detection limit. Flow-through immunoassays have been developed for screening OTA in green and roasted coffee, AFB1 in nuts and ZEA in cereals and feed samples. Although many different rapid strip tests have been developed for detection of major mycotoxins in different food commodities, they are not commonly used in the field and not commercially successful due to problems related to sensitivity, cost, and accuracy.

In addition to the methods described above, several other research methods have potential utility for the analysis of mycotoxins. However, these methods have limited application and have not been widely used outside the research environment as they require further verification and validation by recognized bodies such as AOAC, International Organization for Standardization (ISO) or CEN. There are commercially available test kits which are ready-to-use lateral flow devices (LFD) designed for on-site testing, providing rapid analysis of a wide range of food and feed samples with an assay time of 3 minutes. The test kits are available (62) in a qualitative or quantitative format, which requires a reader to provide objective results and secure a consistent results documentation.

Determination of mycotoxin levels in food samples is usually accomplished by methods (63) that include certain common steps: sampling, homogenization, extraction followed by a clean-up, and finally the detection and quantitation which is performed by many instrumental and non-instrumental techniques.

Chromatography is the most commonly used method used for mycotoxin analysis in food and feed. The earliest chromatographic method was thin layer chromatography (TLC), which is presently still used as a rapid screening method for certain mycotoxins by visual assessment or instrumental densitometry. However, current trends in mycotoxin analysis in food are focused on application of robust, fast, easy to use, and cheap technologies that are able to detect and quantify various mycotoxins with a high sensitivity and selectivity in a single run. To meet those needs, many chromatographic methods such as high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV), diode array (DAD), fluorescence (FLD), or mass spectrometry (MS) detectors and UHPLC or UPLC with reduced column packing material have been developed. Additionally, gas chromatography (GC) coupled with electron capture (ECD), flame ionization (FID), or MS detectors have been used to identify and quantitate volatile mycotoxins. Due to the low volatility and high

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polarity of most mycotoxins, GC analysis often requires a derivatization step; therefore, this method is used rarely in mycotoxins analysis which has been greatly advanced by coupling liquid chromatography techniques to mass-spectrometry (e.g., LC-MS; LC-MS/MS).

Apart from the great advantages of the conventional HPLC methods mentioned above, MS offers several distinct advantages over all LC methods for mycotoxin analysis in food. Basically, the mass spectrometer works by ionizing the molecules, and sorts and identifies them based on their mass-to-charge ratio (m/z). MS offers higher sensitivity and selectivity, as well as chemical structural information by molecular identity of the analyte based on m/z providing the mass spectrum as an ideal confirmatory technique. MS detection reduces time by eliminating the need for error-prone sample derivatization and clean-up steps needed for fluorescence enhancement. Different MS interfaces and analyzers have been used, such as atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), and atmospheric pressure photoionization (APPI). In addition, there are many types of mass analyzers such as quadrupole, time-of-flight (TOF), ion-trap, and Fourier transform-ion cyclotron resonance (FT-ICR). ESI, triple quadrupole, and TOF have been used extensively for mycotoxin analysis. Although the early applications of MS were for the analysis of single mycotoxins, the technique can now simultaneously quantify many hundreds mycotoxins and their metabolites in a single run, making it the current method of choice for detecting multiple mycotoxins in a wide variety of foods.

Since the arrival of modern high-performance LC-MS/MS (liquid chromatography-tandem mass spectroscopy) and GC-MS/MS (gas chromatography-tandem mass spectroscopy) instruments enabling multi-analyte methods for mycotoxin determination have become available, however these are not without substantial cost, with the majority of mycotoxin determination in urine performed recently by LC-MS/MS . However, a major challenge in urine mycotoxin analysis is the extremely low analyte concentrations present following dietary exposure.

Thus, effective, specific, sensitive and accurate methods for mycotoxin detection in urine require appropriate sample preparation protocols to accomplish the desired sensitivity while obtaining acceptable limits of detection (LODs) and quantitation (LOQs). Most of the methods available in the literature are based on traditional extraction techniques such as liquid/liquid extraction (LLE) or solid/liquid extraction (SLE), which have several disadvantages, mainly the high solvent volumes, high amounts of sample, and the long times required for the analysis. In recent years, method simplification and miniaturization are the most important trend in sample preparation allowing the use of low sample and solvent volume, fast analysis, and greater efficiency. Many pages of this monograph could be devoted to discussion relevant to the best method of prep-aration of urine for analysis, while other laboratories use the same “dilute and shoot” method for urines, as they do for bulk samples, blood and dust. In this case, a broad spectrum of spiked control mycotoxins is necessary to confirm matrix effects are being avoided and to enable the analysis to be shown to be linear, despite interferences and inhibition by such compounds that are often found in urine.

Method validation by laboratories undertaking mycotoxin analysis should follow the guidelines established by the EU and other regulatory bodies, including the determination of linearity, matrix effect (ME), limits of detection (LODs), limits of quantitation (LOQs), recoveries, repeatability (intra-day

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precision), and reproducibility (inter-day precision).

Of utmost importance are calibration curves for all mycotoxins analysed – they must be constructed from standard solutions (external calibrators) and in the matrix (matrix-matched calibrations). Matrix-matched calibration curves should be prepared from blank urine samples spiked with standardised mycotoxins before and after extraction, if used. There are limits for the range of these standard curves that should also be observed so that LOQ is also validated.

A final, cautionary note about validating analytical methods for mycotoxins is that reliance on avoidance of foods likely to contain mycotoxins or their metabolites is no guarantee that the urine obtained from "control" subjects will be a genuine baseline. Screening and analysis of the analytes of interest is therefore prudent because mycotoxin producing moulds may contaminate numerous agricultural commodities either before harvest or during storage. A varied diet consisting of different foods may therefore be contaminated with a range of mycotoxins.

It is claimed that naturally occurring mycotoxins have been indicated as causative of a wide array of adverse health effects. The measurement of urinary mycotoxin levels is a means of assessing an individual's exposure, but the development of sensitive and accurate analytical methods for detecting mycotoxins and their metabolites in urine samples is challenging. Urinary mycotoxins are present in low pg/ml concentrations, and the chromatographic identification of their metabolites can be obscured by other endogenous metabolites.

As a result of the advent of the latest generation of high-performance LC-MS/MS instruments, a clear trend towards the development and application of multianalyte methods in mycotoxin biomarker research can be observed. Purification of the analytes is often achieved by using sophisticated sample clean-up approaches with subsequent separation by liquid chromatography and detection using triple-quadrupole analyzers coupled via an electrospray ionization (ESI) interface. However, the latest studies have also successfully applied the so-called "dilute and shoot" approach by omitting any clean-up step. As already stated, this section only provides a short overview and comparison of published multi-biomarker methods, and is expanded further to discuss challenges associated with very low analyte concentrations, sample preparation, matrix effects and a lack of calibrants and certified reference materials, and describes the progress in human exposure assessment studies facilitated by these methods in following sections.

A major challenge in mycotoxin biomarker research are the extremely low analyte concentrations present in biological fluids following dietary exposure. Hence, appropriate sample preparation protocols are crucial to obtain acceptable LODs. This is, however, hampered by the great chemical diversity of analytes typically included in multi-biomarker methods. This issue becomes even more complex once polar conjugates such as glucuronides are included as they are frequently lost during common clean-up approaches such as SPE or IAC procedures.

Accurate mycotoxin analysis in urine, feed and food: GC, MS and others
From the literature

The State-of-the-Art method[74] for testing mycotoxins including aflatoxin, ochratoxin and trichothecenes is mass spectrometry. Gas chromatography (GC) and liquid chromatography (LC), liquid mechanisms, while regarded as accurate and precise, may

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show extreme variable sensitivity due to different biological characteristics of mycotoxins, matrices and instruments. This is especially true for LC-MS where the response can be different depending on ionization techniques used. If fluorescence or UV absorbance can be used for quantitative measurements LC-MS appears to be relegated to use as a confirmatory technique.

If toxins are not volatile, LC-MS is uniquely able to render results that are quantitative and qualitative accurately. These problems are multiplied by attempts at determination of mycotoxins in food given the extreme variability of the food matrices. Specific ionization interfaces are needed to reduce matrix effects and ion suppression. It is possible that MS detectors will show advances that permit low cost, high throughput determination of mycotoxins in food and feed.

Given the concerns about health effects of mycotoxins in food and feed, risk assessment of mycotoxin contamination for both humans and animals require clear identification and reliable quantitation in diversified matrices.[75] With mass spectrometry emerging in the 1970's, we now are seeing a variety of hyphenated techniques that combine chromatography with mass spectrometry. Indeed, LC-MS, or better still LC-MS/MS has become a routine technique.

The challenge of detecting multiple mycotoxins, as is commonly seen in the same sample, requires advanced techniques for each diagnostic run. LC-MS/MS is able to measure different levels of mycotoxins, and their metabolites, that are both free and masked. Newer techniques will likely emerge as multi-dimensional chromatography-MS, capillary electrophoresis-MS and surface plasmon resonance array-MS have become available. Cost of the new advanced techniques will continue to be a factor.

With the enhanced multi-class mycotoxin analysis in food, environmental and biological matrices and LC-MS/MS, the ability to detect mycotoxins has become increasingly precise. This technical advance raises a curious condition, however, in that presence or absence of molds are less frequently identified and correlated with mycotoxin presence.[76] As mentioned, mycotoxins seldom develop alone; various types will be formed in the same foodstuff. Co-occurrence of mycotoxins creates a real problem for assessment of dose-response relationships, not to mention genetic susceptibility such that the mere presence of multiple mycotoxins should be considered as a risk factor but risk itself is not adequate to conclude causation of illness.

A simple question for governing bodies regarding food safety is how one device can provide results that will be sensitive and specific for the wide variety of chemical structures in mycotoxin analysis.[77] An additional challenge remains that heterogeneity of foods demand multiple analytical methods be used at the same time permitting rapid and inexpensive analysis. Ongoing problems include proper collection of representative samples, avoiding secondary contamination after collection, performance of emerging analytical methods, including immunochemical techniques, with validation of methods for those involved with enforcement of standards in regulatory affairs and finally, limitation of current methods.

In an effort to confirm precision of increasingly sophisticated mass spectrometry techniques, a study was done by Gerding in Germany.[78] Food surveys were recorded together with the food frequencies questionnaire followed by LC-MS/MS assessment of urinary biomarkers. The authors looked for 23 separate urinary biomarkers, including trichothecenes (especially DON and its metabolites), T-2

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toxin, HT-2 toxin, aflatoxins and aflatoxin metabolites, ochratoxin A, ochratoxin alpha and others. One or more of a group of six mycotoxins and urinary metabolites were detected in 87% of the samples in a single occurrence. Only DON and its metabolites were detectable in quantifiable amounts. No statistical significance for correlation of staple food intake with urinary biomarker concentration could be provided. This important study supports the commonality of daily exposure of healthy patients to mycotoxins with such mycotoxins being identifiable in urine. The study could not control for a variety of metabolic modifications together with metabolites to provide statistical surety of exposure. Moreover, in normal patients, peak exposures above accepted daily intake levels for DON were routinely observed without evidence of adverse health effects.

These findings were confirmed in a longer-term trial of measurement of urinary biomarkers for aflatoxins in Brazil.[79] 16 volunteers, age 14-55 years old collected first morning urine four times every three months from June 2011-March 2012. Aflatoxin M-1 was found in 61% of samples. Residues of aflatoxin metabolites were not identified in any urine sample. GST was not evaluated. There were no differences in aflatoxin measurement over the four seasons of the study.

Sophisticated measurement of urinary biomarkers shows little or no relationship to development of adverse human health effects.

In a study from Spain,[80] human urine samples were analyzed for 15 mycotoxins and metabolites using a new multi-mycotoxin GC-MS/MS method following salting-out liquid, liquid extraction. 54 urine samples from healthy children and adults in Valencia were analyzed for mycotoxins and were normalized by simultaneous measurement of creatinine. 37 of the 54 samples showed quantifiable values of mycotoxins, finding H-2 toxin, nivalenol and DON. The co-occurrence of these contaminates was seen in 20.4% of samples. 2 of 9 exposed children had levels of DON in urine exceeding international levels without adverse health effects.

Urinary Mycotoxins in Health: Case/Control Studies

In one of the few studies performed looking at occupational exposure of mill workers, an experimental design was adequate to sort out occupational exposure to mycotoxins from diet from three separate grain mills in Germany with matched controls having parallel analysis.[81] Mycotoxins tested by urinary measurements were citrinin, DON, ochratoxin A, and zearalenone.

Immunoaffinity columns and liquid-liquid extraction (ochratoxin) was employed for urine sample clean up prior to liquid chromatography with tandem mass spectrometry (LC-MS/MS) or by high performance liquid chromatography (HPLC). Mycotoxin metabolites analyzed included DON-1, ochratoxin alpha, dihydrocitrinone, alpha- and beta- zearalenone. Urine samples were positive in both groups for citrinin, DON, ochratoxin and zearalenone. DON was found to be the highest concentration in both groups followed by ochratoxin. Mean biomarker levels in urine from mill workers controls were not significantly different, so levels of mycotoxins in urine simply reflected dietary exposure.

Absence of effect of known inhalational exposure on urinary measurements creates a high hurdle for those who espouse significance of urine testing as a reliable marker for illness.

An ongoing problem in measurement of urinary mycotoxins is the possible confounder created by multiple mycotoxins exposure. In a study in South Africa of food and first morning

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urine, sophisticated LC-MS evaluation[82] was able to show correlation of food consumption with presence in morning urine of fumonisins, DON, zearalenone and ochratoxin A. This paper demonstrates the value of multi-biomarker measurements in measuring exposures in populations exposed to multiple mycotoxins.

Assessment of simple sample preparation procedures for evaluating mycotoxins in foods and urine were performed with comparison dispersive liquid-liquid microextraction and salting-out-liquid-liquid extraction of analysis of ten fumonisins mycotoxins in metabolites in urine were compared[83] (see [84] for tandem study). Under optimal extraction techniques, salting out liquid-liquid extractions showed a better accuracy in precision than dispersive liquid-liquid microextraction. Based on these preliminary results a multi-biomarker method and based on salting out liquid-liquid extraction followed by gas chromatography and tandem mass spectrometry was initiated. The method resulted in low limits of detection and quantitation down to 0.12 and 0.25 micrograms per liter respectively.

A follow-up paper[84] from the same group looked at quantitative LC-MS/MS measurement of 11 mycotoxins (aflatoxin, ochratoxin and others in human urine) using dispersive liquid-liquid microextraction methods on ten urine samples from healthy volunteers showed the presence of mycotoxins in low concentration.

This paper from the UK looked at differences in appearance of mycotoxins in urine in healthy adult volunteers and healthy vegetarians.[85] Statistically significant differences were seen with 32% of vegetarians exceeding recommended tolerable daily intakes of mycotoxins.

Ongoing enhancements of LC-MS/MS technology are reported in this study from China looking at zearalenone and its metabolites in urine. 301 urine samples were collected from healthy volunteers of all ages in China with 71% of all samples positive for zearalenone and metabolites. Adolescents had a higher exposure then children, adults and the elderly.[86]

In another study, one of the few that still uses ELISA kits to assay for aflatoxins[87] the study compared urinary aflatoxin measurements in 84 individuals either in a rural or semi-urban community in Nigeria. 99% of urine samples had detectable aflatoxin. Levels were higher in the semi-urban population compared to the rural population. There was no significant difference in mean urinary aflatoxin levels in males/females compared among children, adolescents and adults.

In one of the few studies done comparing mycotoxins found in urine to those in dust, 21 cases who worked in a bread dough factory were compared to 19 individuals who were controls. No reports of illness of either group were found.[88]

In workers, DON and ochratoxin were the most prevalent biomarkers found in 66% and 90% of participants. In controls DON was found in 58% and ochratoxin in 66%. DON was the mycotoxins measured in highest amounts in settled dust samples. Workers in both groups were exposed to several mycotoxins simultaneously, but there was no difference in urine findings in cases and controls. Exposure in the workplace was not felt to be contributing to adverse health effects. However, the workers did have a higher contact with flour dust which revealed a higher exposure to DON. It becomes problematic to institute risk management when the selected biomarker of mycotoxins in urine has no relationship to illness in studies done with control groups and exposed workers alike.

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This is a human biomonitoring study of multiple mycotoxins in urine from Belgium known as the BIOMYCO study.[89] This study design assessed mycotoxin exposure in Belgium adults and children using urine for the measure of exposure. Morning urine was gathered according to a standard study protocol involving 155 children and 139 adults. Urine was analyzed for presence of 33 mycotoxins including aflatoxins, trichothecenes and ochratoxin as well as metabolites using LC-MS/MS methods. DON and ochratoxin and their metabolites were the most frequently detected. A metabolite of DON, deoxynivalenol-15-glucuronide, was the main DON biomarker and was found in all samples. DON itself was detected in 70% of children and 30% of adults. Ochratoxin was found in 51% of children and 35% of adults. Urinary mycotoxins differed significantly based on age and gender in this study. Biomarker analysis showed a clear exposure of a broad segment of a Belgium population to DON and ochratoxin. The concept of risk assessment arises given that young children may need special attention because there is a relatively higher food intake per kilogram of body weight, so it may be worth examining if the biomarkers present in high amounts are a risk factor. No symptoms were presented that correlate with those amounts.

In an interesting study from the UK, assessment of DON in an elderly cohort[90] was taken with 20 patients over the ages of 65 reporting urine findings on two consecutive days. The level of quantification was detected in 90% of elderly men and woman on both days. Dietary assessment of DON suggested only 10% of the elderly exceeded the maximum provisional tolerated daily intake for DON. No data on health or illness in these patients was reported.

In a study without human health assessments, forklift drivers at waste management facilities were assessed for occupational exposure of drivers as well as toxicity of dust collected from filters mounted inside forklifts.[91] Mycotoxin analyses were performed by LC-MS/MS methods. Cytotoxicity was assessed using a filter extract which was analyzed using MTT cell culture. *Aspergillus* species were the predominant organism detected, but no mycotoxins were detected in filter extracts, although those same extracts were either highly toxic or moderately toxic in cell culture. One is left with the question, what in this mixture of dust material besides mycotoxins was creating the cellular injury? Further, if cellular injury were present, did the forklift drivers have evidence of illness? Those questions remain unanswered.

Exposure to mycotoxins is not confined to mammals; in this study of aspergillosis in poultry,[92] consideration of the role of gliotoxin was included. Autopsy was performed on 73 birds, all of which presented with an illness consistent of aspergillosis. A culture was done; chloroform extraction of gliotoxin, thin layer chromatography, and histopathology was performed. *Aspergillus fumigatus* identification was confirmed by PCR. Seven isolates of *A. fumigatus* were obtained in 6 of them. Gliotoxin-like compounds were detected. Though these numbers are less than 10% of the total avian population, the role of gliotoxin in birds is possibly important.

In a different approach to gliotoxin, the authors[93] discuss gliotoxin isolated from *Trichoderma* species as an antibiotic substance involved in biological control of plant pathogenic fungi. Gliotoxin is felt to be a defense molecule thought to have a role in aspergillosis and is used in *Trichoderma*-based bio-fungicides. Gliotoxin has medicinal properties as a potential diagnostic marker and is important in biological crop protection. This

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paper does not assess the claims regarding endogenous production of gliotoxin as an illness-causing agent.

Gliotoxin has a critical role in pathobiology for *Aspergillus fumigatus*. It modulates the immune response and induces apoptosis in different cell types. This fungal metabolite has been subjected to many investigations with a focus on its biosynthetic pathway.[94]

In another study of gliotoxins[95] that looked at the virulence of *Aspergillus fumigatus*. Genes in this cluster include a transcription factor and a non-ribosomal peptide synthetase. Two laboratories have reported gliotoxin to be an important virulence determinate, but three other laboratories showed it was not. The disparity was found to be the immunosuppressive regimen used for mice. Gliotoxin was found to be unimportant when immunosuppression with cyclophosphamide and steroids were used. If the immunosuppression was accomplished with steroids only, gliotoxin was important using virulence methods. These studies indicate that the mouse model is inadequate to evaluate secondary fungal metabolites in human illness. Questions about gliotoxin determination in urine will remain.

With the focus on the gene clusters of *Aspergillus fumigat*us for gliotoxin biosynthesis, several important metabolites produced by the gliotoxin biosynthetic pathway were identified.[96] These metabolites were influenced by either gliotoxin or specific reactions within the pathway. The activity of gliotoxin against animal cells and fungi was often mediated by interference with redox hemostasis. This is an area where glutamic acid would oppose gliotoxins in animals.

This study[97] alludes to overlap of glutathione transferase (GST) in the gliotoxin biosynthetic pathway. Deletion of one of the genes (Glig) from the cluster (identified as a GST) results in cessation of gliotoxin biosynthesis. Return of Glig restored gliotoxin production in vitro. As we investigate gliotoxin more, its role in induction of GST needs to be clarified.

In an earlier gliotoxin study the gliotoxin biosynthetic pathway was examined using a genomics approach. Differential gene activation in this pathway is co-regulated with timing of expression correlating with production of gliotoxin and culture. This is another in vitro study,[98]
one without GST, yet suppression of gene activity in the biosynthetic pathway would protect against gliotoxin production.

Gliotoxin exerts a broad spectrum of immunosuppressive effects in vitro and is detectable in serum of patients suffering from invasive aspergillosis.[99] No comment is made about the correlation of urinary gliotoxin with serum gliotoxin. This study compared isolation of gliotoxin from 158 aspergillus isolates. There were *A. fumigatus*; 27 *A. terreus*, 16 *A. niger* and *A. flavus* had 15. Gliotoxin was identified in 98% of A. *fumigatus* patients with 96% environmental samples. The toxin was also found in 66% of *A. niger*, 37% of *A. terreus* and 13% of *A. flavus*. Culture supernatants of an *Aspergillus fumigatus* strain lacking gliotoxin showed a significantly lower cytotoxicity on macrophage-like cells than T-cells in vitro. Curiously, lack of gliotoxin production in the other *Aspergillus* species had no influence on cytotoxic effect of culture supernatant on these immune cells. The study does not speculate on why gliotoxin would show more toxicity from *A. fumigatus* strains compared to other *Aspergillus* species.

This study examined ochratoxin and citrinin found in blood in 104 blood samples taken from University students in Bangladesh in 2013 and 2014.[100] Ochratoxin was present in all samples. The investigators calculated

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dietary ochratoxin intake among the students and it was found to be lower than the tolerable weekly intake for ochratoxin. The method used for analysis included LC-MS/MS and HPLC techniques. One can question whether blood versus urine analysis is more accurate. This question is not resolved by the literature.

A study [101] from Germany specifically focused on citrinin looking at urine samples from a group of 50 healthy adults (27 females, 23 males). There was a mild increase in urinary citrinin in males compared to females. Finding citrinin and its metabolites in over 80% of all urine samples in healthy patients was instructive.

In another study from Bangladesh,[102] ochratoxin and citrinin were evaluated in pregnant woman in Bangladesh. 54 urine samples were collected from residents of rural and suburban areas for analysis using LC-MS/MS methods together with HPLC. Ochratoxin was found in 93%, citrinin in 87%. There is suggestion of mild difference between urban participants. Urinary biomarkers for ochratoxin and citrinin did not show significant association with intake of food although there were higher levels of citrinin levels with participants who ate more rice.

In an important study[103] looking at piglets that were feed with *Fusarium* toxin contaminated maize, assessing presence of mycotoxins found in blood, liquor and urine with LC-MS/MS, a variety of levels of dietary contamination was noted during 29 days of treatment. Concentrations of zearalenone and DON and their metabolites were analyzed. In urine all analytes were detected in significantly higher concentration compared to serum and liquor. The toxin intake for body weight 3-4 hours before slaughter correlated with the sum of DON metabolites in all three specimens as well as with zearalenone. In the first study reviewed for this report, given the high correlation of dietary DON and the measured DON, the exposure can be evaluated. Serum levels of these toxins were indicative of exceeding the guidance value in feed using regression equations. There was significant individual variation among pigs that needs to be considered.

In a variation of animal studies other researchers[104] looked at zearalenone metabolism in dairy cows. The study design included assessment of zearalenone in blood, milk, urine and bile. Interestingly, the bile concentration of measured zearalenone in cases and controls in contaminated feed in cows were not significantly different, suggesting that rumen fermentation mediated alterations in zearalenone and metabolites were associated with alterations of bile formation and bile turnover.

In variation of normal case /control studies in an Italian study,[105] a group of 55 celiac patients were compared to a control group of 50 healthy subjects with measurement of DON and zearalenone in 105 urinary samples. Markers were detected in 21 celiac patients and 15 controls corresponding to about 34% of the total participants. There was no statistical difference in mycotoxin exposure in the two groups. These findings do not suggest specific regulation of gluten free products as levels of urinary mycotoxins were no different in celiac patients compared to controls.

As discussed, finding ochratoxin in human blood of healthy patients is not unusual. In a patient study in Tunisia, [106] blood samples from healthy subjects were analyzed using HPLC measurements. Ochratoxin was found routinely. An additional study of ochratoxin in blood did not show any correlation with age or gender. The highest ochratoxin plasma levels were found mostly in summer. Ochratoxin levels in populations showed variations from year to year but intra-individual repetition

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showed no specific trend. No correlation of presence of ochratoxin in human plasma was made with abnormalities in health status.

In a study in the Balkans,[107] variations of ochratoxin A in healthy populations were identified. Ochratoxin was measured in 983 samples using HPLC techniques with fluorescent detection. Samples containing ochratoxin above the detection level were found in populations from all Croatian cities at all collecting periods. The highest levels of ochratoxin were in June. While the levels of ochratoxin found in Croatia were lower than other European countries, the study shows that healthy populations of Croatia are exposed to low but seasonally/regionally variable amounts of ochratoxin. 983 samples are the largest study seen in preparation of this review. The conclusions of no evidence of adverse effects from elevated ochratoxin in blood in impressive.

A pediatric population was evaluated in another study[108] comparing subjects from Egypt to Guinea, looking for aflatoxin exposure in young children. Using urinary aflatoxin metabolites in parent toxin samples from Guinea (N=50, age 2-4 years) were analyzed with immunoaffinity clean up, followed by HPLC and fluorescent detection. Aflatoxins were less frequently found in Egyptian children (38%), compared to Guinean (86%) children. These specimens of urine were from healthy children, with less exposure apparent in Egypt compared to Guinea. The study concludes that measures to reduce aflatoxin exposure to both regions are important but unfortunately, to support that conclusion, we have no long-term data to look at the risk of association of exposure to illness.

There is another study from Egypt.[109] Looking at aflatoxin, as it constitutes "a real human threat," this study enrolled 50 healthy breast-feeding mothers and their infants who were exclusively breast feed for at least 4 months. All had thorough lab evaluations including measurement of aflatoxin by HPLC. Forty-eight % of mothers and their infants had been contaminated with aflatoxin found in mothers' blood, mothers' milk and infants' blood. There was no evidence in this study that the contamination was associated with renal or liver function abnormalities.

Summary of control studies:

21 studies covering 2756 controls from children to adults from North and South America, Europe, Asia and Africa. One study showed positive urine mycotoxins of 60% of 15 patients; one showed 66% of 19 patients with the rest showing 80-100% positive.

In specialized groups, there were 11 studies covering 421 controls. The lowest % positive were 38% in Egyptian children, 48% each for nursing mothers and infants; with the rest being > 75% to 100%.

Two papers purport to support the urinary mycotoxin hypothesis

A fundamental question for those health care providers who feel that (i) presence of urinary mycotoxins define a new illness and (ii) use of antifungals will treat the illness is, "What does exposure to mycotoxins actually mean?" If one breathes mycotoxins, there be an immune response adequate to generate the inflammatory responses widely published in CIRS, as described previously in this review, seen in about 25% of patients. All, however, are at risk for a positive urine mycotoxin test. For whom will mycotoxins be ingested, metabolized properly by the human body and excreted harmlessly in the urine? Our multiple cohorts reported herein with good health and positive urine tests showed a marked preponderance of people from around the world that may fall in this category. Or, are mycotoxins generated in

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unknown ways and unknown locations as defined by an unknown case delineation giving a positive urine test.[8,9]

In nearly all instances, mycotoxins will appear in urine, just as they do in controls. How does one assign weight of causation to a biomarker that does not separate cases from controls? Or, are we simply looking at dietary sources of mycotoxins? If so, what is the value of doing urinary testing to diagnose sick people?

As discussed in the section above, a high percentage of control patients show mycotoxins in urine. We saw that not only will trichothecenes, aflatoxin, and ochratoxin routinely appear in urine of controls but their metabolites will as well. These metabolites are readily determined by newer techniques like mass spectrometry, but ELISA testing is fundamentally flawed in that there will be a variety of compounds with similar, but not identical structures, called epitopes, to the quested mycotoxins found in urine. Testing for metabolites of mycotoxins, which one would expect to be mandatory under standard uses of ELISA, would then be skewed as these epitopes would give the false appearance of significance in the urine.

Since metabolites are not reported by two commercial urine mycotoxin test labs in the US, what criteria assist us in ruling out a positive test by presence of a benign metabolite?

We don't know what basis these commercial labs have to define an abnormality as such and not just due to dietary effects that are ubiquitous?

An even greater challenge is what did mycotoxins do on the way through the human body, perhaps through the gastrointestinal tract or the respiratory tract, to get to the urine? Did they set off an immune response, creating a CIRS, or did they metabolize into benign degradation daughters as they were eliminated as waste harmlessly? Mere presence of contaminants in urine is not enough to show causation of illness.

The peer-reviewed literature supporting the use of antifungals and urinary mycotoxins is not non-existent, but certainly is far less robust than what we would expect over the past ten years from proponents of the idea trying to establish its validity. The purpose of peer-reviewed literature is to present ideas in a rigorous fashion, with rigorous bibliographies that are even and unbiased that will allow the skeptical scientist a mechanism to evaluate the evidence presented. Research papers will usually have an abstract and conclusion; unfortunately, many people will only read those two elements of a published paper. Perhaps a better approach is to look at the methods of a study to see if there is any point in reading the study, because if the methods are illogical or incomplete, there is no point in wasting time reading flawed science, much less investing health care dollars in antifungals or the proponent of their use.

We look for a distinct method section in any published paper. The first paper advocating ELISA methods for mycotoxins in urine was published in 2009 by Dennis Hooper and David Straus. This paper appeared in International Journal of Molecular Sciences with a PubMed citation (8). We see an abstract, introduction, results (methods are not in a specific section), with a conclusion without a stand-alone discussion (there is a section called "preparation and evaluation of specimens for mycotoxin detection"). This is not a standard design for a research paper. The paper presents urinary findings for ochratoxin, aflatoxin and trichothecenes with no discussion of metabolites. There is no discussion of any of the known congeners for ochratoxins. There is

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no discussion of epitopes confounding ELISA results found in cases.

The author specifically notes that the experimental design lets them derive qualitative results but not quantitative results. The results claimed that spiked samples created confirmation of the ability of the antibody procedure to detect increasing amounts of toxin; how was this possible if the method wasn't quantitative?

Of vital importance in any kind of test that is being brought to consideration for public use is to compare (i) known cases to (ii) defined controls. Cases are known when they meet a case definition. Hooper and Straus use no case definition. There is no attempt to present a transparent differential diagnosis as no differential diagnosis was presented, with such a process needed to ensure rigor in diagnosis.

No control definition is used. The only control is a "negative control group" as determined by absent or low mycotoxin levels. One wonders if controls were named simply as a result of a negative test because the world's literature, we looked at had no control groups with less than 38% positive, with most over 80-90%. The paper is silent to this concern.

Since people with exposure to WDB must fulfill four layers of case definition to be called a case, we would expect some sort of algorithm to be presented of how controls were shown *not to be exposed*. We are told that specimens from patients with no known toxic mold exposures were tested to develop a set of reference data for a control group. There is no table presenting what methods were used to show absence of microbial amplification in buildings for each control or presence of amplification for each case.

We are not given ages of the 55 controls, gender of the 55 controls or race of the 55 controls. We are not given any human health data regarding the case samples other than "symptoms acknowledged by physicians as being related to mycotoxin mold exposure in and out." This is an egregious error: symptoms are never adequate alone to make a diagnosis without (i) differential diagnosis and (ii) satisfaction of a case definition. It is clear from 25 years of work in the chronic inflammatory response world that the symptoms cited including asthma, memory loss, fatigue, headache, muscle pain or weakness are not specific to exposure to WDB. Indeed, these symptoms are a small portion of the 37 symptoms found in over 30% of CIRS cases as evidenced by published literature beginning in 1997. There is no discussion of validity of symptoms selected by authors in applicability to case definition.

There is no discussion of known biomarkers, well established in peer-reviewed literature, including the US GAO study of 2008; but more importantly published in thousands of cases compared to hundreds of controls beginning in 1998. These publications are not cited in the paper for unknown reasons. Bias as shown by deliberate omission has no basis in science.

Even if the ELISA mycotoxin detection antibodies employed were monoclonal and the author has acknowledged they are not ("specific polyclonal antibodies" is the term used for aflatoxins; monoclonal for ochratoxin A [congener not specified]; and roridin antibodies for trichothecenes); and even if we can ignore metabolites as possible confounders (we cannot); and even if we felt that the size of the study was adequate to compare to mass spectrometry and liquid chromatography; we are then left to guess as to what the control group actually is. Based on the data presented in the prior section, the likelihood of finding 55 consecutive control patients in Texas without mycotoxins in urine approaches a number of (1/2) to the 55^{th} power. We may conclude that

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the likelihood of this control group is reliable is not supported.

The next paper by Joseph Brewer and Dennis Hooper, again with a PubMed citation in 2013 (9), reported the detection of mycotoxins in patients with Chronic Fatigue Syndrome. 10 of 104 cases had building sampling (none of results are reported). Note this paper was published after presentation of a case/control study involving hundreds of patients in which biomarkers for CIRS-WDB were presented (22). Use of a previously published diagnostic and treatment protocol was employed with use of vasoactive intestinal polypeptide (VIP) added to enhance patient correction. As seen in the first paper, these published data are not referenced. The author's use a published case definition of Chronic Fatigue Syndrome from Fukuda but given that CFS has no objective biomarkers, none are presented. CIRS-WDB have a host of published biomarkers over the last 25 years: none were included in either study.

Urinary mycotoxin testing was reported to have been used to compare cases to healthy controls, previously reported. These controls are now identified for the first time by Dr. Brewer as being 28 males and 27 females, age 18-72 years. These were from diverse geographical areas and resided in various areas of the United States. Control subjects were reportedly asked about complaints and/or symptoms related to "mold exposure," but none are reported in a standard data table. It may be assumed the controls had exposure to foods; and airborne mold spores could occur in their daily activity. It is these groups, who nearly always have mycotoxins in urine, as referenced above are found to have trivial levels at best.

There was no delineation of any environmental sampling used to confirm the potential for exposure as required by the US GAO Report of 2008. They do not discuss cases or controls for exposure to water-damaged buildings with musty smells; with visible mold; or with DNA sampling to give accurate delineation of species in genus or fungi present. Without documenting potential, or absence of potential for cases and controls, respectively, no conclusions can be drawn about exposure. Once again, testing for urine mycotoxins (aflatoxins, ochratoxins and macrocyclic trichothecenes) was done, noting that in Hooper's 2009 paper, the antibodies used were against satratoxin and roridin. Extrapolation from these two to DON and others cannot be justified even though there is possible cross reactivity from zearalenone and DON confounding diagnosis for satratoxins.

Testing in cases is done on urine sent in a non-refrigerated container and analyzed at some time after receipt. No documentation is provided regarding stability of clinical samples by whatever delivery method (not sent on dry ice, not sent on wet ice, not sent overnight) such that we don't know stability of the urine substrate.

The statistics presented ignore metabolites and ignore other types of macrocyclic trichothecenes. There is only documentation of qualitative results in cases published by Hooper in 2009 and yet in 2013 the authors now are claiming ELISA data to be both quantitative and specific. Methods do not disclose the source of conversion of the ELISA from qualitative to quantitative. We find the controls used are the same 55 patients without mycotoxins in urine. The same argument of lack of credibility for this finding applies.

Interestingly in Table 2 the control patients are listed as having no aflatoxin; no ochratoxin and no macrocyclic trichothecenes; indeed, no mycotoxins of any kind were detected and yet in Table 3 now we find that controls do have ochratoxins and macrocyclic trichothecenes.

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These results remain confusing, as they appear to be mutually exclusive.

Brewer states, "The environmental histories of these patients for positive exposure to WDB many with visible mold and over 90% of these illnesses tested included residential and workplace." These data are not presented in the paper. Testing was performed only in 10 of 104 patients (data not shown). An analysis of ten cases out of 104 is hardly sufficient to create credibility for history as any experienced mold treating physician knows.

Additionally, this paper quotes the Mitochondrial Disease Foundation as a significant reference. "Mitochondrial deficiency" is listed as the underlying factor causing manifestations including autoimmune disorders, chronic fatigue, neurodegenerative disorders including ALS, MS and Parkinson's disease, depression and psychiatric disorders, together with glycogen disorders among others. There is no basis presented for these claims.

The link to mitochondria, not confirmed for injury to mitoribosomes, claims that mycotoxins cause mitochondrial dysfunction. Whether mitochondria were disrupted in these patients is not clear. One cannot use idle speculation as proof of causation in two patients out of 104.

The conclusions refer to the healthy control population as non-exposed to water-damaged buildings. These are patients with impossible findings in which no mycotoxins are found. Regarding the Chronic Fatigue Syndrome patients, "a majority had prior exposure to WDB," when in fact, data on only 10 out of 104 were alluded to and less than 5 are reported with unconvincing data. We expect clear and convincing exposure data on all patients, both controls and putative CFS cases. Additional unsupported speculation is that mitochondrial dysfunction is a possible cause for the health problems in these patients and such mitochondrial dysfunction may be triggered and accentuated by exposure to mycotoxins.

Summary:

Even if we are presented with impeccable lab results from ELISA and thorough use of standard differential diagnosis (we aren't), based on world-wide control data, and a robust literature on CIRS, there is no basis to ascribe any diagnostic significance to urine mycotoxin testing.

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