

Communication

# Case-Control Study of Nodding Syndrome in Acholiland: Urinary Multi-Mycotoxin Screening

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**Abstract:** This case-control study adds to the growing body of knowledge on the medical, nutritional, and environmental factors associated with Nodding Syndrome (NS), a seizure disorder of children and adolescents in northern Uganda. Past research described a significant association between NS and prior history of measles infection, dependence on emergency food and, at head nodding onset, subsistence on moldy maize, which has the potential to harbor mycotoxins. We used LC-MS/MS to screen for current mycotoxin loads by evaluating nine analytes in urine samples from age- and gender-matched NS cases (n = 50) and Community Controls (CC, n = 50). The presence of the three mycotoxins identified in the screening was not significantly different between the two groups, so samples were combined to generate an overall view of exposure in this community during the study. Compared against subsequently run standards,  $\alpha$ -zearalenol ( $43 \pm 103 \mu\text{g/L}$  in 15 samples > limit of quantitation (LOQ); 0 (0/359)  $\mu\text{g/L}$ ), T-2 toxin ( $39 \pm 81 \mu\text{g/L}$  in 72 samples > LOQ; 0 (0/425)  $\mu\text{g/L}$ ) and aflatoxin M1 ( $4 \pm 10 \mu\text{g/L}$  in 15 samples > LOQ; 0 (0/45)  $\mu\text{g/L}$ ) were detected and calculated as the average concentration  $\pm$  SD; median (min/max). Ninety-five percent of the samples had at least one urinary mycotoxin; 87% were positive for two of the three compounds detected. While mycotoxin loads at NS onset years ago are and will remain unknown, this study showed that children with and without NS currently harbor foodborne mycotoxins, including those associated with maize.

**Keywords:** Nodding Syndrome; mycotoxins; urine; Uganda

**Key Contribution:** This work contributes to the growing body of knowledge implicating frequent mycotoxin exposure as an environmental factor in child growth and health maladies that affect African children.



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## 1. Introduction

Nodding Syndrome (NS) is a brain disorder (tauopathy) of childhood and adolescence that has been present over the past 20–25 years in epidemic form in northern Uganda and South Sudan [1–6]. Internal displacement, severe food shortages, vermiform infection, and lack of medicine and immunization are common to NS-affected populations in these areas. Children with this disease are born normally and undergo unremarkable perinatal development before growth slows and the child becomes physically and mentally stunted, coincident with behavioral changes, periodic head nodding, and convulsions. The short stature and developmental delay seen in some children with NS and its phenotypic variant, Nakalanga syndrome, raise the possibility of neuroendocrine dysfunction [7,8].

In 2001–2002, a team assembled by the World Health Organization (WHO) to investigate an outbreak of NS in then-southern Sudan noted a strong association between

head nodding and ingestion of food (serena, a red sorghum) delivered by the World Food Programme [5]. Plants with direct or indirect neurotoxic potential (ackee, cassava, grasspea, sorghum, vetch, and yams) were ruled out as risk factors, but the possibility that head nodding was associated with food contaminated with pre-harvest or post-harvest mycotoxins (secondary mold metabolites) could not be excluded [5,9]. In northern Uganda (Acholiland), an epidemic of NS (1997–2015) was associated with civil war-related movement of the population to internal displacement camps, where conditions were squalid, infectious disease rampant, and food quality very poor [10]. In March 2014, a case-control study of medical, nutritional, and other risk factors associated with NS among 5–18 year-old Acholi children found a significant case association with reports of prior measles infection; higher monthly onset of NS when food stocks were low; greater use of emergency food delivered by the World Food Programme; and food dependence on moldy plant food, in particular moldy maize, at onset of head nodding [11]. Moldy maize consumed in Africa is frequently contaminated with mycotoxins from the following groups: aflatoxins, fumonisins, trichothecenes, and zearalenones [12–16].

The degree to which mycotoxins as a whole influence global health is not yet fully understood and appreciated, a sentiment echoed by others [17,18]. Simply establishing baseline data of exposure in susceptible populations remains to be done in many regions of the globe. Epidemiological data on foodborne diseases are lacking, particularly in low-income countries, and outbreaks often go unrecognized and unreported [19]. Populations most at risk have limited regulatory oversight or prevention measures to avoid consumption of contaminated food [20], as many rely on subsistence farming and local, unregulated markets [21]. As surveillance studies are completed and the relative burden of mycotoxins comes to light in a given region, this information must be utilized to develop a concerted response among all stakeholders, from producers to consumers, with an emphasis on integration of local voices and infrastructure. Further, the authors stressed that regulation and surveillance are important but that sensitive, cost-effective methods of detection are needed for enforcement of mycotoxin limits under a food safety program.

Relevant biomarkers of mycotoxin exposure with supporting validation and implementation in human subjects across four continents were recently reviewed [22–24]. For the mycotoxins of concern in moldy maize, candidate biomarkers include urinary aflatoxin M1, sphinganine:sphingosine ratio for fumonisins, deoxynivalenol, and zearalenone/ $\alpha$ -zearalenol/ $\beta$ -zearalenol. In the current study, we examined mycotoxin biomarkers in the urine of Acholi children both with and without NS ( $n = 50$ /group). Mycotoxin load reflects exposure to fungal products during the preceding few days. As such, this study aimed to demonstrate that, rather, beyond NS, mycotoxin contamination of food and feed in Africa is a major problem with implications that affect human and animal health and the economy [16,25,26]. Multi-mycotoxin biomonitoring surveys such as this add to the growing database of direct exposure estimates, which will aid in identifying populations vulnerable to development of mycotoxicoses and elucidate the possible contribution mycotoxins make to exacerbating morbidity of regional diseases. This information can provide a framework upon which better, more timely risk assessment and mitigation strategies can be developed for these fungal contaminants.

## 2. Results

Table 1 identifies the mycotoxins detected in the 50 NS and 50 Community Control (CC) samples that were analyzed using a multi-mycotoxin method, which surveyed for nine mycotoxins and their metabolites. Results should be viewed semi-quantitatively, as samples were compared against subsequently run matrix-matched standards.  $\alpha$ -Zearalenol was detected in 93 of the 100 samples; of those, only 15 exceeded the limit of quantitation (LOQ), with mean concentration  $\pm$  standard deviation (SD) of  $43 \pm 103$   $\mu\text{g/L}$ . Other mycotoxins detected in this survey were T-2 (86/100 samples, with 72 above the LOQ and an average concentration of  $39 \pm 81$   $\mu\text{g/L}$ ) and aflatoxin M-1 (48/100 samples, with 15 above the LOQ and an average concentration of  $4 \pm 10$   $\mu\text{g/L}$ ). NS and CC samples were

not significantly different from each other for any of these mycotoxins ( $p > 0.05$ ). Signals for the quantitative and qualitative fragments of zearalenone-14-O-glucuronide were detected, but no standard was commercially available; thus, levels could not be quantified in the 73/100 samples in which it was identified.

**Table 1.** Mycotoxins detected in screen of urine from Ugandan children with Nodding Syndrome and age-and gender-matched Community Controls.

Mycotoxin	Nodding Syndrome (N = 50)			Community Controls (N = 50)			Samples Combined (N = 100)		
	(%) Positive	Mean (µg/L) <sup>a</sup>	Median (Min, Max) (µg/L)	(%) Positive	Mean (µg/L) <sup>a</sup>	Median (Min, Max) (µg/L)	(%) Positive	Mean (µg/L) <sup>a</sup>	Median (Min, Max) (µg/L)
α-Zearalenol	6 (12%)	34 ± 94	0 (0, 317)	9 (18%)	52 ± 112	0 (0, 359)	15 (15%)	43 ± 103	0 (0, 359)
T-2	35 (70%)	29 ± 66	0 (0, 288)	37 (74%)	49 ± 93	0 (0, 425)	72 (72%)	39 ± 81	0 (0, 425)
Aflatoxin M1	7 (14%)	4 ± 9	0 (0, 36)	8 (16%)	4 ± 10	0 (0, 45)	15 (15%)	4 ± 10	0 (0, 45)

<sup>a</sup> Numerical values represent the mean of all samples ± standard deviation.

Ninety-five of the samples were positive for at least one mycotoxin for the 100 Acholi children evaluated, with 87 samples being positive for two of the three quantitatively determined mycotoxins. The remaining mycotoxins in our screening assay (ZEN, β-ZEL, HT-2, FB<sub>1</sub>, FB<sub>2</sub>, and OTA) were not detected in the urine samples above their LOQ. Correlation was run comparing the three mycotoxins to each other in all samples, and none achieved a value of correlation above 0.03, indicating that, within an individual, there was no correlation in mycotoxin concentration for the three mycotoxins detected.

### 3. Discussion

We subjected urine samples from NS and CC children to multi-biomarker analysis for exposure to mycotoxins, which generally reflects recent (days) dietary intake [27]. Since all children at the time of sampling were living in villages under peacetime conditions, instead of the privation and malnutrition that typified their earlier life in IDP camps, no significant differences in mycotoxin content were expected or revealed ( $p > 0.05$ ). However, we found evidence among the 100 samples children that their current (2014) diet exposed them to a range of mycotoxins with immunotoxic, neurotoxic, and genotoxic properties. Another study of this population found high levels of total aflatoxin and ochratoxin in mostly millet, sorghum, maize, and groundnuts in households both with and without children with NS [28]. Similarly to the present study, no significant association was found between dietary mycotoxins and incidence of NS. A limitation of their study also included sampling many years (2014–2015) after the temporal peak of diagnosed NS cases from Ugandan households. However, together, these results demonstrate the need to analyze factors that promote food spoilage in this rural Ugandan community, with the goal of improving food quality to reduce exposure to hazardous mycotoxins as a public health measure.

#### 3.1. Mycotoxins Detected and Their Impacts on Nutrition, Growth, and Child Health

Mycotoxins have more recently been targeted for their effects on nutrition and immunity, particularly as they affect growth and development in infants and children [16,19–21,26,29–33]. Stunting (child's height is two standard deviations or more below the WHO growth reference [20]) and being underweight are manifestations exacerbated by malnutrition in infants and children who are already at a higher risk for the effects of mycotoxin exposure due to their higher rate of food intake per kg body weight [34]. The International Agency for Research on Cancer's Working Group report on mycotoxin exposure in low and middle-income countries provides a comprehensive review on the subject [31]. Their analysis concluded that mycotoxin exposure contributes to child growth impairment independent of, and together with, other risk factors that may cause stunting. Exposure to mycotoxins may thus have played a role in

the stunted growth of NS versus CC children [9]. Aflatoxins, trichothecenes and fumonisins have been the predominant compounds discussed thus far in the literature, but the mechanism of toxicity by which these and other mycotoxins contribute to infant and young child growth impairment need to be further developed, especially in light of the fact that individuals are often chronically exposed to a multitude of compounds in the diet [32,33,35,36]. For example, a study in Tanzania of 6–14 month-old children found urinary fumonisin B<sub>1</sub> (by itself or in combination with aflatoxin exposure) to be negatively associated with child growth [37]. Fumonisins inhibit ceramide synthase, thus altering sphingolipid metabolism and thereby disrupting the function of membrane proteins such as the folate-transporter proteins (folate receptor  $\alpha$  in humans) [38]. Inhibition of folate transport can cause neural tube, craniofacial, and other developmental defects, which have been noted in populations with a history of fumonisin consumption [38,39].

The Sanitation Hygiene Infant Nutrition Efficacy (SHINE) trial in Zimbabwe hypothesized that mycotoxin exposure contributes to child stunting through environmental enteric dysfunction and disruption of the insulin-like growth factor 1 axis [40], pointing to altered nutrient absorption/intestinal function and immunomodulation as possible mechanisms. In another study, average birthweight did not differ among NS cases and controls, but mean body weight at time of examination was significantly lower for NS cases [11]. It is plausible that exposure to immunosuppressive fungal metabolites may set up the children to be more susceptible to secondary conditions, such as infestation with the nematodes *Mansonella perstans* and *Onchocerca volvulus* [41], a risk factor for NS [2,42]. In terms of in utero exposure, studies have demonstrated the transmissibility of mycotoxins across the maternal-fetal barrier [32,43–48]. Mycotoxin presence in breast milk has been reviewed and a summary made of the aflatoxins, ochratoxin A, zearalenone, and fumonisin B1 found to date in that matrix [49]. The authors stressed that we have much to learn about the breadth and actual contamination level of these compounds given recent advances in instrumentation and multi-compound detection methodologies. Further, rate of lactational transfer and the role of biotransformation, from both the mother and infant, will be important in making an accurate risk assessment for exposed infants.

As a whole, no major differences were detected in the 100 Acholi children evaluated, either for an individual or group of mycotoxins in the biomatrix assessed (Table 1). Ninety-five of the urine samples were positive for at least one mycotoxin, with 87 samples being positive for two of the three mycotoxins quantitated.  $\alpha$ -Zearalenol was found in 15 individuals from both the NS and CC groups (Table 1). The mean concentration for the NS group was  $38 \pm 97 \mu\text{g/L}$ , while that for the CC group was  $52 \pm 112 \mu\text{g/L}$ . This difference was deemed not significant ( $t$ -test,  $p = 0.54$ ). Zearalenone-4-*O*-glucuronide was also found in the majority of samples (73/100); combined with  $\alpha$ -zearalenol, these results indicate exposure to zearalenone as the most significant contaminating mycotoxin in the current food supply of the children evaluated. Zearalenone is a non-steroidal estrogenic compound produced mainly by *Fusarium* spp. on grains and is associated with reproductive disorders such as decreased conception, abortion, and hyperestrogenism, but may also be developmentally immunotoxic [50].

Aflatoxins (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>) are produced by *Aspergillus* spp. on peanuts, maize, and other grains common in African diets; they primarily affect the liver, causing hepatic necrosis leading to cirrhosis and cancer if ingested in large enough quantities (acute aflatoxicosis) or chronically consumed over a longer period of time, especially with co-exposure to the hepatitis B virus [51,52]. In addition, aflatoxins have immunosuppressive effects and, due to their ability to bind with DNA, affect protein synthesis [51], an absolute requirement for growth and development. The presence of metabolite aflatoxin M1 in the urine samples from the current study ( $4 \pm 10 \mu\text{g/L}$  of all children (Table 1)) reflects exposure to aflatoxin B1. Echodu and co-authors [28] also found high levels of aflatoxin in foods consumed from Ugandan communities both with and without NS cases. The authors suggest how, despite there being no significant correlation with NS, chronic aflatoxin exposure could act as a cofactor in NS disease development or participate in generally

overwhelming normal developmental processes in children, as found in a recent review on adverse reproductive outcomes in African children exposed to mycotoxins [32].

Trichothecenes (most notably deoxynivalenol (DON), nivalenol, T-2 toxin, HT-2 toxin, and diacetoxyscirpenol) obstruct protein biosynthesis via inhibition of peptidyl transferase; they are found mainly in cereals such as wheat, maize, and barley, and less frequently in sorghum and rice [34,51]. Trichothecenes also disrupt gastrointestinal function and interfere with growth hormone activity. Chronic exposure of animals resulted in decreased food intake and weight gain, altered immune function, and negative developmental sequelae [20,53]. T-2 toxin was frequently detected in samples from the current study (72% of samples), with an average of  $39 \pm 81 \mu\text{g/L}$  in all children (Table 1).

### 3.2. Dietary Exposure to Mycotoxins in African Food Plants

The presence of mycotoxins in the urine reflects food intake of mold-bearing plant materials while subjects were living at home in a time of peace without food shortage. It is reasonable to posit that the mycotoxin load was greater (delayed harvest, poor storage, damp conditions [26]) when these children were subjected to severe food shortages that required them to eat moldy plant components, notably moldy maize, during their wartime internment [11]. An example of similar circumstances was found with retrospective association of T-2 toxin and HT-2 toxin in moldy grains consumed in eastern Siberia at times over the last 100 years and the development of alimentary toxic aleukia in that location [54]. As proof of principle, samples of sorghum (*Sorghum bicolor*) were collected for mycotoxin analysis from a field proximate to an Acholi family with a NS child [55]. Seed samples were classified as “old” (long past harvest,  $n = 4$ ), “young” ( $n = 4$ ), and “neither old nor young” ( $n = 5$ ) by a knowledgeable local community member. It was of interest to analyze samples taken from different harvests because displacement of communities in this region often forced them to scavenge and consume food of questionable quality. Beauvericin was the most prominent mycotoxin detected, at an average concentration of  $552 \mu\text{g/kg}$  in the “old” samples; “young” and “neither old nor young” contained 251 and  $141 \mu\text{g/kg}$ , respectively. Beauvericin is an apolar lipophilic cyclohexadepsipeptide that forms ionophores in plasma membranes. Its ability to inhibit L-type voltage-dependent  $\text{Ca}^{2+}$  channels in a neuronal cell line suggests the possibility that it may increase sensitivity to kainate-induced neurotoxicity (seizures and neuronal damage) [56]. In addition, emodin, alternariol methyl ether, alternariol, zearalenone, deoxynivalenol, aflatoxin G<sub>2</sub>, agroclavine, festuclavine, and dihydroergosine were identified in at least one of the 13 samples. However, a variety of dietary factors examined in another study found edible smut fungus to be inversely linked to NS (non-significant,  $p = 0.065$ ) [11], suggesting that mycotoxins on their own cannot be implicated in disease etiology.

In addition to the toxic effects of individual mycotoxins, co-exposure to numerous compounds at once is likely to be common given the observance of multiple mycotoxins in feed/food surveys done across the globe [32]. This needs to be considered in safety and toxicological evaluations, as a given group of compounds could enhance or reduce the overall toxicity to the organism that consumes them. The potent Group 1 carcinogen aflatoxin B<sub>1</sub>, as well as other members of the aflatoxin and fumonisin families, have been detected with high frequency in African maize in addition to deoxynivalenols and zearalenones; when analysis methodologies allowed, ochratoxin A, moniliformin, 3-nitropropionic acid, cyclopiazonic acid, citrinin, roquefortine C, sterigmatocystin, beauvericin, and the enniatins were variably identified as well [16,57–62]. Ochratoxin A occurs in a wide range of human foods including cereals, coffee, wine, beans, nuts, and meat and milk products from animals consuming contaminated feed [34,51]. It is hepatotoxic, immunosuppressive, teratogenic, and a group 2B possible human carcinogen [63]. Also known as vomitoxin for its emetic effect on animals, DON can cause general gastrointestinal distress, weight loss, and feed refusal/anorexic behaviors. DON is one of the most common mycotoxins assayed for and detected in urinary surveys irrespective of geographic location, as seen in both African [58,61,64] and European [27,65,66] studies. Aflatoxin, ochratoxin, and DON were



all detected in a home survey of stored grains comparing households with and without NS children; no significant difference was found between the two groups in the total average concentration detected [28].

Maize-based porridge samples [12] and maize kernels [13] sampled from Tanzanian villages showed severe contamination with fumonisins and aflatoxins, while zearalenone and DON were also detected. Aflatoxins, fumonisins, trichothecenes, and beauvericin predominated in maize assessed from Burkina Faso and Mozambique [14]. The Republic of Benin analyzed local maize flour and found fumonisins and beauvericin to be present [15]. In Uganda, Sudan, and Tanzania, aflatoxins in millet, sorghum flour and nut products, and aflatoxins and fumonisins in maize, were listed previously as significant detects [16]. PCR analysis of mold species on Tunisian and Egyptian sorghum determined that *Fusarium incarnatum*, *Aspergillus flavus*, and *A. niger* were the main mycoflora present [67]; isolates were able to produce aflatoxins, ochratoxin A, and zearalenone. Sorghum, an imported red variety that was statistically associated with NS in South Sudan [9] but not in Uganda [2,11], is prone to spoilage by *Fusarium verticillioides*; this fungus elaborates fumonisin B1, which blocks sphingolipid synthesis and causes leukoencephalomalacia in horses and hippocampal lesions in laboratory species [68].

Less commonly reported mycotoxins may also be present in food and feed originating in Africa. Dihydroergosine is the principal toxic alkaloid produced by sorghum ergot (*Claviceps africana*) [69] and has been associated with agalactia, suppressed prolactin, feed refusal, and hyperthermia in livestock [70,71]. Sulochrin is produced by *Aspergillus* and *Penicillium* spp. and inhibits eosinophil functions including degranulation [72,73]. Emodin is an anthraquinone produced by *Aspergillus* spp. and other fungal species, contaminating many plants used in herbal medicine in addition to those used as [74]. It has a number of therapeutic properties but can also be hepatotoxic and nephrotoxic and affect reproduction [75]. Emodin was detected previously in maize and feed from Burkina Faso and Mozambique [14]; in that study, the authors emphasized the need to screen for non-regulated compounds such as emodin to maintain a comprehensive picture of mycotoxin exposure in a given environment.

### 3.3. Study Limitations

While we utilized LC-MS/MS in the current study, a number of technical approaches could improve sensitivity and therefore detection of mycotoxins, including use of a more advanced triple quadrupole or time-of-flight mass spectrometer capable of lower detection and quantification limits. In addition, the sample preparation method of “dilute and shoot” has speed and cost advantages along with an ability to recover a chemically diverse array of compounds and their conjugates; however, it was optimized to capture high to moderate concentrations rather than low or background levels [76]. Moreover, sample values here must be considered semi-quantitative as they were compared against subsequently run standards. More advanced methods for mycotoxin exposure biomarkers in urine have been developed since our samples were evaluated; these cleave mycotoxins by glucuronidase incubation, followed by sample clean-up using solid-phase extraction columns and <sup>13</sup>C- or deuterated internal standards [77]. This acknowledges the presence of Phase II conjugates in the urine that have likely escaped detection in methods that analyze only for the parent mycotoxin, thereby underestimating exposure to metabolites with potential toxicity [78].

## 4. Conclusions

We carried out a multi-mycotoxin screen for nine analytes in the urine of children with Nodding Syndrome (n = 50) and healthy Community Controls (n = 50) residing in a remote Acholi village of northern Uganda. Mycotoxins were detected in the urine, including  $\alpha$ -zearalenone, aflatoxin M1, and T-2 toxin, with no significant difference between the two groups. However, because the samples were collected years after onset of NS, a role for mycotoxins in the etiology of NS or associated nematode infection cannot be ruled out specifically as secondary factors that contribute to a generalized decrease in immune

and/or growth function. Mounting evidence that food-derived mycotoxins play a role in altering optimal development in African children and the frequency of their detection in this population call for further investigation with optimized assays to detect mycotoxins, identify their sources, and minimize exposure by avoidance of contaminated food.

## 5. Materials and Methods

### 5.1. Setting

The study was set in the NS epicenter in northern Uganda. Between 1987 and 2006/2008, this region was a civil war zone involving the Lord's Resistance Army (LRA), the Sudan People's Liberation Army, and the Ugandan People's Defence Force. Beginning in 1996, Acholi civilians were moved into internally displaced peoples (IDP) camps guarded by Ugandan government forces. In these camps, there was social breakdown, rising incidence of mental health disorders, HIV/AIDS, alcoholism, malnutrition, and eventual reliance on food aid. They became breeding grounds for malnutrition and deaths resulting from cholera, measles, and other preventable diseases. In 2008, following the expulsion of the LRA, the Acholi began to return to village homelands and rebuild their society. After recognition of NS in Kitgum District in 2008, the Ugandan government registered children, established a government screening and treatment center, and distributed anticonvulsants and nutritional supplementation for affected children.

### 5.2. Study Approval and Informed Consent

Study and instrument design were approved by the Institutional Review Boards of Oregon Health & Science University (Portland, OR, USA) and Oregon State University (Corvallis, OR, USA), in concert with the Research and Ethics Committee of the School of Health Sciences of Makerere University (Kampala, Uganda). The study was approved by the Uganda National Council for Science and Technology, the Office of the President of Uganda, and Chairman, Local Council III, Kitgum District, Uganda. Informed consent was obtained for the collection of urine for large-scale mass spectrometric analysis of mycotoxin content. Approval code: IRB00008470, Approval date 1 November 2013–14 September 2021. Caretakers of children were paid the equivalent of U.S. \$10.00 in local currency to compensate for their time. One half of each subject's biological fluids was banked with the Department of Food Technology and Nutrition, College of Agricultural and Environmental Sciences, Makerere University, Kampala. Permission to transport the other half of each subject's biological fluids to the USA for analysis was granted by the Uganda National Council for Science and Technology.

### 5.3. Urine Sample Collection

Families with and without children with a NS diagnosis were requested by the Village Health Team Leader to visit the designated research study site. The medical status of each child was checked against a list of all NS subjects that recorded the month and year of onset of head nodding. The case-control study enrolled 50 children aged 5–18 years with NS and an equivalent number of seizure-free children from the community matched for age (50/50) and gender (42/50) as Community Controls. Each child gave one sample of free-caught urine in a cup (10–20 mL) during daylight hours, which was immediately placed on ice.

Urine samples were transported frozen by car from Kitgum to Kampala, then separated into two sets. One half of the biological fluids was retained in Uganda, while the balance was transported in a Yeti Tundra Cooler on passenger flights from Entebbe, Uganda to Portland, Oregon. Samples were stored at  $-20\text{ }^{\circ}\text{C}$  prior to mycotoxin analysis.

### 5.4. Chemicals

Acetonitrile (ACN) and methanol were of LC-MS grade (EMD Millipore, Billerica MA USA); acetic acid (LC-MS grade) was purchased from Sigma Aldrich (St. Louis, MO USA). Ultrapure water ( $18\text{ M}\Omega\text{ cm}^{-1}$ ) was obtained from a PURELAB Ultra Genetic system (Elga, Marlow, Buckinghamshire, UK). Zearalenone (ZEN),  $\alpha$ -zearalenol ( $\alpha$ -ZEL),  $\beta$ -zearalenol

( $\beta$ -ZEL), T-2 toxin (T-2), and HT-2 toxin (HT-2) standards came dissolved in ACN, while fumonisin B<sub>1</sub> (FB<sub>1</sub>) and fumonisin B<sub>2</sub> (FB<sub>2</sub>) standards were dissolved in 50:50 water:ACN; all were purchased from Romer Labs (Getzersdorf, Austria). Aflatoxin M1 (AFM1) and ochratoxin A (OTA) (Enzo Life Sciences, Farmingdale, NY, USA) were dissolved after arrival in ACN for dilution and generation of standard curves.

#### 5.5. Sample Preparation-Urine Extraction for Mycotoxins

Urine was prepared using a dilute-and-shoot approach [79]. Urine was removed from the freezer, allowed to reach room temperature, and then centrifuged for 3 min at 10,000 rpm (Eppendorf (Hamburg, Germany)). Supernatant (100  $\mu$ L) was removed and added to 900  $\mu$ L of a 10:90 (*v/v*) ACN:water mixture; the sample was vortexed, then sealed for analysis by LC-MS/MS.

#### 5.6. LC-MS/MS Analysis

Coded samples were subjected to a multi-mycotoxin LC-MS/MS screening assay for 9 mycotoxins (ZEN,  $\alpha$ -ZEL,  $\beta$ -ZEL, T-2, HT-2, FB<sub>1</sub>, FB<sub>2</sub>, AFM1, and OTA) on an ABI/SCIEX 3200 QTRAP LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) via electrospray ionization, with separation performed using a Perkin Elmer (Waltham, MA, USA) Series 200 HPLC connected to an Atlantis T3 column (150  $\times$  3.0 mm, Waters (Milford, MA, USA)) with a 4  $\times$  3 mm security guard cartridge of similar packing [79]. The methodology used was chosen on the basis of available instrumentation and ability to quantify the major mycotoxins of concern that are globally monitored in the human food supply.

Mobile phases consisted of water (A) or ACN (B), each with 0.1% acetic acid, which were run in a gradient program at 600  $\mu$ L/min. Each sample was run in triplicate; each replicate had two injections taken, one each for both positive and negative modes, which captured 9 mycotoxins and mycotoxin metabolites as described in Warth et al. [79] via scheduled (180 s window) multiple reaction monitoring (MRM) of two transitions (quantitative and qualitative) per compound. Dilution solvent (1:9 ACN:H<sub>2</sub>O) served as the blank sample.

#### 5.7. Data Analysis

Qualitative analyses were conducted blind to the group status of each child's specimen using Analyst 1.6.2, MultiQuant 3.0.1 (Applied Biosystems, Framingham, MA, USA) and Excel (Microsoft, Redmond, WA, USA). The presence of a mycotoxin was confirmed when the signal was equal to or greater than the limit of quantitation (LOQ, the concentration at which the analyte had a precision and accuracy that did not exceed greater than 20% of the coefficient of variation [80]), both quantitative and qualitative transitions were present, and retention time was at the expected time in the chromatogram. After samples were evaluated for the presence of mycotoxins, they were quantified against a standard curve with subsequently run standards (matrix-matched standards spiked into urine and diluted as described for samples above) developed in MultiQuant. The limit of detection (LOD) and LOQ for the mycotoxins were as follows: ZEN (10, 50 ng/mL),  $\alpha$ -ZEL (20, 20 ng/mL),  $\beta$ -ZEL (25, 25 ng/mL), T-2 (2.5, 10 ng/mL), HT-2 (0.2, 5 ng/mL), FB<sub>1</sub> (0.1, 2.5 ng/mL), FB<sub>2</sub> (0.5, 2.5 ng/mL), AFM1 (2.5, 2.5 ng/mL), and OTA (0.5, 0.5 ng/mL) (Supplementary Table S1). See Supplementary Table S1 and Supplementary Figure S1 for additional method validation information. After mycotoxin analysis was complete, the code was broken and results compared among NS and CC subjects using a t-test with significance determined as  $p < 0.05$ .

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/toxins13050313/s1>, Table S1. Validation data and Figure S1. Extracted ion chromatograms of mycotoxins detected in urine of Acholi children, as compared to method blank (1:9 ACN:water).



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