MYCOTOXIN EXPOSURES IN KENYAN CHILDREN

by

RUTH NABWIRE WANGIA

(Under the Direction of Jia-Sheng Wang)

ABSTRACT

Mycotoxins are secondary metabolites of *Aspergillus, Fusarium* and *Penicillium* fungi. Even though more than 400 mycotoxins have been identified, Aflatoxins (AFs) and Fumonisins (FNs) are highly toxic with significant public health implications. The International Agency on Research Cancer classified Aflatoxin B_1(AFB_1) as Group I carcinogens and Fumonisins B_1(FB_1) as Group IIB, possibly carcinogenic to humans. More than 25% of the world's food supply is contaminated including cereals, legumes, oilseeds, nuts, milk and meat products, spices, coffee, and tea. The recommended maximum limit for total AFs and FNs in foods is 20 µg/kg and 4mg/kg respectively. Up to 4.5 billion people are exposed through dietary staples and in occupational settings. This dissertation study seeks to assess AFs and FNs exposure among children 6 - 12years using biomarkers. AFB_1-lysine adducts, urinary free FB_1 and sphingolipids are validated biomarkers for AFs and FNs exposure quantified by High Performance Liquid Chromatography. Participants were recruited from Kenyan counties of Siaya and Makueni where naturally co-occurrence of *Aspergillus* and *Fusarium* fungi is documented. All children had detectable levels of AFB_1-lysine adducts in sera, geometric mean (GM) 10.5 (95%CI 9.4 – 11.7) and range 0.65 - 518.9 pg/mg albumin. Makueni county children had higher exposures, GM 14.0

(95%CI 12.5, 15.7) pg/mg albumin, and Siaya's 8.2 (95%CI 7.6, 8.8) pg/mg albumin, $p<0.001$. Children were also exposed to fumonisins. The GM of urinary FB_1 when adjusted for creatinine levels were 0.07 ng/mg for Siaya and slightly higher in Makueni at 0.08 ng/mg, p-value > 0.05. Additionally, Sphinganine and Sphingosine were detectable in 95.07% and 98.94% of the urine samples. Creatinine adjusted mean±SD of Sphinganine and Sphingosine were 1.23±2.18nM, and 4.99±8.3nM while the Sphinganine/Sphingosine ratio was 0.296±0.587. After adjusting for demographic information, socio-economic indicators, farming practices, and household dietary patterns, children with higher AFB_1-lysine adducts and urinary FB_1 were more likely to suffer from growth impairments. Overall, dietary exposure to mycotoxins is a public health issue and efforts geared towards mitigating exposure in children are imperative in regions where corn and peanuts are staple diets.

INDEX WORDS: Children, Mycotoxins, Fumonisins(FNs), Aflatoxins(AFs), AFB_1, FB_1, Adverse health outcomes.

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DEDICATION

This work is dedicated to my beloved parents Judith Ayuma and George H. M. Opot who ensured I had a great start in life. And to the Almighty, with whom everything is possible.

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Chapter 1

INTRODUCTION

Problem Statement

Mycotoxins are naturally occurring metabolites of different fungi species that contaminate food and farm animal feeds throughout the world (CAST 2003; IARC 2002). There are over 400 known mycotoxins with the majority being produced by three fungal genera, namely *Aspergillus*, *Penicillium* and *Fusarium* (CAST 2003; Ismail et al. 2018). In many low- and middle-income countries, the mainstream population are subsistence farmers who grow, store and prepare a large portion of their staple diets. Therefore, exposure to mycotoxins is highly prevalent and most citizen are neither aware of the extent of exposures nor the public health implications. Today, mycotoxins are a leading cause of a majority of global health diseases, environmental problems and significant contributor of food insecurity in developing countries. Ingestion of food contaminated by mycotoxins have serious adverse health effects in human populations in the long-term (IARC 2002, 2015; Wu et al. 2014). For instance, mycotoxins are known to alter the cellular and biochemical functions of the intestine, resulting in micronutrient deficiency, systemic immune suppression and growth impairments in children (Lombard 2014). Exposure to mycotoxins have also been shown to interfere with vaccine efficiency in children ages one to fourteen years old (Githang'a et al. 2019). Given the possible impact associated with adverse health outcomes especially in young children, further basic research and

epidemiological studies are warranted to understand the mechanisms and individual pathways involved in the development and severity of diseases.

The most widely studied mycotoxins in regard to children's health is aflatoxins (AFs). To date, there are no epidemiological studies focused on possible adverse health outcomes in human populations particularly in children as a result of dietary exposure to other mycotoxins including Fumonisins, Deoxynivalenol, T-2 toxin, Nivalenol and Patulin. The paucity of data is mainly due to lack of validated biomarkers and thus, further research should focus on developing and validating biomarkers to measure mycotoxin exposures particularly in human populations. Chapter 8 of this dissertation reports a validation study of the sphingolipid metabolites as plausible biomarkers of fumonisin exposure in children from high exposure regions. Given the limited epidemiological studies in part due to lack of validated biomarkers as it relates to other mycotoxins, this dissertation study reports AFB_1-lysine adducts, urinary free fumonisins B_1 and sphingolipids, as biomarkers of exposure among young children between ages six and twelve years. Co-occurrence of AFs and Fumonisins has been previously reported. Furthermore, household grains and/or flour samples were collected to assess exposure levels in foods products which could possibly corroborate risk of AFs exposure at household level. Moreover, we identify risk factors for AFs exposures and evaluate possible adverse health outcomes associated with AFs exposure.

The study population was recruited from two counties namely Makueni and Siaya in Kenya, East Africa. Makueni is a known hotspot for AFs exposure that has resulted in deaths from AFs poisoning in the past thirty decades. There are no reported aflatoxicosis from Siaya County. Additionally, in a serological survey among adult populations in

Kenya, all archived serum samples collected from inhabitants in Siaya County were below the limit of detection for AFs contamination (Yard et al. 2013). Thus, the selection of Makueni and Siaya Counties as study sites allows for control of variation in AFs exposure levels, climate conditions, poverty and ethnicity. Young children are uniquely vulnerable to AFs exposure due to higher consumption in weaning foods, less developed detoxification mechanisms, higher rates of cell division associated with rapid growth and high intake of food and water per kg body weight (Githanga et al. 2019; Lombard 2014; Njumbe Ediage et al. 2013). It is not possible to eliminate AFs completely due to their natural occurrence and therefore, human exposure is widespread. The main goal of this dissertation study is to perform human biomonitoring study to assess the level of AF and FNs exposure in children populations inhabiting areas of high and low AF exposure. Furthermore, this dissertation study seeks to understand determinants of AFs exposure and adverse health outcomes in early life particularly growth outcomes among children with higher exposure levels.

Working Hypothesis

Children sampled from Makueni County, a historically AFs contaminated hotspot will have higher levels of AFB_1-lysine adducts in sera and higher levels of urinary fumonisins B_1, both validated biomarkers of aflatoxins and fumonisins exposure. Furthermore, children with high mycotoxins exposure are more likely to suffer from adverse health outcomes compared to children with minimal exposures. Moreover, grain and/or flour samples collected from Makueni County are likely to be more contaminated by mycotoxins compared to samples collected from Siaya County.

Specific Aims

The specific aims of the current doctoral study are to;

Aim 1: Bio-monitor aflatoxin exposure in children 6 – 12 years by quantifying AFB_1-lysine adducts using High Performance Liquid Chromatography with Fluoresce Detection methodology

Aim 2: Exposure assessment of urinary fumonisins B_1 as biomarkers of fumonisins exposure

Aim 3: Validate sphingolipid metabolites as plausible biomarkers of fumonisin exposure in Children from high exposure regions.

Aim 4: Identify determinants of household aflatoxin exposure levels including farming practices and food security level.

Aim 5: Quantify aflatoxins in household corn and corn products from rural areas with the purpose of assessing exposure risk.

Aim 6: Evaluate any growth impairments in children with high AFB_1-lysine adducts in their serum and high urinary FB_1.

Study Rationale/Study Significance

Historically, chronic dietary exposure resulting from consumption of food supplies contaminated with mycotoxins is a global problem associated with severe health outcomes including but not limited to hepatoxicity, teratogenicity, and liver carcinogenesis. Since their discovery, numerous studies in experimental animals and humans provided sufficient evidence for AFs carcinogenicity. Symptoms associated with cumulative exposure to

mycotoxins are often non-specific. More recently, exposure to aflatoxins, fumonisins and other mycotoxins in developing nations has been shown to contribute to immune suppression and growth impairment in children. Furthermore, AFs are suspected to interfere with vaccine efficiency making children more vulnerable to increased risk of infectious diseases.

Pediatricians in developing nations could use biomarkers as a tool to monitor exposure and the contributory role of mycotoxins in etiology of diseases. Similarly, diagnostic laboratories could monitor immune markers in correlation with biomarkers of exposure with the specific aim of assessing immune suppression and other cumulative effects of mycotoxin exposure.

The mechanism by which AFs contribute to negative health outcomes in children is currently unknown. However, three hypotheses including immune modulation, disruption of intestinal barrier and interference with growth axis are the three main suspected mechanisms of action postulated in the literature. The basis of this argument is that undernourished children are more likely to experience micronutrient deficiencies, and it follows that these children are also more likely to be susceptible to infectious diseases due to suboptimal micronutrient absorption required for proper biochemical functioning of the human body. Ultimately, affected children are more likely to suffer from growth deficits. Individual exposure assessment to AFs and other mycotoxins among children populations between 6 and 12 years is severely limited. The paucity of data limits further studies in terms of elucidating the role of AFs on adverse health outcomes. Nonetheless, previous epidemiological studies defined study population included children under 5 years' old, pregnant women, adult populations and immune-compromised subpopulations such as

individuals with HIV/AIDS. Screenings for aflatoxin exposure in pregnant women in Nepal, Bangladesh and Ghana provided evidence of in-utero exposure (Groopman et al. 2014; Shuaib et al. 2012). Surveillance studies done in healthy adults in Malaysia reported median AFB_1-lysine adducts of 7.12pg/mg of albumin and a geometric mean of 0.842pg/mg in the United States (Leong et al. 2012; Schleicher et al. 2013). Among HIV/AIDS patients recruited from Ghana and Uganda, it was reported that AFs exposure accelerates immune suppression and disease progression from HIV to AIDS within a shorter incubation period (Jolly 2014; Kang et al. 2015). While these studies have contributed significantly to understanding the negative effects associated with AFs on human health, it is imperative to determine the cumulative effects of aflatoxin exposure in older children before adolescent stage. Therefore, studies aimed at assessing early exposure are imperative to inform policy makers on early interventions to protect public health.

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Chapter 2

LITERATURE REVIEW

Introduction to Mycotoxins

Mycotoxins are naturally occurring metabolites of different fungi species that contaminate food and farm animal feeds throughout the world (CAST 2003; IARC 2002). Today, mycotoxins are a leading cause of a majority of global health diseases, environmental problems and significant contributor of food insecurity in low- and middle-income countries. While there are over 400 known mycotoxins, the majority of mycotoxins are produced by three fungal genera, namely *Aspergillus, Penicillium* and *Fusarium* (CAST 2003; IARC 2002; Ismail et al. 2018). The *Aspergillus spp* fungi mainly produce AFs and ochratoxins, while *Penicillium spp* fungi produce citrinin, patulin and mycophenolic acid. *Fusarium spp* fungi produce Fumonisins and Trichothecenes sub-groups comprised of more than 120 Trichothecenes including T-2 toxin, Nivalenol and Deoxynivalenol/Vomitoxin (CAST 2003). The occurrence of mycotoxins in food products is influenced by environmental factors such as humidity and temperature, type of food, production processes and storage methods. Table 1.1 outlines major mycotoxins, the naturally producing fungi, food groups commonly contaminated and previously reported public health impacts. In many low- and middle-income countries, the majority of the population are subsistence farmers who grow, store and prepare a large portion of their staple diets; and thus, exposure to mycotoxins is highly prevalent (Strosnider et al. 2006).

Ingestion of food contaminated by mycotoxins have serious adverse health effects in human populations in the long term (IARC 2002, 2015; Wu et al. 2014). Specifically, fumonisins are known to decrease complex sphingolipids, while AFs and Deoxynivalenol increase systemic cytokines, and inhibit protein synthesis which results in altered intestinal architecture, inhibition of intestinal regeneration, impaired tight junctions and glucose mal absorption (IARC 2015). Moreover, mycotoxins are known to alter the cellular and biochemical functions of the intestine, resulting in micronutrient deficiency, systemic immune suppression and growth impairments in children (Khlangwiset, Shephard, and Wu 2011; Lombard 2014). Exposure to mycotoxins have also been shown to interfere with vaccine efficiency in children aged one to fourteen years (Githang'a, Wangia, et al. 2019). Given the possible impact associated with adverse health outcomes especially in young children, further basic research and epidemiological studies are warranted to understand the mechanisms and individual pathways involved in the development and severity of diseases.

The most widely studied mycotoxins in regard to children's health is AFs and FNs. To date, there are no epidemiological studies focused on possible adverse health outcomes in human populations particularly in children as a result of dietary exposure to other mycotoxins including Deoxynivalenol, T-2 toxin, Nivalenol and Patulin. The paucity of data is mainly due to lack of validated biomarkers and thus, further research should focus on developing and validating biomarkers to measure mycotoxin exposures particularly in human populations. Given the limited epidemiological studies in part due to lack of validated biomarkers as it relates to other mycotoxins, this dissertation study seeks to assess aflatoxin and fumonisin exposure levels using biomarkers approach, identify risk factors

for exposure to mycotoxins in rural households and evaluate possible adverse health outcomes associated with AFs exposure among young children between six and twelve years.

Overview of Aflatoxins (AFs)

Aflatoxins (AFs) are a group of naturally occurring food contaminants produced by the soil-borne *aspergillus* species of fungi (CAST 2003; IARC 2002). There are about 20 related *Aspergillus spp* that produce four major types of AFs however, *A. flavus* and *A. parasiticus* are the common plant pathogens associated with significant economic losses. Occurrence of different *Aspergillus* strains vary geographically. *A. flavus* occurs worldwide while isolates of *A. parasiticus* fungi are localized in the United States of America, Japan, Australia, Brazil, India, parts of South America, parts of Europe and parts of Africa (IARC 1993, 2002; Okoth 2016). Co-occurrence of both *A. flavus* and *A. parasiticus* has been reported, however *A. flavus* appears to occur exclusively in South East Asia (IARC 2002). AFs were identified in 1961 after the outbreak of turkey X disease that killed 100,000 turkeys in England (Lancaster, Jenkins, and Philp 1961; Sargeant et al. 1961). By 1963, the chemical structures of AFs were elucidated (Asao et al. 1963; Spensley 1963; Kensler et al. 2011). Subsequently, further research was undertaken to elucidate the physical and chemical properties in order to assess utility in detoxification processes. In terms of physical appearance AFs tend to be colorless or pale yellow (Cole and Cox 1981; IARC 1993). Moreover, they are slightly soluble in water, insoluble in non-polar solvents and freely soluble in moderately polar organic solvents such as chloroform, methanol, acetonitrile, and dimethyl sulfoxide. AFs are unstable under ultraviolet light in the presence of oxygen, to extreme pH <3 and >10 and to oxidizing agents (IARC 2002). Chemically,

AFs have closely related structures and form a unique group of highly oxygenated heterocyclic difuranocoumarin compounds.

There are four main types of naturally occurring AFs namely Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1) and Aflatoxin G_2 (AFG_2). These are categorized into two broad categories namely the difurocoumarocyclopentenone series (B-types) and the difurocoumarolactone series (G types) (Wilson 1989; Park et al. 1990). The B-type are characterized by fusion of a cyclopentenone ring to the lactone ring of the coumarin moiety while the G-type contain a fused lactone ring. AFs are intensely fluorescent to ultraviolet light and both B and G classifications are based on emission of blue and green-yellow fluorescent lights respectively (Park et al. 1990; Wilson 1989). Most *Aspergillus spp* produce B-type AFs however, *Aspergillus parasiticus*, *Aspergillus bombys* and *Aspergillus nominus* produce G-type aflatoxin in higher proportions in addition to B types. Moreover, *A. parasiticus* produce both G and B groups of AFs while *Aspergillus flavus* produces only B_1 and B_2 sub-groups. It has been established that *A. flavus* lack the ability to synthesize G group of AFs due to a 0.8- to 1.5-kb deletion in the 28-gene aflatoxin biosynthesis cluster (Probst, Njapau, and Cotty 2007; Probst, Schulthess, and Cotty 2010). The molecular formula of AFB_1 was established as $C_{17}H_{12}O_6$ and AFG_1 as $C_{17}H_{12}O_7$. AFB_2 and AFG_2 are dihydro derivatives of parent AFB_1 and AFG_1 with the molecular formulas $C_{17}H_{14}O_6$, $C_{17}H_{14}O_7$. AFB_1 and AFG_1 possess an unsaturated bond at the 8, 9 position on the terminal furan ring. Epoxidation at the unsaturated bond plays a contributory role in the carcinogenic potency and toxicity of AFB_1 and AFG_1 (Kensler et al. 2011). AFB_2 and AFG_2 are relatively non-toxic unless they are metabolically oxidized to AFB_1 and AFG_1 in vivo.

In a dry state, AFs are stable to heat, up to the melting point and may survive quite severe conditions and processes (Okoth 2016).

Toxicity, carcinogenicity and mutagenicity are in the order $AFB_1 > AFB_2 > AFG_1 > AFG_2$ as shown by experimental studies in animals to illustrate LD_{50}, the lethal dose that causes death in 50% of exposed subjects. AFB_1, a human carcinogen and the most potent genotoxic agent is also mutagenic in many model systems. For instance, Chromosomal aberrations (CA), micronuclei (MN), Sister chromatic Exchange, unscheduled DNA synthesis and chromosomal strand breaks have been reported in rodent and human cells after exposure to AFB_1 (Denissenko et al. 1999; Wang and Groopman 1999).

Other categories of aflatoxin sub-groups are majorly metabolic products of B and G groups. Aflatoxin M_1 (AFM_1) and Aflatoxin M_2 (AFM_2) are hydroxylated metabolites of B-type present in milk; While AFM_1 is cytotoxic, genotoxic and carcinogenic the toxicity of AFM_2 is lower compared to AFM_1 (Creppy 2002; IARC 2002). Aflatoxin P_1 (AFP_1), Aflatoxin Q_1 (AFQ_1), and Aflatoxicol are also metabolic products of B-type present in urine (Cole and Cox 1981; IARC 2002). Other metabolites of AFs spontaneously produced in response to different chemical environments are Aflatoxin B_{2a} (AFB2a), Aflatoxin G_{2a} (AFG_{2a}) and Aflatoxin D_1 (Cole and Cox 1981). Aflatoxins are closely correlated compounds with highly substituted coumarins. The presence of furocoumarin configuration places them among a large group of naturally occurring compounds with significant pharmacological activities (Wogan 1966). The structural formation of all aflatoxin subgroups is shown in Figure 2.1. Overall, all the AFs groups have varying levels of toxicity for both plant and animal life but AFB_1 is established to be the most potent. The toxicity of AFB_1 is 10 times more than that of potassium cyanide, 68 times that of arsenic

and 416 times that of melamine (Li, Zhang, and Zhang 2009). Moreover, their carcinogenicity is over 70 times that of dimethylnitrosamine and 10,000 times that of benzene hexachloride (Li, Zhang, and Zhang 2009). Isolation of aflatoxins from toxic meals and mold substrates was greatly facilitated by the discovery that they were fluorescent in ultraviolet light. The ultraviolet absorption sprectra are very similar with each showing a maximum at 223, 265 and 363mμ. Because of the close structural similarities between the compounds, the infra-red absorption and fluorescence emission are also very similar. Properties of AFs including molecular formula, molecular weight, melting points, UV absorption wavelength and Fluorescence emission are shown in Table 2.1.

Overview of Fumonisins (FNs)

Fumonisins (FNs) are naturally occurring mycotoxins produced by *Fusarium moniliforme* also referred to as *Fusarium verticilliodes* and other related *Fusarium* species (Bezuidenhout et al. 1988; Gelderblom et al. 1988). Fumonisins contaminate major food supplies and thus, exposure occurs mainly through dietary consumption. Fumonisins are possibly carcinogenic and have been classified as Group IIB carcinogens by IARC. Contamination of food supplies and dietary exposure to vulnerable groups in human population is evident. The main route of FNs exposure is via consumption of corn and/or corn products which are highly susceptible to FNs contamination. Human populations in China, South Africa, Italy and the Unites States close to the Mexico-Texas border are exposed to higher levels of FNs from day to day due to overreliance on contaminated corn or corn products as staple diets (Abnet et al. 2001; Chelule et al. 2001; Marasas, Jaskiewicz, et al. 1988). In Tanzania among children less than 6 months of age, consuming

contaminated flours, fumonisin exposure ranged from 0.005 to 0.88 μg/kg BW per day (Magoha et al. 2016). In Somalia, the reported average probable daily intake of fumonisins (FB_1 and FB_2) of 16.70 μg/kg bw/day exceeded the recommended provisional daily maximum tolerable intake of 2 μg/(kg bw)/day by over 835% (Wielogorska et al. 2019). Similarly, estimated daily intake of 0.01 to 0.85 μg/kg of body weight per day for aflatoxin and 2.9 to 310.0 μg/kg of body weight per day for fumonisin has been reported in western highlands of Guatemala (Mendoza et al. 2018). Maternal exposure to fumonisin is associated with neural tube defects(NTD) among populations consuming corn and corn-based products. A higher risk of NTDs is reported among Mexican Americans mainly due to consumption of corn products (Missmer et al. 2006; Gong et al. 2008). FNs may play a role in birth defects by inducing NTDs.

The first report of human related FB_1 poisoning was reported in 27 villages in India, where consumption of sorghum and corn products with high FB_1 resulted in acute foodborne diseases characterized by transient abdominal pain, diarrhea and borborygmus (Rai, Jogee, and Ingle 2015; Shetty and Bhat 1998). Ecological studies in China and South Africa have examined the relationship between dietary exposure to FNs and Esopahgeal Cancer (EC). In China's Linxian and Shangqiu Counties from Henan Province, high- and low-risk EC areas, respectively, the contamination rate of FNs in Linxian corn (48%) was about twice that of Shangqiu corn (25%) (Yoshizawa, Yamashita, and Luo 1994). Similarly, corn samples collected from Haimen and Penlai, high- and low-risk areas for primary liver cancer in China, were analyzed for three consecutive years and higher co-contamination rate of AFs, FNs and trichothecenes was reported in Haimen, that is, upto 10 to 50-fold higher than those from Penlai (Ueno et al. 1997). Moreover, fatal animal

diseases including neurological disorders, and pulmonary edema has been documented in farm animals consuming fumonisins contaminated feed.

FNs were identified and characterized in the late 80s and early 90s shortly after their discovery in 1988 by researchers at Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) in Tygerberg, South Africa (Gelderblom et al. 1988; Marasas 2001). To date, 28 different FNs groups have been characterized and classified into A, B, C and P groups (Rheeder, Marasas, and Vismer 2002). FNs in the A group are acetylated on the amino group, they have low biological activity and are produced in very low quantities (Rheeder, Marasas, and Vismer 2002). FNs in the B group are the most toxic, among which Fumonisin B_1 (FB_1) is the most potent and predominant isomer accounting for more than 60% of the FNs contamination in food products (JECFA, 2018). Fumonisins in the C groups are structurally identical to B groups with the only difference being that C series lack a terminal methyl group on the first carbon. Fumonisins in the P series were the last to be characterized and thus, few studies are published on this subgroup of FNs. Other lesser known FNs may occur naturally in contaminated corn at low levels (<5% of total FNs) and are mostly undetectable with analytical techniques due to complications in the derivatization processes (Rheeder, Marasas, and Vismer 2002).

FB_1 is primarily a diester of propane-1, 2, 3-tricarboxylic acid and penta-hydroxycosane containing a primary amino group known to contribute to the toxicity of FNs (Marasas et al. 2004; Voss, Smith, and Haschek 2007; Humpf and Voss 2004). The structure of FNs is a twenty-carbon backbone, similar to sphingolipids with the primary amine group on the second carbon and two carboxylic acid group at the 14^{th} and 15^{th} carbon (Bezuidenhout et al. 1988; Gelderblom et al. 1988; Marasas et al. 2004). FNs have

structural similarity to the long-chain base backbones of sphingolipids which play a role in membrane and lipoprotein structure and in cell regulation as second messengers for growth factors, differentiation factors, and cytokines (de Nijs et al. 1998; Merrill Jr et al. 2001)

Food Contamination

Aflatoxins Food Contamination

It is estimated that more than 25% of the world's food supply is contaminated by AFs and an estimated 4.5 to 5 billion people are exposed (Williams et al. 2004; Smith et al. 2015). AFs contaminate a variety of food crops including cereals such as corn/corn, pearl millet, rice, sorghum, teff, wheat; Legumes and Oilseeds such as groundnuts/ peanuts, soybeans, sunflower, cottonseed; Root and tuber crops; Spices; Tree nuts mainly almonds, coconut, pistachios, other nuts; Dry fruits; and even coffee and tea (IARC 1993, 2002; Okoth 2016). AFs contamination of staple foods such as corn, peanuts, spices and cassava are prevalent in tropical regions of the world where average temperatures range of 25 – 35ºC. The AFs producing Aspergillus species are prevalent in areas with hot, humid climates, including Sub-Saharan Africa and South East Asia where exposure from dietary staples is likely to be chronic. Countries in temperate and colder climates import foods from areas where AFs exposure is chronic and thus AFs is a worldwide concern.

Aflatoxin contamination can occur at any or every stage of the food supply chain from pre-harvest, post-harvest, storage, marketing and distribution and thus, presents a persistent challenge to food safety. Prevalence and severity of exposure is highly variable and is dependable on soil characteristics, geographic regions, season, household characteristics, farming practices and socio-economic conditions (Kang'ethe et al. 2017;

Mahuku et al. 2019; Wild 2007). The extent of AF contamination of food supplies also varies depending on factors such as temperature, water, nutrient availability, plant density, and whether the Aspergillus strain is toxic or non-toxic. Furthermore, drought stress has been associated with increased Aspergillus growth and AF accumulation in food supplies. *A. flavus* and *A. parasiticus* thrive in arid conditions because they are both semi-thermophilic and semi-xerophytic, i.e., tolerant to relatively high temperatures and arid conditions (Okoth 2016). AF accumulation in food supplies continues post-harvest at household levels and are highly dependent on post-harvest activities including storage choices, drying/threshing, and pest damage control. AFs not only contaminate agricultural food supplies but is also detectable in edible tissue/fleshy meats, milk and eggs from farm animals which are mainly raised on AFs contaminated feed (Alpsoy, Agar, and Ikbal 2009). Unfortunately, conventional cooking methods neither decontaminate nor reduce AFs in contaminated food.

The European Codex Alimentarius Commission recommends a maximum limit of 4µg/kg for total AFs in all cereal foods intended for human consumption (European Commission 2006). The United States Food and Drug Administration (USFDA) recommends maximum limit in foods for human consumption of 20 µg/kg. For animal feed however, the maximum limit for AFs exposure range from 100 to 300µg/kg given that about 6% of the AFs has been confirmed to be transferred to food products (USFDA 2000). Around the world, standards for food and animal feed have been adapted based on recommendations by the European Union's Codex Alimentarius and the United States Food and Drug Administration's with maximum limits range from 4 – 20µg/kg among different countries (Wu, Guclu, and Montoya 2012; Wu, Stacy, and Kensler 2013).

Nonetheless, given that aflatoxins are ubiquitous and naturally occurring, countries such as Brazil, India, Iran, and Jordan have less strict standards, for instance, higher maximum limit of 30µg/kg while Nepal and China set a maximum limit for cereals at 40µg/kg (Wu, Guclu, and Montoya 2012; Wu, Stacy, and Kensler 2013).Wu et al reported that most countries prefer to trade with countries whose regulatory limits are similar, for instance, the United States is the largest corn exporter to Canada, Latin America and Middle East nations who have all set maximum AFs limit similar to United States at 20µg/kg. Unfortunately, these maximum limits are not protective against adverse health outcomes in the long term (Wu, Stacy, and Kensler 2013). Therefore, it is important to limit AFs exposure as low as reasonably possible.

Fumonisins Food Contamination

FNs are known to contaminate corn and/or corn products, a major staple diet to more than 5 billion of the world's population with great variation influence by regional factors and location. Fumonisins are mostly naturally occurring and invisible to the naked eye, thus regulatory agencies are required to protect public health. The United States Food and Drug Administration (USFDA) set maximum limits of 2mg/kg for degermed dry milled corn products in human foods, 3mg/kg for corn intended for popcorn snack and 4mg/kg for dry milled corn (USFDA 2001). In comparison, the European Food Safety Authority (EFSA) set FNs maximum limit at 0.4mg/kg in corn based foods for direct human consumption which is ten times lower than USFDA's maximum limits (European Commision 2006). FNs do not fluoresce, thus most analytical techniques incorporate a pre-column derivatization using σ-phthaldialdehyde and naphthalene-2, 3 dicarboxaldehyde (NDA) (Sydenham and Shephard 1996; Turner, Subrahmanyam, and Piletsky 2009).

Analytical techniques including high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), Thin-layer chromatography (TLC), High Performance Liquid Chromatography with Fluorescence Detection (HPLC-FD), Mass Spectrometry, post-hydrolysis gas chromatography, and immunochemical techniques have been developed to quantify FNs in food items (Sydenham and Shephard 1996; Turner, Subrahmanyam, and Piletsky 2009). While all these methodologies have been optimized for surveillance of fumonisins contamination of food products, implementation is not particularly easy due to limited expertise especially in low and middle- income nations where contamination of food supply is not heavily regulated.

In the United States, FB_1 was present in corn samples collected from grocery stores in the range 0.6 – 1 mg/kg (Sydenham et al. 1991). Home brewed beer in Transkei region of South Africa had detectable levels of AFs in the range of 38 – 1066ng/mL whereas in the United States, 29 different brands of beer were positive for FB_1 in the range 0.3 – 12.7ng/mL (Hlywka and Bullerman 1999; Shephard et al. 2005). In south Africa, especially EC endemic regions, up to 94% of corn collected from households were contaminated with FB_1 in the range 0 – 118 mg/kg (Gelderblom et al. 1988).

In a recent study, corn and corn-based samples analyzed using HPLC coupled with tandem mass spectrometry (LC-MS/MS), 86% was contaminated with both free and hidden FNs with the highest contamination levels evident in dried corn kernels, mean levels of 15,737 µg/kg and 30,785 µg/kg for free and total fumonisins, respectively while frozen, and fresh corn kernels had lower contamination levels of less than 200 µg/kg of total FNs (Hu et al. 2019). In a different Chinese study, corn products, wheat flour and corn oil collected from the province of Shadong were analyzed by isotope dilution ultra HPLC–

tandem mass spectrometry (Li et al. 2015). Fumonisins were detected in 98.1% of the corn products, with the average total level of 369.2 µg/kg while only 6.2% of the wheat flour samples were contaminated with FB_1, with concentrations ranging from 0.3 to 34.6 µg/kg (Li et al. 2015). Moreover, FB_2 and FB_3 were detectable in corn samples but neither was detectable in wheat flour and corn oil samples (Li et al. 2015).

Contamination of additional food products including wheat, rice, sorghum, millet, soy beans and other cereal based grains have also been documented despite paucity of FNs surveillance data in regard to these food items (IARC 1993, 2002; JECFA 2018). For instance, in a multi-mycotoxin analysis of wheat, corn and rice products using isotope labelled internal standard and liquid chromatography/tandem mass spectrometry with positive ionization; the total FBs in corn products, median of 37.1mg/kg with range 27.2–47.0 mg/kg and 48.4 (22.8 - 130) mg/kg in wheat based products but not detectable in rice and rice products (Andrade et al. 2017). Similarly, high total FNs contamination levels exceeding 10,000 µg kg in both white and yellow corn samples but non-detectable FNs in wheat products and sorghum has also been reported (Wielogorska et al. 2019). In Argentina, 91 samples of wheat-based products tested using HPLC-MS/MS, fumonisins (FB_1 + FB_2) in quantifiable samples ranged from 0.05 ng/g to 18.9 ng/g. Although FB_1 was more prevalent, FB_2 was found in higher levels than FB_1. (Cendoya et al. 2019) Overall, fumonisin prevalence was high, but concentrations were far below EU or USFDA limits set for corn and corn-based products (Cendoya et al. 2019). Heavy contamination in Brazilian dry soybeans with range 138-1495 µg/kg and 178-552 µg/kg for FB_1 and FB_2, respectively, has been reported given that there are limited regulatory measures in Brazil (Garcia et al. 2016). Surprisingly, the presence of FB_2 is date palm fruits

have been confirmed in Egypt with contamination levels ranging from 4.99 to 16.2 µg/kg, by way of liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Abdallah, Krska, and Sulyok 2018). In Western highlands of Guatemala, fumonisin was detected on 52% of farms at 400 to 31,000 µg/kg (Mendoza et al. 2018). In Nigeria, cultivation of improved varieties of corn, pearl millet and sorghum yielded produce with relatively low FNs exposure levels, range 5 - 2860 µg/kg for corn, 6 - 29 µg kg pearl millet and 5 - 1340 µg kg for sorghum with lower levels attributed to good agricultural practices initiated by International Institute for Tropical Agriculture (IITA) (Vismer et al. 2015). Additionally, heterogeneous contamination levels by FB_1, FB_2 and FB_3 have been reported in gluten-free foodstuffs intended for individuals with celiac disease (Cano-Sancho et al. 2012; Dall'Asta et al. 2009; Esposito et al. 2016). It is possible that FNs decompose when subjected to high heat because detectable levels of FNs have been found in green coffee, maximum value of 164 µg/kg, while non-detectable in both roasted and instant coffee when quantified by UHPLC-MS/MS, during roasting (Nielsen et al. 2015). Nonetheless, studies with corn did not yield reduction in FNs exposure levels after convectional cooking methods.

Weaning Foods

The World Health Organization recommends 6 months of exclusive breast feeding to promote positive health outcomes for both child and mother (WHO 2017; Zielinska and Hamulka 2018). However, in both developed and developing countries, many mothers do not exclusively breast feed their children for 6 months because of one or more of the following reasons; a) insufficient breast milk, b) perceived inadequate maternal nutrition, c) maternal employment and, d) lack of support among other reasons (Kavle et al. 2017;

Zielinska and Hamulka 2018). Therefore, weaning infants on commercial baby formula, cow milk and other milk products, cereal and legume-based baby foods, groundnut-mixed and or homemade blend of different food ingredients occurs before six months of age. These weaning foods have been shown to be contaminated by a myriad of different mycotoxins. Moreover, studies have demonstrated that as breastfeeding decreases with age, the level of AF metabolites and other mycotoxin markers increases as infants are introduced to weaning foods (Mahfuz et al. 2019; Shouman et al. 2012). The recommended maximum concentration of AFs in weaning and complementary foods for infants and young children is 0.1ng/ml for AFB_1 and 0.025ng/ml for AFM_1 (European Commission 2006). Unfortunately, detectable levels of AFs and other mycotoxins have been found in weaning foods around the world.

The use of milk during weaning also widely accepted. The role of milk in human nutrition is well known and most infants are introduced to cow milk before the age of one year (Adejumo et al. 2013; Donath and Amir 2002). Nonetheless, the presence of AFM_1 in cow milk and other milk products has been reported worldwide. AFM_1 appears in milk because animal feed contaminated with AFB_1 and unfit for human consumption is usually processed and converted to feed for farm animals (Ahlberg et al. 2018; Kang'ethe and Lang'a 2009; Kuboka et al. 2019). As a result, there is worldwide documentation of AFM_1 in milk samples. First off, in Japan, bulk raw milk randomly sampled had AFM_1 levels of 11 ng/l in January, 7 ng/l in February and 5ng/l in June of 2004 (Sugiyama, Hiraoka, and Sugita-Konishi 2008). Higher exposure levels were recorded in Kerman, Iran, where 72 milk sample from 6 dairy farms were 100% positive of AFM_1 in the range of 10 – 410ng/l (Rohani, Aminaee, and Kianfar 2011). Likewise, in other 2 studies done in Iran, 45 samples

of pasteurized milk purchased from different supermarkets were positive for AFM_1 with ranges of 8.8 – 64ng/l (Riahi-Zanjani and Balali-Mood 2013; Ghazani 2009). AFM_1 in the range of 10 – 630ng/l has been reported in Ultra Heat Treated (UHT) milk purchased from retail outlets in 5 Turkish cities (Temamogullari and Kanici 2014; Unusan 2006). In Nigeria's Ogun State, AFM_1 in cow milk and milk products sampled locally were reported in the range of 204 – 400 ng/l (Atanda et al. 2007). Not surpringly, in Kenya where mycotoxin exposure is prevalent, higher contaminations levels range of 0 – 2,550 ng/l of AFM_1 in milk samples collected from low- and middle-income areas of Nairobi was recently reported (Ahlberg et al. 2018; Kagera et al. 2019; Kuboka et al. 2019).

Detectable levels of AF metabolites have also been reported in commercial baby formulas purchased from stores and or supermarkets (Oliveira et al. 1997; Zhang et al. 2018). Home-made cereal and/or legume based weaning foods comprised of peanuts, corn, sorghum, and finger millet tend to have higher AFs contamination levels (Kumi et al. 2014; Magoha et al. 2016; Zhang et al. 2018). Furthermore, children raised in communities where corn and peanuts are dietary staples are continuously exposed to AFs throughout their childhood and into adulthood. Collectively, contamination of weaning food products is prevalent and thus multiple efforts must be undertaken to mitigate AF contamination of not only weaning foods but all food supplies especially in warm and humid regions of the world.

Weaning foods have also been found to be contaminated by fumonisins. Weanimix, a traditionally home-made blend made of groundnuts, corn and beans for children, specially prepared for weaning in West Africa was found to be contaminated by FNs, mean 4.7mg/kg, range 0.74 – 11.0mg/kg, quantified using fluorometric methods (Kumi et al.

2014). Similarly, corn flour samples used for weaning in Tanzania contained detectable fumonisins median 124 μg/kg with range 48-1224 μg/kg; when quantified by HPLC (Magoha et al. 2016).

Toxic Effects of Aflatoxins

Since their discovery in the 1960s as the major cause of the Turkey X disease which caused more than 100,000 deaths of turkey poults fed AF contaminated peanut meals (Sargeant et al. 1961; Spensley 1963), numerous studies have established AFs as carcinogens, immune-toxicants and mutagens. Among the four naturally occurring AFs namely AFB_1, AFB_2, AFG_1 and AFG_2, AFB_1 is the most potent and is the primary risk factor for hepatocellular carcinoma (HCC). The toxicity, carcinogenicity and mutagenicity of these AFs are in the order $AFB_1 > AFB_2 > AFG_1 > AFG_2$ as shown by experimental studies in animals. AFB_1 has a LD_{50} ranging from 1 – 50 mg/kg for most animals, however, pigs, rainbow trouts and ducklings are highly susceptible to AFs exposure with a LD_{50} of <1mg/kg (IARC 1993). LD_{50}, is the lethal dose that causes death in at least 50% of exposed subjects. In human populations, adverse health outcomes including aflatoxicosis, hepatotoxicity, nephrotoxicity, immune suppression, growth faltering, and carcinogenesis have been linked to dietary exposure of AFs as shown in disease outcome pathways figure 2.5. Moreover, various reports documented the presence of AFs in foods associated with Reye's syndrome patients in New Zealand, Thailand, Czechoslovakia, and the United States (Peraica et al. 1999).

Consumption of food products such as corn with AFs exceeding 200μg/kg results in aflatoxicosis; a disease characterized by jaundice, edema, sudden liver failure and even deaths. Aflatoxicosis has been reported in India, Kenya and Tanzania with case fatality

rates ranging from 39 to 50 percent (Kamala et al. 2018; Narsimha Reddy and Raghu Raghavender 2007; United States CDC 2004). In Kenya for example, contamination of corn by AFs is a recognized public health problem, which has resulted in over 600 human deaths due to aflatoxicosis related poisonings (Wangia 2017). In the unlikely circumstances of acute exposure that results in aflatoxicosis intervention measures such as use of novasil clay that decrease gastrointestinal absorption are therapeutic (Phillips 2002, Afriyu-Gwayu 2007). Administration of phenobarbital also enhances detoxification processes in the liver and thus, protects against AFB_1-induced toxicity and carcinogenicity (IARC 2012). Short-term exposures to high levels of AFs are rare events, however, consistent exposure to low quantities of AFs has been implicated in immune suppression, increased susceptibility to infectious diseases, anti-nutritional properties, infertility, growth faltering and carcinogenesis of HCC (IARC 1993, 2002; Okoth 2016).

Dietary exposure to AFs is generally chronic over a long period of time and the most established adverse health outcome associated with AFs exposure is development of primary liver cancers as discussed under carcinogenesis. HCC is a leading cause of cancer deaths with close to 30% of HCC cases attributed to dietary exposure to AFs. Moreover, AFs are genotoxic where once activated in the liver by the Cytochrome P450 cells, AFB_1-epoxide binds to DNA forming guanine adducts which have been shown to induce G to T mutations, including the TP53 codon 249. Furthermore, AFs are immune suppressants and are more likely to interfere with the innate immune system cell-mediated immunity and phagocytic cell functions (Bondy and Pestka 2000). The presence of AFs in liver and jejunum cells was associated with increased production of immune cells namely macrophages, CD4+ and CD8+ T cells (Kraieski et al. 2017). Epidemiological studies

assessing the impact of AFs exposure on immunity have also been reported. In Kenya, a recent study in children age 1 - 14 years reported that children with AFB_1-lysine adducts of more than 20pg/mg of albumin did not develop sufficient hepatitis B surface antibodies despite immunization during childhood (Githang'a, Wangia, et al. 2019). A study in the Gambia with 323 children age 3 – 8 years reported significantly higher AFB_1-Lysine adducts among children who had Hepatitis B surface antibodies and were positive of *Plamodium falciparum* (Allen et al. 1992; Wild et al. 1992). In a systematic review, dietary exposure to AFs and other mycotoxins was associated with reduced levels of serum Immunoglobulin A, reduced levels of hepatitis B surface antibodies, lower amounts of antibodies to pneumococcal antigen 23, but positive link to asexual parasites (Githang'a, Anzala, et al. 2019). It is worth noting that studies reviewed by Githang'a, Anzala, et al. 2019 were cross-sectional studies, with few participants and are not considered representative of the study populations where they were recruited. Moreover, the studies reviewed used different parameters to assess immune response. Further studies, preferably longitudinal or prospective studies, are warranted to further evaluate the role of mycotoxin exposures on children's immune system.

The mechanism of toxicity has been partially studied. Enzymes involved in both activation and detoxification processes of AFs are polymorphic and may influence toxic insult (Eaton and Gallagher 1994). AFs are relatively innocuous before metabolism and biotransformation, and a great diversity in metabolism of AFs among different species exist. For instance, GST polymorphisms have been associated with risk of various cancers as well as adverse health outcomes including pregnancy loss in Japanese women (Sata et al. 2003; Strange and Fryer 1999). Recently, exposure to AFs during pregnancy was shown

to be detrimental to the developing fetus. Adverse birth outcomes including preterm birth, low birth weight, intrauterine growth restriction and still births have also been reported (Khlangwiset, Shephard, and Wu 2011; Lombard 2014). In animal studies conducted in pregnant rats, mice, hamsters, swine, quails and rabbits exposed to AFs contaminated diets; decreased fetal weight, compromised live births, smaller litter sizes are consistently reported while none of these adverse birth outcomes are evident in controls. Furthermore, impaired organ development and skeleton anomalies were observed in a dose-dependent manner in pregnant rabbits and rats dosed with 0 – 300 µg/kg bw/day of AFs (El-Nahla et al. 2013; Wangikar, Dwivedi, and Sinha 2004). Taken together, these animal studies provide evidence that AFs are potent teratogens and needs further research.

Absorption, Distribution, Metabolism and Excretion of Aflatoxins

The exposure route for AFs is mainly dietary through consumption of AFs contaminated food, however, inhalation and dermal exposure routes have also been reported in occupational settings (IARC 2015; Viegas, Viegas, and Oppliger 2018; Wangia, Tang, and Wang 2019). As small lipophilic compounds, AFs are readily absorbed in the gastrointestinal tract in the small intestines and enters the liver through the hepatic portal vein after which it is widely distributed in the blood stream and throughout the body tissues (Ramos and Hernandez 1996; Wogan, Edwards, and Shank 1967). Normally, the distribution of toxicants is highly dependent on cardiac output and regional blood flow, vascular permeability, tissue perfusion rate, and the ability of the toxicant to bind plasma proteins (Dohnal, Wu, and Kuca 2014; Wogan, Edwards, and Shank 1967). Nonetheless, early experimental studies with animal models showed that AFs are majorly deposited in the liver where they undergo biotransformation (Toledo et al. 1987; Bhattacharya, Prabhu,

and Aboobaker 1989; Daniels et al. 1990). AFs are relatively innocuous before metabolism and biotransformation by cytochrome P450 enzymes which converts AFs into mutagenic and carcinogenic intermediates.

Overall, AFB_1 metabolism occurs in two phases. In phase I, AFB_1 undergo oxidation reactions mediated by Cytochrome P450 (CYP450) isoenzymes namely CYP1A2, CYP3A4, CYP3A5 and CYP3A7 (Bbosa et al. 2013; Eaton and Gallagher 1994). The metabolic processes of phase I reactions are catalyzed by cytochrome P450 largely available in the liver. AFB_1 may undergo different oxidation pathways such as o-dealkylation, keto-reduction, hydroxylation and epoxidation to produce hydrophilic compounds with varying levels of toxicity which are easily excreted. The oxidation pathways *O-dealkylation* produce AFP_1; *hydroxylation produces* AFM_1, AFP_1, AFQ_1, or AFB_{2a} metabolites; *ketoreduction* produces aflatoxicol, while *epoxidation* yields the most potent metabolite AFB_1-8, 9-epoxide (Bbosa et al. 2013; Dohnal, Wu, and Kuca 2014; Wu et al. 2009). AFM_1, AFP_1 and AFQ_1 are detoxification metabolites excreted in urine or bile and also validated biomarkers of AFs exposures as shown in Figure 2.4. The isoenzyme CYP3A4 which is predominant in the liver has the greatest capacity in the activation of AFB_1 into AFB_1-8, 9-epoxide and AFQ_1 metabolites, followed by CYP3A5, CYP3A7 and CYP1A2 has the least capacity; nonetheless, CYP1A2 converts AFB_1 to AFM_1, a small amount of AFQ_1 and the AFB_1-8, 9-epoxide (Bbosa et al. 2013; Dohnal, Wu, and Kuca 2014; Wu et al. 2009). Specifically, CYP1A2 and CYP3A4 activates AFB_1 to AFB_1-8, 9-endo-epoxide and AFB_1-8, 9-exo-epoxide respectively. The AFB_1-8, 9-epoxide is known to bind to guanine at the N^7 position, thus forming the AFB_1-N^7- adduct which may initiate point mutations at codon 249 of the human p53 tumor suppressor gene (Eaton and

Gallagher 1994; Wang and Groopman 1999). The epoxides are later detoxified by glutathione-S-transferases(GST) to products that can be easily excreted in urine, bile and feces. In fetal livers, CYP3A7 which is closely related to CYP3A4 catalyze the formation of epoxide at similar rate to adults. Unfortunately, the expression of GSTs is limited and thus, rapid detoxification of epoxides in severely limited in children. The expression of CYP3A5 is dependent on ethnicity, about 40% of African Americans do not express this enzyme and thus may influence AFs susceptibility. Thus, toxicity of AFs is highly variable depending on species, sex, age and nutritional status of the exposed. Therefore, individual and interspecies variation is dependent upon the proportion of AF metabolized to the 8, 9-epoxide relative to other non-toxic metabolites.

During metabolic Phase II, the reactive AFB_1-8, 9-epoxide undergoes detoxification processes through two pathways namely glutathione S-transferase (GST) mediated pathway and/or non-enzymatic processes (Bbosa et al. 2013; Guengerich and Johnson 1999). In the GST mediated pathway, the AFB_1-8, 9-epoxide is conjugated by glutathione (GSH) to form AFB_1 glutathione conjugate, which is excreted in urine as mercapturic acid (Bbosa et al. 2013; Dohnal, Wu, and Kuca 2014; Kensler et al. 2011; Wu et al. 2009). Alternatively, a non-enzymatic process which involves hydrolysis of the AFB_1-8, 9-epoxide to AFB_1-8, 9-dihydrodiol is initiated rapidly (Bbosa et al. 2013; Guengerich and Johnson 1999). AFB_1-8, 9-dihydrodiol then undergoes a base-catalyzed ring opening to form dialdehyde phenolate ion (Bbosa et al. 2013; Dohnal, Wu, and Kuca 2014). The dialdehyde from AFB_1 and AFG_1 form Schiff bases with primary amine groups such as those in lysine to form protein adducts (Sabbioni 1990; Sabbioni et al. 1987). Further, the AFB_1 aldehyde reductase (AFB_1-AR) catalyzes the dialdehyde phenolate ion

to dialcohol and is excreted in urine (Sabbioni 1990; Scholl et al. 2006). When AFB_1-8, 9-epoxide does not undergo phase II processes, it reacts with the DNA adducts at N^7 atom of guanine to form pro-mutagenic aflatoxin-N^7-guanine adducts. Often, aflatoxin-N^7-guanine adducts are unstable, undergoes depurination and are excreted in urine. Some of the DNA adducts however, are resistant to DNA repair processes resulting in gene mutation which can form cancerous tumors (Bbosa et al. 2013; Eaton and Gallagher 1994; Wogan, Kensler, and Groopman 2012). Alternatively, the AFB_1-8, 9-epoxide can cumulatively bind to serum albumin and form AFB_1-lysine adducts. The role of the enzyme epoxide hydrolase in hydrolysis of AFB_1-8, 9-epoxide has been investigated in terms of its role in the development of HCC in AFs exposed populations. Its role in carcinogenesis is however limited (Guengerich and Johnson 1999). The metabolism pathway of AFB_1 are shown in Figure 2.3 and Figure 2.4. More than 90% of AF metabolites from detoxification processes are excreted in urine, bile, and feces, while part of the AF metabolites is irreversibly retained in the liver, binds to tissues and or circulated in blood which cause further adverse health effects (Dalezios and Wogan 1972). The metabolic pathways of AFs are important because exposure assessment in both human and animal studies are based on AF metabolites that are by-products of metabolism.

Biomarkers for Exposure Assessment

Biomarkers are molecular, biochemical or cellular alterations that are measurable in biological media including serum, urine, fecal matter, hair and/or nails to assess exposure of a particular toxin, the resulting biological effect and the specific species susceptibility to toxicity (National Research Council 1987). In early studies, dietary exposure to AFs was estimated by analysis of contamination levels in food samples

combined with dietary intake surveys and food frequency questionnaires. However, AF contamination in food is typically heterogeneous and does not provide an accurate measure of actual exposure to individuals in a population. The route of AFs exposure is mainly dietary; however, exposure can occur in utero, dermally and through inhalation in occupational settings (Hernandez-Vargas et al. 2015; Wangia, Tang, and Wang 2019). Thus, biomarkers of AFs exposure were developed to integrate all exposure routes and provide accurate assessment particularly for populations at risk. In the 1970s, several epidemiological studies showed significant correlations between dietary intake of AFs with high incidence of HCC in South East Asia and West Africa (Groopman, Hall, et al. 1992; Groopman, Jiaqi, et al. 1992). At the time, no biomarkers were available for both AFB_1 exposure and other risk factors such as hepatitis B virus (HBV) which significantly made it difficult to establish a causal association. The development of biomarkers for AFs were successful in part due to extensive data available on their characteristics, metabolism, adduct formation processes, and mechanism of toxicity (Bbosa et al. 2013; Kensler et al. 2011). Validated biomarkers for AFs exposure include AFM_1, AFP_1, AFQ_1, AFB_1-mercapturic acid, AFB_1-N^7-Gua and AFB_1-lysine as shown in Figure 2.3 and Figure 2.4.

AFM_1 is quantifiable in both urine and breast milk samples. AFP_1, AFQ_1, AFB_1-mercapturic acid and AFB_1-N7-Gua are detectable in urine samples. AFB_1-N^7-Gua is a confirmed genotoxic marker of AFB_1 exposure. It has been established that AFB_1 forms adducts with DNA at N^7-guanine which are often rapidly excised and excreted in the urine with a half-life of about 8 hours (Kensler et al. 2011). Therefore, Aflatoxin-N^7-guanine adducts reflects short term aflatoxin exposure within 24 – 48 hours of dietary exposure. AF biomarkers in urine have been used in human populations at risk not only because of the

non-invasive nature of urine collection, but also because the metabolites reflect daily dietary variation of AFs contaminated food produces. However, urinary biomarkers vary significantly and have very short half-life. Nonetheless, laboratory studies using animal models have demonstrated that urinary AFB_1-N^7-guanine level is highly correlated with AFB_1 dose level, liver AFB_1-DNA adduct level and HCC incidence (Eaton and Gallagher 1994). Moreover, AFB_1 biomarkers were further validated by evaluating the modulatory effects of chemo-preventive agents such as Oltipraz, and green tea polyphenols (Egner et al. 2001; Luo et al. 2008; Wild and Hall 2000). While studies have demonstrated significant association between integrated AFs biomarker levels and HCC, the use of biomarkers is still limited in terms of establishing a relationship between exposure and the risk of disease. Given the multi-stage processes involved in carcinogenesis, dose response studies are still warranted.

AFB_1-lysine in serum albumin permits the measurement of more chronic exposure to aflatoxin given a longer half-life of about 2-3 months (Sabbioni 1990; Scholl et al. 2006). The AFB_1-lysine adducts are proteolytic digests of serum and can be easily quantified by different analytical methods. Another closely related AFB_1-lysine adducts biomarker is AF-albumin biomarker, quantified from plasma using Enzyme Linked Immunosorbent Assays (ELISA) and expressed in picogram (pg) AF-lysine equivalent per mg albumin. The AF-albumin biomarker in plasma is normally about 4.7 times AFB_1-lysine adducts in serum albumin (McCoy et al. 2005; Scholl et al. 2006). The use of AF-albumin biomarker is common in population-based studies conducted in low- and middle-income countries due to affordability, ease of use and high degree of sensitivity of the ELISA method. Nonetheless, ELISA has lower specificity for AFB_1-lysine adducts due to incomplete

proteolysis digestion, formation of adducts with different amino acids, and other structurally related AFs such as AFG_1 (Scholl et al. 2006; McCoy et al. 2005). Thus, up to five times of AFs concentrations are reported in studies that use ELISA. Other methods that have been developed and validated for use in the quantitation of aflatoxin metabolites in biological samples are Isotope Dilution Mass Spectrometry, Thin Layer Chromatography, High- Performance -Liquid-Chromatography, Liquid Chromatography Mass Spectrometry, Radioimmunoassay and Immunohistochemistry. Each of these methods have inherent strengths and limitations that affect sensitivity, specificity and sample throughput (McCoy et al. 2005). In this dissertation studies, we use High Performance Liquid Chromatography with Florescence Detection (HPLC-FD) to quantify AFB_1-lysine adducts in serum (Qian et al. 2010; Wang et al. 2001), because the methodology offers high specificity, sensitivity and high sample throughput. Albumin is the only serum protein which activated AF metabolites bind specifically on lysine to a significant extent (Sabbioni 1990; Scholl et al. 2006). AFB_1-lysine in human serum is highly stable, a reliable indicator of long-term exposure to AFs, and an effective biological response marker for aflatoxin-related human diseases in high-risk populations.

There are numerous exposure routes to mycotoxins as shown in Figure 2.2. Human exposure to AFs and other mycotoxins can occur in utero, via breast milk, through weaning foods and throughout an individual's lifetime (Watson, Gong, and Routledge 2017). Aflatoxin exposure occurs in utero and is dominant throughout childhood as weaning foods are introduced to children in high aflatoxin exposure regions (Turner 2013; Turner et al. 2005). Early life exposure to mycotoxins such as AFs has been associated with adverse health outcomes such as low birth weight, immune suppression, and growth impairments

(Lauer et al. 2018; Githanga et al. 2019; Khlangwiset, Shephard, and Wu 2011). In cases of continuous exposure, liver injury and development of hepatocellular carcinoma during adulthood are extensively studied and documented. The major metabolites of AFB_1 used as bioamarkers are summarized in Figure 2.3 and 2.4. Dietary AFs and their metabolites are detectable in biological matrix including blood products, urine, and breast milk.

In utero Exposure

Human epidemiological studies have been done to assess in utero exposure to AFs especially in the Middle East, South East Asia and Sub-Saharan Africa. For example, In a Gambian study with pregnant women, AFs exposure was lower before 16 weeks, GM of AFB_1- lysine adducts of 34.5pg/mg increased significantly as the pregnancy progressed to 41.8 pg/mg of albumin (Castelino et al. 2014). Likewise, in a different study, pregnant women from the Gambia in West Africa provided sera from umbilical cord and maternal venous blood at delivery; 97% (29/30) of maternal venous blood and 70% (21/29) of cord sera were positive for the aflatoxin-albumin adduct (Wild et al. 1991). Overall, aflatoxin albumin adducts were ten times lower in cord sera compared to maternal venous blood samples which indicates that though aflatoxin metabolites do not easily cross the placenta barrier, fetal liver have limited capacity to metabolize AFs (Wild et al. 1991). Similarly, studies conducted in Bangladesh and Nepal reported a 100% detection rate for AFB_1 - lysine adducts in cord blood samples tested and a median of 27.41pg/mg albumin. Taken together, these studies corroborate the evidence that fetal liver have biochemical capacity to convert AFs crossing the placenta into toxicologically active compounds (Groopman et al. 2014). Furthermore, Cytochrome P540 3A7, the fetal form of CYP3A4 which transforms AFs to epoxide has been confirmed to be active in fetus but the GST responsible

for detoxification processes are not fully developed at this time (Kitada et al. 1989). Collectively, these studies provide evidence of early exposure to AFs in utero and limited detoxification capacity in fetus.

AFM_1 in Breast Milk

AFM_1 is a mono-hydroxylated metabolite of AFB_1 that is secreted in milk. It can be used as an exposure biomarker of AFB_1 to confirm AFs exposure in early life. Even though AFM_1 is genotoxic, it is about ten times less toxic compared to AFB_1 (Creppy 2002; IARC 2002). The evaluation of AFM_1 in human breast milk serves as a good biomarker for post-natal exposure of AFs to infants in regions of high aflatoxin exposure. AFM_1 present in breast milk indicates exposure over the previous 24 hours. AFM_1 is a validated biomarker and its use was first reported in 1987 (Wild et al. 1987). Since then, numerous studies have been conducted in Africa, the Middle East and other parts of the world which reports the presence of AFM_1 in breast milk. In North Africa for example, among 388 Egyptian lactating mothers, 36% of breast milk samples tested was positive for AFM_1 with a median 13.5 pg/ml(Polychronaki et al. 2006). Likewise, in another study by same authors, AFM_1 distribution varied by season with highest mean levels of 64pg/ml reported in the summer months and lowest mean exposure levels of 8pg/ml in winter months (Polychronaki et al. 2006; Polychronaki et al. 2007). Another Egyptian cross-sectional study of 150 mother-infant dyads reported that 98 (65.3%) of breast milk samples tested positive of AFM_1 (Tomerak et al. 2011).

In sub-Saharan Africa, among 50 samples of breast milk from lactating mothers collected in Ogun State Nigeria, 82% of samples was contaminated with AFM_1 in the range of 3.49 – 35ng/ml (Adejumo et al. 2013). In Sudan, 54.3% (51/95) of breast milk samples

collected were positive of AFM_1 with a range of 0.007 - 2.561 ng/ml (Elzupir et al. 2012). In this study, consumption of peanut butter among lactating mothers was significantly associated with high AFM_1 in breast milk among the Sudanese nursing mothers. Peanuts are known to be highly susceptible to aflatoxin contamination. Studies of AFM_1 in breast milk samples collected from Iran suggests that aflatoxin exposure is often higher in rural areas compared to urban areas. For instance, one study recruited 80 lactating women from two urban clinics in Isfahan Iran, and only one breast milk sample was contaminated by 6.8ng/L of AFM_1 (Jafarian-Dehkordi and Pourradi 2013). In a different study, 91 lactating mothers were recruited from urban areas and 91 from rural areas in Tabriz Iran; none of the samples from urban areas was positive for AFM_1 whereas 21.9% (20/91) of samples from rural areas were positive of AFM_1 (Mahdavi et al. 2010). In other rural areas of Iran, AFM_1 in breast milk samples were reported to range from 2 – 39.3 ng/l (Jafari et al. 2017; Maleki et al. 2015). These studies suggest that food handling processes in rural areas of Iran may promote AFs contamination and therefore, women in rural areas are more likely to expose their infants to AFM_1 metabolites during breast feeding.

In Lebanon, among 111 breast milk samples collected from lactating mothers, 93.8% of samples were positive of AFM_1 with range 0.2 to 7.9 ng/L (Elaridi et al. 2017). In a different study in rural and urban areas of Famagusta District in Cyprus, 80% (40/50) of breast milk samples were found to be contaminated with AFM_1 with the mean of 7.84 ± 1.72 ng/l (Kunter et al. 2017). In the United Arab Emirates, among 140 lactating mothers recruited from special care baby unit or post-natal wards, 92% of breastmilk samples were positive for AFM_1 when analyzed by HPLC (Abdulrazzaq et al. 2003). In Turkey, several epidemiological studies have quantified AFM_1 in breast milk samples; one study of 61

breast milk samples, 13.1% were detectable of AFM_1 range 5.10 – 6.90 ng/L (Keskin et al. 2009). Similarly, a study in Ankara found AFM_1 in breast milk samples range 60.90 – 299.99ng/l and a 100% detection rate (Gurbay et al. 2010). In other studies, breast milk samples collected from mothers in hospitals in Eastern Turkey reported AFM_1 with a range of 1.3 – 13.0 ng/l (Atasever et al. 2014; Kilic Altun, Gurbuz, and Ayag 2017).

Additionally, AFM_1 in breast milk samples has been quantified in South America and some European Countries.. In Colombia and Southern Brazil, more than 90% of breast milk samples were positive for AFM_1 with range of 0.9 – 18.5ng/l (Diaz and Sanchez 2015; Ishikawa et al. 2016). Studies from Europe, especially Italy, breast milk samples test positive for Ochratoxin A and only rarely for AFM_1 , an indication of higher consumption of wheat based products which are more susceptible to Ochratoxin A exposure compared to AFs (Galvano et al. 2008; Turconi et al. 2004). Taken together, these studies provide sufficient evidence of the widespread nature of AFs worldwide and the need to advise pregnant and lactating women on moderate consumption of food products such as corn, peanuts and/ or their products to limit possible transfer of AF metabolites to their infants.

Biomarkers in Urine and Serum

AFM_1 and AFG_1 in serum, urinary AFB_1, AFG_1 and AFM_1, and AFG_2 in milk even though quantifiable and are indicative of exposure, their concentrations are sometimes not correlated with dietary intake (Smith et al. 2017; Smith, Stoltzfus, and Prendergast 2012). The metabolites are majorly used for short term exposures of less than 24 hours. The advantage of these biomarkers is the ability to provide daily variation of dietary aflatoxin exposure levels. AFM_1 and AFB_1-N^7-guanine in urine and AFB_1-Lysine in serum are validated biomarkers of AFs exposure and are often highly correlated with dietary intake

of AFs. To date, these biomarkers have been used in human populations at risk to determine AFs exposure. An early study in the Philippines reported the presence of AFM_1 in urine of human subjects (Campbell et al. 1970).This study in the Philippine was the first to employ biomarkers in biomonitoring of aflatoxin exposure among human populations at high risk. Another early study was done in Zimbabwe where AFM_1 metabolites was detected in 4.3% of 1228 urine samples collected with an average concentration of urinary AFM_1 of 4.2 ng/ml (Nyathi et al. 1987). In China, a good correlation between AFM_1 in urine and dietary intake of AFB_1 contaminated foods over a 3-day period of time was reported (Zhu et al. 1987).

In biomonitoring studies using Aflatoxin B_1-N^7-guanine adducts, a study in the Gambia reported a strong correlation between Aflatoxin B_1-N^7-guanine adducts and dietary intake of AFB_1 in 20 individuals (Groopman, Hall, et al. 1992). Additionally, among 983 urine samples collected from Kenya, Aflatoxin B_1-N^7-guanine adducts were positive of 12.6% of the samples (Autrup et al. 1987). Sera from the United Kingdom and Japan have also been reported be positive of AF metabolites (Tsuboi et al. 1984; Wilkinson, Denning, and Morgan 1988). Overall, AFs exposure levels are significantly high in individuals residing in low-and middle-income economies while very low to non-detectable levels of AF metabolites are reported in Europe, Japan and the United States.

Mechanism of Fumonisins Toxicity

The primary mechanism of FNs toxicity is established to be disruption of sphingolipid synthesis *de novo* by way of FB_1's inhibitive action of the enzyme ceramide synthase (Merrill Jr et al. 2001; Riley and Merrill Jr 2019; Voss, Smith, and Haschek 2007). Sphinganine and sphingosine are synthesized *de novo* from palmitoyl-COA and serine

substrates through a myriad of different enzymes including serine palmitoyl transferase, 3-ketoreductase, ceramide synthase and desaturase (Dupre and Siskind 2018; Lynch 2018). Due to structural similarity between FNs and the long chain sphingolipid backbones, FB_1 has the ability to compete with sphingolipids to bind and inhibit ceramide synthases (Dupre and Siskind 2018; Lynch 2018; Zitomer et al. 2009). Moreover, the ability of ceramide synthase to recognize amine and tricarboxylic acid chain groups favors binding to FB_1. Inhibition of the enzyme ceramide synthase interferes with the sphingolipid metabolism causing free sphingolipid bases and their 1-phosphates to accumulate in cells (Gardner et al. 2016; Gelineau-van Waes et al. 2012; Merrill Jr et al. 2001). Since ceramide is a precursor to more complex sphingolipids, the loss of *de novo* ceramide synthesis triggered by FB_1 would result in a decreased pool of these sphingolipids, which play important roles in cell membrane integrity (Riley and Merrill Jr 2019). Overall, Accumulation of intracellular free sphinganine is associated with alteration of the sphinganine to sphingosine ratio, and thus, past studies monitored these changes to confirm FNs exposure.

A close relationship between Sa accumulation and the expression of toxicity in liver and kidney has been established (Delongchamp and Young 2001; Enongene et al. 2002). Once accumulated, free sphingolipid bases persist in the kidneys much longer than FB_1 (Enongene et al. 2002; Voss et al. 2002; Voss, Smith, and Haschek 2007). An oral dose of FB_1 insufficient to increase the concentration of free Sa (0.1 mg/kg BW per day) can prolong the half-life of free Sa in urine of rats after they have been taken off diets that contained a dose sufficient to cause free Sa (1 mg/kg BW per day) to accumulate in urine which was confirmed in mice treated by oral gavage (Enongene et al. 2002; Voss et al. 2002; Voss, Smith, and Haschek 2007). FB_1-induced increases in free sphingolipid bases

and toxicity are both reversible, although elimination of free Sa from the liver is faster than from the kidney (Enongene et al. 2002; Voss et al. 2002; Voss, Smith, and Haschek 2007). Accumulated Sa is metabolized to Sa- 1- phosphate (SaP) and then cleaved into a fatty aldehyde and ethanolamine phosphate (Merrill et al. 1999; Merrill Jr et al. 2001).

Sphingolipids are also involved in signaling pathways, cell recognition and modulating interactions with receptors and integrin (van Meer and Hoetzl 2010; Merrill Jr et al. 2001; Riley and Merrill Jr 2019). Therefore, other processes mediated via glycosylphosphatidylinositol-anchored proteins associated with lipid rafts and endosome-mediated retrograde transport of molecules to the Golgi apparatus are also disrupted with alterations is sphingolipids balance (Riley and Merrill Jr 2019; Voss, Smith, and Haschek 2007). Other consequences associated with disruption of de novo synthesis pathway of sphingolipid metabolism include inhibition of the protein kinase C, activation of phospholipase D, inhibition of Na+/K+ ATPase and induction of dephosphorylation of retinoblastoma protein (Kim et al. 2018; Liu et al. 2019; Merrill Jr et al. 2001).Ultimately, once complex sphingolipid are depleted, inhibition of cell growth occurs and ultimately induction of apoptosis (Liu et al. 2019).

The second mechanism of toxicity involves Oxidative Stress (OS) which refers to the disturbance of pro-oxidant and/or antioxidant balance in preference to oxidants resulting in cell damage (Betteridge, 2000; Tsalamandris *et al.*, 2018). In *vivo*, alongside with sphingosine and sphinganine accumulation, the FB_1 subacute exposure to one-day-old male broiler chicks induce liver oxidative stress (Poersch et al. 2014). During sphingolipids synthesis, the presence of FB_1 prevents the acylation of ceramide synthase to dihydroceramide (Wangia et al. 2019). This inhibition promotes accumulation of

sphinganine, a highly toxic compound that increases oxidative stress, and promotes impairment in cell cycle regulation, apoptosis and or necrosis (Marasas et al. 2004; Riley and Merrill Jr 2019). Additionally, exposure to FNs is known to induce oxidative stress (OS) in form of lipid peroxidation (Hassan et al. 2010; Kouadio et al. 2005; Stockmann-Juvala et al. 2004). Increase in lipid peroxidation and production of reactive oxygen species from FB_1 treatment, which appear to be preceded by caspase activation, has been linked with neurotoxicity *in vitro* (Riedel et al. 2016; Stockmann-Juvala et al. 2004). For neuronal cell lines, exposure to FB_1 is associated with lipid oxidation and necrotic cell death in mouse GT1-7 hypothalamic cells, rat C6 glioblastoma and human SH-SY5Y cells (Stockmann-Juvala et al. 2004). In human intestinal Caco-2 cells, exposure to FB_1 from the concentration 10 μM of FB_1 showed the capacity to induce lipid peroxidation, thus altering the cell membrane and ultimately cell death (Kouadio et al. 2005). In female Sprague-Dawley rats, extracts of *Aquilegia vulgaris* have been shown to counter FB_1 effects by increasing the antioxidant capacity, inhibiting lipid peroxidation and scavenging free radicals (Hassan et al. 2010). For cells to oppose OS, the endoplasmic reticulum stress is activated and leads to autophagy activation for protecting cells *in vitro* and *in vivo* from FB_1 exposure (Singh and Kang 2017). This FB_1 induced autophagy is suggested to be facilitated by activation of ERN1-MAPK8/9/10 signaling pathway (Singh and Kang 2017; Yin et al. 2016). Kim et al. demonstrated that mice exposed to 2.5mg/kg of FB_1 per body weight consecutively for 4 days experienced activation of both apoptosis and autophagy (Kim et al. 2018). Moreover, increased expression of inositol-requiring enzyme 1 (IREI-α), Jun N-terminal kinase (p-JNK), Caspase-3, and LC3I/II is observed (Kim et al. 2018; Singh and Kang 2017; Yin et al. 2016). Pro-oxidants are made of reactive oxygen species

(ROS), oxygen based metabolic products with unpaired electrons which include few radicals including superoxide (O_2^-) and hydroxyls (OH^-)(Betteridge 2000; Tsalamandris et al. 2018). Different enzymes such as xanthine oxidase, lipoxygenase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cyclooxygenase, and uncoupled-endothelial NO synthase (eNOS) are all known to generate ROS in mammalian cells (Betteridge 2000; da Silva, Bracarense, and Oswald 2018; Ostry et al. 2017; Tsalamandris et al. 2018). Moreover, heme oxygenase, superoxide dismutase (SOD), glucose-6-phosphate dehydrogenase, catalase, and glutathione peroxidase (GSHP) are anti-oxidant enzymes which counter ROS effects to limit cellular damage (Tsalamandris *et al.*, 2018).

Absorption, Distribution, Metabolism and Excretion of Fumonisins

Little to no FB_1 is detectable in plasma and tissues in animal models, an indication of limited absorption. Absorption is mostly negligible, but FNs are rapidly distributed and eliminated. While it is clear that FNs are excreted in bile, there is little to no evidence of the metabolism in vitro or in vivo. In murine models when FB_1 is administered intraperitoneally or intravenously, FB_1 elimination is rapid with no evidence of metabolism by either cytochrome P450s, microsomal enzymes or any other enzymes (Marasas, Kellerman, et al. 1988; Merrill Jr et al. 2001). Toxicokinetic experiments in rodents show liver and kidneys retain most of un-metabolized FB_1. The kidneys likely retain more than 10 times compared to the liver (Gelderblom et al. 2008; Voss, Smith, and Haschek 2007). It has been postulated that FB_1 is hydrolyzed in the gut by microbial degradation and excreted in faeces but not in urine or bile (Merrill et al. 1999). The elimination kinetics of FB_1 administered orally has not been well described and not fully accounted for (Delongchamp and Young 2001). Ultimately, FNs is excreted in the faeces, either

unchanged or with loss of tricarboxylic acid side chains.

Animal studies established that orally administered FB_1 is eliminated rapidly from circulation. Free FB_1 is recoverable from both urine and faecal samples (Chelule et al. 2001; Shephard, Van Der Westhuizen, and Sewram 2007). There are methodologies developed to quantify free FB_1 in faeces for short term exposure, however, use of faeces in large scale epidemiological studies may not be feasible (Chelule et al. 2001; Shephard, Van Der Westhuizen, and Sewram 2007). An alternative method proposed and developed involves assessment of FNs accumulation in human hair for long term exposure to FNs (Sewram et al. 2003). Nonetheless, use of hair samples in quantification of FNs metabolites is not yet validated. In the study by Sewram et al, small sample size and unclear correlation between dietary exposure to FNs and biomarkers severely limited the strength of evidence.

Fumonisin Biomarkers of Exposure Assessment

Corn is the third most important cereal grain for human consumption in the world after wheat and rice and is a staple food in many regions. To date, there is a limited understanding of the role of fumonisin in human disease in part due to limited availability of validated biomarkers for human exposure. In traditional epidemiology, FNs exposure would be typically estimated from food frequency questionnaires or surveillance data on FNs contamination of food products. There have been iterations to incorporate biomarkers in exposure assessment and determine disease risks associated with chronic exposure to FNs. Biomarkers are molecular, biochemical or cellular changes measurable in biological matrix such as serum, urine, faecal matter, hair and/or nails to assess exposure of a

particular toxin, the resulting biological effect and species susceptibility to toxicity (National Research Council, 1987). The use of biomarkers in FNs exposure assessment limits problems of confounding, effect modification and exposure estimation errors. Biomarkers are integral in establishing the etiological role of a toxin in both human and animal disease outcomes (Ryan *et al.*, 2007). Non-invasive bio specimens such as urine, blood, faeces are preferred in molecular epidemiological studies.

Development and validation of biomarkers is dependent on understanding the metabolism and mechanism of FNs toxicity. Biologically plausible biomarkers must be validated for accuracy, reproducibility, and specificity (Cai, Tang, and Wang 2007; Ryan et al. 2007; Wangia et al. 2019). Due to lack of major metabolite and low bioavailability of FNs, free FB_1 in urine and sphingolipid metabolites have been evaluated as biomarkers of FNs exposure (Shephard, Van Der Westhuizen, and Sewram 2007). The initial processes of validating biomarkers involve establishing a good correlation between FNs exposure and levels of proposed biomarkers in serum, urine and or target organs for objective evaluation.

To date, urinary free FB_1 and Sphingolipid metabolites are the only validated biomarkers for FNs exposure in human populations. For both free FB_1 and sphingolipid biomarkers, urinary creatinine (mg/dL urine) is normally used to adjust the fumonisin concentration to control for inter individual variation in urine concentration (Gong et al. 2008). The use of free FB_1 in urine as biomarkers for fumonisins exposure was first proposed in 1998, 10 years after the isolation of fumonisins from *F. moniliforme* fungi. Shetty and Bhat proposed the use of urinary FB_1 as a biomarker of exposure in humans (Shetty and Bhat 1998). Since them, urinary free FB_1 has been used in human

biomonitoring studies in Ghana and Guatemala. In an intervention study designed to evaluate the binding capacity of calcium montmorillonite clay to fumonisins, reduced levels of free FB_1, 1.02 (95% CI 0.45 – 1.59) ng/mg creatinine in human urine recruited from the Ashanti Region of Ghana was observed among participants assigned the high dose, (3.0g clay capsule administered per day) which was significantly lower than 6.29 (95% CI 3.75 – 8.84) ng/mg creatinine of the placebo group after a period of 10 weeks (Robinson et al. 2012). Though significant reductions were observed in this study, FB_1 levels in food consumed by study participants were not measured and hence could not be correlated with urinary excretion patterns. Similarly, a study in Guatemala inferred a dose response relationship between consumption of FB_1 contaminated food products and urinary levels of free FB_1. In individuals with low, medium and high corn consumption, the mean urinary FB_1 were 44.0 (95%CI 22.3-86.8) pg FB_1/mg creatinine, 92.3 (95%CI 54.7-155.7) pg FB_1/mg creatinine & High 133.9 (95%CI 78.5-228.3) pg FB_1/mg creatinine respectively when quantified using LC-MS (Gong et al. 2008).

Sphingoid bases namely, Sphinganine (Sa), Sphingosine (So) and the Sphinganine to Sphingosine ratio (Sa/So) are widely used biomarkers for fumonisins exposure. Both Sa and So are highly variable and thus, Sa/So ratio is preferred as a biomarker given the low variability. Sphingolipid based biomarkers have long been evaluated in animal studies including ducks, foals, pigs, mink, rodents, and vervet monkeys (IARC 1993, 2002; Riley 1994).

These animal studies enabled the evaluation of sensitivity, specificity, accuracy, and reliability of sphingolipid-based FNs exposure biomarkers. Furthermore, data obtained from animal studies allow for assessment of both intra- or inter-individual variability,

background levels, relationship of biomarkers to external dose or to disease status as well as feasibility for use in large population-based human studies (Enongene et al. 2002; Shephard et al. 1994; Shephard, Van Der Westhuizen, and Sewram 2007). Thus, FB_1-induced biochemical elevations of Sphingolipids including Sphinganine (Sa), Sphingosine (So), Sa-1-Phosphate (SaP) and So-1-Phosphate (SaP) and their ratios (Sa/So or SaP/SoP) in tissues and body fluids are plausible biomarkers of FNs exposure (Cai, Tang, and Wang 2007; Wangia et al. 2019). The sphingolipid-based biomarker is reliable in assessing dose response relationships, especially in animals, however, the correlation between the biomarker and human exposure to FNs remained conflicted until validation studies were undertaken (Shephard, Van Der Westhuizen, and Sewram 2007; Wangia et al. 2019).

FN biomarkers have also been used in animal studies to assess the efficacy of adsorbent materials in binding dietary FB_1 (Alberts, van Zyl, and Gelderblom 2016; Denli et al. 2015). In early studies, urinary Sa/So ratios were established to be highly correlated with FNs intake in rats fed with diet containing 0.5 to 15 mg/kg of FB_1 and FB_2 for 7 days (Solfrizzo, Avantaggiato, and Visconti 1997). Similarly, a statistically significant correlation between urinary Sa/So and FN contents of the diets were observed, even though the total FN levels were less than 5 mg/kg in a study where rats were fed fumonisin contaminated diets for 2 – 3 weeks (Merrill et al. 1997). One study in ducks chronically dosed with FN-containing extracts revealed that effects of FNs on serum Sa/So was linear. Specifically, when the cumulative dose fed to ducks was below 100-200 mg FB_1 whereas it saturated when the cumulative dose fed was higher (Tran et al. 2006). In rodents, disruption of sphingolipid metabolism in liver and kidney occurs at a FB_1 dose below those that induce morphologic injury (Voss et al. 2002; Voss, Smith, and Haschek 2007).

Moreover, a close correlation between the incidence and severity of liver pathology and the increase in free Sa was reported (Delongchamp and Young 2001; Riley and Voss 2006). In rainbow trout, dietary FB_1 exposure after AFB_1-initiation induced a dose-dependent increase in liver tumor incidence, which was paralleled by an increase in free Sa and Sa/So in liver (Carlson et al. 2001).

Validation of biomarkers majorly involves studying to what extent a given biomarker reflects FNs exposure. Since sphingolipids were proposed as biomarkers of FNs exposure, several studies have been done to evaluate its validity. Sphingolipid biomarkers have been validated using robust HPLC-fluorescence and LC/MS methods as biomarkers of fumonisin exposures and are used in human populations to monitor FNs exposures (Cai, Tang, and Wang 2007; Wangia et al. 2019). Validity of exposure biomarkers means that there is a consistent link between the biomarker level and the extent of FNs exposure. Validation of biomarkers provide opportunity to assess if developed biomarkers offers high throughput and are appropriate for use in population studies.

Studies in human populations have been done in Burkina Faso, China, South Africa, south America, Spain and Portugal to explore the potential and validity of sphingolipid-based biomarkers as detailed in Table 7.5. Serum and urine levels of sphingolipid bases were analysed in the plasma and urine of male and female volunteers consuming a staple diet of home-grown corn from Transkei region of the Eastern Cape and KwaZulu-Natal province, South Africa and in the Bomet district, western Kenya (Van Der Westhuizen et al. 2003). No significant differences were found in the Sa/So ratios between males and females within the regions nor between the different regions. They speculated that the ratio is not sensitive enough to act as a biomarker for FN exposure in humans at these levels of

contamination in corn. Nonetheless, more recent studies have shown that urinary based sphingolipid biomarkers are reported to be more sensitive than serum in response to both acute and chronic exposure to FB_1 (Cai, Tang, and Wang 2007; Shephard, Van Der Westhuizen, and Sewram 2007; Wangia et al. 2019). In a recent study, urinary sphingolipid metabolites were validated as biomarkers for FNs exposure in children's population (Wangia et al. 2019). The choice of which biomarker to use depends on the goal of the study. For instance, one study aimed at evaluating the effectiveness of green tea polyphenols in mitigating tumorigenic effects of FB_1 monitored changes in the levels of free urinary FB_1, serum and urinary sphingolipids (Xue et al. 2015). In epidemiological studies that seek to study aetiology of diseases as it relates to exposure of FNs, integrating use of biomarkers and questionnaires will provide a true representation of actual exposures.

Aflatoxin Related Adverse Health Outcomes

Numerous diseases have been linked to consumption of aflatoxin contaminated diets including carcinogenesis, aflatoxin poisoning, immune suppression and growth impairments in children as described in the subsections below. The summary of confirmed diseases with their possible mechanistic pathways is shown in Figure 2.4.

Carcinogenesis

The International Agency on research for cancer (IARC) found sufficient evidence for the carcinogenicity of naturally occurring mixtures of AFs and AFB_1 (IARC 1993, 2002). This section discusses studies in human populations, experimental systems using cell lines and laboratory animals that corroborate the observation that AFs are carcinogenic.

Human Studies

Since their discovery more than 50 years ago, the association between AFs exposure and human liver cancers have been examined using ecologic, cross-sectional, case-control and cohort studies in high-risk populations. Liver cancer is the sixth most common cancer worldwide, with an estimated 782,000 new cases diagnosed in 2012 alone (World Cancer Research Fund 2018). Moreover, liver cancer is the second most common cause of death from cancers and is more common in men than women (World Cancer Research Fund 2018). Nearly 90% of liver cancers occur in South East Asia and Sub Saharan Africa with China alone accounting for over 50.5% of the total cases (World Cancer Research Fund 2018). The most common form of liver cancer in adults is the Hepatocellular Carcinoma (HCC) which begins in hepatocytes (Bulatao-Jayme et al. 1982; Jepsen and Kissmeyer-Nielsen 2008). The main route of exposure to AFs in human population is dietary intake, thus proposed regulations for maximum limits in food items range from 5 to 20μg/kg for total AFs and even lower for AFB_1 alone in most countries. Significant positive correlations exist between mortality rates of primary liver cancer and AFs intake. While no acceptable daily intake is given by the Joint Food Expert Committee on Food Additives and Agriculture/World Health Organizations (JEFCA), human intake should be reduced to the lowest practical level.

In early descriptive studies, incidences of primary liver cancer were consistently related with dietary intake of AFs. In Swaziland for example, out of 2, 583 food samples tested for AFs contamination, 120 were highly contaminated with AFs (Peers et al. 1987). Significant correlations between liver cancer incidence and daily consumption of AFs contaminated foods was reported in this particular study (Peers et al. 1987). Similarly, early

studies in Kenya reported that the estimated consumption of AF contaminated food was highly correlated with liver tumor incidence (Peers and Linsell 1973; Autrup et al. 1987). Conversely, a cross-sectional survey in 48 sites across China found no association between AFs intake and primary liver cancer but a strong correlation between HBsAg prevalence and primary liver cancer (Campbell et al. 1990). Case control studies done in Philippines, China and Nigeria assessed the role of AFs in liver tumorigenesis. For instance, among 90 hospital cases of confirmed primary liver cancer in the Philippines, cases were more likely to have consumed diets with high AFs contamination levels compared to controls in their lifetime (Bulatao-Jayme et al. 1982). In another hospital-based case-control study consisting of 107 Chinese patients with primary liver cancers matched with orthopedic patients in the same hospital, the frequency of consuming of AFs contaminated diet in the past 20 years was evaluated and there was no significant difference in dietary patterns with controls recruited from orthopedic wards (Lam et al. 1982). Collectively, these early studies evaluated the role of AFs contaminated diets on liver carcinogenesis, but exposure assessment was done using dietary questionnaires marred with recall bias and more often, the studies were initiated after disease onset.Another significant limiting factor was limited evaluation of the effects of hepatitis virus in the populations at risk All these factors limited the strength of studies which further pushed the need for studies based on individuals as units were urgently required. Evidence from these studies however prompted further research on the role of AF intake in development of primary liver cancers. Cohort and or Longitudinal studies were initiated shortly afterwards.

The most notable early cohort study conducted in China, it was reported that the average annual intake of AF in heavily contaminated villages was 6.0mg per person in

areas with high mortality rate (372/100,000) from primary liver cancers, while a region considered of low AFs contamination, AFs intake was estimated at 0.64mg per person with cancer mortality rate of 32.8/100,000 per year (Yeh, Mo, and Yen 1985; Yeh et al. 1989). In the same vein, another cohort study included the evaluation of Hepatitis B virus infection status, consumption of AFs contaminated foods, and presence of Aflatoxin B_1-N^7-guanine adducts in urine and reported that all were positively associated with HCC cases (Wild et al. 1993). This study reported a multiplicative effect between AF intake and HBsAg status, but there is a possibility that misclassification of HBsAg status affected the results. Overall, inappropriate methodologies, inaccurate diagnosis of liver cancers, lack of validated biomarkers for exposure assessment and limited controlling for chronic hepatitis virus infections also severely limited early studies. Collectively, these early human studies done in the 1980s and 1990s informed future research

Beginning 1990s, new improved biomarkers for AFs exposure in individuals, improved ability to measure hepatitis infection and cancers, and better study designs able to control for numerous confounding factors were initiated. In a hybrid ecological cross-sectional study conducted in Taiwan, China; HCC was significantly associated with urinary AFs metabolites after adjusting for age, sex and HBsAg in multivariate regression analysis (Hatch et al. 1993). In another cohort study established in January 1986 and followed up to March 1990, male residents of Shanghai, China aged between 45 - 64 years were followed, in HBsAg positive individuals, the RR for developing cancers was 60.1 (95% CI 6.4 – 561.8) while only 1.9 (95% CI 0.5 – 7.5) in HBsAg negative participants with detectable aflatoxin B_1-N^7-guanine adducts after controlling for HBsAg positivity, chronic liver disease, smoking and alcohol use (Ross et al. 1992). This study however was limited

given the short follow-up period and it is possible that participants who developed liver cancer already had tumors. In an extended analysis of a previously established cohort by Ross et al 1992, of 364 cancer cases identified, 55 were diagnosed with HCC and confirmed by biopsy, the risk for HCC was higher among people with AFB_1-N^7-guanine adducts in urine and other AFs metabolites in urine were higher in cases than in controls (Qian et al. 1994). In a prospective cohort study in the Penghu Islets of Taiwan, up to 4,691 men and 1,796 women between the ages 30 to 60 years old were recruited from areas with highest incidence rates of HCC (Chen et al. 1996). Blood samples were collected for screening of hepatitis virus and AFB_1-lysine adducts, questionnaires were administered to collect information on socio-demographic factors, dietary patterns and medical history of participants; and a two stage screening process incorporating serological markers and ultrasonography was used in diagnosis of HCC (Chen et al. 1996). Using logistic regression with age and sex adjustment, the OR for an association between AFB_1-lysine adducts and HCC among 13 cases was 3.2 (95% CI, 1.1 – 8.9); however, when other covariates were added including HBsAg positive status, family history of liver cancer and cirrhosis, the OR for HCC increased to 5.5 (95% CI, 1.2 - 23) (Chen et al. 1996).

In another cohort study carried out in seven townships of Taiwan, including three from Penghu Islets, 56 cases of HCC were matched with 168 controls without liver cancer or liver diseases, serum samples were assayed for HBsAg and alpha-fetoprotein and various markers of liver function (Wang et al. 1996). Using conditional logistic regression models, the OR was 4.6 (95% CI 2.0 – 10) for liver cancer in subjects with detectable levels of AF-albumin adducts while little to no effect of AF biomarkers among HBsAg-positive subjects was reported; nonetheless, strong effects among HBsAg positive subjects

was observed (Wang et al. 1996). In a nested case control study which is an extended cohort study of Wang et al 1996, about 79 HBsAg positive cases of HCC were identified between 1991 and 1997 and matched for age, gender, residence and date of recruitment with one or two randomly selected HBsAg positive controls; collected blood samples from 149 participants in total were analyzed for HBV, HCV, AFB_1-lysine adducts and GST M1 vs T1 genotypes (Sun et al. 2001). In conditional logistic regression models, increase in HCC incidence was significantly correlated with AFB_1-lysine adducts while GST genotypes were associated with a decreased risk of HCC. However, an interaction between AFB_1-Lysine adducts and GSTT1 genotype was found, indicating an increased risk for HCC for individuals who were GSTT1-null genotype (OR = 3.7, 95% CI 1.5 – 9.3) (Sun et al. 2001). In a nested case-control study of 50 HCC patients recruited from Taiwan, all study subjects were positive for urinary metabolites AFM_1 and 43% AFB_1-N^7-guanine adducts (Yeh et al 1997). Moreover, a significant correlation between dietary intake of various foods and levels of urinary AFM_1 was reported (Yeh et al 1997). Additionally, among HBsAg positive participants, AFM_1 was significantly associated with elevated risk of HCC and after controlling for confounding factors, 16 subjects with both AFB_1-N^7-guanine adducts and AFM_1 in their urine samples had an OR of 12 (95%CI, 1.2 - 117) (Yeh et al 1997). Likewise, Lu et al 1998 investigated the role of HBV and AFs in the etiology of liver cancer among males in Qidong, China in a nested case control analysis. Among HBsAg positive participants, AFB_1-lysine adducts was significantly higher among cases than controls (Lu, Kuang, and Wang 1998). Similarly, Sun et al 1999 reported that among 145 men with chronic HBV, 22 developed HCC at the end of the study; subjects with family history of HCC, anti-HCV positive and more than 3.6 ng/L of urinary AFM_1 had an increased risk of

developing HCC (Sun et al. 1999). A small-scale study carried out in a Nigerian hospital recruited 22 HCC cases and 22 controls from gastroenterology ward to study the role of HBV and AFs on HCC, the authors report high AFs detectable in cases and HBsAg positive in most patients, which are both risk factors for HCC (Olubuyide, Maxwell, Akinyinka, et al. 1993; Olubuyide, Maxwell, Hood, et al. 1993). In western and central Sudan, a case-control study to assess the association between peanut butter consumption as AFs source, HBsAg status and the GSTM1 genotype in HCC etiology was assessed among 115 cases and 199 controls (Omer et al. 2004; Omer et al. 2001). Peanut butter consumption was associated with an increased risk of HCC in central Sudan but a very high risk in western Sudan. Moreover, GSTM1 genotype was a strong effect modifier and excess risk was concluded to be due to AFs contamination in peanut butter and the OR in the highest quartile of peanut butter exposure among GSTM1-null subjects was 17 (95% CI , 2.7 – 105) (Omer et al. 2004; Omer et al. 2001). Despite improved methodologies, studies in human populations to assess the role of AF in carcinogenesis were still marred with limitations. Dietary questionnaires at population levels provide crude assessment of AFs exposure and could not account for individual variation of exposure. Moreover, biomarkers of AFs exposure only reflect exposure in a recent period, these may not necessarily reflect exposures during the etiologically relevant period. Furthermore, the role of HBsAg virus in etiology was not clearly defined and it was not clear if the presence of liver related diseases obscures the etiological role of AFs in carcinogenesis.

Collectively, these studies show that the degree by which AFB_1 contributes to HCC is influenced by Hepatitis B and/or C virus infection, nutritional status, metabolic polymorphisms and age. A greater multiplicative risk for developing hepatocellular

carcinoma in individuals with concomitant exposure to both AFs and hepatitis B virus has been established (Wogan, Kensler, and Groopman 2012; Wu, Stacy, and Kensler 2013). Molecular epidemiological studies of populations at high risk for liver cancer show that hepatitis B virus and dietary exposure to AFB_1 are the two major risk factors for liver cancer.

Cancer Studies in Experimental Animals

The first report of AF induced tumor was confirmed in rats shortly after the Turkey X disease outbreak in England (Lancaster, Jenkins, and Philp 1961). Young rats fed groundnut diets with AFs in the range of 3000 - 4000 μg/kg for a period of three weeks then returned to normal diets developed carcinosarcoma and/or hepatocellular carcinoma; another group of male rats one year old fed the same diet for thirty-nine weeks developed HCC and adenocarcinomas (Butler and Barnes 1966). A group of six male rats administered a mixture of AFs intratracheally at doses of 0 μg/kg or 300 μg/kg twice per week for 30 weeks developed squamous cell carcinomas, hepatoma and renal-cell adenoma (Dickens, Jones, and Waynforth 1966). Female Wistar rats exposed to 7650 μg/kg of AFs in diets developed HCC, some developed nodular hepatic hyperplasia while others developed cystic bile-duct hyperplasia in their lifetime (Carnaghan 1965). Groups of 25 male and 25 female Fischer rats fed semi-synthetic diets containing 0, 15, 300 and 1,000 μg/kg AFB_1 of more than 99.5% purity for 52 weeks or until tumors developed reported that HCC incidence increased for both sexes in a dose dependent manner, and time to tumor development decreasing as the dose was increased (Wogan and Newberne 1967). In addition to HCC, hepatocellular adenomas and preneoplastic liver lesions were reported. Groups of 30 male and 30 female Fischer rats administered with AFB_1 in dimethyl

sulfoxide (DMSO) at 0 or 80 μg/kg orally by gavage for 5 days reported that all males died within 2 weeks while female mortality at 35 weeks was 11/30; the surviving females to 82 weeks developed hepatocellular adenomas and preneoplastic liver lesions (Wogan and Newberne 1967). Newberne et al exposed groups of rats to diets containing AFs in the range of 5 – 5,000μg/kg and observed incidence of hepatomas in a dose dependent manner from 20 weeks (Newberne and Rogers 1973; Newberne and Suphakarn 1977). Eight-to-nine-weeks old MRC rats were administered 0 or 20 μg/kg AFB_1 in drinking water five times a week for 10 weeks; AFB_1 produced HCC tumors, cholangiocarcinomas, hyperplastic hepatic nodules and cystadenomas at death of at 90 weeks when the experiment was ended (Butler, Greenblatt, and Lijinsky 1969). Female Patron rats fed diets mixed with 10,000 μg/kg AFB_1, and 200 μg/kg AFB_2 for one to nine weeks developed HCC while others developed adenomas of the kidney, stomach carcinomas, and tumors in the lung and salivary glands when they became moribund or died (Butler and Barnes 1966). Male Wistar rats weighing 150-200g fed diets containing AFB_1 at 0, 250, 500 or 1000 μg/kg for 21 weeks then maintained on a basal diet till death, an increased incidence of HCC, hyperplastic liver nodules, renal cell tumors were all observed in a dose-dependent manner (Epstein, Bartus, and Farber 1969). Groups of male rats of unspecified strains and male Fischer rats, weighing about 100g injected subcutaneously with 0 or 20 μg/kg of AFB_1 twice per week for up to 65 weeks developed sub-cutaneous sarcomas, none in controls (Dickens and Jones 1965; Wogan, Edwards, and Newberne 1971). A high incidence of HCC was also found at all dose levels in groups of 10-20 male Fischer rats administered AFB_1 in 0.05ml DMSO at 0, 25, 37.5 or 70 μg/kg sacrificed between 25 and 78 weeks (Wogan, Edwards, and Newberne 1971). Groups of male Fischer rats weighing

average 80g fed semi-synthetic diets containing 0, 1, 5, 15, 50 or 100 μg/kg of AFB_1 also developed dose-and time related increase in HCC incidence and preneoplastic liver lesions (Wogan and Paglialunga 1974). In another study, groups of male Wistar Rats weighing 80 - 120 g administered AFB_1 in 0.1DMSO by gastric intubation at 0 and 50 μg/animal two times a week for four weeks, and then 0 and 75 μg/animal of AFB_1 in 0.15ml DMSO two times a week for 10 weeks, AFB_1 reportedly induced HCC and hepatocholangiocellular carcinomas in 70% of the rats (Kalengayi, Ronchi, and Desmet 1975). In 63-day-old male Charles River rats fed AFB_1 contaminated diets at 0 or 1000 μg/kg for 15 weeks, an unspecified number of rats were killed at 8 weeks and the surviving animals maintained on a control diet until 88 weeks, when sacrificed, small foci of vacuolated hepatocytes were observed in treated rats after 16 weeks and HCC after 68 weeks with the cumulative incidence of HCC reported to have reached 40% at the end of 88 weeks (Nishizumi et al. 1977). The early studies in experimental animals had major shortfalls since the strain, sex, and age of the rats used in experiments were not specified in most cases.

Studies in experimental animals conducted after 1980 provided descriptive details of Groups of male and female Sprague Dawley rats fed diets with AFB_1 ranging 5 – 7.5μg/kg for 22 months developed sarcomas, parenchymal liver damage and pre-malignant liver lesions (Fong and Chan 1981). Three-week-old Fischer 344 rats, were fed diets containing 0 μg/kg and 176 μg/kg of AFs mixture comprised of 150 μg/kg AFB_1, 18 μg/kg AFB_2 and 8 μg/kg AFG_1 or the corresponding ammoniated products; at 92 weeks when the experiment ended,12/12 males, 11/12 females had developed HCC, 6/12 males and 10/12 females developed neoplastic hepatic nodules and preneoplastic foci of altered hepatocytes were observed while neither tumors nor preneoplastic liver lesions were seen in rats fed

AFs ammoniated products (Norred and Morrissey 1983). In a similar study, Fischer 344 rats fed AFs contaminated diets developed preneoplastic foci of altered hepatocytes but not in rats fed ammoniated diets (Frayssinet and Lafarge-Frayssinet 1990; Manson and Neal 1987). These studies provided early evidence that AF toxicity can be reduced by ammonization. In groups of seven-weeks old male and female Wistar rats treated with 0, 100 μg/male animal and 75 μg/female animal, malignant hepatomas accompanied by cholangiocellular adenomas appeared 386 days in males while 417 in females, pre-cancerous lesions were observed in all treated rats from 184 days after cessation of AFB_1 administration (Zawirska and Bednarz 1981). This is an indication that male species are more susceptible to AFs toxicity compared to females. In a different study, groups of 66 and 120 female Wistar rats weighing on average 200 g were given a single dose of 0 or 5,000 μg/kg bw AFB_1 in olive oil by gavage; 29 rats died in the first few days after AFB_1 treatment, and more died in-between, foci of altered hepatocytes were observed in surviving rats at 104 weeks (Bannasch et al. 1985). Groups of 56 male six-weeks-old Fischer 344/CDF rats exposed to 0 or 25 μg/animal AFB_1 in 0.1 DMSO five times a week for 2months, or 0, or 70 μg/animal AFB_1 in 0.15 DMSO nine times in two weeks (Soffritti and McConnell 1988). The animals were sacrificed in groups of 6 at 2, 6, 10, 14, 22, 32 and 47 weeks after the last treatment with AFB_1, and all surviving rats killed after 66 weeks. Preneoplastic hepatic foci, neoplastic hepatic nodules and HCC increased in number and size with time in a dose dependent manner while no neoplastic or preneoplastic lesions was observed in controls (Soffritti and McConnell 1988). In groups of transgenic mice, a single intraperitoneal administration of AFB_1 of 6μg/kg bw, adenomas and hepatocellular carcinomas were detected in 5 out of 12 mice (Schnur et al. 1999). Treatment of AF

contaminated diets with 200kPa of ammonia reduced carcinogenic potential of contaminated diet while treatment with ammonia at 300kPa eliminated induction of tumors at 12 months (Frayssinet and Lafarge-Frayssinet 1990). In trouts fed diets contaminated with a mixture of AFs at 180µg/kg treated by ammoniation or untreated, trouts fed untreated contaminated diets had a higher incidence of hepatocellular carcinoma (Brekke et al. 1977). These studies provided early evidence that ammoniation significantly reduces the carcinogenic potential of AFs.

Compared to rats, mice are relatively resistant to AFs related toxicities. No tumors were reported in a series of experiments where three strains of mice namely Swiss, C3HfB/HEN and C57BI/6NW were fed AFB_1 contaminated diets at 1000 µg/kg (Wogan 1969). Groups of 8 male and 8 female A/J mice strain , six-to-eight weeks old were administered 5,000, 12,500 and 25,000 µg/kg intraperitoneally, at 24 weeks when the experiment was terminated, treatment with AFB_1 resulted in 100% lung adenomas in survival animals at all dose levels (Stoner et al. 1986). In another study, one-to-seven day old inbred F1 mice (C57B1 X CH3), a single dose of 2,000 µg/kg bw AFB_1, 55% died within 4 days and all mice developed hepatomas at every dose level of 250, 1000, 2000 and 6000 µg/kg bw AFB_1 (Vesselinovitch et al. 1972). Two-to-three month old transgenic mice of C57B1/6 nature were administered AFB_1 intraperitoneally at 0 and 250 µg/kg bw as a single dose five times at equal monthly intervals or 2,000 µg/kg bw in three weekly doses (Sell et al. 1991). When the experiment was terminated at 15 months, multiple liver nodules of different sizes, hepatocellular adenomas and HCC cases were reported. Likewise, 2 groups of 16 STCF1 mice, nine weeks of age, were administered 0 and 6,000 µg/kg bw AFB_1 intraperitoneally after partial hepatectomy, HCC and hepatocellular

adenomas (Dix 1984) were observed after 55 weeks in a dose dependent manner.

Studies in other species including ducks, trouts and hamsters have yielded similar results. For instance, seven-day old Khaki Campbell ducklings fed diets with groundnut meal containing 35 μg/kg of AFB_1 developed liver tumors after 14months while no liver tumors were observed in controls (Carnaghan 1965). Furthermore, a group of rainbow trouts fed AFB_1 contaminated diet at 3.7 - 42 μg/kg developed hepatomas in a dose-dependent manner with the highest incidence observed in rainbow trouts fed 42 μg/kg AFB_1 contaminated diets (Sinnhuber et al. 1968). Male Syrian golden hamsters, 4 weeks old were administered 2,000 μg/kg bw AFB_1 dissolved in DMSO by gavage for five days a week for 6 weeks, and a high incidence of cholangiocellular tumors was observed (Moore et al. 1982). Moreover, a male rhesus monkey injected intramuscularly by a mixture of AFs at 50 μg five days in a week for one month followed by 100μg for 11 months and then administered orally by gavage at 200 μg on a daily basis for 4.5 years developed HCC (Gopalan, Tulpule, and Krishnamurthi 1972). A female rhesus monkey treated the same except that the oral dose was 100μg developed metastasizing intrahepatic bile-duct carcinoma, an indication that female species may be more susceptible to AFs toxicity (Tilak 1975). In different experiments, a mix of rhesus, cynomolgus and African monkeys, 47 in total received AFB_1 dissolved in DMSO either intraperitoneally or orally at concentrations ranging from 100 - 800 μg/kg bw for two months or longer, 13 monkeys developed one or more malignant neoplasms, 2 developed HCC, 3 developed liver angiosarcomas, 2 developed osteogenic sarcomas, 6 adenocarcinomas of the bile duct, two pancreatic adenocarcinomas, one undifferentiated pancreatic tumors and one papillary carcinoma of the gall bladder; monkey without tumors had evident liver damage

characterized by toxic hepatitis, cirrhosis and hyperplastic nodules (Sieber et al. 1979). Carcinogenicity has also been observed in toads, tree shrews, and hamsters with administration of AFB_1 or a mixture of AFs (IARC 1993, 2002). All species exposed to AFB_1 or a mixture of AFs produced tumors. Collectively, these studies established the carcinogenic role of AFs leading to the designation of AFB_1 as a group 1 carcinogen and AFs mixtures as carcinogens irrespective of the route of exposure.

Carcinogenicity of AFB_1 metabolites including AFM_1, AFQ_1, and AFB_2 has been reported even though at lower toxicities. For example, weanling Fischer rats administered 0 or 25μg/rat of synthetic AFM_1 with more than 95% purity by gastric intubation five days a week for 2 months; one out of 30 rats developed HCC while 8 developed preneoplastic liver lesions after 96 weeks (Wogan and Paglialunga 1974). In a similar experiment, male Fischer rats fed diets with AFM_1 at 0, 0.5, 5 or 50 μg/kg; liver neoplasms were found in rats dosed at 50 μg/kg AFM_1 , an indication that even though AFM_1 play a role in carcinogenesis, the incidence of liver tumors is lower compared to AFB_1 (Hsieh, Cullen, and Ruebner 1984). Similarly, groups of seven weeks-old male Fischer 344 rats were fed semi-synthetic diets containing 0, 0.5, 5 or 50 μg/kg of AFM_1 up to 21 months of age (Cullen et al. 1987). The number and size of foci of altered hepatocytes induced by the high dose AFM_1 were significantly less compared to those produced by the same dose of AFB_1 (Cullen et al. 1987). AFQ_1 has also been shown to produce HCC in rainbow trouts (Hendricks et al. 1980). Moreover, incidence of HCC in rats and rainbow trouts treated with aflatoxicol was about half of that in rats treated with AFB_1 at the same dose level (Nixon et al. 1981; Schoenhard et al. 1981). In rats treated with 20μg/kg AFB_2, hepatic nodules were found but neither hepato-carcinomas nor renal-cell tumors were observed

(Butler, Greenblatt, and Lijinsky 1969). In male Fischer rats administered 0, 50 or 100 μg/animal of AFB_2, there was increased incidence of preneoplastic liver lesions but no hepatocellular carcinoma (Wogan, Edwards, and Newberne 1971). In trouts, AFB_2 had very little hepatocarcinogenic effects at 20 μg/kg (Ayres et al. 1971). In summary, oral administration of AF mixtures and AFB_1 in different strains of rats, hamsters, salmon, trout, ducks, tree shrews and monkeys induce benign and malignant tumors in the liver. Oral administration of AFB_1 does not induce tumors in mice while intraperitoneal administration increased the incidence for lung adenomas. In rats, oral administration of AFB_1 induced renal cell tumors, and intraperitoneal administration in pregnant and lactating mice induced benign and malignant tumors in mothers and their offspring. Oral administration of AFB_2 in rats induced liver adenomas, and intraperitoneal administration induced a low incidence of HCC. Oral administration of AFG_2 induce hepatocellular adenomas, and renal cell tumors in rats while in trouts, liver cell tumors were reported. At equal dose levels, AFM_1 and AFG_1 induced tumors at a lower incidence compared to AFB_1.

Overall, the evidence for carcinogenicity in experimental animals is sufficient for AFB_1, AFG_1, and AFM_1 but limited for AFB_2 and inadequate for AFG_2. Since 1999, the focus of AFs research shifted from studies on hepatic carcinoma to agricultural concerns mainly due to international trade restrictions of certain food products. Moreover, the carcinogenicity of AFB_1, its metabolites and a mixture of AFs was already established while there was still paucity on data of other adverse health outcomes associated with AFs exposure including aflatoxicosis, immune suppression and growth impairments.

Aflatoxicosis

In the tropical regions of the world, hot and humid conditions provide ideal environments for *aspergillus* fungi to thrive and produce AFs. Contamination of staple diets such as corn, peanuts, legumes, spices, meat and milk products are prevalent in many low- and middle-income countries. More than 25% of the world's food supply is contaminated by AFs and an estimated 4.5 to 5 billion people are exposed. The dose and duration of exposure define toxicity. Aflatoxicosis is the poisoning that results from ingestion of aflatoxin contaminated food within a short period of time. The clinical manifestations of aflatoxicosis include jaundice, hemorrhage, acute liver damage, vomiting, abdominal pain, pulmonary Edema, and fatty infiltration and necrosis of the liver (Mwanda, Otieno, and Omonge 2005; Ngindu et al. 1982). In severe cases, deaths have been reported with a case fatality ranging from 39 – 50%. The earliest case of human aflatoxicosis was reported in India in 1974 where 397 people from 180 villages of Gujerat and Rajasthan were affected by a hepatitis characterized by jaundice, rapidly developing ascites, portal hypertension and a high mortality rate as106 died (Krishnamachari et al. 1975; Krishnamachari et al. 1977). The disease was associated with the consumption of corn heavily contaminated with *A. flavus*. During the outbreak a large number of dogs that invariably shared the food of affected households developed ascites and icterus and died within 2-3 weeks of the onset (Krishnamachari et al. 1975; Krishnamachari et al. 1977). In 1995, acute hepatic encephalopathy in Malaysian children was reported after consumption of 3mg/kg aflatoxin contaminated noodles (Lye et al. 1995).

Globally, aflatoxicosis incidences are frequently reported in Kenya, East Africa, particularly in Makueni County and other counties in close proximity. Over 600 deaths

have been documented from recurrent outbreaks of aflatoxicosis in Kenya even though it is suspected that there are many more undocumented deaths (Wangia 2017). An assessment of peer-reviewed literature on aflatoxicosis was evaluated with special focus on Kenya where aflatoxin contamination is a recognized public health problem (Wangia 2017). The very first documented poisoning was reported in ducklings in a white settler farm in former rift valley province. Over 16,000 ducklings died after consuming groundnut feed that was contaminated with AFs (Peers and Linsell 1973). In 1977, dogs and poultry died in large numbers in Nairobi, Mombasa and Eldoret after they were fed with AFs contaminated grains (FAO/WHO/UNEP 1977). The very first documented deaths of humans from consumption of aflatoxin contamination corn in Kenya was reported in 1981, where up to 12 people died in Machakos after consuming aflatoxin-contaminated corn (Ngindu et al. 1982). Prior to the human deaths, numerous deaths had been reported for dogs, and poultry, shortly, after which 20 people visited health facilities for symptoms related to acute hepatitis (Ngindu et al. 1982). The patients were managed conservatively, 8 patients recovered while 12 of the patients developed hepatic failure and died between 1 to 12 days after admission at the hospitals (Ngindu et al. 1982). In 1987, up to 3 people in Meru North suffered from acute effects related to consumption of aflatoxin-contaminated corn and eventually died (Autrup et al. 1987). Between 1988 and 2000, there is very limited documentation of aflatoxin related outbreaks in Kenya. It is known that many cases go unreported (Strosnider et al. 2006), as many villagers choose not to go to the hospital unless the symptoms worsen. In 1981, the meantime from onset of aflatoxin related symptoms to hospital admissions was about 10.2days (Ngindu et al. 1982). The reported cases in the literature are mainly for individuals who sought medical attention from local health

facilities and there is a great possibility that many aflatoxin poisoning related cases go unreported. Nonetheless, intermittent exposure to AFs in low quantities over a long period of time increases the risk of developing hepatitis and liver related diseases.

An additional 16 human deaths were reported in Maua, Meru County after consumption of moldy corn in 2001 (Probst, Njapau, and Cotty 2007). Moreover, in 2002, large numbers of dogs and poultry succumbed to death in Coast region after consumption of contaminated feed. The worst and most reported outbreak of aflatoxicosis happened in 2004 in Makueni, Kitui, Machakos and Kwale Counties of Kenya where about 331 patients were admitted after showing symptoms such as abdominal discomfort, jaundice, and low-grade fever (Azziz-Baumgartner et al. 2005; Daniel et al. 2011; Lewis et al. 2005). The Kenya's Ministry of Health invited the United States Center for Disease Control and the World Health Organization to probe into the aflatoxicosis outbreak (United States CDC 2004). Meanwhile, 125 people died shortly after hospital admissions which is about 40% Case Fatality Rate (Lewis et al. 2005). The results revealed that the deaths were attributed to high aflatoxin levels found in corn grains collected from the affected households where AFs levels of up to 8,000µg/kg was quantified in samples despite the maximum limit set as 10 µg/kg in foods for human consumption (Daniel et al. 2011; Azziz-Baumgartner et al. 2005). Numerous studies have been completed after the 2004/2005-aflatoxicosis outbreak. Makueni County is particularly worst affected due to plant stress from drought, which is established to promote colonization of food items with the *A. flavus* fungi (Okoth 2016; Probst, Schulthess, and Cotty 2010). Moreover, food insecurity in the region encourages farmers to store food supplies in hot humid households instead of an outdoor granary for the fear of theft. It is well established that storage of food supplies particular corn under

sub-optimal conditions promote the proliferation of AFs producing *aspergillus* fungi to thrive. Therefore, aflatoxicosis is a recognized public health problem in semi-arid parts of Kenya. The government of Kenya has in the past provided replacement corn to affected household (United States CDC 2004). In the past few years since the 2004/2005 outbreak, the county government is tasked with similar efforts and in July 2017, the county government of Makueni provided 10,600 bags of corn consignments that had been tested for aflatoxin contamination to educational institutions in response to drought conditions and high aflatoxin contamination (Nzioka 2017). However, long term solutions that will ensure food safety and total eradication of aflatoxin contamination of corn in Makueni and other counties must be initiated.

AFs contamination can occur when the crops are in the fields or post-harvest period which guide the form of agricultural interventions to be undertaken. Pre-harvest strategies that have been implemented include field inoculation with non-toxic aflatoxigenic fungi, use of pesticide and irrigation to minimize plant stress and vulnerability to attack by the aflatoxin producing fungi (Bandyopadhyay et al. 2016; CAST 2003). During post-harvest, adequate drying of corn to less than 10% moisture content is ideal followed by storage where airflow is adequate. Other strategies include dilution where highly contaminated grain is mixed with grain of low contamination to avoid economic losses that may ensue from destroying the corn. In Mexico, decontamination is through nixtamalization, where corn is soaked and cooked in limewater before processing into food products. The process is very efficient and results in over 90% reduction of aflatoxin in corn. There are other efforts such as chemoprevention and use of drug therapy to either increase detoxification processes within the body or prevent the formation of biological metabolites that initiate

disease outcomes. These processes are expensive and could result in long-term side effects. Moreover, use of enterosorbents such as novasil clay to bind the aflatoxin for easier elimination in stool has also been proposed as a short-term intervention during aflatoxicosis outbreaks (Afriyie-Gyawu et al. 2008; Williams et al. 2004). In summary, aflatoxicosis presents a significant burden to many communities who practice subsistence farming and thus successful mitigation of AFs contamination calls for multiple key players in Agriculture, Public Health and Community Involvement.

Integration of diversified dietary options such as promotion of consumption of bananas, sweet potatoes, sorghum or millet based stiff porridge meals are local solutions that can be easily implemented to mitigate dietary exposure to AFs and long-term health effects. Similar efforts of diversified diets have been done in China and significantly led to reduction of human exposures. For example, the successful cost-efficient intervention in China in the 1980s involved shifting from corn as a staple to rice, which is less likely to be contaminated by AFs (Chen et al. 2013; Groopman et al. 2014). To date, fewer people suffer from aflatoxicosis and the incidence of primary liver cancer has significantly diminished. Similar interventions can be promoted in Kenya and other countries' that are over reliant on corn as a dietary staple.

Immune Suppression

The immune system is highly modulated by different mycotoxins.Aflatoxin poisoning can also take the form of chronic sub-symptomatic exposure where exposures occurs in small quantities over a long period of time and have been shown to have immunological effects. While studies in human populations evaluating the effects of mycotoxins on the immune system are very limited; immune suppression characterized by

decrease in the levels of CD4+ and CD8+ T cells has been linked to AF exposures (Jiang et al. 2005; Turner et al. 2003). AFs are more likely to interfere with the innate immune system, particularly the cell-mediated immunity and phagocytic cell functions (Bondy and Pestka 2000; Guilford and Hope 2014). For instance, it has been shown that the presence of AFs modulated immune cells in the jejunum and liver tend to induce an immune response, thereby increasing the production of macrophages, CD4+ and CD8+ T cells (Kraieski et al. 2017). In a study in the Gambia, exposure to AFs was strongly associated ($p = 0.006$) with reduced salivary secretory Immunoglobulin A (sIgA) levels, but not with T-cell, B-cell, mucosal secretions or vaccine responses (Turner et al. 2003). In HIV positive patients, high AFB_1-lysine adducts have been reported to suppress immunity and accelerate progression to AIDS disease (Jolly et al. 2011; Jolly 2014; Kang et al. 2015). It has also been postulated that AFs may increase HIV viral load via immune suppression characterized by decreasing subsets of immune cells as CD4+ Tregs, perforin expressing CD8+ T cells and B cells (Jolly 2014). In study populations recruited from Ghana and Uganda, HIV positive patients more likely had high AFB_1-lysine adducts and lowest percentage of Tregs which often facilitates immune hyper activation associated with HIV and a higher rate of viral replication (Kang et al. 2015; Obuseh et al. 2011). Moreover, lower percentage of perforin-expressing CD8+ T cells found in HIV infected individuals with high AFB_1-lysine adducts indicates impaired immune function which could lead to higher HIV viral loads (Jolly et al. 2006; Obuseh et al. 2011). Immunomodulatory effects of AFs by monocytes and macrophages have been documented in numerous animal studies including expression of Pro-inflammatory cytokines (– TNF-alpha, IL-1Beta, IL-6 and

IFN-Y) and regulatory cytokine (IL-10) were upregulated in pigs fed AF contaminated diets (Dugyala and Sharma 1996).

Given the limited studies on AFs exposure on immunomodulation, this section also evaluated how other mycotoxins interfere with the immune responses. Fumonisins (FNs) induced immunotoxicity involves aspects of innate, cellular and humoral immunity (Bondy et al. 2012; Bondy and Pestka 2000). T-2 toxin can decrease the function of the innate immune system by promoting the depletion of leukocytes and blood cells in lymphoid organs (Adhikari et al. 2017; Feijo Correa et al. 2018; Guilford and Hope 2014). Moreover, exposure to T-2 mycotoxins inhibits erythropoiesis in the bone marrow and spleen, impairs antibody production, reduces the proliferative response of lymphocytes, hinders the development of dendritic cells and diminishes immunoglobulin and cytokine {IL-2, IL-5} levels (Adhikari et al. 2017; Tsai et al. 2016). Repeated exposure to T-2 toxins has been shown to cause immune suppression and decrease resistance of exposed animals to a variety of diseases such as Marek's disease in chicken (Kufuor-Mensah et al. 2016). Low-dose exposure to DON triggers immune responses, whereas a high dose leads to leukocyte apoptosis and subsequent immunosuppression (Akbari et al. 2017). For pigs, consumption of DON contaminated feed impairs immune response, induced by porcine reproductive and respiratory syndrome virus live attenuated vaccine (Savard, Gagnon, and Chorfi 2015). In bovines, rumen microbes have the capability of detoxifying the immune-modulating mycotoxin deoxynivalenol (DON) preventing any alterations in leucocyte, granulocytes and lymphocyte counts when animals are fed high protein diets (Danicke et al. 2016). In bovine macrophages, exposure to penicillium mycotoxins reduce the expression of Interleukins including IL-1-alpha, IL-6, IL-12-alpha, IL-10, IL-23 and TGF-Beta (Oh et al.

2015). Moreover, the Penicillium mycotoxin patulin suppresses Toll-like receptor- and RIG-I/MAVS-dependent cytokine production through GSH depletion, mitochondrial dysfunction, the activation of p62-associated mitophagy, and p62-TRAF6 interaction (Tsai et al. 2016). Alternaria mycotoxins induces formation of ROS which interacts with DNA topoisomerase to generate DNA- strand breaks thereby increasing macrophage autophagic activity and senescence, and changes in macrophage morphology in various human macrophage cell models (Solhaug, Eriksen, and Holme 2016). It has also been shown that exposure to DON results in allergic sensitization through stress or damage of the intestinal epithelial cells (Bol-Schoenmakers et al. 2016).

Mycotoxins rarely occur in isolation and co-occurrence with other mycotoxins is prevalent. Co-exposures to AFB_1 and FB_1, an/ or co-occurrence of DON and FB_1 increased oxidative product ROS and stimulated bio-molecular oxidative damage in spleen mononuclear cells (Guilford and Hope 2014). Another study showed that fusarium mycotoxins namely deoxynivalenol (DON), nivalenol, zearalenone (ZEA), and fumonisin B_1 (FB_1) either individually or in mixtures activate distinct antimicrobial defense mechanisms possessing the potential to alter the intestinal microbiota through diminished antimicrobial effects (Wan et al. 2013). In a different study, a mixture of *aspergillus* and *penicillium* mycotoxins were shown to cause pulmonary hemorrhage as well as cytokine and chemokine production in a murine model (Rosenblum Lichtenstein et al. 2015). In another study, chronic exposure to low levels of mycotoxins (DON, ZEA, & AFB_1) in dairy cows reported an increase in serum immunoglobulin A {IgA}(Jovaisiene et al. 2016). Micronutrients and other organic substances have been shown to reduce the immunomodulatory impact of mycotoxins. Identified mechanisms of action include

oxidative stress and glutathione depletion which occurs in a dose dependent manner (Guilford and Hope 2014; Tsai et al. 2016). Generation of oxidative stress is a significant factor in the pathophysiology of mycotoxin-related immune suppression. This has been observed in dendritic cells, peripheral blood mononuclear cell, and alveolar cells where a decrease to glutathione was correlated with increased Th2 response (Guilford and Hope 2014). Oxidative stress ultimately contributes to excess inflammation which further contributes to immunopathology and disease progression (Tsai et al. 2016). It is also suspected that exposure to mycotoxins such as DON may affect the NF-kB pathway and ultimately interfere with anti-oxidative and immune defense systems (Xie et al. 2018). Recently, the intestinal barrier has been seen as an emerging target in toxicological assessment of mycotoxins. A compromised barrier function is associated with non-specific inflammatory response and an overstimulation of the gut associated immune system. Akbari 2017 evaluated the role of DON in the etiology of various chronic intestinal inflammatory diseases and reported that DON can induce both immunostimulatory as well as immunosuppressive responses depending on dose, frequency and duration of exposure (Akbari et al. 2017). Basic toxicological endpoints for immunotoxicity testing exists for murine models and are widely accepted, however, there is no agreement on any immunotoxicity testing strategies for mycotoxins (Bondy and Pestka 2000). Studies aimed at evaluating the immunomodulatory role of mycotoxins in the past monitored changes on specific aspects of the immune system specifically the innate immune responses and non-specific immune parameters (Bondy and Pestka 2000; Park et al. 2015). Innate immune responses are important for pathogen elimination and adaptive immune response activation (Tsai et al. 2016). Despite limited methodologies to evaluate immune activity triggered by

mycotoxin exposure, use of the sheep red blood cells to monitor antibody responses is used as a marker of humoral immunity after mycotoxins exposure (Bhatti et al. 2018). Other studies have evaluated cellular responses of cytokines, chemokines and lipopolysaccharide to study evaluate modulation associated with mycotoxin exposures (Grover and Lawrence 2017; Mayer et al. 2017). Studies that evaluate innate or non-specific immunity tend to examine natural killer cells activity and macrophage function; and Cellular immune changes have also been studied by observing lympho-proliferative responses to T-cell antigens and delayed-type hypersensitivity responses.

Birth Outcomes

Various studies use different body fluids including maternal serum, breast milk and umbilical cord to quantify aflatoxin metabolites, an indication of exposure and their associated birth outcomes. The most reported birth outcomes include low birth weight, small for gestation age, stillbirths and jaundice. In an early study on 125 babies in rural Kenya, AFs were detected in 53% of mothers' blood, and the mean birth weight of female children born to mothers whose blood tested positive for aflatoxin were 255 g lower than those born to mothers with no aflatoxin detected in the blood (De Vries, Maxwell, and Hendrickse 1989). In another study in the United Arab Emirates, presence of AFs metabolites in cord blood and maternal blood samples were associated with low birth weight, $p = .0001$ (Abdulrazzaq et al. 2004). Similarly, in Tehran, Iran; maternal exposure to aflatoxin, quantified in breast milk to range from 0.3 – 26.7ng/l was inversely associated with length of infants at birth, $p<0.01$(Sadeghi et al. 2009). Moreover, a significant inverse relationship between AFM_1 levels in maternal breast milk and the height-for- age *z*-scores (HAZ) in infants 90–120 days old ($\beta = -.31$, $p < .015$) has been reported. The children

whose mothers were AFM_1 positive had lower HAZ and weight-for-age *z*-scores (WAZ) than children born to mothers with no detectable AFM1 (Mahdavi et al. 2010). In a cross sectional study with 785 pregnant women in Ghana, those with high AFs exposure exceeding 11.34pg/mg albumin were two times more likely to give birth to babies weighing less than 250g, with an increasing likelihood for pre-term delivery, stillbirths and small for gestational age children after adjusting for socio-demographic indicators (Shuaib et al. 2010; Shuaib et al. 2012). In a more recent prospective cohort study to evaluate the association between maternal exposure during pregnancy and lower birth weight among 220 mother infant dyads recruited from Mukono district, Uganda; mothers with high aflatoxin exposure were more likely to have babies with low birthweights, p=0.04 and smaller head circumference, p=0.03 (Lauer et al. 2018). Similarly, in an ongoing prospective cohort study, in Banke Nepal; maternal serum AFB_1-lysine adduct concentration was significantly associated with small for gestation age at birth but not with other birth outcomes including low birth weight and pre-term birth (Andrews-Trevino et al. 2019). Moreover, genetic changes such as differential DNA methylation was observed in growth factor genes namely FGF12 and IGF1; immune-related genes namely CCL28, TLR2 and TGFBI in white blood cells of infants exposed to AFs in-utero (Hernandez-Vargas et al. 2015).

Nonetheless, there are studies that have found no association between AFs exposure and birth outcomes. For example, a study in Uganda did not find any significant association between maternal AFs exposure (ln AFB_1-lysine) and infant length, Weight-for-Length Z-score (WLZ), and Length-for-Age-Zscore (LAZ) (Lauer et al. 2018). Furthermore, even though exposure is widely documented in pregnant women, none to date has found positive

association between AFs exposures and intrauterine growth restriction (Smith et al. 2017). Nonetheless, there is need for rigorous human studies on relationship between maternal AF exposure and pregnancy outcome. Overall, given the variation in methodologies, assessment of aflatoxin in different biological fluids and different birth outcomes assessed, it is important to interpret these data cautiously. These studies however reinforce the need for early interventions to reduce aflatoxin exposure, especially during critical periods of fetal and infant development. Unfortunately, the risk of exposure to AFs increases significantly post-natal from breast milk, to weaning foods and further increases if the children's staple diets include corn and peanut based foods.

Anti-Nutritional

Food products can be both therapeutic and/or toxic. Food mutagens such as AFB_1 are well tolerated if optimum antioxidant nutrients are available and consumed on a regular basis. Polyphenols including vitamin E, Curcumin, carotenoid, flavonoids and Vitamin C have also been shown to protect cells against genotoxic damage either solely or in combination (Ahmad, Sheeba, and Afzal 2004; Alpsoy, Agar, and Ikbal 2009; Alpsoy and Yalvac 2011). Vitamin A is a fat-soluble micronutrient essential for immunity, cellular differentiation, maintaining epithelial surfaces, and vision. Vitamin E is an antioxidant, and also contributes important role to immune function. Retinoid and Beta-carotene inhibit mutagenicity, production of Chromosomal Aberration and DNA adduct formation by AFB_1 (Bhattacharya, Francis, and Shetty 1987; Huang et al. 1982). Furthermore, retinoid also inhibit the formation of AFB_1-DNA adducts in a dose dependent manner. Formation of AFB_1-N^7-DNA adducts can be reduced by intake of specific nutrients including Vitamin A, C, E, and selenium. Likewise, selenium inhibits AF induced hepatocarcinogenesis (Shi,

Hew, and Ong 1995). Vitamins A,C,E, and selenium are antioxidants and their protective effects against AF toxicity especially that the levels of Chromosomal Aberration and Sister Chromatid Exchanges were lowered suggesting a protective role of vitamins against genotoxic damage (Ahmad, Sheeba, and Afzal 2004; Alpsoy, Agar, and Ikbal 2009; Alpsoy and Yalvac 2011). In human lymphocyte cells exposed to AFB_1, genotoxic and mutagenic damage caused by 5uM concentration of AFB_1- was reversed when Vitamins A, C and E was added, moreover, the frequency of Sister Chromatid Exchanges decreased which demonstrates the protective effect of these micronutrients in a dose dependent manner (Alpsoy, Agar, and Ikbal 2009). Relatively high AF exposure of more than 0.5mg/kg is cytotoxic and promotes lysis of red blood cells, which is an indication that AFs exposure may contribute to low hemoglobin, and low iron absorption which are hallmarks of anemia (Andretta et al. 2012; Verma and Raval 1991). A Ghanaian study with 785 pregnant women as participants reported 1.85 increased odds of developing anemia among pregnant women in the highest quartile of AFs exposure (Shuaib et al. 2010). Proposed mechanisms include decreased capacity of intestinal walls to absorb iron, decreased erythropoiesis from chronic inflammation and hepcidin upregulation which results in reduced iron availability (Shuaib et al. 2010; Smith et al. 2017). Although it is biologically plausible that AF exposure is associated with anemia, doses used in experimental animals are high and may not be relevant in human populations. Populations at risk of frequent AF exposure often have low diet diversity, micronutrients are deficient and when diets are deficient in Vitamins A, C, E and selenium, all which are known to protect again AF related toxicities, increased risk for adverse health outcomes is paramount (Smith et al. 2017). For example, in a study to evaluate the role of micronutrients, and AF exposure on HIV/AIDS disease progression,

reduced levels of Vitamin A and E was associated with high levels of oxidants, particularly in the lymphocytes which could promote viral activation and increase in HIV viral load (Obuseh et al. 2011). It is also reported that dietary exposure to AFs may contribute to increases in HIV viral load through liver injury because AF targets parenchyma and biliary tract cells of the liver (Jolly 2014). AFs may also contribute to HIV progression indirectly by micronutrient malnutrition, which impairs the immune system by suppressing immune function.

Growth Impairments

Dietary exposure to AFs is implicated in growth impairment among children in low- and middle- income countries and is a significant public health problem (Khlangwiset et al. 2011; Wild 2007). Although the general mechanism of growth faltering associated with dietary exposure to AFs and other mycooxins is not clear, chronic inflammatory enteropathy of the small intestines mucosa which promotes poor digestive and barrier functions is also believed to contribute to growth retardation to some extent (Hoffmann, Jones, and Leroy 2015; Khlangwiset, Shephard, and Wu 2011; McMillan et al. 2018). The classical use on anthropometric parameters to assess nutritional status is children is widely accepted because of the non-invasive nature of data collection (de Onis 2015). Often, a child's weight and height are measured and used to calculate anthropometric parameters including weight for age z-score (WAZ), Height for age Z-score (HAZ) and Weight for Height Z-score (WHZ) (de Onis and Onyango 2008; WHO 1986). The indicators of growth impairments namely HAZ, WAZ, and WHZ are compared to World's Health Organization's (WHO) reference values to determine any growth deficits specific to age and sex. The Multicenter Growth Reference (MGR) was purposely designed and included

children from a diverse set of countries namely Brazil, Ghana, India, Norway, Oman and the USA(de Onis and Onyango 2008; WHO 1986). Therefore, the parameters used as standards for reference have considerable built-in ethnic, genetic and cultural variation in regard to how children are nurtured, which further strengthens the standards' universal applicability(de Onis et al. 2012; de Onis and Onyango 2008; de Onis et al. 2007). According to the WHO MGR, a z-value less than -2 SD for WHZ, WAZ and HAZ are classified as wasting/thinness, underweight and stunting. Z-score values less than -3 SD are a sign of severe malnutrition and intervention actions are promptly warranted (de Onis 2015; WHO 1986). It is imperative that genetic factors and possible impact of infectious diseases is controlled for to promote comparability of the many studies geared towards mycotoxins exposures and growth impairments.

Stunting is the most commonly reported form of chronic malnutrition and has been associated with chronic aflatoxin exposure (de Onis and Branca 2016). Childhood stunting is based on low height-for-age z-score (HAZ) and is defined as a condition when the child's height for his/her age is two standard deviations or more below a WHO Multicenter growth reference mean (de Onis and Branca 2016; WHO 1986). A HAZ of -2 indicates that the child is stunted, and a HAZ value of -3 indicates that the child is severely stunted. Stunting is a well-established risk marker of poor child development with significant consequences that last beyond childhood years (Ahlberg et al. 2018; Ricci et al. 2006). Stunting has been established to contribute to increased vulnerability to infectious diseases, cognitive impairments, lower school achievements, life-time earnings, increased health problems, and decreased productivity (Ahlberg et al. 2018; Ricci et al. 2006). In addition to stunting, being underweight for one's age, dangerously thin for one's height (wasted) and deficient

in vitamins are hallmarks of malnutrition. To date, studies focused on examining the role of mycotoxin exposure and growth impairment are not conclusive.

Nonetheless, in thoroughly evaluating published literature, studies with well-defined sample sizes, appropriate estimate of aflatoxin exposure in individual study subjects, proper dose assessments, outcome measurements and plausible multivariate analyses that implicate mycotoxins in growth deficits were found (IARC 2015; Khlangwiset, Shephard, and Wu 2011; Smith et al. 2015). For example, in Togo and Benin, studies in the early 2000s reported a significant association between aflatoxin exposure and growth impairment in young children (Gong et al. 2004; Gong et al. 2002; Gong et al. 2003; Castelino et al. 2015). In focus is a cohort of 480 children aged 9 months to 5 years in these two countries that reported the prevalence of stunting and underweight as 33% and 29%, respectively (Gong et al. 2002; Gong et al. 2003). Additionally, clear dose-response relationships were found between mean aflatoxin-albumin adducts and lower HAZ and WAZ scores (Gong et al. 2002; Gong et al. 2003). Similarly, a different study in Kenya among children 6 to 9 years, found a significant association between aflatoxin-albumin adducts and impaired child height after adjusting for age, sex and possible infection status (Castelino et al. 2015). In another 8-month longitudinal study in 200 children aged between 16 and 37 months, a significant negative association between height velocity, but not weight, and mean aflatoxin-albumin adducts was reported in Benin (Gong et al. 2004). Moreover, a difference of 1.7 cm over the 8-month study period in adjusted height between the highest and lowest aflatoxin-albumin adducts quartile was observed (Gong et al. 2004). Following 138 Gambian neonates for 1 year, Turner et al. (2007) reported that reducing maternal exposure to AFs from 110 to 10pg/mg could result in

weight increase by 800g and height increase by 2cm on average (Turner et al. 2007). In a Nigerian study by McMillan et al, a significant correlation between HAZ and AFB_1-lysine supports the causal role of aflatoxin and stunting (McMillan et al. 2018).

Some studies have assessed exposure levels in weaning foods. For example, a study in Kenya reported a significant association between aflatoxin exposure and wasting, p=0.002 among children less than three years consuming weaning gruel made of aflatoxin contamined weaning flours (Okoth and Ohingo 2004). AFs in weaning flours was used a proxy for aflatoxin exposure (Okoth and Ohingo 2004). In a cluster randomized control trial done in Kenya's Meru County, reducing aflatoxin exposure by providing households with non-contaminated corn lowered AFs serum levels by 27% at end line compared to controls, however, this reduction was not associated with improved linear growth (Hoffmann, Jones, and Leroy 2015; Hoffmann, Jones, and Leroy 2018). Another recent study conducted in Kenya estimated the extent of growth reduction among children below three years exposed to AFM_1 from milk and reported an average reduction of -0.34 on HAZ, thereby contributing a total of 2.7% of childhood stunting (Ahlberg et al. 2018). These studies show that aflatoxin exposure in a younger group of children is more likely to contribute to growth impairment due to the higher sensitivity of growth inhibitory effects associated with aflatoxin exposure.

Not a lot of studies have been done in terms of other mycotoxins.One study reported significant negative association between urinary fumonisins and both stunting and underweight in children below 3 years (Chen et al. 2018; Shirima et al. 2015). In terms of other mycotoxins, there is a possibility that chronic low dose exposure of children to DON can adversely affect growth trajectories and the maximum TDIs for children is set in the

range of 1 to 3µg/kg bw (Canady et al. 2001). To date, there are no human epidemiological studies directed towards Trichothecenes related growth effects. Limited studies are mainly attributed to lack of validated biomarkers for biological monitoring. Taken together, these studies report consistently a significant association between mycotoxin exposures, particularly aflatoxins and growth deficits.

On the contrary, a number of studies have also found no significant association between AF exposure and growth impairments. A cohort study in Gambia among 472 older children between 6 and 9 years old did not report any association between aflatoxin exposure and HAZ or WAZ; however, a weak association was reported for WHZ (wasting) (Turner et al. 2003). In Cameroon, a study conducted of 220 children between the ages 1.5 to 4.5 years reported no association between the different malnutrition categories namely stunting, wasting and underweight with mycotoxin exposure (Njumbe Ediage et al. 2013). Similarly, in a longitudinal study completed in Tanzania with 166 children between the ages of 6 to 14 months, a negative association between aflatoxin-albumin adducts and stunting did not reach statistical significance (Shirima et al. 2015). In a different longitudinal study completed in Nepal, aflatoxin exposure in children less than 3 years was not associated with LAZ, WAZ (underweight), and WLZ (thinness/wasting) (Mitchell et al. 2017). In a Tanzanian cohort study of younger children below 3 years there was no association between aflatoxin exposure and stunting, underweight or wasting/thinness (Chen et al. 2018; Shirima et al. 2015).

The mechanisms underlying mycotoxin exposure and impaired growth are still speculative. However, intestinal function damage, reduced immune function, and alteration in the insulin like growth factor axis caused by the liver damage are the suggested

hypotheses for the mechanism of toxicity (Watson, Gong, and Routledge 2017; Castelino et al. 2015; Hoffmann, Jones, and Leroy 2015). Moreover, there must be a specific threshold under which toxic AF metabolites contribute to altered function and ultimately growth impairment which is yet to be determined. It has been established that aflatoxin associated immune suppression is likely to increase susceptibility to infectious diseases. Moreover, enteropathy attributed to aflatoxin related toxic damage on the intestinal epithelium severely limits nutrient uptake. Finally, liver toxicity associated with mycotoxins exposures may damage production of insulin like growth factor pathway proteins negatively affecting child growth. Nonetheless, further studies aimed as assessing growth inhibitory pathways associated with immune modulation, reduced insulin like growth factor (IGF-1) and AF induced enteropathy are warranted to provide robust and conclusive scientific data on the mechanism of toxicity. In conclusion, given the adverse health outcomes particularly growth deficits in children exposure to mycotoxins, exposure must be kept as low as reasonably achievable.

Fumonisins Related Adverse Health Outcomes

Developmental and embryogenesis toxicity

The brain and the spinal cord which make up the central nervous system is formed from the neural tube during mammalian embryogenesis (Detrait et al. 2005). In the presence of $FB_{1,}$ the neural tube does not close during mammalian embryogenesis (Detrait et al. 2005; Gelineau-van Waes et al. 2012). FB_1 interferes with the functionality of the high affinity folate transporter thereby inhibiting folate metabolism and uptake which is a key element during the neural tube formation (Gelineau-van Waes et al. 2009; Lumsangkul

et al. 2019; Marasas et al. 2004; van Gool et al. 2018). Developmental and embryogenesis toxicity have also been confirmed where, exposure to FB_1 inhibited folate uptake in the embryos and placenta of LM/Bc mice (Waes et al. 2009). Thus, FB_1 is a known risk factor for neural tube defects which is partially recoverable by folate supplementation (Lumsangkul et al. 2019; van Gool et al. 2018). In human populations, maternal exposure to FB_1 via contaminated corn diet has been correlated with neural tube defects among infants in Texas, Mexico, Guatemala, and South Africa (Detrait et al. 2005; Marasas et al. 2004; van Gool et al. 2018; Waes et al. 2009).

Equine Leukoencephalomacia and Porcine Pulmonary Edema

Formerly known as '*moldy corn poisoning*', Equine Leukoencephalomalacia (ELEM) is a neurological disorder in equids characterized by paralysis, impaired coordination, aimless circling, blindness, and ultimately death (Haliburton and Buck 1986; Ahangarkani, Rouhi, and Gholamour Azizi 2014). Equids are the only species reported to suffer from the neurological syndrome ELEM which occurs naturally when horses are exposed to feed with at least 10 mg/kg of FB_1+FB_2+FB_3 and is widely reported during equine breeding (Voss, Smith, and Haschek 2007). Equids are the most sensitive species to FNs toxicity as evident by numerous field outbreaks in several countries (Haliburton and Buck 1986; Wilkins et al. 1994; Vendruscolo et al. 2016). Even though ELEM outbreaks were recognized and reported as early as 1891, dedicated studies in search of the causative agent were intensified after '*mouldy corn poisonings'* reported in 1970 (Haliburton and Buck 1986; Marasas, Kellerman, et al. 1988). In 1970, the first isolates of *F. moniliforme* from mouldy corn fed to horses were isolated, and what followed was 18 years of dedicated research to identify the causative agent of '*mouldy corn poisonings'*, currently known as

ELEM (Marasas 2001). In addition, ELEM is associated with decrease in cardiac function due to disruption of ceramide synthase enzyme which inhibits L-type calcium in myocardial cells (Bertero et al. 2018; Smith et al. 1999). The underlying mechanism of action linked to ELEM is not completely understood, however, scientists point to either alteration of sphingolipids, or disruption of myelin synthesis (Ahangarkani, Rouhi, and Gholamour Azizi 2014; Al-Dissi 2015; Voss, Smith, and Haschek 2007)

Following consumption of FNs contaminated feeds, pigs are known to develop porcine pulmonary edema (PPE) characterized by cyanosis, respiratory distress, hydrothorax and development of acute pulmonary edema which leads to death in pigs (Haschek et al. 2001; Smith et al. 1999; Voss, Smith, and Haschek 2007). These clinical signs appear in about 4 – 7 days after FNs exposure and are experimentally reproducible by intravenous or oral administration of FB_1 to pigs (Haschek et al. 2001; Voss, Smith, and Haschek 2007). Normally, the activity of pulmonary macrophages responsible for elimination of pathogens in pigs is highly compromised with exposure to FB_1 (Haschek et al. 2001; Upadhaya, Park, and Ha 2010). In 1950s, higher incidence of PPE was reported in Europe, however, research on PPE intensified after an outbreak of a fatal disease in pigs was reported in 1989 in mid-west and south-east parts of the United States of America (Haschek et al. 2001; Voss, Smith, and Haschek 2007). Like ELEM, PPE is induced by an acute failure in the functionality of the left sided part of the heart following alteration of So/Sa concentration in the heart, and inhibition of L-type calcium channel (Smith *et al.*, 1999). PPE is a species-specific disease to pigs and has not been reported in any other species after exposure to FNs.

Organ- and Species- Dependent Fumonisins Toxicity

Exposure to FNs are known to either inhibit or activate enzymes involved in signaling pathways (Smith 2018; Voss et al. 2002). The resulting oxidative stress, lipid peroxidation and peroxisome proliferation can induce different levels of toxicity depending on the dose, exposed organ, species differences, sex, and immune status (Bertero et al. 2018; Ahangarkani, Rouhi, and Gholamour Azizi 2014; Upadhaya, Park, and Ha 2010). In laboratory studies using experimental animals, rabbits are established to be more sensitive to FNs toxicity compared to mice due to higher risk of nephrotoxicity and hemorrhage in the brain compared to mice (Ahangarkani, Rouhi, and Gholamour Azizi 2014; Voss, Smith, and Haschek 2007) . Therefore, rabbits and equids can be said to be the most sensitive to FNs toxicity due to the neurological effects associated with exposure to FNs. The USFDA set a maximum limit of 5 mg/kg of FNs in feeds intended for equids and rabbits (USFDA, 2001). FNs toxicity is also dependent on sex of the species as female rodents have been shown to be more likely to develop tumors when exposed to FNs compared to male controls (Lemmer et al. 2004). Poultry including chicken, duck and turkeys tend to be less sensitive to FNs (Poersch et al. 2014). The USFDA's maximum limit for poultry being raised for slaughter is set at 100 mg/kg (USFDA, 2001). In testing diets of poultry with high FNs concentrations of 100 - 400 mg/kg of FB_1 hepatomegaly, liver necrosis, weight loss, and biliary hyperplasia are manifested in a dose dependent manner (Lumsangkul et al. 2019; Poersch et al. 2014). Farm animals including sheep, goats, cow and dairy cattle are likely to suffer adverse health outcomes characterized by deep lesions in the liver, gastrointestinal tract, nervous system and lungs; however diagnosis of FNs induced toxicity is difficult in ruminants due to limited toxicity signs (Ahangarkani, Rouhi, and Gholamour Azizi 2014;

Upadhaya, Park, and Ha 2010). Therefore, the USFDA recommends feed for breeding ruminants should not contain more than 30 mg/kg of FNs (USFDA, 2001). These recommendations corroborate the evidence that FNs toxicity varies significantly depending on species.

Toxico-kinetics studies indicated that FNs get absorbed in the gastrointestinal tract and is rapidly distributed in blood (Ahangarkani, Rouhi, and Gholamour Azizi 2014; Voss, Smith, and Haschek 2007). Both Liver and kidneys are highly susceptible to FNs toxicity, however, the kidneys are confirmed to be the most sensitive to FNs toxicity (Demirel, Alpertunga, and Ozden 2015; Dupre and Siskind 2018; Voss, Smith, and Haschek 2007; Yin et al. 2016). In one study, cell lines of hepatocytes, lymphocytes, kidney cells, and splenocytes were exposed to FNs; kidney cells develop cytotoxicity the fastest compared to other cell lines (Gutleb, Morrison, and Murk 2002).

FNs toxicity is species-specific. Equids are highly susceptible to FNs toxicity with rapid development of the neurological disorder Equine Leuko-Encephalomalacia (ELEM). Swine are also sensitive to FNs toxicity and likely to develop Porcine Pulmonary Edema (PPE) after consumption of FNs contaminated feed. In humans, epidemiological studies have linked higher incidence of esophageal cancer to consumption of FNs contaminated corn. The US FDA recommends that foods for human consumption should have total FNs levels between 2 to 4 mg/kg (USFDA, 2001).

Epigenetics

Epigenetics refer to heritable and reversible changes to a gene without alteration in the DNA sequence, yet these changes affect a gene's competence or efficiency of

expression (Huang et al. 2019; Meyer 2018). Epigenetic alterations advance the development of diseases such as cancers, neurological disorders, diabetes, asthma and cardiovascular conditions (Huang et al. 2019) Aberrant epigenetic modifications predispose DNA to become more susceptible to environmental factors. This may lead to activation of gene expression such as the oncogenes and or silencing expression of the tumor suppressor genes (TSGs) in particular (Huang et al. 2019; Meyer 2018).

Epigenetic alterations may be modulated by FNs exposure specifically occur at three levels namely histone modification, DNA methylation and regulation of non-coding RNA. Dose- and time-dependent effects of FB_1 on global histone modifications are evident in NR-52E rat kidney epithelial cells (Sancak and Ozden 2015; Gardner et al. 2016). In a different study, significant accumulation of sphinganine-1-phosphate (Gelderblom et al. 2008) in nuclear cells decrease histone deacetylase, the enzyme that removes acetyl groups from an amino acid on a histone action (Gardner et al. 2016). These studies show FNs capability in altering epigenetic events through global histone modifications.

In terms of DNA methylation, epigenetic changes in mammalian DNA is mainly characterized by DNA methylation occurring in cytosine and adenosine (Bayoglu et al. 2017; Huang et al. 2019). For instance, in CpG islands of gene promoter, DNA methylation suppresses gene transcription, which results in gene silencing (Demirel, Alpertunga, and Ozden 2015; Bayoglu et al. 2017; Huang et al. 2019). CpG islands are DNA regions with much higher GC frequency of more than 50% compared to the other regions (Huang et al. 2019). In HK-2 cells, FB_1 is shown to induce changes on the global DNA methylation (Bayoglu et al. 2017; Demirel, Alpertunga, and Ozden 2015). While Clone 9 rat kidney cells and the NRK-52E liver epithelial cells did not show significant dose related effects

on DNA methylation when exposed to FB_1 (1-50 μM) over 24-hours (Bayoglu et al. 2017; Demirel, Alpertunga, and Ozden 2015), CpG promoter specific genes such as VHL gene in both cells; *c-Myc* in Clone 9 and p16 gene in NRK-52E, were methylated (Bayoglu et al. 2017; Demirel, Alpertunga, and Ozden 2015). For HepG2 cells, FB_1 at 200 μM was shown to increase hypomethylation in DNA by downregulating the activities of DNA methyltransferases and upregulating MBD2 (Bayoglu et al. 2017; Chuturgoon, Phulukdaree, and Moodley 2014; Demirel, Alpertunga, and Ozden 2015). DNA methylation significantly contributes to chromatin instability and tumorigenesis.

Oxidative stress associated with FB_1 toxicity is speculated to also contribute to indirect genotoxic action on mammalian cells, mediated by the cytotoxic effects on DNA, protein synthesis and mitochondrial injury (Theumer et al. 2010; da Silva, Bracarense, and Oswald 2018). Nonetheless, studies in animal models show no visible adducts formation detectable after exposure to FB_1, which further corroborates lack of direct genotoxicity associated with FB_1 exposures (Howard et al. 2001). In the past two decades, researchers have confirmed the carcinogenicity and tumor promotion activities of FB_1 in murine models (Howard et al. 2001; Lemmer et al. 2004; Riedel et al. 2015). In male Fischer 344 rats, pre-treatments with FNs initiate regenerative cell proliferation, loss of tumor necrosis factor alpha and promotion of tumorigenesis (Voss et al. 2002). Observational studies in human populations have consistently reported that chronic dietary exposure to FNs through consumption of contaminated corn is linked to esophageal cancers in South Africa and Iran. These studies prompted the move by many scientists to evaluate the carcinogenic properties of fumonisins in experimental animals.

Carcinogenesis

Lifetime studies in rats fed diets containing FNs yielded a high incidence (80%) of liver tumors, while no lesions were observed in control animals (Gelderblom et al. 1991). Liver has been shown to be the main target organ in the FB_1-treated rats where a greater percentage developed primary HCC (Gelderblom et al. 1991; Howard et al. 2001; Voss et al. 2002). A potential mechanism for FB_1-induced hepatocarcinogenesis has been proposed. FB_1 can regulate cell cycle through the pathway of cyclin D1 stabilization which is associated with activation of protein kinase B and inhibition of glycogen synthase kinase activity (Ramljak et al. 2000). FB_1 is thus an animal carcinogen and long-term exposure to FB_1 induced liver and kidney tumors in different animal models. FB_1 is also a potent tumor promoter in rats after initiation with diethylnitrosamine and AFB_1 (Gelderblom et al. 1991; Gelderblom and Marasas 2012; Gelderblom et al. 2008). Cancer-promoting activity occurred at levels that neither induced measurable liver and/or kidney toxicity nor initiate carcinogenesis.

Although FB_1 is carcinogenic, it lacks genotoxic effects in various assays *in vitro* and *in vivo*. The principal effect of FB_1 in normal and transformed human cells has been shown to be anti-proliferative, resulting from increased apoptotic cell death as opposed to decreased cell proliferation. Apoptosis induced by FB_1 was found to be *via* pathways of tumor necrosis factor alpha and caspase activation (Jones et al. 2001; Sharma, He, and Sharma 2006).

Mice deficient of ceramide synthase which is inhibited by FB_1, have been shown to spontaneously develop liver tumors which supports the science that disruption of sphingolipid profile can potentiate carcinogenesis via disruption of cell cycle regulation

(Chuturgoon, Phulukdaree, and Moodley 2014; Howard et al. 2001). The current understanding of FB_1 induced mechanism of carcinogenesis involves sphingolipid signaling pathways. This indicates that the balance between intracellular concentrations of sphingolipid effectors that protect cells from apoptosis and the effectors that induce apoptosis determines cellular response (Merrill Jr et al. 2001; Riley and Merrill Jr 2019). The balance between the rates of apoptosis and proliferation is a critical determinant in hepato- and nephrotoxicity and tumorigenesis in animal models (Dragan et al., 2001; Howard et al., 2001a; Voss et al., 2001). Therefore, contamination of food supplies for human consumption should be as low as reasonably possible to mitigate the increased risk of tumorigenesis associated with exposure to FNs.

Summary of Literature Review

Mycotoxins are naturally occurring metabolites of different fungi known to contaminate food, particularly cereal based, and farm animal feeds throughout the world. To date, up to 400 mycotoxins have been identified, isolated and characterized. The maximum limit for total aflatoxins and fumonisins in foods destined for human consumption are 20 μg/kg and 4mg/kg respectively. In many low- and middle-income countries, the mainstream population are subsistence farmers who grow, store and prepare a large portion of their staple diets; and thus, exposure to mycotoxins is highly prevalent. The main route of mycotoxins exposure is via consumption of corn and/or corn products which are highly susceptible to contamination. Today, mycotoxins are a leading cause of a majority of global health diseases, environmental problems and significant contributor of food insecurity in developing countries.

AFs are established Group I carcinogens while FNs are possibly carcinogenic to humans (Group 2B). AFs groups have varying levels of toxicity for both plant and animal life but AFB_1 is established to be the most potent. Overall, Toxicity, carcinogenicity and mutagenicity are in the order $AFB_1 > AFB_2 > AFG_1 > AFG_2$ .The toxicity of AFB_1 is 10 times more than that of potassium cyanide, 68 times that of arsenic and 416 times that of melamine, and its carcinogenicity is over 70 times that of dimethyl nitrosamine and 10,000 times that of benzene hexachloride.

Ingestion of food contaminated by mycotoxins have serious adverse health effects in plant life, animal health and human populations in the long term. Dietary exposure to mycotoxins is generally chronic over a long period of time and the most established adverse health outcome is development of primary liver cancers and esophageal cancers for AFs and FNs respectively. Various reports documented the presence of AFs in foods associated with Reye's syndrome and kwashiorkor and aflatoxin poisonings named aflatoxicosis. Fumonisin exposures contributed to fatal animal diseases including neurological disorders and pulmonary edema in farm animals consuming fumonisins contaminated feed. The primary mechanism of FNs toxicity is established to be disruption of sphingolipid synthesis *de novo* by way of FB_1's inhibitive action of the enzyme ceramide synthase (Merrill Jr et al. 2001; Riley and Merrill Jr 2019; Voss, Smith, and Haschek 2007). The mechanism of aflatoxin toxicity is linked to the binding of the bio-activated AFB_1-8, 9-epoxide to cellular macromolecules.

The first report of human related FB_1 poisoning was reported in 27 villages in India (Rai, Jogee, and Ingle 2015; Shetty and Bhat 1998). Additionally, FNs may play a role in birth defects by inducing neural tube defects (NTDs) in regions where maternal exposure

is prevalent due to consumption of corn and corn-based products. A higher risk of NTDs was previously reported among Mexican-Americans mainly due to consumption of corn products (Missmer et al. 2006; Gong et al. 2008). Communities in China, South Africa, and Italy are exposed to higher levels of FNs from day to day due to overreliance on contaminated corn or corn products as staple diets. Additional adverse health outcomes including aflatoxicosis, hepatotoxicity, nephrotoxicity, immune suppression, growth faltering, and carcinogenesis have been linked to dietary exposure of mycotoxins.

The use of biomarkers in mycotoxins exposure assessment limits problems of confounding, effect modification and exposure estimation errors. Biomarkers are integral in establishing the etiological role of toxins in both human and animal disease outcomes (Ryan *et al.*, 2007). Use of non-invasive bio specimens such as urine, blood, faeces for biomonitoring are preferred in molecular epidemiological studies. This dissertation study focused on aflatoxin and fumonisins contamination of food supplies, determining exposure assessment among children using biomonitoring approaches and evaluation of adverse health outcomes. Specific focus on growth impairment among children with high levels of mycotoxins in their biological media, particularly serum and urine. Both AFs and FNs producing fungi are widespread in the African continent particularly Kenyan Counties of Siaya and Makueni where our study participants were recruited. The selection of Makueni and Siaya Counties as study sites allows for control of different ethnic and genetic differences, poverty levels, climate conditions and variations in mycotoxins exposures.

Study Gaps

The most widely studied mycotoxins in regard to children's health is aflatoxins (AFs) and fumonisins(FNs). To date, there are no epidemiological studies focused on

possible adverse health outcomes in human populations particularly in children as a result of dietary exposure to other mycotoxins including Fumonisins, Deoxynivalenol, T-2 toxin, Nivalenol and Patulin. The paucity of data is mainly due to lack of validated biomarkers and thus, further research should focus on developing and validating biomarkers to measure mycotoxin exposures particularly in human populations. Given the limited epidemiological studies in part due to lack of validated biomarkers as it relates to other mycotoxins, this dissertation study reports AFB_1-lysine adducts, urinary free fumonisins B_1 and sphingolipids, as biomarkers of exposure among young children between ages six and twelve years.

Exposure to mycotoxins have also been shown to interfere with vaccine efficiency in children aged one to fourteen years (Githang'a, Wangia, et al. 2019). Given the possible impact associated with adverse health outcomes especially in young children, further basic research and epidemiological studies are warranted to understand the mechanisms and individual pathways involved in the development and severity of diseases. Epidemiological studies assessing the impact of AFs exposure on immunity ae urgently needed.

Young children are uniquely vulnerable to mycotoxin exposures due to higher consumption in weaning foods, less developed detoxification mechanisms, higher rates of cell division associated with rapid growth and high intake of food and water per kg body weight (Githanga et al. 2019; Lombard 2014; Njumbe Ediage et al. 2013). More recently, the role of mycotoxins particularly aflatoxins and fumonisins on immune modulation properties, possible vaccine efficiency interference and growth deficits among children living in low- and middle- income countries have gained more traction and are timely.

Our study is the first to determine exposure assessment among children 6 – 12 years as previous epidemiological studies defined study population included children under 5 years' old, pregnant women, adult populations and immune-compromised subpopulations such as individuals with HIV/AIDS.

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Table 2.1. Health impacts of Mycotoxins

Mycotoxins	***Fungi***	***Food Products***	***Health Impacts***
Aflatoxins	*Aspergillus flavus, Aspergillus parasiticus*	Corn, peanuts, cereals, oil seeds, dry fruits, spices, cottonseed and tree nuts	Hepatotoxicity, bile duct hyperplasia, hemorrhage, carcinogenesis
Fumonisins	*Fusarium moniliforme, Fusarium verticilliodes, Fusarium prolifratume*	Corn and corn products	Leukoencephalomecia, Porcine pulmonary edema, Human esophageal cancer.
Ochratoxin A	*Aspergillus ochraceus Penicillium verrucosum Penicillium viridicatum*	Cereals, legumes, coffee beans, wheat, barley, oats, dried meats and fruits, coffee, wine	Tubular necrosis of the kidneys, porcine nephropathy, liver damage, teratogenesis, kidney tumors
Patulin	*Aspergillus clavatus Penicillium expansum and other Penicillium & Aspergillus spp.*	Grapes, apples, other fruits	Brain & lung edema, Lung hemorrhage, Paralysis, Convulsions, & Carcinogenesis
Trichothecenes	*Fusarium moniliforme, Fusarium equiseti, and other Fusarium spp.*	Wheat, corn, barley, oats	Digestive disorders, oral lesions, edema hemorrhage of the Stomach, heart, intestines, lungs, bladder, &kidney.

Table 2.2. Chemical properties of aflatoxins

	Molecular Formula	***Molecular Weight***	***Melting Point***	***U.V Absorption @***		***Fluorescence Emission***
				265nm	363nm	
AFB_1	$C_{17}H_{12}O_6$	312	268 - 269	13,400	21,800	425
AFB_2	$C_{17}H_{14}O_6$	314	286 - 289	9,200	14,700	425
AFG_1	$C_{17}H_{12}O_7$	328	244 - 246	10,000	16,100	450
AFG_2	$C_{17}H_{14}O_6$	330	237 - 240	11,200	19,300	450

Table 2.3. Reported Aflatoxin Poisonings in Kenya 1960 - 2010

Year	Who/	Number	Location	Contamination	Effect	Reference
1960	Ducklings	16,000	Rift valley	Groundnut feed	Death	Peers & Linsell,1973
1977	Dogs Poultry	Large numbers	Nairobi, Mombasa Eldoret	Contaminated grains	Death	FAO/WHO/ UNEP, 1977
1981	Humans	12	Machakos	Corn	Death	Ngindu, et al., 1982
1984/85	Poultry	Large numbers	Poultry farms	Imported corn	Death	Ngindu et al., 1982
1988	Human	3	Meru North	Corn	Death and acute effects	Autrup et al., 1987
2001	Humans	3 & 26	Meru North Maua	Mouldy corn	Death	Probst, et al., 2007

2002	Poultry & Dogs	Large numbers	Coast	Contaminated feed	Death	Njapau et al., 2007
2003	Humans	6	Thika	Mouldy corn	Death	Onsongo, 2004
2004	Humans	331	Makueni & Kitui	Aflatoxin contaminated grains	125 deaths	Lewis et al., 2005
2005	Humans	75	Machakos, Makueni, & Kitui	Aflatoxin contaminated corn	75 cases with 32 deaths	Eduardo Azziz-Baumgartner et al., 2005
2006	Humans	20	Makueni, Kitui, Machakos	Contaminated corn	10 deaths	Muture & Ogana, 2005
2007	Humans	4	Kibwezi, Makueni	Contaminated corn	2 deaths	Wagacha & Muthomi, 2008

2008	Humans	5	Kibwezi, Kajiado, Mutomo	Contaminated corn	3 cases, 2 deaths	Muthomi et al., 2009
2010	Humans	n/a	29 districts in Eastern Kenya	Suspected contaminated corn	unconfirmed	Muthomi et al., 2010

Table 2.4. Aflatoxin M_1 in Breast Milk collected from Lactating/Nursing Mothers

Description of study population	*Detection rate	Mean/median/range	References
388 Egyptian mothers	36%	median 13.5 pg/ml	Polychronaki et al. 2006
388 Egyptian mothers	>50%	mean levels of 64pg/ml & 8pg/ml in the summer and winter	Polychronaki et al. 2007
150 mother-infant dyads	65.3%		Tomerak et al. 2011.
50 Nigerian Mothers	82%	range of 3.49 – 35ng/ml	Adejumo et al. 2013.
Sudanese mothers	54.3%	range of 0.007 - 2.561 ng/ml	Elzupir et al. 2012.
Tabriz Iran, 91 recruited from urban areas and 91 from rural areas.	21.9%	None of the samples from urban areas was positive for $AFM_{1,}$ range from 2 – 39.3 ng/l for samples from rural areas	Mahdavi et al. 2010. Jafari et al. 2017 Maleki et al. 2015.

111 samples collected from mothers in Lebanon	93.8%	range 0.2 to 7.9 ng/L	Elaridi et al. 2017
Famagusta District in Cyprus	80%	mean of 7.84 ± 1.72 ng/l	Kunter et al. 2017.
United Arab Emirates, 140 lactating mothers	92%		Abdulrazzaq et al. 2003.
Western Turkey	13.1%	range 5.10 – 6.90 ng/L	Keskin et al. 2009.
Ankara, Turkey	100%	range 60.90 – 299.99ng/l	Gurbay et al. 2010.
Eastern Turkey		a range of 1.3 – 13.0 ng/l	Atasever et al. 2014; Kilic Altun, Gurbuz, and Ayag 2017.
Colombia and Southern Brazil,	>90%	range of 0.9 – 18.5ng/l	Diaz and Sanchez 2015; Ishikawa et al. 2016.

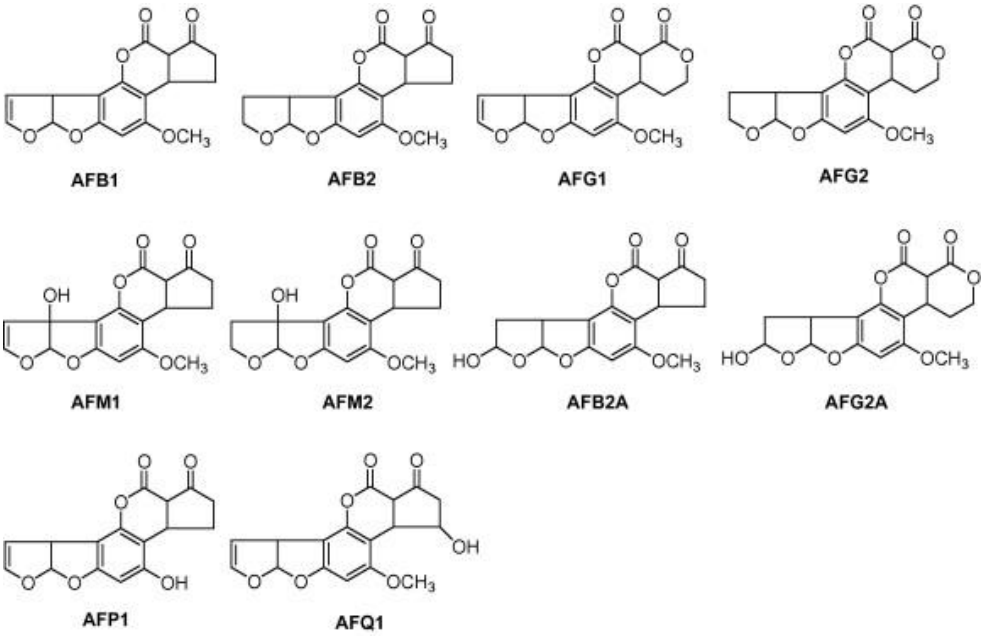
*% of positive samples for AFM_1

Table 2.5. Mycotoxins exposure and Growth Impairments

Population	Results	Study
Benin 16-37months (n=200)	Significant negative correlation ($p <$ .0001) between *AF-alb adduct and height increase over 8-month study period	Gong et al. (2004)
Benin & Togo 9months - 5 years (n = 480)	Negative correlation between individual AF-alb adduct and HAZ, WAZ, and WHZ (p = .001, .005, .047, respectively)	Gong et al. (2002, 2003)
Children ages 6 – 9 years (n = 472)	Significant association between AF-alb adduct and WHZ, (p = .034).	Turner et al. (2003)
Gambia/Infants to 1-year- olds (n = 138)	reduction of maternal AF-alb from 110 to 10 pg/mg resulted in 0.8 kg increase in weight & 2 cm increase in height of children within first year of life	Turner et al. (2007)
Iran/Lactating mothers (n = 160: 7 preterm delivery mothers, and 153 with full- term infants)	Significant association between AFM1 in maternal breast milk and height at birth of infants ($p < .01$)	Sadeghi et al. (2009)

Iran/Lactating women (n = 182: 91 from urban areas in Tabriz and 91 from rural area)	Significant lower scores in HAZ and WAZ of infants born to AFM1-positive mothers ($p < .05$)	(Mahdavi et al., 2010)
Ghana/Pregnant women and their infants (n = 785)	Odds ratio of having low-birth-weight babies in the highest AF-alb adduct quartile mothers = 2.09 ($p = .007$)	Shuaib et al. (2010)
Kenya/Children ages 3–36 months (n = 242)	Significant association between numbers of children who were wasted(53.8%) & were being fed flour contaminated with aflatoxin ($p = .002$)	Okoth and Ohingo (2004)

*AF-alb is aflatoxin-albumin adducts



Adapted from Li, Zhang, and Zhang 2009

Figure 2.1. Chemical structure of key aflatoxins

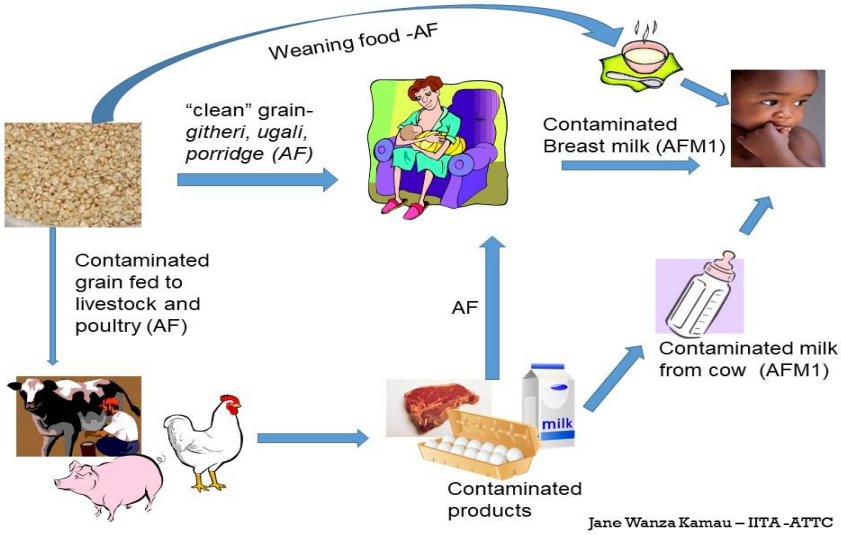


Figure 2.2. Multiple routes of mycotoxin exposures

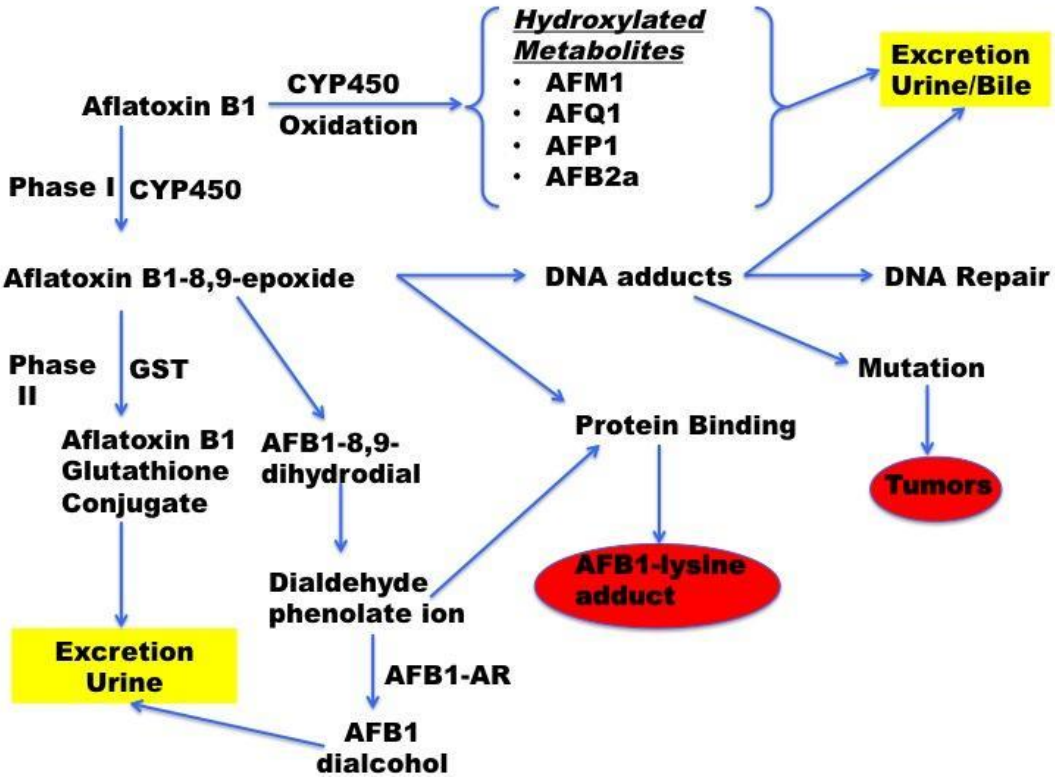


Figure 2.3. Metabolic Pathways of AFB_1

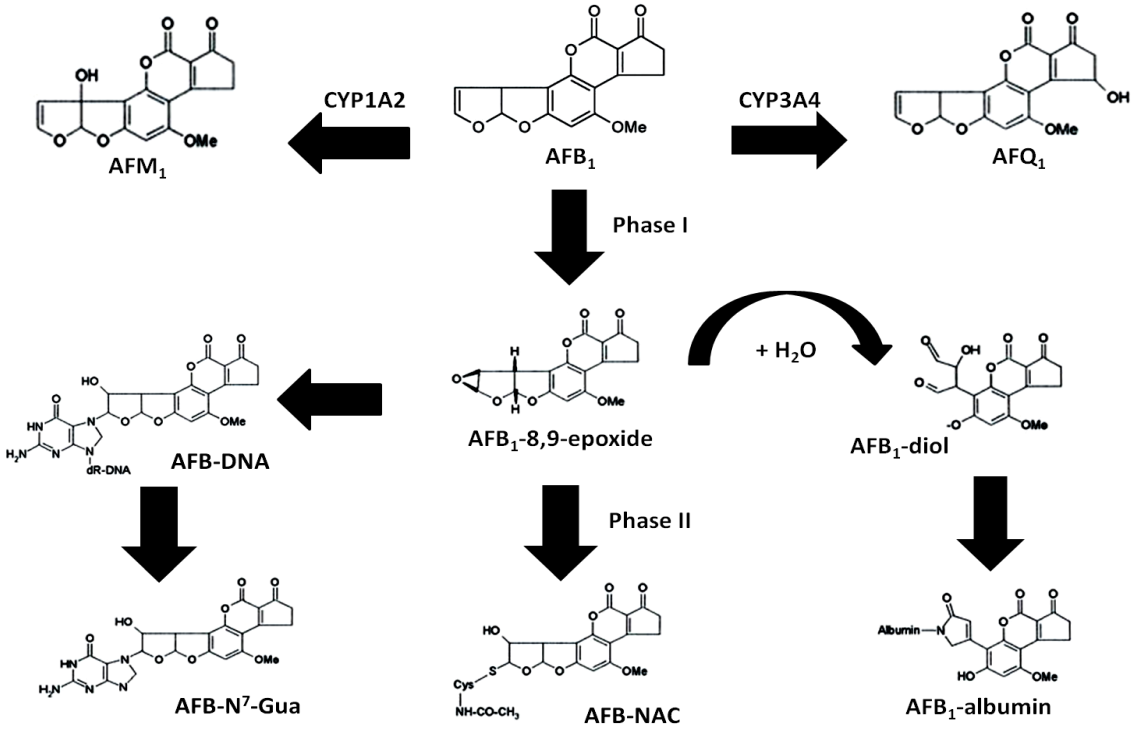
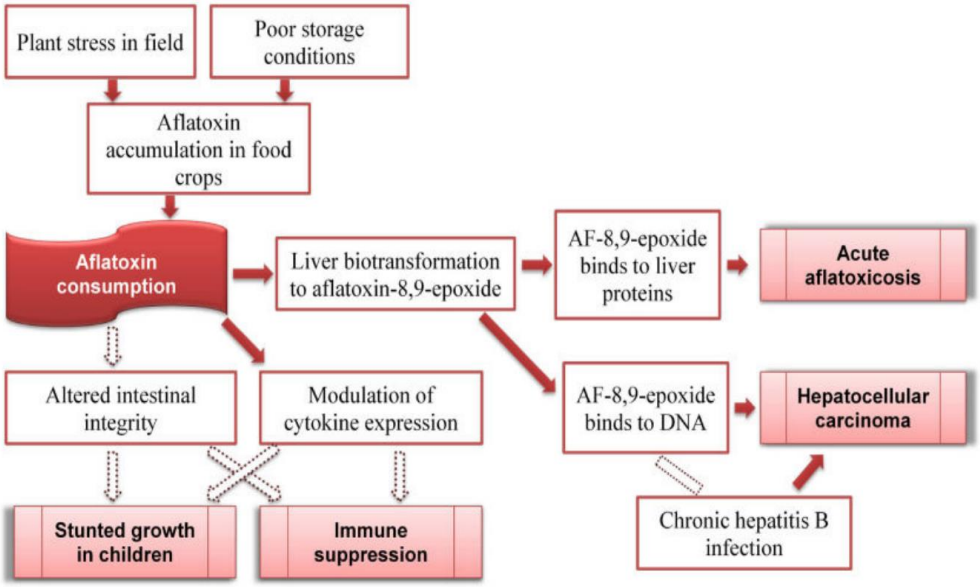


Figure 2.4. Metabolites and Biomarkers of AFB_1



Adapted from Wu, 2010, 2011

Figure 2.5. Aflatoxin disease pathways

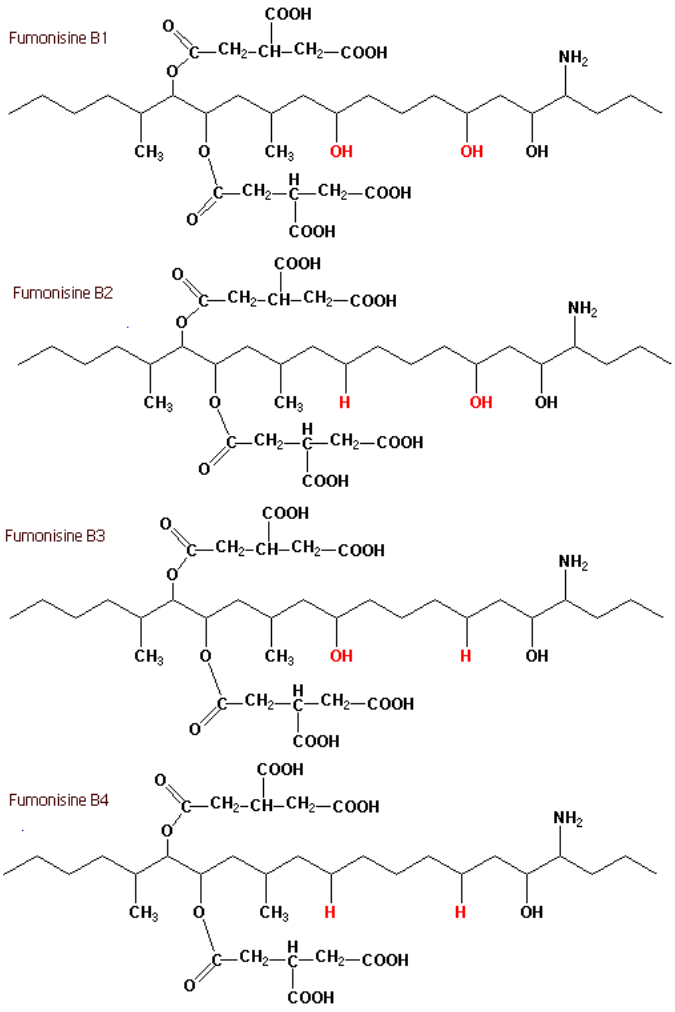


Figure 2.6. Chemical structures of B-series-fumonisins

Chapter 3

AFLATOXIN EXPOSURE IN CHILDREN AGE 6 – 12 YEARS: A STUDY PROTOCOL OF A RANDOMIZED COMPARATIVE CROSS-SECTIONAL STUDY IN KENYA, EAST AFRICA.[1]

[1] Wangia, D.P. Githanga, J.S. Wang, and O.A. Anzala. 2019. 'Aflatoxin exposure in children age 6–12 years: a study protocol of a randomized comparative cross-sectional study in Kenya, East Africa', *Pilot and Feasibility Studies*, 5:141. This is an Author's Original Manuscript of an article published Open Access by BMC Part of Springer Nature on November 29, 2019, available online at https://pilotfeasibilitystudies.biomedcentral.com/articles/10.1186/s40814-019-0510-x. Reprinted here with permission of publisher.

Abstract

Aflatoxins (AFs) are naturally occurring fungal metabolites produced by the Aspergilla species of fungi. The staple food grain corn (*Zea mays)*, is highly susceptible to AF contamination. In Kenya, contamination of corn supplies by AFs is a recognized public health problem which has resulted in over 600 human deaths. Human exposure to AFs can occur in utero, via breast milk, through weaning foods and throughout an individual's lifetime. Recent epidemiological studies have shown that exposure to AFs in early life through diet is a contributing factor to immune suppression, micronutrient deficiency, possible vaccine interference and impaired growth in children. However, these results remain inconsistent and inconclusive due to lack of randomized controlled studies. A randomized school-based cross-sectional study was designed to study AF exposure levels and associated health effects in children between ages 6 and 12 years. Participants were recruited from primary schools within Siaya and Makueni Counties of Kenya, East Africa. The Joint Ethics Committee of the University of Nairobi and Kenyatta National Hospital in Kenya approved the research protocol and procedures for the study. Both parental consent and child assent were obtained before enrollment in the study. Parents were requested to provide household grain samples and fill out questionnaires detailing their socio-demographic information, household dietary patterns, farming practices and knowledge of AF contamination. Blood samples were collected from children participants and sera were prepared for analysis of AFB_1-lysine which is one of the validated biomarkers for AFs exposure. This protocol describes a school-based, cross-sectional study whose objective is to comparatively evaluate the role of AF exposure on adverse health

outcomes in children. Specifically, effects of cumulative AF exposure on nutritional status, immune markers and growth parameters will be assessed.

Trial Registration

This study is not a clinical trial, rather a cross sectional study aimed at providing baseline data on AF exposures in children who live in presumably high versus low AF exposure regions. Results from the study can be used to design interventions and/or prospective cohort studies aimed at studying adverse health effects associated with cumulative AF exposure through diets.

Key Words: Aflatoxins, Children, Adverse Health Outcomes, Immune Suppression, Growth Impairments

Introduction

Aflatoxins (AFs) are a group of naturally occurring mycotoxins produced by the common fungus *Aspergillus flavus* and the closely related *Aspergillus parasiticus* fungi (CAST 2003; IARC 1993, 2004). Up to 4.5 billion people around the world are exposed to AFs through the diet and or via occupational exposures during grain handling (Williams et al. 2004; Liu and Wu 2010). AFs are common food contaminants and present a persistent challenge throughout the food chain. Food staples frequently contaminated with AFs include corn, peanuts, rice, cassava, spices and other food items (CAST 2003; IARC 2004, 1993). There are up to 14 different groups of naturally occurring AFs, but the commonly studied groups include B_1, B_2, G_1 and G_2 (CAST 2003; IARC 2004). Aflatoxin B_1 (AFB_1) is the most biologically potent because it is a confirmed human carcinogen classified into

Group I by the International Agency for Research on Cancer (IARC 1993, 2004). AFB_1 is a major risk factor associated with primary liver cancers as evident in many studies conducted in Africa and South East Asia (Wang, Huang, et al. 2001; Kew 2013; Magnussen and Parsi 2013). Moreover, AFs are immune-toxicants and has been associated with immune suppression in human populations (Jiang et al. 2005a; Githanga et al. 2019). In children, recent epidemiology studies provide evidence that cumulative exposure to AFs in low concentrations contribute to micronutrient deficiency, possible vaccine interference, immune suppression and growth impairments (IARC Working Group Reports 2015; Khlangwiset, Shephard, and Wu 2011; Obuseh et al. 2011). The adverse health outcomes associated with AF exposure may persist into adulthood if neither interventions nor corrective measures are undertaken. To date, comprehensive data on AF exposure is limited and thus assessment of adverse health outcomes is further hindered.

Contrary to long term exposure to AFs where adverse health outcomes occur over time, dietary exposure to AFs exceeding 200µg/kg in the short term can be fatal due to aflatoxicosis (Kamala et al. 2018; Ngindu et al. 1982; Narsimha Reddy and Raghu Raghavender 2007). Aflatoxicosis is a medical condition characterized by jaundice, bile duct proliferation, edema, sudden liver failure and ultimately death within twenty-four hours of consumption of AF contaminated corn (Mwanda, Otieno, and Omonge 2005; Ngindu et al. 1982; United States CDC 2004; Narsimha Reddy and Raghu Raghavender 2007). In Kenya, AF contamination in corn supplies is a recognized public health problem, which has resulted in more than 600 documented human deaths attributed to aflatoxicosis (Wangia 2017; Lewis et al. 2005; Daniel et al. 2011). The United States Food and Drug Administration (USFDA) recommends that food destined for human consumption should

not exceed total AFs of 20µg/kg (USFDA 2000). European Union's Codex Alimentarius recommends 15µg/kg (European Commission 2006), while Kenya, given its troubled past with deaths associated with aflatoxicosis stipulated a strict recommendation of 10 µg/kg (East African Community 2013). These recommended exposure levels are significantly difficult to implement due to widespread subsistence farming which promotes higher AF exposure because corn produced in farms are consumed directly without prior testing for AF contamination levels.

Methods

The study design is school-based and cross-sectional to provide a snapshot of AF exposure levels amongst children recruited from Siaya and Makueni Counties. Cross-sectional study design was preferred due to its ability to generate factual information on AF exposure among children widespread over two geographic locations. To ensure samples are representative of both Makueni and Siaya Counties, primary schools without feeding programs were randomly selected in different constituencies per county. The selection of Makueni and Siaya County was made according to previous studies which reported high AF exposures in Makueni County while Siaya's exposure levels were below the level of detection (Yard et al. 2013; Ngindu et al. 1982). Makueni County lies in Kenya's former Eastern Province while Siaya County forms one of the six counties in former Nyanza province (Yard et al. 2013). Siaya County is hot and humid with temperatures averaging 21 to 25ºC while annual precipitation ranges between 1000 – 1750 mm annually (CIAT 2016). Conversely, the climate of Makueni County is predominantly semi-arid characterized by long dry seasons interspersed by an annual rainfall of about 500mm and ambient temperature ranges between 18 and 24ºC in the cold season and

between 24º C and 33 ºC in the hot season (Kilonzo et al. 2014). Makueni County's aridity has been implicated to be a significant contributing factor to AF contamination as a result of undue crop stress (Ngindu et al. 1982; Kilonzo et al. 2014). The Luo ethnic groups are the main inhabitants of Siaya County while the Akamba community inhabits the high exposure regions of Makueni County (Kenya Bureau of Statistics 2015). The estimated poverty rate in Makueni and Siaya Counties are 64% and 48% respectively compared to the national average of 47% as of 2009 (CIAT 2016; Kenya Bureau of Statistics 2015). Overall, the choice of Makueni and Siaya Counties as study location provides control for differences in weather conditions, ethnicity and poverty levels.

Due to limited food diversity in Sub-Saharan Africa, our study populations depend on corn and corn based products for daily energy requirements (Ekpa et al. 2018; De Groote and Kimenju 2012). The dietary staples in Kenya include corn based meals comprised of solid corn meal, commonly known as ugali in Swahili, porridge, roasted and boiled corn cobs, and or corn boiled with beans (De Groote and Kimenju 2012). Moreover, each household is estimated to consume corn based meals at least two times a day because ugali is often served during lunch and/or dinner in accompaniment with a mix of vegetables and/or any kind of stew (Bukania et al. 2014; De Groote and Kimenju 2012). Consuming a variety of foods from several food groups is a recommended approach to achieve necessary nutritional requirements and limit excessive exposure to AFs through the diet (Kennedy 2009). Diet diversification has been proposed to be instrumental in mitigating AF exposure and the associated adverse health outcomes. The International Food Policy Research Institute proposed that households with a score less than 4.5 can be categorized as low dietary diversity, a score between 4.5 and 6 have medium dietary diversity while a

score greater than 6 can be considered both high and good dietary diversity (Vhurumuku 2014). In our study populations, past studies reported low dietary diversity with a score of less than 4 food groups each day (Wangia et al. 2019; Bukania et al. 2014). Therefore, food insecurity is paramount in our study population due to low agricultural productivity that is characterized by unreliable rains, limited access to farm inputs and hired labor which increases the cost of food production significantly (CIAT 2016). Nonetheless, small-scale holder farmers undertake corn cultivation for household consumption despite low agricultural output and increased risk of AF contamination.

Justification of Makueni and Siaya as Study Setting

In Kenya's former Eastern Province, high level of AF contamination is concentrated in Makueni County as evident in multiple aflatoxicosis outbreaks previously reported (Ngindu et al. 1982; United States CDC 2004). In 2004, a severe aflatoxicosis outbreak characterized by 317 cases of acute hepatic failure and subsequent 125 deaths were reported (Azziz-Baumgartner et al. 2005; Mwanda, Otieno, and Omonge 2005; Probst, Njapau, and Cotty 2007). The Kenyan Ministry of Health worked collaboratively with experts from the United States Center for Disease Control and Prevention to assess risk factors associated with the outbreak (United States CDC 2004; Daniel et al. 2011). While the cases were inhabitants of Makueni, Kitui and Machakos Counties, inhabitants of Makueni County were the most affected, accounting for almost 50% of the cases (Lewis et al. 2005; Muture and Ogana 2005). Consumption of AF contaminated corn was linked to aflatoxicosis (Daniel et al. 2011; Probst, Njapau, and Cotty 2007).This study confirmed the exclusive role of aflatoxin poisonings among human populations due to exposure to high contamination levels through dietary sources.

In a serological survey to evaluate regional variation of AF exposure in Kenya, 78% of archived serum samples had detectable AF levels (Yard et al. 2013). The highest AF exposure was recorded in the former Eastern province with a median of 7.87pg/mg aflatoxin-albumin adducts while in the former Nyanza province; the aflatoxin-albumin adducts were below the limit of detection (<LOD) in human serum samples evaluated. The regional variation of AF exposure levels as reported by Yard et al (Yard et al. 2013) is exhibited in Figure 3.1. The AF levels were much lower in all the other 5 provinces.

Sample size determination

According to the Kenya Population census of 2009, Siaya County had a population of 842,304 while Makueni County had 884,527 people (Kenya National Bureau of Statistics 2010). The sample size was determined using Krejcie and Morgan's methodology (Krejcie and Morgan 1970).

$$s = X^2NP[1-P] \div d^2[N-1] + X^2P[1-P]$$

Where;

s, is the required sample size; X^2= 3.841, is the table value of chi-square for 1 degree of freedom at 95% confidence interval

N, is the population size; P, is the population proportion which is assumed to be 0.50 since this provides maximum sample size and d, is the degree of accuracy expressed as a proportion (0.05)

According to Krejcie and Morgan's formula, the estimated sample size was determined to be 393 School children per county (Krejcie and Morgan 1970). The estimated total number of participants required to achieve the study objectives was 786 across different schools

from 2 counties. Participants were recruited from randomly selected primary schools in Makueni and Siaya Counties in Kenya.

Sampling and Human Participants

Makueni is confirmed to have the highest prevalence of AF exposure among Kenya's 47 counties spread across former eight provinces. In contrast, Siaya which is considered to be a low exposure region, has had few studies conducted in the region. Siaya and Makueni counties were chosen based on AFs exposure levels reported on published literature (Yard et al. 2013; Muture and Ogana 2005; Lewis et al. 2005). Schools were randomly selected per constituency at the county level as long as the school did not have feeding programs and were located at least three kilometers from a shopping center to limit the possibility of consuming market or store-bought corn products. In Siaya County, schools allow a one-hour lunch break where students go home for lunch and come back for afternoon classes. In Makueni, however, students bring lunch from home in the morning and have half-hour lunch break, before resuming classes in the afternoon. Participants enrolled in the study were randomly selected using Kish Grid Method to avoid selection bias (Leslie 1949). School administrations were requested to convene parent meetings at a convenient time and location for participants. Community leaders including local area chiefs and members of the county assembly were invited to the meetings. The chairperson of the Parent Teachers Association was in attendance and presided over the meetings. The study purpose and significance were explained to school officials, teachers, support staff and parents. The participants were given ample opportunity to ask questions during the meeting, before subject recruitment and during the study to ensure full disclosure of study components. Informed consent forms were explained to parents in their local dialect and

all their questions were addressed before being asked to provide consent and fill out questionnaires.

The five-part questionnaire administered collected basic information including participants' age, sex, weight, height, mid-upper arm circumference; socio-demographic factors including marital status, education levels, living conditions, occupations of both the respondent and their spouse, household income levels, information on home ownership and living conditions. Participants also reported farming practices which entail whether they grow or buy corn for household use, use of pesticides and/or fertilizers during farming, extent of AF knowledge and how each household stores corn and other food supplies after harvest. In addition, the questionnaire collected information on dietary intake of different food groups, number of meals in a day, choice of food and nutrition practices. Information collected from questionnaires provided sufficient information to enable analysis of socio-economic status and respective poverty levels. Moreover, these possible confounding factors can be easily controlled in statistical analyses. After the meeting at various schools, the researchers crosschecked all consent forms and questionnaires for accuracies. In cases where consent was not clear, the individual parent was contacted to confirm their decision. In addition, parents were required to provide additional consent by checking YES or NO on the questionnaire form to allow international shipping of samples, use of media files, and storage of bio specimens and publication of anonymized data summaries. Parents were also asked to provide about 150g of household corn flour or kernels for AF measurements of household food. Only healthy children between the ages of six and twelve with no current active medication were enrolled in the study. After obtaining assent, the study personnel obtained anthropometric measurements including height, weight and mid-upper

arm circumference, 6-8ml of venous blood and 15ml of urine samples. Collection of urine samples was discontinued due to budget constraints and the labor-intensive nature of collecting urine from children. Figure 3.2 shows a flow diagram showing the study participants recruitment and enrollment.

Discussion

This is the first study designed to establish a baseline AF exposure in children between the ages of six and twelve years. To date, studies to establish AFs exposure have been completed in populations of children below the age of 5 years, adults between the ages of 18 and 65 years, vulnerable populations of pregnant women, immunocompromised groups including individuals suffering from HIV/AIDS, and hepatitis B virus infections (Wang, Huang, et al. 2001; Khlangwiset, Shephard, and Wu 2011; Turner et al. 2005; Williams et al. 2010). Past studies tend to address specific adverse health outcomes including immune suppression, micronutrient levels, possible growth impairment and risks of carcinogenesis without controlling for related health outcomes. For instance, studies aimed to assess the effect of AF exposure on children's growth in Benin and Togo prioritized possible growth impairments and micronutrient levels in blood plasma while immune markers were not evaluated (Gong et al. 2002; Gong et al. 2004). Conversely, a study in Ghana prioritized evaluation of immune suppression as it relates to AF exposure while neither micronutrient markers nor growth indicators were considered (Jiang et al. 2005). Studies in farm animals and human sub-populations have reported downstream effects associated with AF exposure, malnutrition, immune suppression, vulnerability to infectious diseases and consequently growth impairments (Githanga et al. 2019; Owaga et al. 2011; Khlangwiset, Shephard, and Wu 2011). Intervention studies such as use of

enterosorbents like Novasil Clay to bind AF adducts in human blood has been evaluated for safety, palatability and efficacy (Awuor et al. 2017; Mitchell et al. 2014; Afriyie-Gyawu et al. 2008; Wang et al. 2008). In a phase II clinical trial, 180 adults aged 18 to 58 years at risk of aflatoxicosis who received Novasil Clay capsules significantly had lower AFB_1-lysine adducts in both blood and urine samples in a dose dependent manner (Wang et al. 2008). Other interventions involving use of green tea polyphenols (Luo et al. 2008), and chlorophyllin (Egner et al. 2001) have been shown to reduce AF-biomarkers and may also form basis for future prospective studies. The current study protocol provides a multifaceted approach in addressing adverse health outcomes linked to dietary exposure to AFs while controlling for social, economic and demographic factors. While the mechanism by which AFs contribute to adverse health outcomes is currently unknown, it is suspected to be biological (Hoffmann, Jones, and Leroy 2015).

Given the multifaceted aspects of this study, lessons that may be valuable to other scientists seeking to undertake research projects in low-or middle-income countries are outlined herein. First, plan the study at least 2 to 3 years in advance before actual fieldwork. This will give the study team sufficient time to prepare, apply for ethics justification, follow up different requirements, and obtain additional permissions required to conduct field studies involving human subjects. Our study's ethical approval was prolonged because it involved children. In order to prepare for successful fieldwork for research projects, travel to focus country of interest to build social capital. Moreover, investigators seeking to accomplish fieldwork in low- and middle-income countries will be more successful if collaborative efforts are fostered with local scientists. It is also important to familiarize oneself with myths and beliefs in a community as blood draw is a contentious issue in some

communities. In our study populations, a common belief that blood products can used for financial profit by researchers was prohibitive. Moreover, study administrators must make it clear to participants that their samples will be shipped for analysis to a different location and obtain consent for shipment which must be approved a priori by the ethics committee. The paperwork associated with additional approvals for sample shipment, export and import can be extensive and thorough. Last but not least, remuneration of field workers can exponentiate overhead costs.

The strength of the study is its multifaceted approach in assessing health effects in children exposed to AFs through the diet. In addition, recruiting subjects from both a high and a low exposure region is instrumental in elucidating the role of AF exposure specifically on micronutrient deficiency, immune suppression and possible growth impairments. Study limitations include; researchers are not ethically allowed to contact participants in the future and thus, study results cannot be used to analyze behavior change, or determine cause and effect. Moreover, we expect AF exposures to be high in May, June and July because it is rainy, humid, and most farmers have a surplus of harvested grains from the earlier season which are highly susceptible to AF contamination. Nonetheless, this is not guaranteed as factors such as community education programs to promote AF awareness, diet diversification and food security in some regions where study participants were recruited may be associated with decreased AF exposures. Based on results from this study, a prospective cohort study will be valuable in further studies aimed at explaining adverse health effects associated with AF exposure.

This paper describes a protocol of the first school-based randomized cross-sectional study aimed at assessing health effects associated with exposure to AFs through the diet.

Whether or not a relationship is found between AF exposure and adverse health outcomes, the results can be used to prioritize AF control efforts not only in Kenya but other developing countries. In conclusion, exposure to AFs and other mycotoxins in low- and middle-income countries has been shown to contribute to immune suppression and growth impairment. Mycotoxins are also suspected to interfere with vaccine efficiency making children more vulnerable to increased risk of infectious diseases. Pediatricians and clinicians in developing nations should pay attention to the role of not only AFs but other environmental factors that influence health in their practice to better serve their populations.

Declarations

The Joint Ethics Committee of the University of Nairobi and Kenyatta National Hospital in Kenya approved the research protocol and procedure for study referenced P741/12/2017. Study purpose, objectives and expected outcomes were explained in local dialect to participants and all concerns were addressed before enrollment. Both parental consent and child assent were obtained from participants. Only participants who agreed to publication of de-identified data were enrolled in the study. Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study. Only study investigators will have access to the final data set. The authors declare that they have no competing interests.

Authors' Contributions -RNW conceptualized the study and drafted the manuscript. DPG, OAA, JSW and RNW reviewed the manuscript for accuracy. All authors read and approved the final manuscript.

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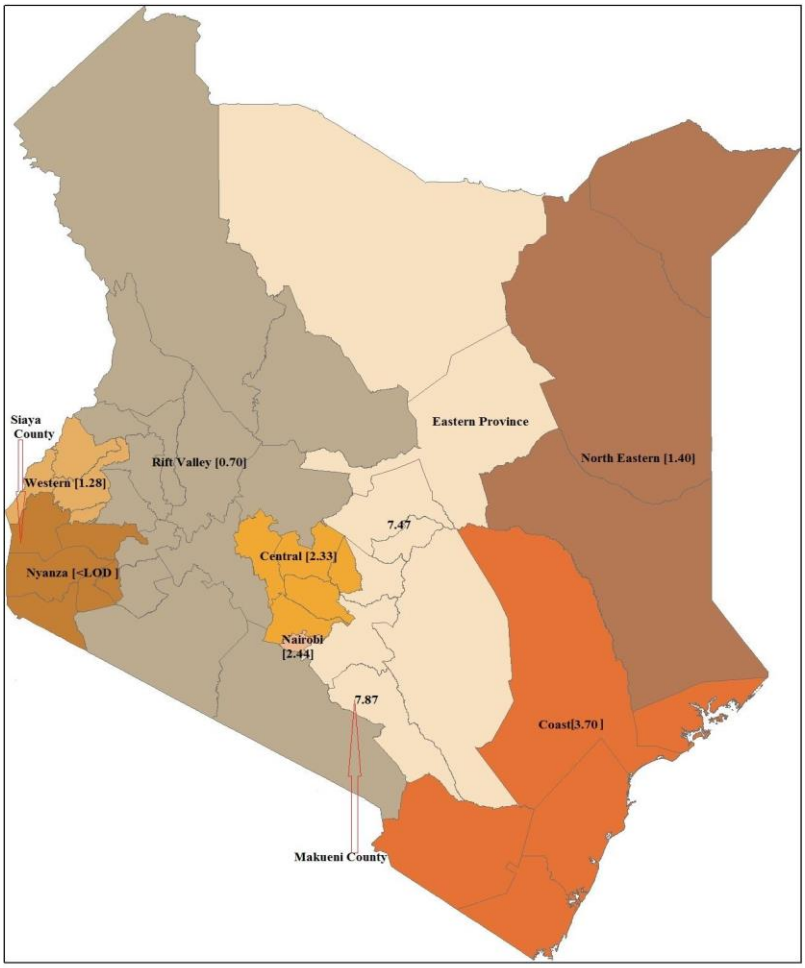

Figure 3.1. Regional Variation of Aflatoxin Exposure by Kenya provinces

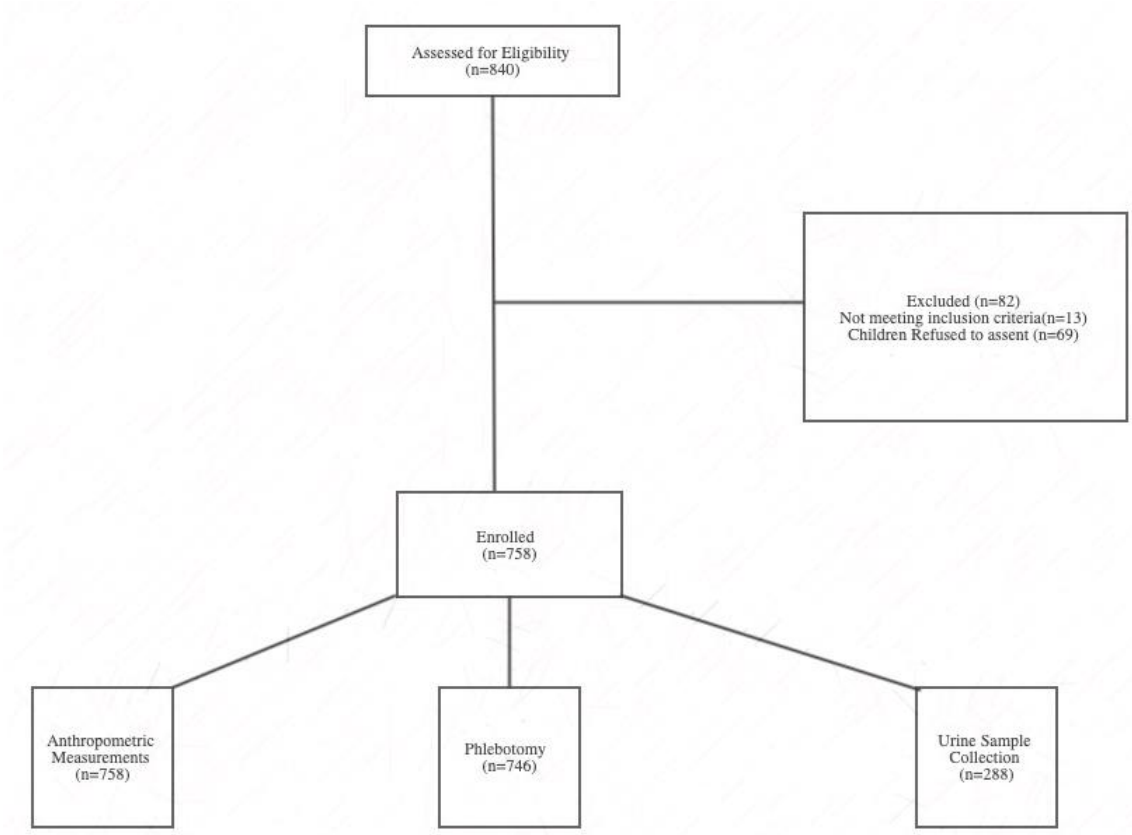

Figure 3.2. Study Participants Recruitment

Chapter 4

AFLATOXIN CONTAMINATION IN HOUSEHOLD CORN FOR HUMAN CONSUMPTION IN KENYA, EAST AFRICA[2]

[2] Nabwire, Wangia Ruth, James Ombaka, Christopher Peyton Dick, Christian Strickland, Lili Tang, Kathy Siyu Xue, and Jia-Sheng Wang. 2019. 'Aflatoxin in household corn for human consumption in Kenya, East Africa', *Food Additives & Contaminants: Part B*: This is an Author's Original Manuscript of an article published by Taylor & Francis in *Food Additives & Contaminants* on November 27, 2019, available online at the Taylor & Francis Ltd web site: www.tandfonline.com and the link to the article - https://www.tandfonline.com/doi/full/10.1080/19393210.2019.1690053. Reprinted here with permission of publisher.

Abstract

The objective of this study is to determine the occurrence and level of Aflatoxins (AFs) contamination in freshly harvested corn for human consumption in rural Kenya. Corn kernels and freshly milled corn flour (n=338) were collected from households in Siaya and Makueni counties. While both counties are representatives of different environmental and climate conditions, Makueni County is the area with reported outbreaks of aflatoxicosis. Samples were analyzed for AFB_1, AFB_2, AFG_1 and AFG_2 using Ultra High-Performance Liquid Chromatography with Fluorescence detection. AFs were detected in 100% of the samples with the range of 2.14 – 411 µg/kg. The geometric mean of total AFs in all samples from Makueni County is 62.5 µg/kg with 95% CI: 53.7, 71.4 while in Siaya County is 52.8 µg/kg with 95% CI: 44.0, 61.7. This study showed that AFs contamination is prevalent in corn based foods in the region.

Keywords: Aflatoxins; AFB_1; total aflatoxins; household corn; rural Kenya;

Introduction

Aflatoxins (AFs) are a group of mycotoxins commonly produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* under favorable warm and humid climate (Villers 2014). They were discovered and characterized in 1960s after the reported death of more than 100,000 turkey poults fed on AFs contaminated feed in England (Kensler et al. 2011). Since their discovery, AFs have been associated with acute and chronic toxicity

in both animal and human populations (IARC 1993, 2002). While 16 structurally related AFs have been characterized, only four major groups namely AFB_1, AFB_2, AFG_1 and AFG_2 are extensively studied due to common occurrence in food supplies. AFs contaminate agricultural products including corn, peanuts, sorghum, rice, cassava, spices, and nuts (IARC 1993, 2002). Therefore, AFs contamination pose a significant food safety issue and potential risks to human and animal health (IARC Working Group Reports 2015; CAST 2003). *A. flavus* produce AFB_1 and AFB_2 while *A. parasiticus* mainly produce AFG_1 and AFG_2 and is confirmed to produce all four major AFs (Cole and Cox 1981). AFB_1 is the most potent mycotoxin and the International Agency for Research on Cancer (IARC), classified AFB_1 as Group I human carcinogen due to sufficient evidence from animal and human epidemiology studies that associated AFB_1 exposure to increased risk of developing primary liver cancers (IARC 1993, 2002)

In humans, acute toxicity resulting from exposure to high levels of AFs in the diet was reported in India with a case fatality rate of 10% in humans and 100% in dogs (Tandon et al. 1977). In Kenya, aflatoxicosis was reported in 1981 which was also preceded by deaths of farm animals (Ngindu et al. 1982). More recently, consumption of AFs contaminated grain caused aflatoxicosis in large human populations with case fatality rate of 40% in Kenya and 50% in Tanzania (Azziz-Baumgartner et al. 2005; Daniel et al. 2011; Kamala et al. 2018). Aflatoxicosis is characterized by vomiting, jaundice, abdominal pain, edema, convulsions, sudden liver failure and ultimately death (Mwanda, Otieno, and Omonge 2005). While acute toxicities associated with exposure to high levels of AFs are rare events worldwide, cases occur and are concentrated in high risk regions such as Makueni County of Kenya. Cumulative exposure to low quantities of AFs through the diet

over a period of time is more widespread and is the leading cause of liver cancer in adult populations in the developing world (Kew 2013; Magnussen and Parsi 2013). In children populations, exposure to AFs through weaning foods and the diet is associated with immune suppression, micronutrient deficiency and possible growth impairments (IARC Working Group Reports 2015; Githanga et al. 2019). Populations that rely on corn products as staple food need to be assessed for ultimate health consequences associated with chronic consumption of AFs contaminated corn.

AFs are closely regulated in most countries with maximum limits ranging from 5 - 20µg/kg for food destined for human consumption (Wu, Stacy, and Kensler 2013; USFDA 2000). Kenya, given its troubled past with aflatoxicosis has a maximum limit of 10µg/kg in corn and corn products (East African Community 2013). The European Union's standard is the strictest with maximum limit of 2 µg/kg for AFB_1, and 4 µg/kg for total AFs (European Commission 2006). Despite good agricultural practices combined with rigorous regulation, it is common to find detectable levels of AFs in food commodities. AFs contamination of food supplies present a continuous challenge throughout the food chain and is a major risk factor for food insecurity in low and middle-income countries.

In this study, Ultra High Perfomance Liquid Chromatography with Fluorescence detection (UHPLC-FD) is preferred for simultaneous analysis of AFs contamination in corn products because of high sensitivity and specificity (Wacoo et al. 2014; Alshannaq and Yu 2017). The purpose of this study is to determine the occurrence and level of AFs contamination in freshly harvested corn for human consumption collected from rural Kenya using the UHPLC-FD method.

Materials and Methods

Sampling

This study is part of the larger cross-sectional study aimed at establishing AFs exposure levels in children between the ages of six and twelve years. The overall objective of the study is to comparatively assess if dietary exposure to AFs contributes to micronutrient deficiency, immune suppression and growth impairment. The research and study protocols were reviewed and approved by the Joint Ethics Committee of the University of Nairobi and Kenyatta National Hospital in Kenya. Randomized multistage stratified sampling was used in the identification of schools, and selection of study participants from Siaya and Makueni counties. All participants concern and questions were addressed before subject recruitment. Informed consent was explained in local dialect and parents who agreed to study procedures were asked to complete a questionnaire. Parents who provided informed consent were asked to provide 150 grams of household corn and/or flour used for daily meal consumption. A total of 338 samples of corn products (173 milled flour samples and 165 corn grain samples) were collected and prepared for UHPLC-FD analysis. Milled flour samples were sieved through a 1.0mm sieve while the corn grain samples were first ground into fine texture using a Ninja Professional 1100-Watt Blender (Euro-Pro Operating LLC, Newton, MA), then passed through a 1.0 mm sieve. All samples were weighed into sealable plastic storage bags, labelled and stored under refrigeration at 4 °C until analysis.

Chemicals and Reagents

AFB_1, AFB_2, AFG_1, and AFG_2 standards were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). HPLC grade water was purchased from Avantor

Performance Materials (Center Valley, PA) and methanol was purchased from Honeywell (Morristown, NJ). Sep-pak Classic C18 Cartridges were purchased from Waters Corporation (Milford, MA). An extraction solution was prepared using a ratio of 70:30 methanol: HPLC grade water. Individual AFs standards including AFB_1, AFB_2, AFG_1, and AFG_2 were dissolved in dimethyl sulfoxide (DMSO) and used as stock solutions. A standard curve was created from dilutions of the stock solutions in concentrations of 0.0, 1.0, 2.5, 5.0, 10, 20, and 40 µg/kg.

Sample Extraction and Cleanup

Due to the heterogenous nature of AFs contamination, each sample was thoroughly mixed and multiple scoops randomly taken from different parts of the plastic bag. This method has been used in previous studies conducted in rural Kenya (Daniel et al. 2011; Lewis et al. 2005). Up to 5 grams of each sample was weighed into a centrifuge tube and 25ml of 70% MeOH added, then vortexed for 1 minute. The suspension was centrifuged for 5 minutes at 4000rpm and filtered through Whatman No.1 Filter paper. A portion of 2.0 ml filtrate was transferred into a polypropylene tube and diluted with 3.6ml HPLC water. Cleanup was done through a Sep-Pak cartridge and syringe barrel in the fume hood. Samples were eluted by 1.0 ml MeOH and dried using Labconco Centrivap concentrator (Kansas City, MO). The samples were reconstituted with 25%MeOH, centrifuged and filtered before transferring 30µl into a UHPLC vial for analysis.

UHPLC Conditions

The Thermo Scientific Dionex UltiMate 3000RS UHPLC system was used for separation of target analytes. Excitation and emission wavelengths for fluorescence detection were set at 360 nm and 435 nm, respectively, and 362 nm and 450 nm for UV

detection. The mobile phase A consisted of 10%Methanol and 90%HPLC grade water while B contained 100% Methanol. The flow rate was 0.4ml/min and column temperatures were maintained at 50 °C. For each sample, 10 µL was injected to the Acclaim column (Acclaim RSLC 120C18 2.1x150 mm, 2.2 µm 120Å, Thermo Scientific, Waltham, MA). Total run is 25 minutes (min) with the gradient at 0.0min specified for 95A:5B, at 6.0min 50A:50B, at 10.0min 5A:95B, and from the fifteenth minute, the program is specified to run 95A:5B until end. Control samples were prepared in duplicates every day. In the first step of sample processing, milled corn samples with known AF concentration was spiked with 5.0 µg/kg of AFB_1. In addition, during UHPLC-FD analysis, 2 blanks of 25% methanol, 2 standards containing 2.5µg/kg of AFB_1 and 2 standards containing a mixture of AFB_1, AFB_2, AFG_1 and AFG_2 in equal concentrations were used for quality controls.

Method Validation

Method validation parameters reported herein are selectivity, linearity, the limit of detection (LOD), and limit of quantification (LOQ). The performance of the method was in accordance with the criteria of the Commission Regulation EC No 401/2006 (European Commission 2006). Parameter used for selectivity is retention times with averages of 8.31, 8.72, 9.16, and 9.46 minutes for AFG_2, AFG_1, AFB_2 and AFB_1 respectively. The calibration curves used to quantify AFs were determined by linearity assumption. Linearity was accomplished by injecting in duplicates standard solutions of AFG_2, AFG_1, AFB_2 and AFB_1 at concentrations of 0.0, 1.0, 2.5, 5.0, 10, 20, and 40 µg/kg and then constructing standard curves. Linearity parameters were based on the regression coefficients (r^2) of the standard curve of each AFs group which were over 0.998. The LOD and LOQ were defined as 3.3 and 10 times the standard deviation respectively, divided by the slope of the

calibration curve for each mycotoxin (Firdous et al. 2014; Janic Hajnal et al. 2017). The parameter used to validate accuracy is the recovery rate of AFs after spiking milled corn flour samples with known AFs levels while relative standard deviation was used to validate precision as shown in Table 4.1.

Statistical Methods

Statistical analyses were accomplished by use of SAS software version 9.4 (Cary, North Carolina) and Microsoft Excel 365 Office. Any value below the LOQ were excluded from statistical analysis. The concentrations reported in this study were adjusted for recovery of each AF group according to Table 4.1. Total AFs were calculated by summing AFB_1, AFB_2, AFG_1 and AFG_2. Results were analyzed by ANOVA assuming a level of significance at $p<0.05$ and 95% confidence Interval. Significant differences in contamination levels were evaluated according to post-hoc Tukey's honestly significant differences and 2 sample t-test assuming unequal variances.

Results

A detection rate of 100% for total AFs was found in all 338 samples. AFB_1 is the most dominant in all samples contributing up to 97.1% of total AFs. The next is AFG_1, then AFB_2, and AFG_2 contributed the least percentage to total AFs. Up to 128 (37.9%) samples did not have detectable levels of AFG_1. The geometric mean of AFB_1 in all samples (338) is 57.9 µg/kg with 95% CI: 51.0, 64.7. The median is 59.3 µg/kg and the range of AFB_1 is 1.69 – 404 µg/kg. The geometric mean of AFG_1 in detectable samples is 0.58 µg/kg with 95% CI: 0.43, 0.73. The median is 0.62 µg/kg and the range of AFG_1 is 0.01 – 8.38 µg/kg. The geometric mean of AFB_2 in all samples (n=338) is 0.40 µg/kg with 95% CI: 0.31, 0.48.

The median is 0.45µg/kg and the range of AFB_2 is 0.06 – 5.83 µg/kg. The corn flour and kernels were least contaminated by AFG_2. The geometric mean of AFG_2 in all samples (n=338) is 0.17 µg/kg with 95% CI: 0.08, 0.25. The median is 0.23 µg/kg and the range of AFG_2 is 0.01– 7.01 µg/kg. When the four groups of AFs were combined, the geometric mean of total AFs in all samples (n=338) is 59.6 µg/kg with (95% CI: 52.8, 66.5). The median is 61.8 µg/kg and the range of AFB_1 is 2.11 – 411 µg/kg. These results are summarized in Table 4.2. The geometric mean of total AFs in all samples from Makueni County is 62.5 µg/kg with (95% CI: 53.7, 71.4). The median is 63.0 µg/kg and the range of total AFs in samples from Makueni County is 5.77 – 411 µg/kg. The geometric mean of total AFs in all samples from Siaya County is 52.8 µg/kg with (95% CI: 44.0, 61.7); median is 58.1 µg/kg and the range of total AFs in samples from Siaya County is 2.14 – 251.87 µg/kg. These results are summarized in Table 4.3. Corn kernels appear to be more contaminated by AFB_1 compared to corn flour. In Siaya County, the corn kernels' geometric mean is 66.6 µg/kg with (95% CI 50.9, 82.3); median is 66.5 µg/kg with the range of 1.69 – 247 µg/kg which is significantly higher than AFB_1 levels in flour samples whose geometric mean is 41.4 with (95% CI 34.5, 48.2). The median is 40.5 µg/kg with the range of 13.9 – 118 µg/kg, ($p<0.0001$). While AFB_1 in kernel samples from Makueni have higher contamination levels than flour samples from the same region, these results are not statistically significant ($p > 0.05$). In flour samples collected from Makueni, the geometric mean of AFB_1 is 59.0 µg/kg with (95% CI: 45.0, 73.0), the median is 65.8 µg/kg with the range of 4.17 – 403 µg/kg. In Siaya, AFB_1 contamination levels of flour samples have geometric mean of 41.4 µg/kg with (95% CI: 34.5, 48.2), a median of 40.5 µg/kg with a range of AFB_1 from 13.9 – 118 µg/kg. Regional variation of total aflatoxins and AFB_1 is

shown in Figure 4.2 and the mean levels of AFB_2, AFG_2 and AFG_1 are shown in Figure 4.3.

The geometric mean of AFB_1 in corn kernels from Siaya County is 66.6 μg/kg with 95% CI: 50.9, 82.3, the median is 66.5 μg/kg with a range of 1.69 – 247 μg/kg. The geometric mean of AFB_1 in corn kernels from Makueni County is 62.5 μg/kg with (95% CI: 51.8, 73.1), the median is 60.6 μg/kg with a range of 14.0 – 338 μg/kg. These results are shown in Table 4.4. Overall, 95.3% of all corn samples exceeded the United States Food and Drug maximum limit of 20 μg/kg for total AFs in household grain for human consumption. If considering Kenya Bureau of Standards maximum limit of 10 μg/kg, 97.5% of the corn sample exceeded the limit and are considered unfit for human consumption. Up to 25% (84/338) of the samples had total AFs contamination levels higher 100μg/kg, which is ten times over the Kenya Bureau of Standards maximum limit. The current study reports a maximum level of total AFs contamination to be 411 μg/kg, a value over 40 times the maximum limit. These data are summarized in Table 4.4

Discussion

Corn and corn products are highly susceptible to AFs contamination. The geometric mean of total AFs in all samples (n=338) is 59.6 μg/kg with (95% CI: 52.8, 66.5). The geometric mean of total AFs in all samples from Makueni County is 62.5 μg/kg with (95% CI: 53.7, 71.4) while in Siaya County is 52.8 μg/kg with (95% CI: 44.0, 61.7). In rural parts of south and southwestern Ethiopia, total AFs in corn samples were generally above 24 μg/kg with a reported maximum of 513 μg/kg (Getachew et al. 2018). In another study conducted in Ethiopia, AFs contaminated complementary foods intended for consumption by young children 5 years and under with a mean range of 0.3 – 9.9 μg/kg (Ayelign et al.

2018). In Serbia, the highest reported levels of AFB_1 in corn samples is 8.8 µg/kg with overall mean contamination levels of 0.53 µg/kg of AFB_1 (Torovic 2018). In Tunisia, mean total AFs reported were 11.08 ± 8.84 µg/kg mainly contributed by AFGs (Jedidi et al. 2017). These studies show that AFs contamination is still prevalent in corn based foods in low- and middle- income countries and presents a significant challenge to food safety. The Kenyan population rely on corn as a staple, with an estimated consumption rate of 400 g/person/day (Kilonzo et al. 2014). It has been estimated that Kenyans are exposed to AFs in the range of 4.3 – 554 ng kg^{-1} bw day^{-1} whereas on average, Australians and Americans are exposed to 0.8 ng kg^{-1} bw day^{-1} and 0.26 ng kg^{-1} bw day^{-1} respectively from AF contaminated corn and corn based products (Wambui et al. 2017). This shows that the Kenyan population is highly susceptible to AF exposure through the diet and are at a greater risk of developing adverse health outcomes in adulthood if early measures to mitigate AF exposure do not take in effect. Previous studies were mainly conducted in Makueni County due to past aflatoxicosis outbreaks linked to corn grain contamination by AFs (Daniel et al. 2011; Lewis et al. 2005) In a study conducted in Kibwezi of Makueni County, 45% of households consuming corn kernels were exposed to AFs at levels ranging from 18 to 480 µg/kg (Kilonzo et al. 2014). A 3-year (2005 – 2007) cross sectional survey conducted in Makueni and Kitui counties found that the overall geometric mean of AFs was 17.8 µg/kg in household corn samples (Daniel et al. 2011). In a different survey, AFs contamination levels were found in corn samples with the range of 0.98 – 722 µg/kg (Mahuku et al. 2019). In the current study, the total AFs ranged from 2.14 – 411 µg/kg.

In another regional survey conducted in 6 additional counties of Kenya, AFs contamination was detected in 49% of the corn flour samples with reported ranges of 2.0

– 710 µg/kg, and higher levels of AFs contamination were found in purchased corn compared to household corn (Mutiga et al. 2015). In Makueni County, however, AF contamination is often higher in homegrown corn compared to corn bought from vendors at the market (Daniel et al. 2011; Kilonzo et al. 2014). During aflatoxicosis outbreak years, AFs contaminations up to 48,000 µg/kg in 2005 and 24,400 µg/kg in 2006 were reported in household corn samples (Lewis et al. 2005; Daniel et al. 2011; Azziz-Baumgartner et al. 2005). The findings of the current study show very high AF contamination levels in household corn for human consumption. To date, there's no documented evidence of any aflatoxicosis cases in Siaya County, and thus, no documented surveillance studies from the region. While none of the surveillance studies previously conducted in Kenya reported AF contamination of corn products from Siaya County, this study reports contamination levels of more than five times the maximum limit of 10.0 µg/kg (East African Community 2013).

This study shows that rural households should take careful consideration on how corn products are stored to prevent AFs contamination. The higher contamination levels found in Makueni County can be attributed to severe flooding in the region during the growing season when sampling took place. This could also be an indication of limited capacity of small-scale holder farmers to adapt to climate change (Muema et al. 2018; Famine Early Warning Systems 2018). Higher AFs contamination has also been reported in Serbia's corn growing regions and Europe due to erratic weather and potential effects of climate change (Janic Hajnal et al. 2017; Battilani et al. 2016; Kos et al. 2018). AFs contamination is highly variable by region. In a country wide serological survey, Yard et al found that the highest AFs exposure levels were reported in Eastern Province where Makueni County is located whereas in Nyanza Province where Siaya County is located,

AFs exposure levels in human populations were below the limit of detection (Yard et al. 2013). In the current study, 19% (18/96) of samples collected from Siaya County were corn kernels and or flour mixed with either sorghum, millet and or cassava which tend to have lower AFs contamination levels compared to corn (Sirma et al. 2015). In Makueni County, however, 100% of the samples were pure corn kernels and/or pure corn flour. Moreover, Siaya's climatic conditions are favorable for cultivation of other grains compared to Makueni, an indication of opportunities to access diversified diets especially in Siaya (CIAT 2016). AFs contamination often starts when the crops are still in the field. Mahuku et al conducted a study to assess the prevalence of AFs contamination in physiologically mature corn from farms in 6 counties of Kenya (Mahuku et al. 2019).The pre-harvest corn was found to be contaminated by AFs with the highest levels of contamination found in Embu (196.3 ± 1202 µg/kg) and Makueni (39.0 ± 132 µg/kg) counties. The mean AFs contamination levels in Kisii (28.5 ± 72.7µg/kg) and Homabay counties (24.5 ± 94.9µg/kg) were more than two times the maximum limit while the lowest levels were found in Machakos (10.5 ± 16.5 µg/kg) and Migori (12.7 ± 24.9 µg/kg) counties (Mahuku et al. 2019). In a different study, pre-harvest corn samples collected from Kakamega and Bungoma counties contaminated with AFB_1 levels were below the maximum limit of 10 µg/kg, with the highest recorded level of 17 µg/kg (Alakonya, Monda, and Ajanga 2009). The low AFs levels could be attributed to heavy rainfall and limited crop stress in the corn growing region of Kakamega and Bungoma Counties (CIAT 2016). These studies show that AF contamination is present before corn is harvested and good post-harvest strategies should be implemented to prevent further accumulation of AFs.

AFs contamination is common in most households in rural Africa and presents a significant risk to food security. In the current study conducted in June and July of 2018, freshly harvested corn was sampled from Makueni and Siaya Counties for analysis. AFB_1, the most potent AFs contributed up to 97.1% of total AFs. A high prevalence of the novel S-morphology *A. flavus* have been shown to be dominant in Kenya and is known to produce high concentrations of AFB_1 (Mutegi, Cotty, and Bandyopadhyay 2018; Probst, Njapau, and Cotty 2007). In a different study conducted in Tunisia, 85.7% of corn samples were contaminated with AFGs, which suggests the involvement of *A. parasiticus* fungi, whose occurrence was confirmed by species- specific polymerase chain reactions (Jedidi et al. 2017). Several solutions have been recommended and used to mitigate AFs contamination. In Kenya and other sub-Saharan Africa countries, the International Institute of Tropical Agriculture (IITA) is championing use of AflaSafe, a form of biological control to mitigate AFs contamination of corn before harvest (Bandyopadhyay et al. 2016). In addition to preharvest prevention of AFs contamination, post-harvest methods such as effective sorting, proper drying, and storage that limits AFs producing fungi to thrive are effective strategies in controlling AFs contamination (Bandyopadhyay et al. 2016; IARC Working Group Reports 2015). Manual sorting is commonly practiced in Kenya, but subsistence farmers tend to sell healthy looking kernels and store the discolored kernels for own consumption (Bandyopadhyay et al. 2016).This increases the predisposition of consuming aflatoxin contaminated corn in households. Moreover, industries that perform automatic sorting use discarded kernels to manufacture animal feed which ultimately gets in the food chain through consumption of animal products such as milk and meat. Effective control of

AFs contamination of food supplies is resource intensive and will require expertise that may not be available for small scale holder farmers in most of the developing world.

Conclusions

In the current study, the presence of AFs in food products in households indicates an elevated risk of exposure to populations in Siaya and Makueni Counties. Due to the widespread nature of AFs contamination in Kenya, it is important to educate small scale holder farmers on prevention and mitigation of AFs contamination. This should be done in addition to good agricultural practices combined with frequent surveillance.

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Table 4.1. Method Verification Parameters

Analyte	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%)	RSD (%)	*Retention Time
AFB_1	0.036	0.12	89.4	8.56	9.46
AFB_2	0.001	0.01	94.1	8.73	9.16
AFG_1	0.002	0.02	87.7	6.95	8.72
AFG_2	0.001	0.01	92.3	9.26	8.31

Table 4.2. The Levels (μg/kg) of AF contamination in corn products (n=338).

Variable	GM (95%CI)	Mean ± SD	Median	Range
AFB_1	57.9 (51.0 – 64.7)	76.2±63.9	59.3	1.69 – 403
AFG_1	0.58 (0.44 – 0.73)	0.99±1.11	0.62	0.01 – 8.38
AFB_2	0.40 (0.31 – 0.48)	0.66±0.79	0.45	0.06 – 5.83
AFG_2	0.17 (0.08 – 0.25)	0.47±0.79	0.23	0.01 – 7.01
Total	59.6 (52.8 – 66.5)	77.9±64.3	61.8	2.14 – 411

Table 4.3. Difference in AFs contamination levels between kernels and flour samples

	n	GEOMEAN (95% CI)	Mean ±SD
All samples	338	59.6 (52.8 – 66.5)	77.9±64.3
Flour	173	60.2 (50.0 – 70.2)	79.4±67.6
Kernels	165	59.0 (49.7 - 68.3)	76.3±60.9
Makueni Samples	242	62.5 (53.7 – 71.4)	83.1±70.1
AFB_1 in Flour	120	59.0 (44.9 – 73.1)	85.6±78.2
AFB_1 in Kernels	122	62.5 (51.8 – 73.1)	77.4±59.8
Siaya Samples	96	52.8 (43.9 – 61.7)	64.7±44.2
AFB_1 in Flour	53	41.4 (34.5 – 48.2)	47.4±25.4
AFB_1 in Kernels	43	66.6 (50.9 – 82.3)	82.0±51.9

Table 4.4. Distribution of total AFs in corn products from Kenya, East Africa.

Sample Matrix	Number of Samples in the Range (µg/kg)						
	<9.9	10.0 – 19.9	20.0 – 39.9	40.0 – 59.9	60.0 – 79.9	80.0 – 99.9	>100
Flour	4	8	38	35	24	20	45
Kernels	1	3	19	36	39	28	39
All Samples	5	11	56	71	63	48	84

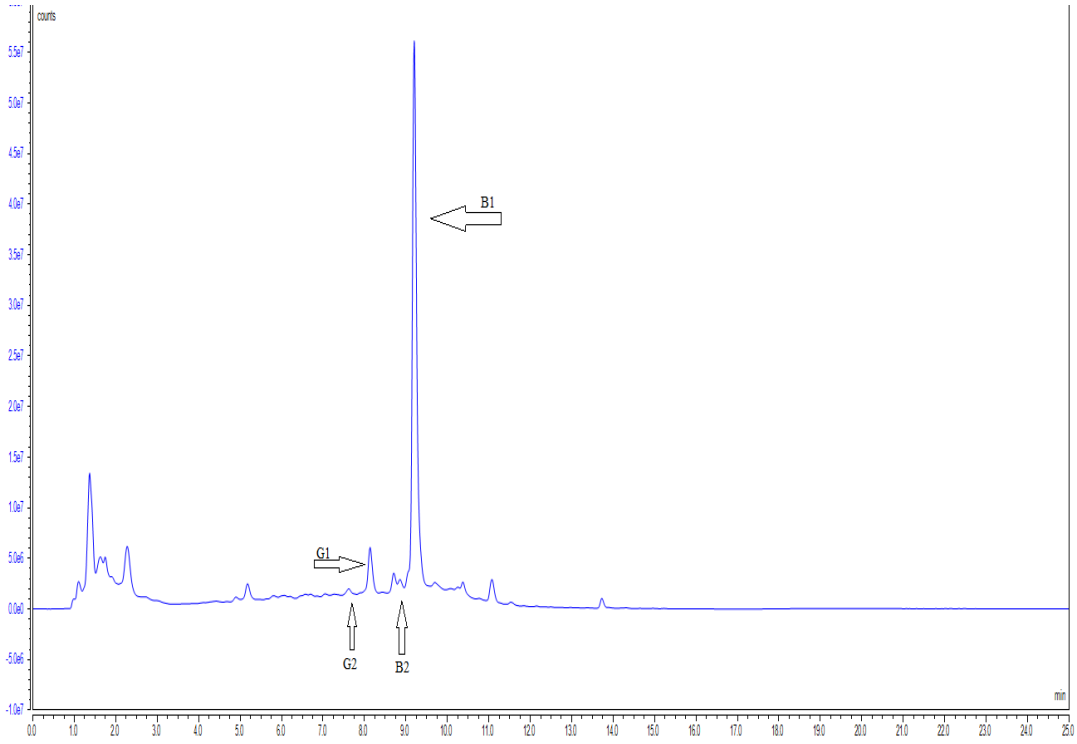

Figure 4.1. Sample Chromatogram for AFG_2, G_1, B_2 and B_1

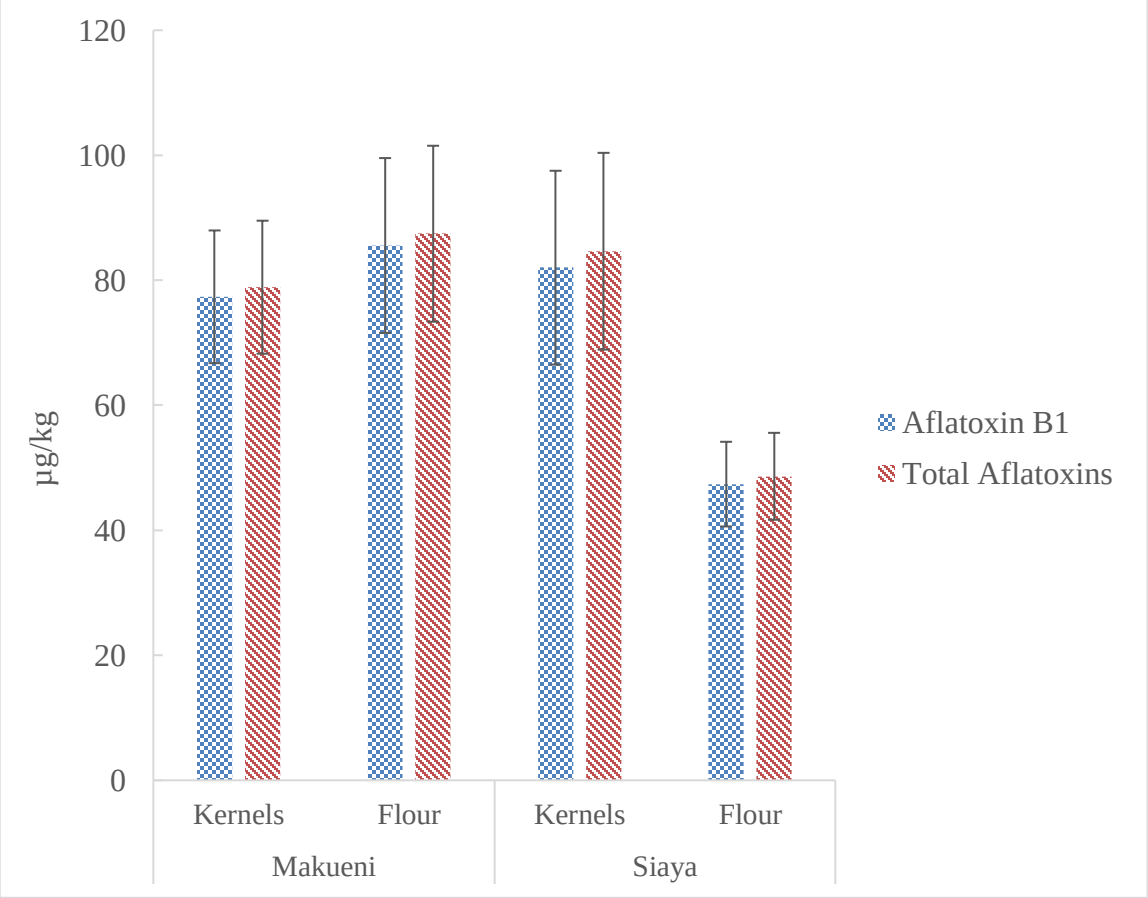

Figure 4.2. Aflatoxin levels by Region and Type of Sample

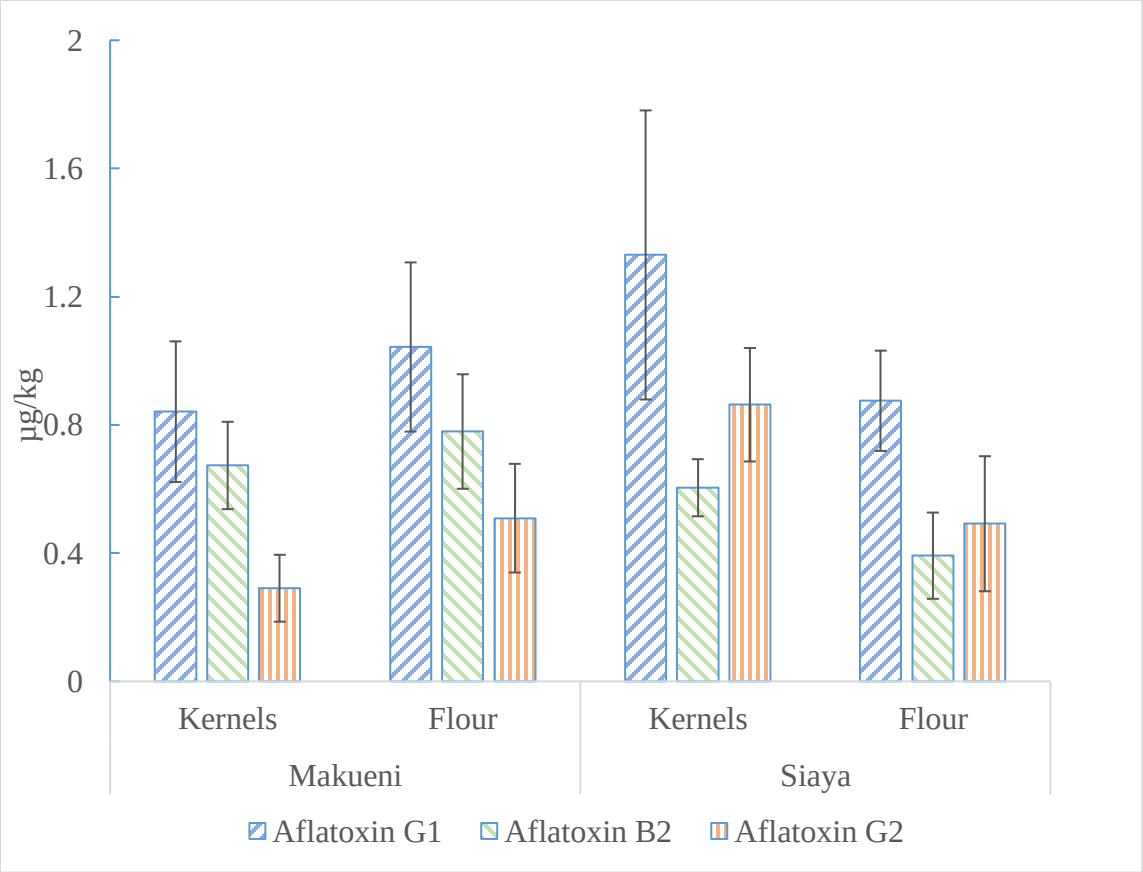

Figure 4.3. Mean levels of AFs B_2, G_1 and G_2

Chapter 5

DETERMINANTS OF AFLATOXIN EXPOSURES IN KENYAN CHILDREN [3]

[3] Ruth Nabwire Wangia-Dixon, Trang Ho Thu Quach, Xiao Song, James Ombaka, David Peter Githanga , Omu Aggrey Anzala, and Jia-Sheng Wang . To be submitted to The Journal of Mycotoxin Research

Abstract

Mycotoxins are naturally occurring food contaminants produced by fungi in the *Aspergillus, Penicillium* and *Fusarium* genera. Aflatoxins (AFs) are a group of mycotoxins known to contaminate staple food items including cereals, legumes, milk, meat products, spices, and tree nuts. The maximum limit for total AFs for foods destined for human consumption range from 2 - 20 μg/kg. In children, continuous consumption of aflatoxin contaminated food products is linked to immune suppression, vaccine interference and growth faltering while in adult populations, carcinogenesis in the liver has been established. In this study, we assess the determinants of AFs contamination in children recruited from households of Makueni and Siaya Counties. A five-part questionnaire was used to collect basic demographic information, socio-economic indicators, farming practices, household dietary patterns, and health status of family members. Aflatoxin B_1 lysine adducts in children's sera and total aflatoxins in household food samples were analyzed by High- Performance Liquid Chromatography with Fluorescence detection. Chi-squared tests and Kruskal-Wallis tests were used to evaluate the associations. Overall, children from low income households had the highest AFs exposure, p-value=0.0029. There was no significant difference in children's aflatoxin exposure levels between middle- and high-income households. Smaller family size, having more than three meals per day, and good farming practices were associated with low aflatoxin exposures $p<0.001$. Therefore, despite widespread contamination of AFs in rural households, the worst affected are individual households with higher levels of poverty.

Keywords: aflatoxins, children, determinants, socio-economic factors.

Introduction

Aflatoxins are a group of naturally occurring food contaminants produced by the soil-borne *aspergillus spp* fungi mainly *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins contaminate a variety of food crops including cereals, legumes, oilseeds, nuts, milk, meat products, spices, coffee and tea (Bandyopadhyay, Kumar, and Leslie 2007; CAST 2003). It is estimated that more than 25% of the world's food supply is contaminated by aflatoxins and up to 4.5 billion people are exposed (Smith et al. 2015; Williams et al. 2004). The aflatoxins producing *Aspergillus* species are prevalent in areas with hot, humid climates, including Sub-Saharan Africa and South East Asia (Okoth 2016). Additionally, countries in temperate and colder climates import foods from areas where aflatoxin exposure is chronic and thus aflatoxins is a worldwide concern (Wagacha and Muthomi 2008; Wu 2015; Wu, Guclu, and Montoya 2012). Aflatoxin contamination can occur at any or every stage of the food supply chain from pre-harvest, to post-harvest stages including drying, storage, marketing and distribution (Dembedza et al. 2019; Kang'ethe et al. 2017; Villers 2014). Therefore, aflatoxin contamination of food supplies presents a persistent challenge to food safety.

Mycotoxin contamination of food supplies is a major barrier to trade. Aflatoxins is estimated to contribute up to 670 million dollars in lost revenues in Africa (Okoth 2016; Wagacha and Muthomi 2008). The easily accessible European market has a strict maximum limit of 4 µg/kg for total aflatoxins for foods (European Commission 2006). When trade occurs, the least contaminated foods are exported leaving the highly contaminated products for household consumption by the population that is already at a greater risk of aflatoxin exposures (Lewis et al. 2005; Strosnider et al. 2006). Furthermore,

subsistence farmers sell better looking grain locally and store low grade products for own household consumption in many parts of Africa (Strosnider et al. 2006; Wagacha and Muthomi 2008). Moreover, rejected contaminated foods are often converted to feed for farm animals and ultimately, aflatoxin metabolites are present in meat and milk products (Kana et al. 2013; Kang'ethe and Lang'a 2009; Martins, Mendes Guerra, and d'Almeida Bernardo 2007). Therefore, in Africa and many parts of the developing world, access to safe and nutritious foods is almost impossible due to widespread contamination of food supply by aflatoxins (JECFA 2018; Moser and Hoffmann 2015; Wagacha and Muthomi 2008). In a recent study, exposure to aflatoxins was shown to interfere with hepatitis B vaccine efficiency, with children who had higher aflatoxin-albumin adducts did not develop protection against Hepatitis B viral infections (Githang'a, Anzala, et al. 2019; Githang'a, Wangia, et al. 2019). The aim of the current study is to determine factors that make populations at risk to aflatoxin contamination, with the goal of informing appropriate policy action to protect public health.

Since their discovery in the 1960s, aflatoxins have been established to be carcinogenic, mutagenic, immunotoxic, genotoxic and a major contributor to growth faltering in children in low and middle- income countries (IARC 2002, 2015).There are four main types of naturally occurring Aflatoxins namely Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1) and Aflatoxin G_2 (AFG_2) which are broadly classified as difurocoumarocyclopentenone series (B-types) or difurocoumarolactone series (G types) (Wilson 1989; Park et al. 1990). Aflatoxins are intensely fluorescent to ultraviolet light and both B and G classifications are based on emission of blue and green-yellow fluorescent lights respectively (Asao et al. 1963; Wilson 1989; Park et al. 1990). Toxicity,

carcinogenicity and mutagenicity are in the order $AFB_1 > AFB_2 > AFG_1 > AFG_2$ as shown by experimental studies in animals to illustrate LD50, the lethal dose that causes death in 50% of exposed subjects (Li, Zhang, and Zhang 2009). Nonetheless, AFB_1 is the most potent with significant adverse health and economic effects. When compared to different toxic compounds, AFB_1 is 10 times more toxic than potassium cyanide, 68 times than arsenic and 416 times more toxic than melamine (Li, Zhang, and Zhang 2009; Wogan, Edwards, and Newberne 1971).

Main determinants of aflatoxins and other mycotoxins exposure levels in rural households include environmental factors, farming practices, food production processes and socio-economic status (Gong et al. 2003; Jolly et al. 2006; Muema et al. 2018; Shuaib et al. 2012; Wild et al. 2000). In this study, we evaluate the major determinants of aflatoxins exposure in Kenyan children recruited from Siaya and Makueni Counties of Kenya. Makueni county is a known hotspot for aflatoxins exposures and deaths from aflatoxin related poisonings have been reported (Awuor et al. 2017; Daniel et al. 2011; Lewis et al. 2005; Yard et al. 2013). On the contrary, no prior documentation of aflatoxins both in food supplies and in human biological specimens have been reported from Siaya County. In a serological survey however, aflatoxins were detectable in all archived serum samples from Makueni while aflatoxin exposure levels were below the limit of detection for samples collected from Siaya (Yard et al. 2013). The choice of Makueni and Siaya Counties as study locations provide control for differences in weather conditions, ethnicity, variation in aflatoxins exposure and poverty levels.

Methods

This study forms part of a larger cross-sectional study designed to establish the extent of aflatoxins exposure in children population and the associated health outcomes. Detailed description of the study setting, rationale, and significance is provided in the study protocol (Wangia et al. 2019). The Joint Ethics committee of the University of Nairobi and Kenyatta National Hospital of Kenya approved the study protocol and procedures. Randomized multistage stratified sampling design was used in the identification of schools, and selection of study participants, mainly children between the ages of six years and twelve years and their parents/guardians. The school administrations of selected schools were requested to convene parent meetings at a convenient time and location. The study purpose and significance were explained to school officials, teachers, support staff and parents at the meetings presided by the chairperson of parents and teacher's association of each respective school. The meetings were open to the public, and community leaders were in attendance. Full disclosure of study details was given to all participants before completing informed consent forms. Parents who filled out informed consent forms were invited to complete a five-part questionnaire that collected basic demographic information, socio-economic indicators, farming practices, household dietary patterns, and health status of family members.

In addition to parental consent, children's assent was required and about 6-8ml of blood was obtained for aflatoxin biomonitoring. Children's sera were analyzed for AFB_1-lysine adducts using High Performance Liquid Chromatography with Fluorescence Detection as reported previously (Kang et al. 2015; Wang et al. 2001). For statistical analyses, SAS v9.4 (Cary, NC), R version 3.6.1 (Vienna, Austria) and Microsoft Excel 365

Office statistical tools were used. The level of significance was set at p-value ≤ 0.05. Characteristics of study population was summarized with proportions for categorical variables and with median and interquartile ranges (IQR) for continuous variables. Distribution of AFB_1-lysine adduct by different variables was summarized as continuous (geometric mean and median) and categorical variables (proportion). Descriptive statistics, t tests and Analysis of Variance for the variable AFB_1-lysine classified by different categorical variables were determined. Kruskal Wallis test was used to determine significant associations between categories if any. Chi-squared tests were applied to accept or reject independence between AFB_1-lysine and different variables. In the tests for independence, the global hypothesis test revealed that the groups were significantly different from each other.

Results

The baseline characteristics of children and mothers in the study population is shown in Figure 5.1. Generally, up to 811 mothers were interviewed with respondents varying in age. The median age of mothers enrolled in the study is 36 years Interquartile Range (30, 43) while the average age by which most mothers had their first child is 20.3 years. More than 85% of mothers started weaning children before the recommended age of 6 months. The average number of children per woman are 4 children, Interquartile Range (3 - 5). The overall median family size is made up of 6 people, Interquartile Range (5 - 7) with bigger family sizes of up to 7 in Siaya County. An overall 79.8% of mothers were married and only 28.4% had more than 8 years of formal education.

The majority of the participants (62.3%) were farmers while others were teachers, nurses, police officers, other civil servants, petty traders and/or unemployed. The study

population was made up of subsistence farmers, who grow food mostly for household use. Among these, 72% use pesticides and/or fertilizer during farming. After harvest, 39.4% in Makueni and 93.0% in Siaya store maize in maize sacks. Even though 59.8% of participants were knowledgeable about aflatoxins, more participants in Makueni were knowledgeable on how to identify aflatoxins and good agricultural practices known to mitigate widespread aflatoxin contamination in food products. While more than half (51.3%) of survey respondents reported low monthly incomes of less than KES 5000 (~USD 50), all things considered, most participants perceive their life to range from fair to good. There were significant differences among the variables between Makueni and Siaya Counties, <0.001.

The distribution of aflatoxin contamination is highly variable by County. In the current study, among children recruited from Siaya County of Nyanza province, the median levels of AFB_1-lysine adduct were 9.01 pg/mg albumin; and 13.19 pg/mg albumin among children of Makueni county of Eastern Province as shown in Figure 5.1. These exposure levels are significantly higher than previously reported AFB_1-lysine adducts among adult population in other regions in Kenya. A great variation of aflatoxins exposure levels observable by school and village of residence where sampling took place with higher levels of exposures evident in Makueni County as shown in Figure 5.2. Greater variation in exposure levels was observed in Makueni. For instance, schools within villages with lowest exposure levels such as median level of 3.79pg/mg of albumin 95%CI (2.78, 4.79) while villages with highest level of exposure of 36.47 pg/mg of albumin were in Makueni (Table 5.3). All schools within villages in Siaya had median exposure levels below 12.0pg/mg

albumin (Figure 5.2 and Table 5.3).Moreover, all children were exposure to aflatoxins irrespective of Age as shown in Figure 5.4.

Aflatoxin exposure is widespread in both counties irrespective of socio-economic status (Figure 5.3). However, higher exposure levels are evident in Makueni County across all socio-economic strata compared to Siaya. Additionally, when evaluated in terms of contamination levels in household grain, low income households had the highest aflatoxins exposure, p-value=0.0029 while there was no significant difference between middle- and high-income households. In Makueni, lower income households are worse off with high aflatoxins in household grains and corresponding higher AFB_1-lysine adducts among children, compared to Siaya County, p-value=0.0036.

The main determinants of aflatoxin exposure are shown in Table 5.2. Overall, number of children and family size as determinants of aflatoxins exposure is confounded by regional differences, p-value > 0.05. When stratified by county, the effect of family size is more pronounced in Makueni county, p-value =0.0295. Families with less than 4 children and less than 6 family members on average had lower aflatoxins levels in sera compared to bigger families. Families who have less than three meals a day have higher aflatoxins exposure, marginally significant for Makueni County, p-value=0.0704.

Farming households had lower aflatoxins exposure while households that rely on purchased maize generally had higher aflatoxins exposure levels. Nonetheless, aflatoxins contaminations levels of 59.45µg/kg is exceedingly high at almost 6 times more than the recommended maximum limit of 10 µg/kg. The effect of good agricultural practices in farming is more pronounced in Makueni County, p-value=0.0001. Families that grow maize have lower aflatoxins exposure, an indication of small production volumes and

insufficient produce, and therefore, less likelihood for storage, p-value=0.0792, marginally significant overall but very significant for Makueni County p-value=0.0236. Farming households that reported use of pesticides and/or fertilizer, which serves as an indicator for good farming practices reported lower household aflatoxins levels even though not statistically significant. Maize storage in sacks is associated with high aflatoxins exposure, and is more paramount for Makueni county, p-value=0.0257. Aflatoxin knowledge and ability to identify aflatoxins was also associated with lower levels of mycotoxins contamination among households.

Discussion

The frequency of aflatoxin exposures is dependent on geographic location, household wealth, agricultural practices, and a combination of dietary diversity and food sufficiency. Aflatoxin exposure has been shown to vary by geographic location in a previous study in Kenya (Yard et al. 2013). The results of the current study indicate that children below the age of 12 years are more susceptible to higher aflatoxin exposures compared to adolescents and adults aged 15 – 64 years (Figure 5.1).In the current study, exposure levels among children below 12 years were exceedingly high with median levels of 9.01 pg/mg albumin and 13.19 pg/mg albumin for Siaya and Makueni Counties respectively. In a previous study, 600 sera samples obtained from participants aged 15 – 64 years were analyzed for AFB_1-lysine adducts using High Performance Liquid Chromatography with isotope-dilution tandem mass spectrometry and exposure levels were significantly lower when compared to the present study (McCoy et al. 2005).

As shown in Figure 5.1, AFB_1-lysine adducts in participants from other regions within Kenya were significantly lower with median values of 3.70, 2.44, 2.33, 1.40, 1.28

and 0.70 pg/mg albumin for Coast, Nairobi, Central, North Eastern, Western and Rift Valley provinces respectively (Yard et al. 2013). Furthermore, sera from Eastern province, where Makueni is located had the highest AFB_1-lysine adducts, median of 7.87pg/mg albumin while none of the samples from Nyanza, where Siaya forms part of six counties had detectable AFB_1-lysine adducts (Yard et al. 2013). Lower levels of AFB_1-lysine in Siaya County schools and villages are attributed to favorable weather conditions compounded by good agricultural practices which are associated with lower mycotoxin exposure levels. Furthermore, schools within villages of Makueni County with low levels of aflatoxins exposure reported having participated in previous aflatoxins training and awareness campaigns, and/or use of AFlasafe as biocontrol in addition to good agricultural practices (Bandyopadhyay et al. 2016; Mutegi et al. 2018).

Differences in aflatoxin contamination levels could also be attributed to ethnicity, as Siaya County is mainly inhabited by Luo while Makueni by the Akamba communities (Wangia et al. 2019). Ethnicity and village location have been previously reported as predictors of aflatoxin exposure in households (Jolly et al. 2006). Similar differences in exposure levels attributed to ethnicity has been previously reported in Gambia, Thailand, Bangladesh and Malaysia (Groopman et al. 2014; Leong et al. 2012). These studies indicate that ethnicity can influence aflatoxin metabolism with respect to genetic polymorphism in cytochrome P450 and glutathione-S-transferase enzymes (Wild et al. 1992). Nonetheless, further studies are warranted to determine differentiation or polymorphism in the CYP450 genes in respect to aflatoxins metabolism among different ethnicities.

In addition to biomonitoring, food samples provided by study participants were highly contaminated by aflatoxins. Higher levels of contamination were reported in

Makueni with geometric mean of total aflatoxins in all samples from Makueni County of 62.5 μg/kg with (95% CI: 53.7, 71.4) while in Siaya County is 52.8 μg/kg with (95% CI: 44.0, 61.7) was reported (Nabwire et al. 2019). Good farming practices and use of fertilizers are also associated with lower aflatoxin exposure levels. In a previous study, the levels of aflatoxins in maize samples were 3.9 times higher than 10μg/kg in farms that used fertilizers at planting, $p<0.05$(Njeru et al. 2019). In Makueni County, drought conditions stress plants and render them susceptible to aflatoxins contaminations, further exacerbated by late harvest, and high ambient humidity (Probst, Njapau, and Cotty 2007; Probst, Schulthess, and Cotty 2010; Wagacha and Muthomi 2008). In addition to hot and humid climate conditions, other determinants of aflatoxins exposure include erratic weather patterns as previously reported in Kenya and Serbia (Janic Hajnal et al. 2017; Nabwire et al. 2019). Makueni County is particularly worst affected due to plant stress from drought, which is established to promote colonization of food items with the *A. flavus* fungi (Okoth 2016; Probst et al. 2010). Taken together, aflatoxin contamination is variable by geographic conditions, weather patterns of the specific locale and good agricultural practices.

In the past, the socio-economic status of participants and the level of AFB_1-lysine have been statistically related, but the various studies do not report consistent findings (Adejumo et al. 2013; Egal et al. 2005; Shirima et al. 2015). In the current study, aflatoxins exposure is predominant with higher exposures evident in children recruited from Makueni County compared to Siaya County irrespective of socio-economic status.

In a study conducted in Western Kenya, the socio-economic and agronomy factors that promote co-occurrence of aflatoxins and fumonisins were limited aflatoxin related knowledge of prevention strategies, food insecurity and poverty (Mutegi, Cotty, and

Bandyopadhyay 2018; Mutiga et al. 2015; Njeru et al. 2019). Participants who have higher incomes are less likely to experience high aflatoxins in household food products. A previous study among women in Meru Kenya reported that women with higher household expenditure, better food security, and land ownership, use of fertilizers and higher level of education which translates to higher household incomes are generally exposed to low aflatoxins levels (Hoffmann, Jones, and Leroy 2015; Leroy, Wang, and Jones 2015). Moreover, epidemiological studies in Ghana by Shuaib et al established that individuals with higher incomes and education levels more likely have access to relevant information and may be aware of health risks linked to consumption of aflatoxins contaminated food products (Jolly et al. 2011; Obuseh et al. 2010; Shuaib et al. 2012).Therefore, having sufficient financial resources improves an individual's ability to afford higher quality foods because safe foods are likely to cost more. It is possible that participants in our study may not have the purchasing power to pay extra for nutritious foods and instead settle for the cheaper commodities.

Number of children and family size are determinants of household aflatoxins exposure. A small study in Ghana found a significant negative association between household size and aflatoxins contamination levels (Jolly et al., 2006). Family size is a surrogate for available financial resources per head and bigger families have to stretch available resources and thus, is significantly associated with higher aflatoxin exposures. Seasonal variation in aflatoxins contamination levels has been previously reported where aflatoxins tend to be lower in the dryer season compared to rainy season (Jolly et al. 2015; Gong et al. 2004). The months of May, June and July are part of the growing season; therefore, participants are likely eating freshly harvested crops from previous seasons.

It has been established that storing produce for more than 3 months is associated with higher household aflatoxins contamination (Hell et al. 2000). Traditionally, maize and other food products were stored in outdoor elevated granaries with adequate aeration to limit proliferation of fungi and aflatoxin contamination (Azziz-Baumgartner et al. 2005; Strosnider et al. 2006). However, food insecurity, lack of awareness and limited technological advancement have contributed to non-conventional methods of storing food products which further promotes aflatoxin contamination. In the current study, after harvest, 39.4% in Makueni and 93.0% in Siaya store maize in maize sacks. In Makueni, 46.8% prefer agro-bags, a multi-layered hermetic storage bag designed to reduce post-harvest losses associated with damage from pests and insects to further prevent contamination by aflatoxins (Dembedza et al. 2019). Farming households had lower aflatoxins exposure while purchased maize had higher aflatoxins exposure levels. It is possible that households that purchase maize are more likely to be exposed to higher mycotoxin exposures. This is mainly because traders hoard maize waiting for prices to go up. A negative association between the price of commercial maize brands and aflatoxin contamination levels have been previously reported (Moser and Hoffmann 2015). A study in Ghana reported that participants purchasing over 20% of food products, storing about 25% of produced maize experienced significantly higher levels of aflatoxins contamination (Jolly et al. 2015). The storage of cereal grains in large quantities over long periods of time promote fungal proliferation given the hot humid weather conditions and sub-optimal storage capacity in the developing world, thereby promoting proliferation of mycotoxin generating fungi (Hell et al. 2000; Jolly et al. 2015). In farming households, the volume of production is generally small and therefore, not much of the produce is left over for long-

term storage. This is an indication that food insecurity is prevalent in these rural households.

Being knowledgeable about aflatoxins is not significantly associated with aflatoxins in household grain, an indication that despite being knowledge, participants may not have expertise to prevent aflatoxins contamination (Leroy, Wang, and Jones 2015). For example, in a Kenyan study with fairly literate participants, there was no significant associations between knowledge of mycotoxins and reduction or prevention strategies (Njeru et al. 2019). Moreover, food insecurity in the region encourages farmers to store food supplies in hot humid households instead of an outdoor granary for the fear of theft, and storage under sub-optimal conditions promote the proliferation of aflatoxins producing *aspergillus* fungi to thrive.

Conclusion

In Africa and most parts of the developing world, access to safe and nutritious foods is almost impossible due to widespread contamination of food supply by not only aflatoxins but also other mycotoxins. In our study populations where corn is a staple for many households, participants recruited from Makueni County were more likely to have higher aflatoxin exposure levels compared to participants who reside in Siaya County. The main determinants of aflatoxins exposure in children include environmental factors, farming practices, food production processes and socio-economic status. Number of children and family size are also determinants for aflatoxins exposure. Intervention strategies to mitigate aflatoxin exposures must focus on poor households and/or communities that rely on subsistence farming for dietary staples.

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Table 5.1. Baseline Characteristics of children and mothers in the study population

Characteristics		N (%)
Age of child	Median (IQR)	9.0 (8.0 to 11.0)
Age of the mother	Median (IQR)	36 (30 to 43.0)
Sex of the child	Male	372 (45.9)
	Female	439 (54.1)
Wean start at month	Median (IQR)	6.0 (5.0 to 6.0)
No. meal/day	Median (IQR)	3.0 (2.0 to 3.0)
Number of children	Median (IQR)	4.0 (3.0 to 5.0)
Family Size	Median (IQR)	6.0 (5.0 to 7.0)
Marital Status	Married	657 (82.3)
	Not Married	141 (17.7)
Formal Education	Pre/Primary	565 (70.7)
	Secondary/Post	234 (29.3)
Household Income	Low	422 (54.9)
	Middle	202 (26.3)
	High	145 (18.8)
Living Conditions	Good	93 (11.9)

	Fair	547 (69.9)
	Poor	142 (18.2)
Mothers Occupation	Farmer	513 (63.2)
	Other	298 (36.7)
Spouse Occupation	Farmer	359 (44.3)
	Other	451 (55.7)
Grow maize	Yes	719 (90.9)
	No	72 (9.1)
Pesticide or fertilizer use	Yes	567 (72.0)
	No	220 (28.0)
Aflatoxin knowledge	Yes	474 (59.8)
	No	319 (40.2)
Identify aflatoxins	Yes	433(47.8)
	No	474 (52.3)

†IQR – Inter Quartile Range; #not married includes single and widowed mothers, †Formal education is based on the Kenya's 8 years of primary, 4 years of secondary and 4 years of post-secondary education, thus pre/primary refers to all mothers who got formal education for 8 years or less and secondary/post refers to all mothers who received more than 8 years of formal education. ⁕the respondents perception of their daily life in general, ǂOther occupation included teachers, nurses, police officers, other civil servants and petty traders††Household Income amounts in Kenya Shillings(KES), 100KES = 1USD – cutoffs based on frequency distribution of incomes in populations sampled where mothers who reported monthly income of less than KES 5,000 were categorized as low, >5,000 ≤10,000 were categorized as middle income and monthly income levels of more than KES 10,000 were categorized as high income earners.

Table 5.2. Determinants of /Association between log serum aflatoxin lysine adducts

Mother characteristics	n	Median (95%CI) pg/mg albumin	Geomean (95%CI) pg/mg albumin	Mean pg/mg albumin	X^2	p-value
Weaning Start						
< 6 months	254	10.76 (9.06, 12.46)	10.64 (9.51,11.91)	20.08	0.16	0.69
>6 month	464	10.36 (9.03, 11.70)	10.41(9.54, 11.37)	23.12		
No. Meal/day						
<3	281	9.42 (8.12,10.73)	9.43 (8.49,10.48)	19.60	4.52	0.03
>=3	459	11.29 (10.01,12.57)	11.03 (10.1,12.04)	23.01		
Marital status						
Married	602	10.19 (9.06, 11.31)	10.26(9.51, 11.06)	21.11	0.50	0.48
Not married	131	12.27 (10.44,14.10)	10.74(9.27, 12.44)	19.58		
Formal Education						
Pre/primary	512	10.08(8.98,11.18)	10.06(9.27,10.93)	19.46	6.41	0.09
Secondary/ post	222	11.67(9.82,13.51)	10.33(9.02, 11.83)	22.44		
Household Income						

Low	383	9.90 (8.59, 11.20)	9.65 (8.84,10.54)	18.23	4.56	0.10
Middle	189	9.75 (8.41,11.10)	9.59 (8.50,10.82)	17.35		
High	133	13.32 (9.50,17.13)	12.14 (10.0,14.74)	29.11		
Living Conditions						
Good	84	11.72 (9.87,13.57)	11.52 (9.27,14.30)	24.99	3.75	0.15
Fair	502	9.75 (8.51,10.98)	9.96 (9.16,10.84)	21.51		
Poor	131	12.28 (10.15,14.41)	11.29 (9.93,12.85)	16.70		
Grow Maize						
Yes	662	10.28 (9.20,11.36)	10.24 (9.53,11.01)	21.19	1.55	0.21
No	64	11.93 (10.29,13.56)	12.27 (9.98,15.09)	21.65		
Pesticide/Fertilizer Use						
Yes	527	10.39 (9.11,11.66)	10.21 (9.42,11.06)	21.21	0.47	0.49

No	195	10.44 (8.90,11.98)	11.11 (9.76,12.64)	21.59		
Aflatoxin knowledge						
Yes	448	11.90 (10.64,13.17)	11.74(10.72,12.85)	25.46	11.97	0.0005
No	280	9.33 (8.02,10.64)	8.48 (7.71, 9.33)	13.14		
Identify aflatoxins						
Yes	409	11.50 (10.18, 12.82)	11.3(10.25,12.46)	25.96	2.96	0.09
No	315	9.62 (8.38, 10.86)	9.23 (8.44, 10.09)	14.02		

Table 5.3. AFB_1-lysine Adducts for All Children by School Clusters.

*School	Median (Range) pg/mg albumin	GM (95% CI) pg/mg albumin
School 1	3.82 (0.65, 518.93)	4.86 (3.58,6.59)
School 2	11.56 (0.69, 132.15)	11.52 (9.40,14.11)
School 3	12.96 (6.22, 32.19)	12.91 (11.69,14.26)
School 4	14.41(1.77, 192.74)	13.62 (11.29,16.44)
School 5	16.93 (1.59, 320.59)	18.11 (11.34,28.91)
School 6	28.45 (0.84, 495.23)	33.37 (24.03,46.34)
School 7	43.19 (4.16, 269.64)	36.47 (25.2,52.8)
School 8	5.60 (0.85, 53.46)	5.78 (4.73,7.05)
School 9	5.66(1.03, 88.98)	5.91 (4.99,7.00)
School 10	9.37 (0.81, 87.94)	8.94 (6.44,12.42)
School 11	11.02 (0.86, 265.75)	9.15 (8.14,10.28)
School 12	11.87 (1.15, 49.84)	10.44 (9.02,12.08)

*Each school is located in different villages, identity of schools is restricted for ethical/privacy reasons

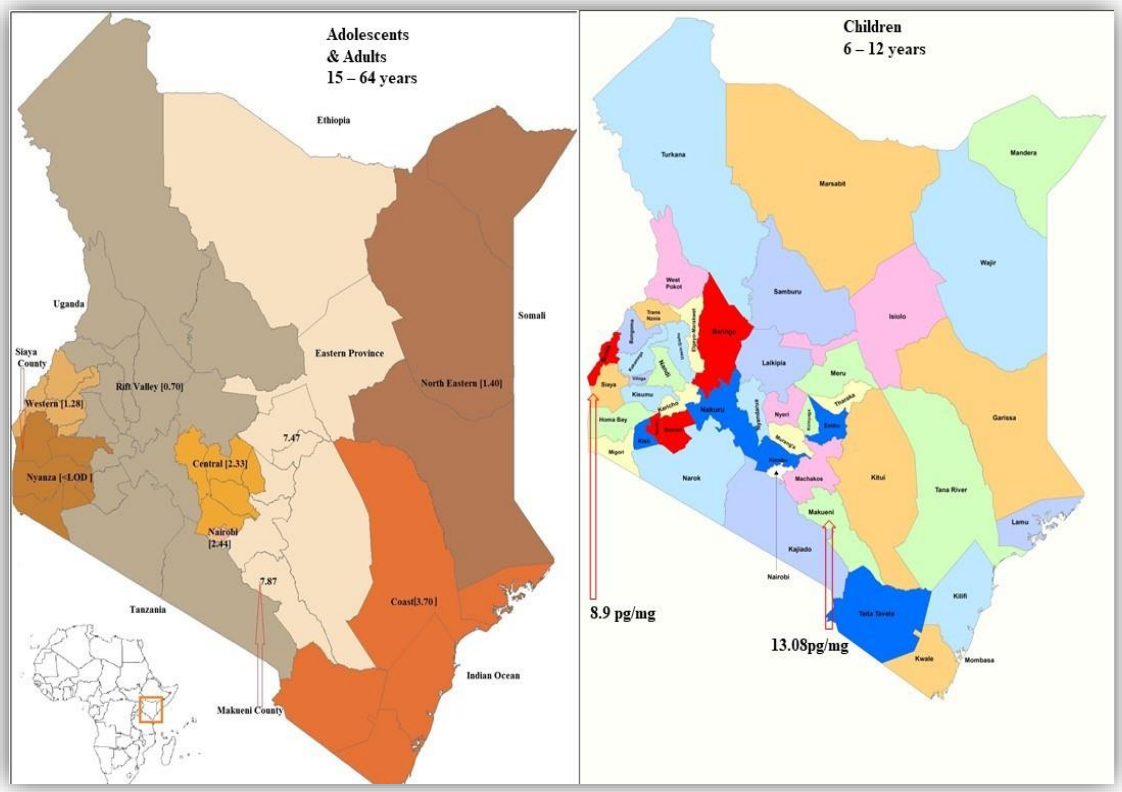

Figure 5.1. Regional Variation of AFB$_1$-lysine Adducts in Children

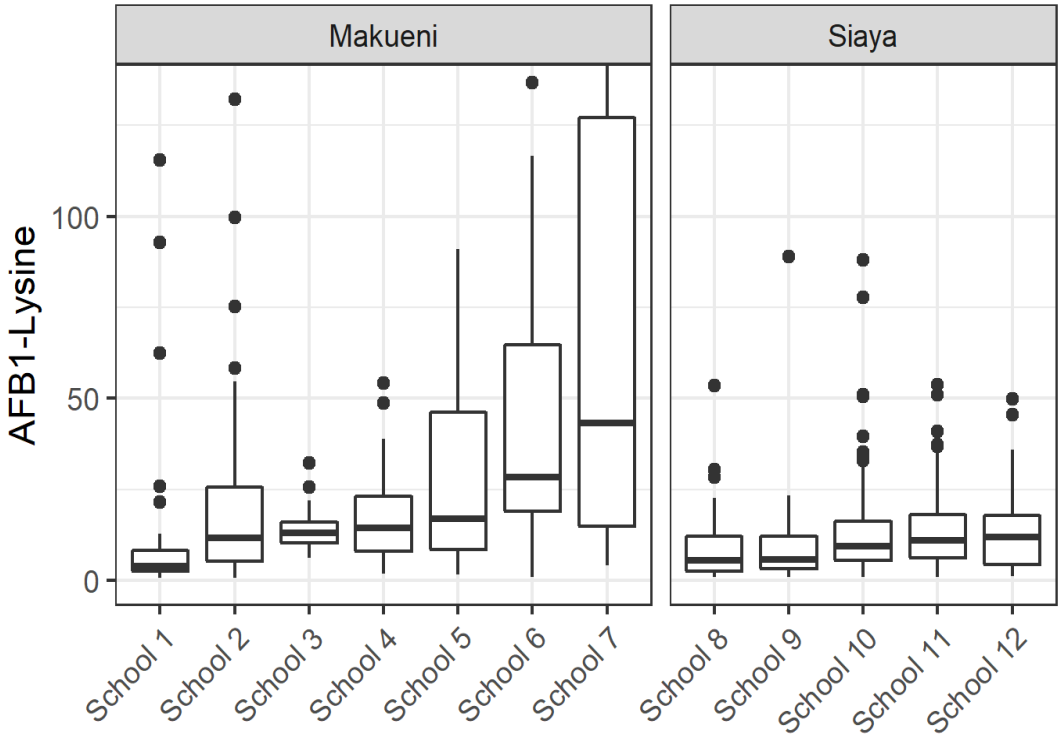

Figure 5.2. Variation of Aflatoxin exposure by school/village of sampling

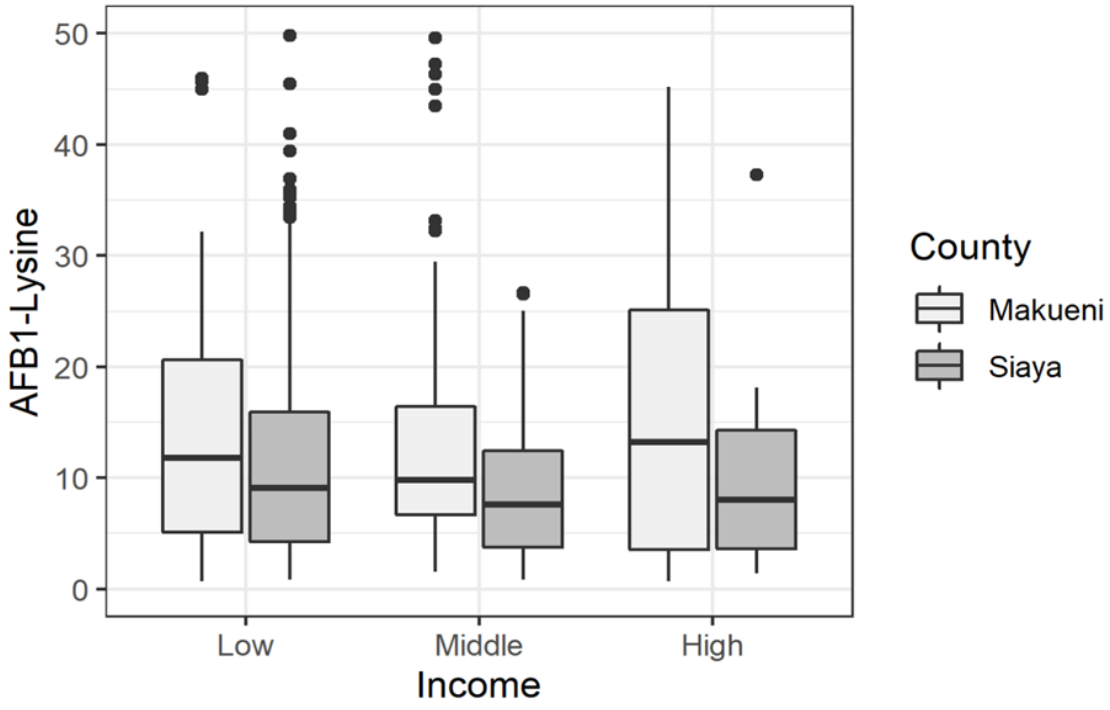

Figure 5.3. Aflatoxin exposure and Socio-Economic Status

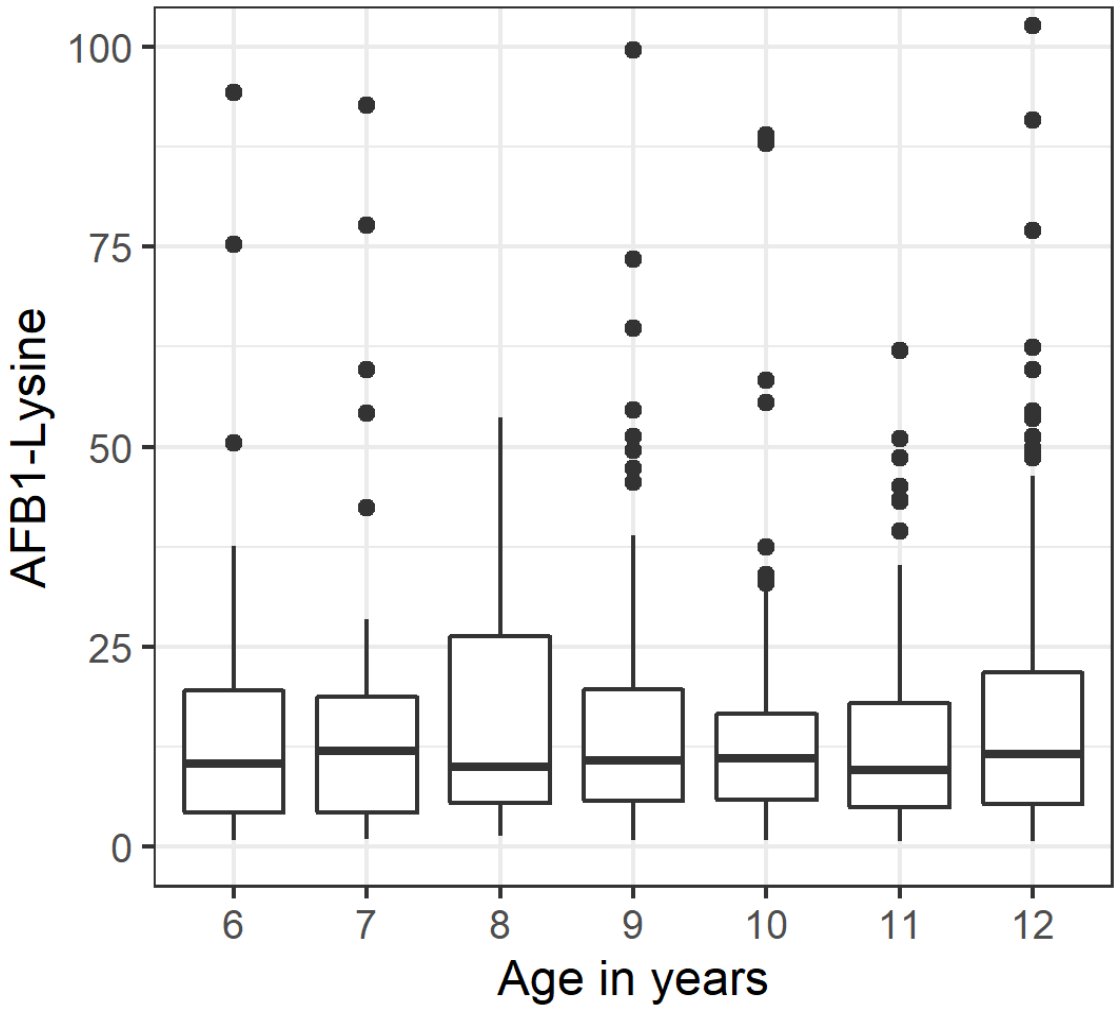

Figure 5.4. Variation of aflatoxin exposure with age

Chapter 6

NUTRITION AND GROWTH OUTCOMES ARE AFFECTED BY AFLATOXIN EXPOSURE IN KENYAN CHILDREN [4]

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Abstract

Aflatoxin exposure, malnutrition and growth impairment in children present significant public health problems in low- and middle- income countries. Recent epidemiology studies have shown that dietary exposure to AFs in early life contribute to impaired growth in children. However, these results remain inconsistent and inconclusive due to limited studies in high versus low AFs exposure regions. In this study, we conducted a cross-sectional study in Kenya, East Africa to evaluate the association between AFs exposure, malnutrition and growth impairment in children aged 6 to 12 years. AFB_1-lysine adducts was quantified using HPLC to assess AFs exposure. All children (n=746) had detectable levels of adducts in serum, ranged 0.65 - 518.9 pg/mg albumin with a geometric mean (GM) of 10.5 pg/mg albumin, and (95%CI 9.4 – 11.7). AFB_1-lysine adducts in children recruited from Makueni county, a high AFs exposure region, had GM of 14.0 pg/mg albumin with (95%CI 12.5, 15.7) and is significantly higher than Siaya's 8.2 pg/mg albumin with (95%CI 7.6, 8.8), ($p<0.001$). Adjusted multivariate analysis revealed that socio-economic indicators, farming practices, and household dietary patterns are associated with growth outcomes. Increasing one unit of AFB_1-lysine is associated with decreasing Weight-for-Age-Z score (WAZ) by -0.004, p=0.054) overall. In both Siaya and Makueni counties among children ages 6 – 9years, WAZ decreases by -0.15, p-value=0.013 and WHZ decreases by -0.43, p-value=0.041. Additional growth parameters did not reach statistical significance. These data suggest that efforts must be put in place to control for aflatoxin exposure in order to achieve better growth outcomes.

Keywords: AFB_1-lysine, Malnutrition, Stunting, Underweight, Wasting

Introduction

Aflatoxins (AFs) are naturally occurring secondary metabolites of *Aspergillus flavus, Aspergillus parasiticus* and other *Aspergilla* strains (CAST 2003; IARC 2002). AFs were originally discovered in the 1960s after the outbreak of the Turkey X disease in England which contributed to deaths of more than 100,000 turkey poults fed meals containing peanuts imported from Brazil (Lancaster, Jenkins, and Philp 1961; Sargeant et al. 1961). To date, up to 16 structurally related AFs have been identified and characterized, among which aflatoxin B_1 (AFB_1) is the most potent (Asao et al. 1963; Kensler et al. 2011; Spensley 1963). AFs contaminate a variety of food crops including cereals, legumes, oilseeds, nuts, milk, meat products, spices, coffee and tea (CAST 2003; IARC 2002). Overall, an estimated 25% of food crops are contaminated by AFs and other mycotoxins (Daniel et al. 2011; Sarma et al. 2017). The maximum limit of AFs in food for human consumption varies for different countries but the range is 2 – 20µg/kg for various foods (European Commission 2006; USFDA 2000; Wu, Guclu, and Montoya 2012). Significant economic losses have been reported. The United States spends an estimated 500 million USD in controlling AFs in peanuts, corn and other crops while the African continent loses up to 670 million USD annually in earned revenue due to rejection of AFs contaminated foods (Wu 2015; IARC 2015; JECFA 2018).

Worldwide, up to 4.5 billion people are unintentionally exposed to AFs through dietary sources. AF exposure can occur *in utero*, via breast milk, through weaning foods and throughout an individual's lifetime (Groopman et al. 2014; Shuaib et al. 2012). Other routes of exposure include inhalation and dermal exposures in occupational settings

(Wangia, Tang, and Wang 2019). AFB_1 is an established Group I human carcinogen linked to primary liver cancers (IARC 1993, 2002). More than 50% of primary liver cancer cases occurring in South East Asia is attributed to consumption of AFs contaminated food products, which is further confounded by Hepatitis B virus infection (Palliyaguru and Wu 2013). AFs are also immune toxicants, teratogens and mutagens, especially when exposure occurs in low quantities over a long period of time (IARC 1993, 2002). Short term exposure to AFs exceeding 20µg/kg is linked to AF poisoning named aflatoxicosis. Incidences of acute aflatoxicosis in animals and humans have been reported worldwide with the severity of poisoning dependent on species, age, sex and nutritional status of the exposed subjects. In animals, AFs toxicity results in gastrointestinal dysfunction, reduced feed utilization, anemia, jaundice and in most cases, hepatic necrosis (Di Gregorio et al. 2017; Sarma et al. 2017). Clinical manifestation of aflatoxicosis in human populations include haemorrhage, jaundice, liver cirrhosis, liver failure and ultimately death. The case fatality rate of aflatoxicosis reported in human populations ranges from 39 – 50% (Kamala et al. 2018; Ngindu et al. 1982). Human deaths from aflatoxicosis have been reported in India, Kenya and Tanzania (Kamala et al. 2018; Narsimha Reddy and Raghu Raghavender 2007; Probst, Njapau, and Cotty 2007). Human hepatocarcinogenesis resulting from long term exposure to AFs in low quantities has been well documented (Liu and Wu 2010; Magnussen and Parsi 2013). Additionally, rapid progression of infectious diseases such as HIV to AIDs has been reported in Ghana and Uganda (Jolly et al. 2011; Kang et al. 2015), and is majorly attributed to immune suppression and impaired liver function modulated by chronic exposure to AFs.

Recent epidemiology studies provide evidence that dietary exposure to AFs in early life is a contributing factor to malnutrition and impaired growth in children (IARC 2015). The classical use of anthropometric parameters to assess malnutrition in children is widely accepted because of the non-invasive nature of data collection. A child's weight and length/height is used to calculate weight-for-age-z-score (WAZ), length/height-for-age Z-score (HAZ) and Weight for Height Z-score (WHO 1986; de Onis and Onyango 2008). For younger children below 36 months of age, length is measured instead of height. Underweight, stunting and wasting are defined by WAZ, HAZ, and WHZ only if these scores are less than -2 standard deviations. A study done in the Gambia with infants aged 6, 12 and 18 months reported that one unit increase of AF albumin adducts was associated with decreases in WAZ, length-for-age-z-score (LAZ), and weight-for-length-z-score (WLZ) (Watson et al. 2018). In a different longitudinal study done in Tanzania, the negative association between AF exposure and growth parameters did not reach statistical significance, but co-exposure with fumonisins contributed to decreased LAZ (Shirima et al. 2015). In children from Nepal below the age of 36 months, mean AFs exposure of 3.62pg/mg was not associated with LAZ, WAZ, or WLZ (Mitchell et al. 2017). In Nigeria, a significant association between higher AFB_1-lysine exceeding 4.5pg/mg compared to controls 0.8pg/mg was observed in children aged 6 – 48 months whose WHZ and HAZ were below -2 SD (McMillan et al. 2018). In a cohort of 480 children aged 9 months to 5 years in Togo and Benin, the prevalence of stunting and underweight associated with AFs exposure were reported to be 33% and 29%, respectively (Gong et al. 2002; Gong et al. 2003). Children who are underweight, stunted and/or wasted are significantly at risk of dying from infectious diseases, increased health problems, cognitive impairments, lower

school achievements, reduced life-time earnings, and decreased productivity (Ahlberg et al. 2018; Black et al. 2008). The dangers of AFs exposure to public health are greater than earlier suspected. Therefore, studies that seek to assess the impact of AFs on malnutrition and growth faltering are timely.

To date, some cross-sectional, longitudinal, and cohort studies between AF exposure and childhood growth impairments have reported significant association. Nonetheless, a dose-response relationship is yet to be established. The thresholds at which chronic exposures to AFs initiate adverse health outcomes in children is currently unknown. In the past, a number of studies have used proxy parameters such as putative contamination levels in weaning flour, corn products and other foods as a measure of exposure (Ayelign et al. 2018; Magoha et al. 2016; Okoth and Ohingo 2004). However, biomarkers of exposure are more useful in determining biological effective dose to be used in evaluating the role of the toxicant against specific health outcomes. Studies with well-defined sample sizes, appropriate estimate of AF exposure in individual study subjects using biomarkers approach, proper dose-response assessments, and use of plausible multivariate analyses that control for confounding factors are warranted. Past studies have put emphasis on exposure to younger children less than 5 years. Our study is the first to recruit older children between the ages of 6 – 12 years and complete a comparative assessment of exposure in regions historically known to have high and low levels of AFs contamination. We conducted a randomized school based cross-sectional study in Kenya, East Africa to determine AFs exposure using biomarkers, and evaluate their association with nutrition and growth outcomes.

Methods

Setting of the study

The study design is cross-sectional to provide a snapshot of AF exposure levels in children populations recruited from Siaya and Makueni Counties of Kenya, East Africa. Detailed description of study setting is provided in the study protocol (Wangia et al. 2019). Briefly, a cross-sectional study design was preferred to generate factual information on AF exposure among children aged 6 – 12 years widespread over two geographic locations. The selection of Makueni and Siaya Counties was based on a previous serological survey of AFs exposure levels which reported high AF exposures in Makueni County while Siaya's exposure levels were below the level of detection among adolescents and adults aged 16 – 64 years (Yard et al. 2013). Makueni County lies in Kenya's former Eastern Province while Siaya County forms one of the six counties in the former Nyanza Province (Wangia et al. 2019). Makueni county is a known hotspot for AFs exposures where deaths from AF poisonings have been previously reported (Lewis et al. 2005; United States CDC 2004). On the contrary, no prior documentation of AFs either in food supplies or human biological specimens have been reported from Siaya County. The choice of Makueni and Siaya Counties as study locations provide control for differences in weather conditions, ethnicity, and poverty levels.

Study participants

The Joint Ethics committee of the University of Nairobi and Kenyatta National Hospital of Kenya approved study protocol. Up to seven schools were randomly selected per constituency in the two counties. Inclusion criteria for schools included no feeding programs to guarantee that all meals were provided in the home, and to further limit the

possibility of consuming commercially purchased food supplies. School administrations of selected schools convened parent meetings at a convenient time and location. The meetings were open to the public and community leaders were in attendance. The study purpose and significance were explained to school officials, teachers, support staff and parents at the meetings presided by the chairperson of parents and teachers' association of each respective school. All questions and concerns were addressed during the meeting, before subject recruitment, during the study. Full disclosure of study details was given to all participants before informed consent forms were administered. Parents who filled out informed consents were invited to complete a five-part questionnaire that collected demographic information, socio-economic indicators, farming practices, household dietary patterns, and health status of family members. Parents were asked to provide the name and class of their children only if their children were healthy with no current medication and were aged between six and twelve years. After the meeting, the researchers cross checked all forms for accuracies and compiled a list of children as provided by the parents. We only contacted children whose parents had provided prior consent for assent. A child could refuse study procedures without consequence even if their parents had provided prior consent. Children who wanted to be part of the study without parental consent were turned down to their disappointment. Anthropometric measures including height, weight and mid upper arm circumference was measured in addition to 6 - 8 ml of venous blood obtained by phlebotomists.

Laboratory Analyses

AFB_1-lysine adducts, a validated biomarker for AFs exposure in human populations was quantified using High Performance Liquid Chromatography with

Florescence Detection as described previously (Qian et al. 2010; Scholl et al. 2006; Wang et al. 2001). Thawed human serum samples were placed in 56˚C water bath for 30 minutes to deactivate any pathogens present. Serum albumin and total protein were quantified as described before (Kang et al. 2015; Qian et al. 2013). An aliquot of 150 µl serum samples were digested by Pronase in the ratio1:4; pronase: total protein, for 3 hours in a water bath maintained at 37˚C. The samples were then loaded onto an Oasis Max cartridge from Waters Co. (Milford, Ma, USA) and purified via solid phase extraction over a vacuum chamber manifold. The cartridge was sequentially washed and eluted with 2% formic acid in methanol. The eluents were evaporated to dryness and reconstituted with 150 µl 25% methanol prior to HPLC injection. HPLC analysis was carried out on a 1200 liquid chromatography system (Agilent Technologies Wilmington, DE, USA), at excitation/emission of 470/405 nm. Chromatographic separation was achieved using Zorbax Eclipse XDB-C18 column (5 µm particle size, 250 X 4.6 mm). The mobile phase consisted of 20mM ammonium phosphate monobasic (pH 7.2) and 100% methanol to achieve separation within 25 minutes at flow rate of 1.0 ml/min. The detection limit of this method is 0.4pg/ml. For every injection, 100µl of samples processed were used. AFB_1-lysine binds to blood albumin to a significant extent following metabolism with high stability in human serum when stored properly (Qian et al. 2010; Scholl et al. 2006; Wang et al. 2001). Final AFB_1-lysine concentration in albumin was obtained by manual integration and calculated using calibration curves then adjusted for albumin content.

Statistical Analyses

Characteristics of study population were stratified by county and summarized with proportions for categorical variables and with median and interquartile ranges (IQR) for

continuous variables. Distribution of AFB_1-lysine adduct by county was summarized as both continuous (geometric mean and median) and categorical variables (proportion). Weight for age z-score (WAZ), Height for age Z-score (HAZ) and Weight for Height Z-score (WHZ) were determined in reference to the World Health Organizations (WHO) Multicenter Growth Reference. This reference included children from a diverse set of countries including Brazil, Ghana, India, Norway, Oman and the USA (WHO 1986; de Onis and Onyango 2008).The Multicenter Growth Reference provides considerable built-in ethnic, genetic and cultural variation in regard to how children are nurtured, which further strengthens the standards' universal applicability. We used "zscorer" R package to calculate child anthropometry z-score based on the WHO Child Growth standard. We performed univariate linear regression to estimate the crude coefficient between AFB_1-lysine adduct and multiple children's growth indicators (weight, height, BMI, MUAC, HAZ, BAZ, WAZ, MAZ). We also performed multiple linear regression to estimate adjusted coefficient between AFB_1-lysine adduct and growth indicators (weight, height, BMI, MUAC, HAZ, BAZ, WAZ, MAZ). AFB_1-lysine adduct, child-related variables (age of child, sex, wean start at month, the number of meals per day, feel hungry when go to bed, giving snack, and eat after bed time), and other variables (age of mother, age of mother when having the first child, the number of children, marital status, education level, living condition, house's wall, roof type, family size, income, mothers' occupation, spouse's occupation, smoking/alcohol use, grow corn, pesticide fertilizer use, corn storage, aflatoxin knowledge, identify aflatoxin, flour content, source of flour, food source, cost of food affect purchases) were considered for final model selection. We determined variables to include in our final models with lowest AIC values using stepwise algorithm in both

directions. If the final models have not included AFB_1-lysine adduct, we would add this variable in the final model to explore the relationship between AFB_1-lysine adduct and multiple growth indicators .To explore the association between child-related variables and other growth indicators z-score (HAZ, BAZ, WAZ), we also presented univariate and multivariate linear regression with smallest AIC value.

AFB_1-lysine was not normally distributed and therefore, the Log AFB_1-lysine was used in all univariate, bivariate and multivariate analyses. Log AFB_1-lysine adducts was used as continuous variables in multiple linear regression models. In Siaya County, 484 participants were enrolled mainly due to greater mobilization efforts by the ground team, prior participation in previous research efforts and generally greater involvement in community led initiatives compared to lower participation of 327 recruited from Makueni. In statistical analyses, missing entries and incomplete data were excluded from analyses and thus, values in tables may not add up to 811. Multiple imputation was used strategically in sensitivity analyses to address incomplete data and missing entries (Schomaker and Heumann 2014). The total number of children who assented to study procedures are 746, less than our proposed 786 determined in the study protocol (Wangia et al. 2019). The level of significance was set at p-value ≤ 0.05. SAS v9.4 (Cary, NC) and R version 3.6.1 (Vienna, Austria) statistical tools were used for data cleanup and analyses.

Results

Baseline characteristic of children in the study population stratified by county of residence is detailed in Table 6.1. Overall, 54.1 % of children enrolled in the study were female. The sex distribution was different per county with more male children, 49.8%

enrolled from Makueni County compared to Siaya at 43.2%. The median age of children enrolled in the study is 9 years, Inter Quartile Range (IQR) (8, 11) years. The median weight and height of the children enrolled in the study are 28 kg and 135.6 cm respectively. The proportion of children weighing less than 28 kg and shorter than 135 cm is 44.12% (330/748) and 48.66% (364/748) respectively. About 10.4 % of children were stunted, underweight and/ or too thin for their age. There is a significant difference between counties, p-value <0.001 for all the variables (Table 6.1).

Aflatoxin exposure is predominant and widespread in the areas of study. All children had detectable levels of AFs in serum, range 0.65 - 518.9 pg/mg albumin and Geometric mean 10.5 pg/mg albumin 95%CI (9.4 – 11.7). Generally, up to 67.6% of children had AFB_1-lysine in their serum exceeding 5pg/mg of albumin (Figure 6.1), an indication of widespread dietary exposure to AFs in both Siaya and Makueni Counties. More children in Siaya County had AFB_1-lysine less than 5pg/mg of albumin compared to Makueni's(Table 6.2, Figure 6.2 and Figure 6.3). Children with high AFB_1-lysine adducts exceeding 20pg/mg of albumin were mostly from Makueni County (Figure 6.2), up to 36.42%, which is more than double Siaya County's 15.33%(Figure 6.3). Overall, the geometric mean of AFB_1-lysine adducts in children recruited from Makueni county at 14.03 pg/mg albumin (95%CI 12.50, 15.74) is significantly higher than Siaya's 8.2 pg/mg albumin (95%CI 7.6, 8.8), p<0.001(Table 6.2).

Across all age groups, higher exposures were evident in Makueni County with exposure levels being two or three times that of children from Siaya County. Children who were 8 years of age had the highest exposure levels in Makueni, median and range 20.39

(1.46 - 495.23) pg/mg of albumin (Table 6.3). For Siaya County, children who were 10 years had the highest exposure, median and range 10.86 (0.86-88.98) pg/mg of albumin.

Stunted children from Siaya County had high AFB_1-lysine adducts in serum as shown in Figure 6.4. Underweight children in Makueni County have the highest AFB_1-lysine adducts as shown in Figure 6.5 compared to other indicators. Children with low BMI- for age Zscore, and low mid-upper arm circumference in Makueni County had the highest aflatoxins exposure (Figure 6.6 and Figure 6.7)

Overall, in univariate and multivariate linear regression analyses, increasing one unit of AFB_1-lysine is associated with decreasing Weight-for-Age-Z score (WAZ) by -0.004, p=0.054), and decreasing BAZ by -0.006 (-0.011 to -0.002, p=0.008) (Table 6.4). In children recruited from Makueni County, WAZ decreases by -0.08 (-0.21, 0.04), p-value = 0.044, WHZ decreases by -0.01 (-0.38, 0.35) p-value = 0.354 and BAZ decreases by -0.09 (-0.34, 0.16 p-value = 0.470 while none of the indicators are significant for Siaya County with every increase in AFs exposure. Among children below nine years in Siaya County, the HAZ decreases by – 0.27 (-0.54, -0.01, p-value=0.049, WAZ decreases -0.07 (95%CI -0.28, 0.13), p-value=0.479. In both Siaya and Makueni counties among children ages 6 – 9years, WAZ decreases by -0.15 (95%CI-0.28, -0.03), p-value=0.013 and WHZ decreases by -0.43 (95%CI-0.83,-0.02), p-value=0.041(Table 6.5). Additional growth parameters did not reach statistical significance.

Discussion

Dietary exposure to AFs is widespread in both Siaya and Makueni counties. Overall, about 67.6% of the children had AFB_1-lysine adducts exceeding 5.0pg/mg of albumin. Furthermore, children recruited from Makueni County were susceptible to higher

exposure levels exceeding 20pg/mg of albumin similar to previous studies (Githang'a et al. 2019; Turner et al. 2003). In a previous study, AFB_1-lysine adducts in individuals between the ages 15 – 64 years from Coast, Nairobi, Central, North Eastern, Western and Rift Valley provinces were significantly lower with median values of 3.70, 2.44, 2.33, 1.40, 1.28 and 0.70 pg/mg albumin for (Yard et al. 2013). In the current study, children population aged 6 - 12 years have higher AFs exposures, Geometric mean 10.5 pg/mg albumin 95%CI (9.4 – 11.7) and range 0.65 - 518.9 pg/mg albumin. A previous study among children between the ages of 1 – 14 years in Makueni County reported higher exposure levels, geometric mean of 20.4pg/mg albumin, mean 19.98pg/mg albumin and range 0.74 – 901.15 pg/mg albumin which are significantly higher compared to the current study (Githang'a et al. 2019). Another study conducted in Uganda, AFB_1-lysine adducts were higher in children below 3 years at GM 9.7pg/mg (95%CI 8.2 - 11.5) (Asiki et al. 2014). In Gambia, sera of 466 of 472 children 6–9 years of age were tested for the level of aflatoxin-albumin adducts, 93% were positive, GM 22.3 pg/mg, (95%CI 20.3–24.5), range 5–456 pg/mg (Turner et al. 2003). In the study by Turner et al, aflatoxin-albumin adduct levels were not significantly related to the age or sex of the children, unlike the current study where AFs is dependent on age but not sex. Taken together, these studies indicate that children are more susceptible to higher AFs exposure compared to adolescents and adults. Toxicant exposure levels are relative to body weight and thus, expected to be higher in children compared to adults, which is partly attributed to developing organs and limited capacity for detoxification of xenobiotic including AFs.

Dietary exposure to AFs and other mycotoxins has been implicated in malnutrition and growth impairment in children. In the current study, increasing one unit of AFB_1-lysine

is associated with decreasing Weight-for-Age-Z score (WAZ) by -0.004, (95% CI -0.009 to 0.001, p=0.054), and decreasing BAZ by -0.006 (95% CI -0.011 to -0.002, p=0.008). Moreover, younger children between the ages of 6 - 12 years appear to suffer worse outcomes. WAZ decreases by -0.15 (95%CI-0.28, -0.03), p-value=0.013 and WHZ decreases by -0.43 (95%CI-0.83,-0.02), p-value=0.041 with every increase in AFB_1-lysine exposure among this age group. A different study in Kenya among children 6 to 9 years, found a significant association between AF-albumin adducts and impaired child height after adjusting for age, sex and possible schistosomiasis infection status; the growth faltering was attributed to altered expressions of the insulin-like growth factor genes P = 0.052. (Castelino et al. 2015). Likewise, a different study reported a significant association between AF exposure and wasting, p=0.002 among children less than three years in Kenya. This study however quantified AFs in weaning flours, which was used a proxy for AF exposure (Okoth and Ohingo 2004). In another study conducted in Kenya, growth reduction estimation for children below three years exposed to AFM_1 from milk in average have an effect of -0.34 on HAZ, contributing a total of 2.7% of childhood stunting (Ahlberg et al. 2018). In Benin, a significant negative association between height velocity, but not weight, and mean AF- albumin levels was reported in an 8-month longitudinal study in 200 children aged between 16 and 37 months (Gong et al. 2004). Moreover, a difference of 1.7 cm over the 8-month study period in adjusted height between the highest and lowest AF-albumin quartile was observed (Gong et al. 2004). Following 138 Gambian neonates for 1 year, Turner et al. reported that reducing maternal exposure to AFs from 110 to 10pg/mg could result in weight increase by 800g and height increase by 2cm on average (Turner et

al. 2007). In a Nigerian study by McMillan et al, a significant correlation between HAZ and AFB_1-lysine supports the causal role of AF and stunting (McMillan et al. 2018).

On the contrary, a number of studies have also reported no association between AF exposure and growth impairments. A cohort study in Gambia among 472 older children between 6 and 9 years old did not report any association between AF exposure and HAZ or WAZ; however, a weak association was reported for WHZ, an indicator for wasting (Turner et al. 2003). In Cameroon, a study conducted of 220 children between the ages 1.5 – 4.5 years reported no association between the different malnutrition categories namely stunted, wasted and underweight with mycotoxin exposure (Njumbe Ediage et al. 2013). Furthermore, in a longitudinal study completed in Tanzania with 166 children between the ages of 6 - 14 months, a negative association between AF-albumin and stunting did not reach statistical significance, even though co-exposure with fumonisins was implicated in growth impairments (Shirima et al. 2015). In another Tanzanian cohort study of children aged 36 months or less, there was no association between AFs exposure and stunting, underweight or wasting/thinness, however, significant negative association was reported between urinary fumonisins, stunting and underweight in children aged 0 – 36 months (Chen et al. 2018; Shirima et al. 2015). Similarly, a different longitudinal study completed in Nepal, AF exposure in children less than 3 years was not associated with LAZ, WAZ (underweight), and WLZ (thinness/wasting) (Mitchell et al. 2017). In a cluster randomized control trial done in Meru County of Kenya, reducing AF exposure by providing households with non-contaminated corn lowered AFs serum levels of pregnant women by 27% (Hoffmann, Jones, and Leroy 2015; Hoffmann, Jones, and Leroy 2018), however, this reduction was not associated with improved linear growth. Taken together, these results

indicate the pervasive nature of AFs exposure and regulatory measures must be undertaken to protect public health.

In summary, dietary exposure to AFs and other mycotoxins is widespread, however their role on children's growth impairment is not conclusive. While mechanisms by which AF exposure contributes to growth impairment have been proposed, further studies are urgently needed. The suggested mechanisms of action include AFs induced immune suppression, which is likely to increase susceptibility to infectious diseases; and/or chronic inflammatory enteropathy of the small intestines mucosa which severely limits nutrient uptake, and/or liver toxicity aggravated by continuous exposure to AFs may ultimately damage production of insulin like growth factor pathway proteins (Hoffmann, Jones, and Leroy 2015; Khlangwiset, Shephard, and Wu 2011; McMillan et al. 2018). The proposed mechanisms are suggested to work in a synergistic manner thereby contributing to growth retardation among children living in low- and middle- income countries.

Conclusions

In developing countries, malnutrition is one of the most pervasive risk factors for human morbidity and mortality. Stunting, wasting and underweight are major indicators of continuing malnutrition and has been associated with chronic AF exposure. These parameters are well-established risk markers for poor child development with significant consequences that last beyond childhood years. The current study adds to the mounting evidence on the role of AFs and other mycotoxins in growth impairment among children. The effect is adverse in children due to limited detoxification capacity of xenobiotic and higher sensitivity of growth inhibitory effects at a younger age. Despite numerous targeted interventions to curb malnutrition, the problem is persistent in low- and middle-income

countries due to numerous interacting factors including but not limited to sub-optimal nutrition, socio-economic status, high prevalence of infectious disease, and exposure to environmental contaminants. In order to sufficiently address chronic malnutrition and growth impairments, efforts must be put in place to control for mycotoxin exposure in order to achieve better growth outcomes. Nonetheless, exposure to AFs and other mycotoxins must be kept as low as reasonably achievable. Further studies to evaluate specific thresholds under which toxic AF metabolites contribute to immune modulation, disruption of intestinal barrier and interference with growth axis are timely and warranted.

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Table 6.1. Characteristics of children in the study population by county

Characteristics		**Overall N(%)**	**Makueni (N=327) N (%)**	**Siaya (N=484) N(%)**	**p-value**
Sex	Male	372 (45.9)	163 (49.8)	209 (43.2)	**0.062**
	Female	439 (54.1)	164 (50.2)	275 (56.8)	
Age	6	97 (12.0)	44 (13.6)	53 (11.0)	**0.012**
	7	82 (10.2)	40 (12.4)	42 (8.7)	
	8	105 (13.0)	37 (11.5)	68 (14.1)	
	9	120 (14.9)	42 (13.0)	78 (16.1)	
	10	134 (16.6)	41 (12.7)	93 (19.3)	
	11	115 (14.3)	44 (13.6)	71 (14.7)	
	12	153 (19.0)	75 (23.2)	78 (16.1)	
Height	Median (IQR)	135.6 (124.9 to 153.1)	136.1 (126.1 to 154.0)	135.2 (124.0 to 152.4)	**0.021**
Weight	Median (IQR)	28.0 (25.0 to 35.0)	28.0 (23.2 to 33.2)	29.0 (25.0 to 35.0)	**0.033**

BMI	Median (IQR)	15.6 (13.5 to 17.9)	14.8 (13.1 to 16.8)	16.1 (14.3 to 18.3)	**<0.001**
MUAC	Median (IQR)	18.0 (17.0 to 20.0)	18.0 (17.0 to 20.0)	18.0 (17.0 to 20.0)	**0.023**
Height-for-Age-Z score (HAZ)	Low	77 (10.4)	16 (5.0)	61 (14.4)	**<0.001**
	Normal	542 (72.9)	246 (77.1)	296 (69.8)	
	High	124 (16.7)	57 (17.9)	67 (15.8)	
BMI-for-Age-Z score (BAZ)	Low	77 (10.4)	16 (5.0)	61 (14.4)	**<0.001**
	Normal	542 (72.9)	246 (77.1)	296 (69.8)	
	High	124 (16.7)	57 (17.9)	67 (15.8)	
Weight-for-Age-Z score (WAZ)	Low	77 (10.4)	16 (5.0)	61 (14.4)	**<0.001**
	Normal	542 (72.9)	246 (77.1)	296 (69.8)	
	High	124 (16.7)	57 (17.9)	67 (15.8)	
MUAC-for-Age-Z score (MAZ)	Low	77 (10.4)	16 (5.0)	61 (14.4)	**<0.001**
	Normal	542 (72.9)	246 (77.1)	296 (69.8)	
	High	124 (16.7)	57 (17.9)	67 (15.8)	

Table 6.2. Distribution of AFB_1-lysine adducts in children by County

	Siaya (n=327)	Makueni (n=419)	†P-value
ǂGeometric mean (95%CI)	8.17 (7.59 to 8.81)	14.03 (12.50 to 15.74)	0.001
ǂMedian (Range)	9.01 (0.81 to 265.74)	13.19 (0.65 to 518.93)	0.001
ǂ*AFB_1-lysine Adducts by Age Clusters of Children, Geometric Mean* (95%CI)			
6 years	7.51 (4.76,10.26)	13.01 (3.61, 22.40)	0.001
7 years	7.37 (2.73, 18.33)	12.85 (-0.24 , 25.94)	0.055
8 years	7.56 (4.30,10.81)	21.60 (-8.24, 51.44)	0.014
9 years	8.08 (6.04,10.12)	17.20 (0.25, 34.15)	0.005
10 years	8.91 (5.63,12.19)	11.84 (-6.12,29.79)	0.175
11 years	8.37 (5.84,10.89)	12.08 (-15.00,39.16)	0.034
12 years	8.73 (1.44,16.01)	13.32 (-2.09, 28.73)	0.047

ǂAFB_1-lysine adducts expressed in pg/mg albumin †significant differences between the child ages

Table 6.3. Aflatoxin B_1-lysine adduct levels in children by Age Groups

Age In Years	Overall Median (Range) pg/mg albumin	Makueni Median (Range) pg/mg albumin	Siaya Median (Range) pg/mg albumin
6	10.37 (0.83-142.84)	12.94(0.83 - 142.84)	7.54 (1.15-37.32)
7	11.96 (0.89-192.74)	14.20(0.89-192.74)	6.52(1.73-77.72)
8	10.00 (1.33-495.23)	20.39(1.46 - 495.23)	6.84(1.33-53.68)
9	10.72 (0.86-320.59)	17.43(1.77-320.59)	9.28(0.86-45.51)
10	11.07 (0.86-358.31)	11.77(1.56 - 358.31)	10.86(0.86-88.98)
11	9.57 (0.66-448.40)	10.95(0.66 - 448.40)	8.98(0.85-51.06)
12	11.57 (0.65-518.93)	13.19(0.65 - 518.93)	9.79(0.81-265.75)

Table 6.4. AFB_1-lysine and growth indicators in Kenya 2018.

Results from univariate and multivariate linear regression models

Outcome	N	Univariable coefficient	Multivariable coefficient
Weight (kg)	547	0.003 (-0.012 to 0.018p=0.715)	0.001 (-0.012 to 0.013, p=0.956)
Height (cm)	547	0.042 (0.011 to 0.073, p=0.008)	0.024 (0.004 to 0.044, p=0.017)
BMI (kg/m2)	547	-0.009 (-0.016 to -0.002, p=0.014)	-0.003 (-0.011 to 0.004, p=0.361)
Mid-Upper-Arm Circumference (MUAC)	543	-0.001(-0.004 to 0.004, p=0.987)	-0.001 (-0.004 to 0.003, p=0.730)
Height-for-Age-Z score (HAZ)	547	0.005 (0.001 to 0.008, p=0.004)	0.003 (0.000 to 0.007, p=0.042)
BMI-for-Age-Z score (BAZ)	547	-0.006 (-0.011 to -0.002, p=0.008)	-0.003 (-0.007 to 0.002, p=0.216)
Weight-for-Age-Z score (WAZ)	357	-0.004 (-0.009 to 0.000, p=0.054)	-0.002 (-0.006 to 0.003, p=0.481)
MUAC-for-Age-Z score (MAZ)	543	-0.001 (-0.003 to 0.001, p=0.394)	-0.000 (-0.002 to 0.002, p=0.931)

In adjusted models, N=547 because all missing entries in any category were excluded from the analyses. Moreover, no reference values from World Health Organizations were available for children older than 120 months and thus were excluded from analyses.

Table 6.5. Growth Parameters outcomes when stratified by County and Age

Growth parameters	†Overall, (p-value)	Siaya, (p-value)	Makueni, (p-value)	Overall <9years, (p-value)	Siaya < 9 years, (p-value)	Makueni < 9 Years, (p-value)
Weight-for-Age-Zscore (WAZ)	-0.13 **(0.019)**	0.03, (0.686)	-0.08, (**0.044**)	**-0.11,** (**0.049**)	-0.07, (0.479)	-0.04, (0.546)
Height-for-Age-Zscore (HAZ)	0.10 (**0.069**)	-0.05, (0.625)	0.03, (0.769)	-0.08, (0.337)	-0.27, (0.049)	0.13, (0.224)
Weight-for-Height-Zscore (WHZ)	0.03 (0.818)	0.12, (0.598)	-0.01, (0.354)	-0.17, (0.437)	0.01, (0.970)	-0.12, (0.651)

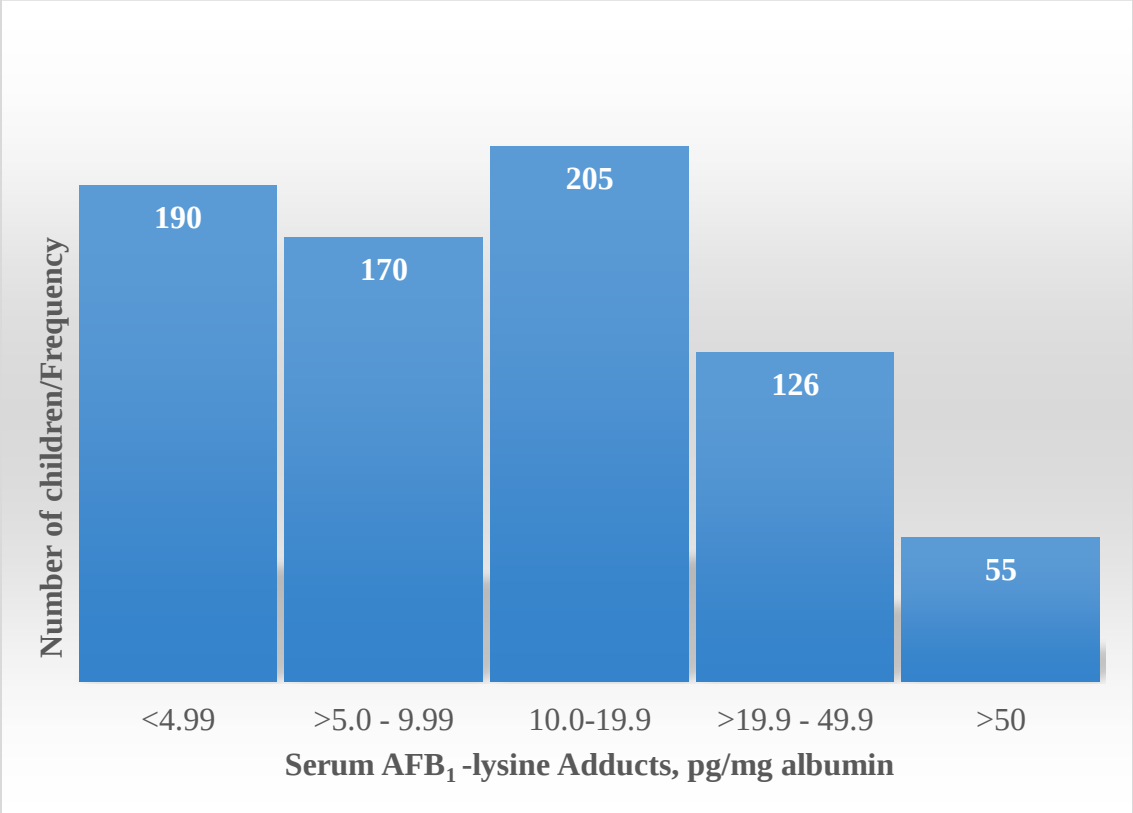

Figure 6.1. Distribution of AFB_1-lysine adducts in all children 6 - 12 years

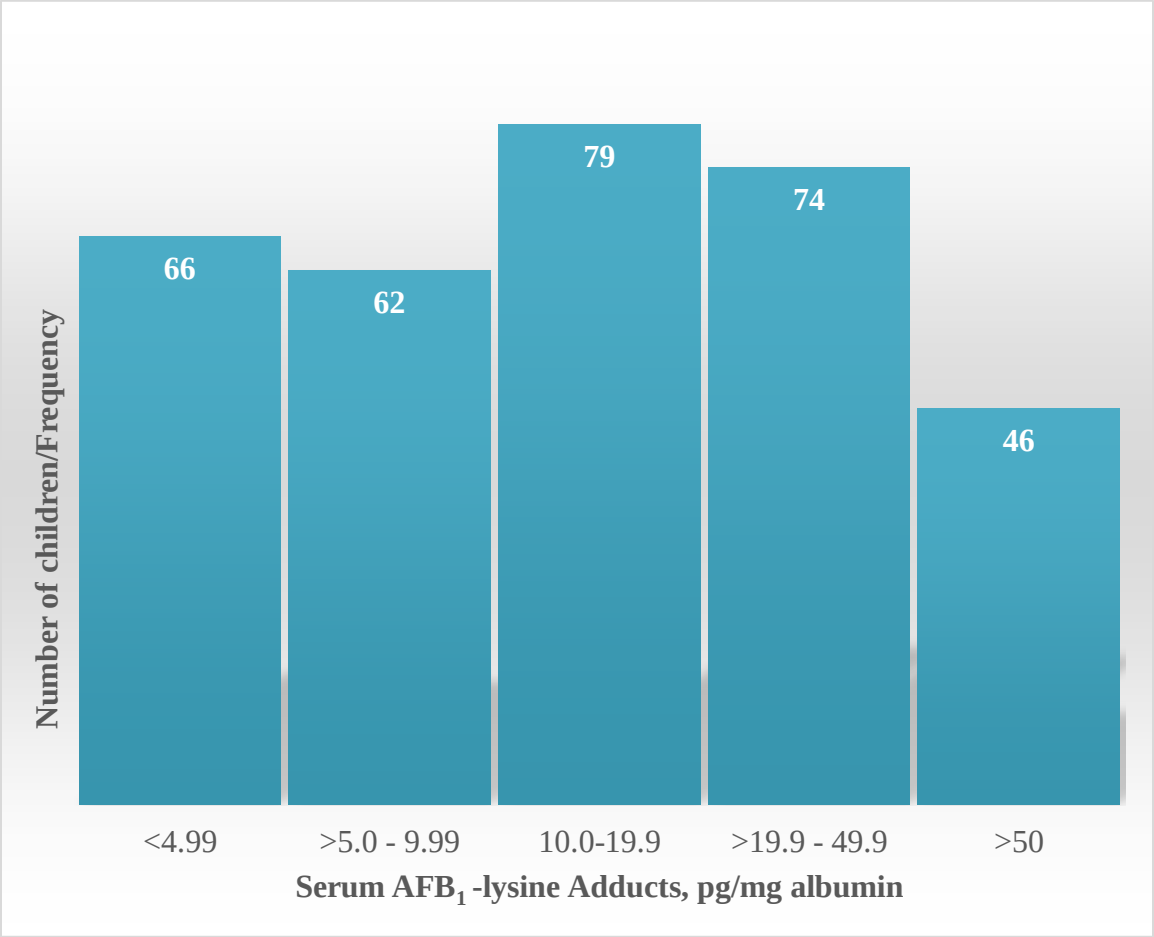

Figure 6.2. AFB_1-lysine adducts in Makueni County children

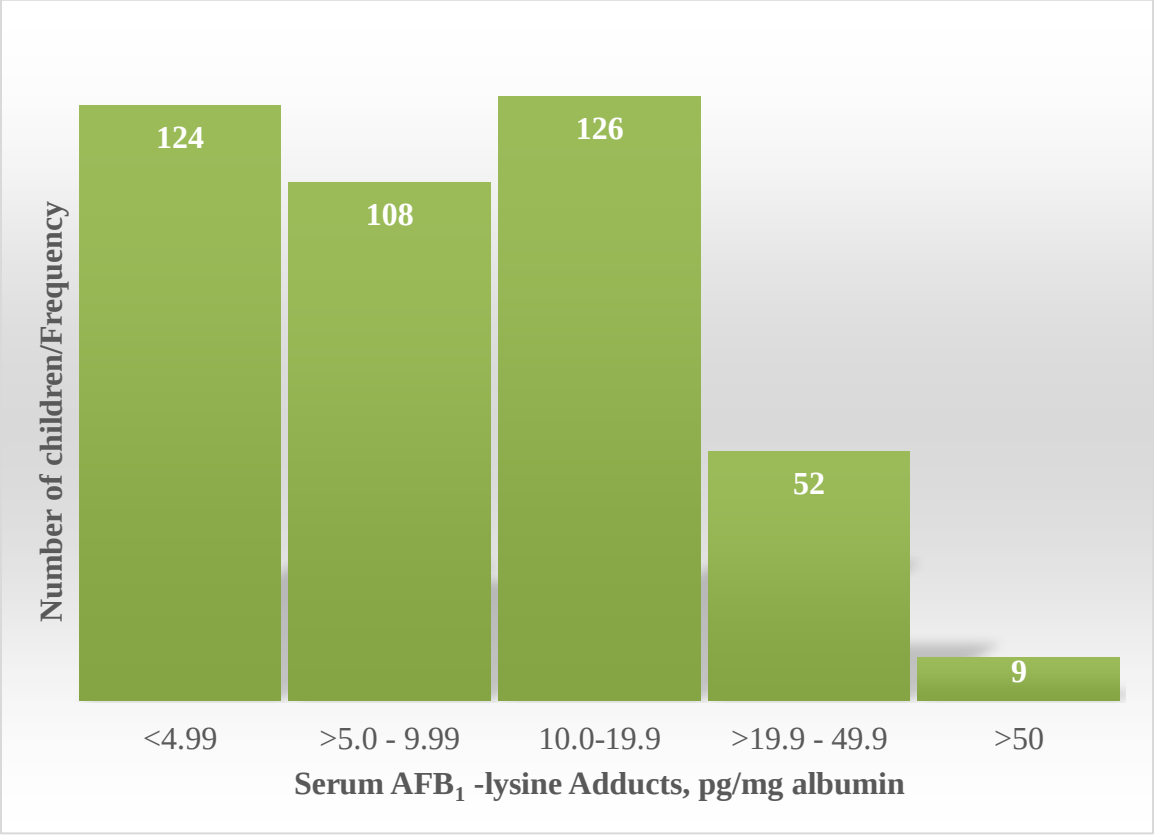

Figure 6.3. AFB_1-lysine adducts in Siaya County children

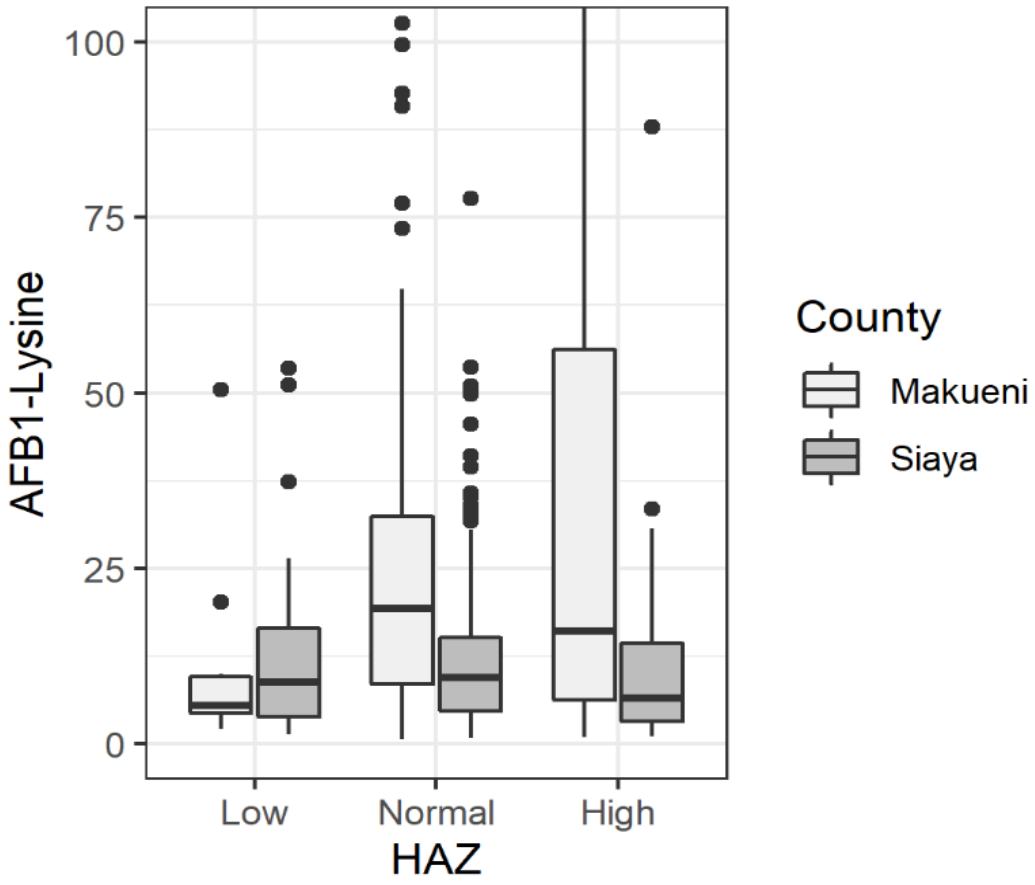

Figure 6.4. AFB_1-lysine adducts and Height for Age Z-score (HAZ)

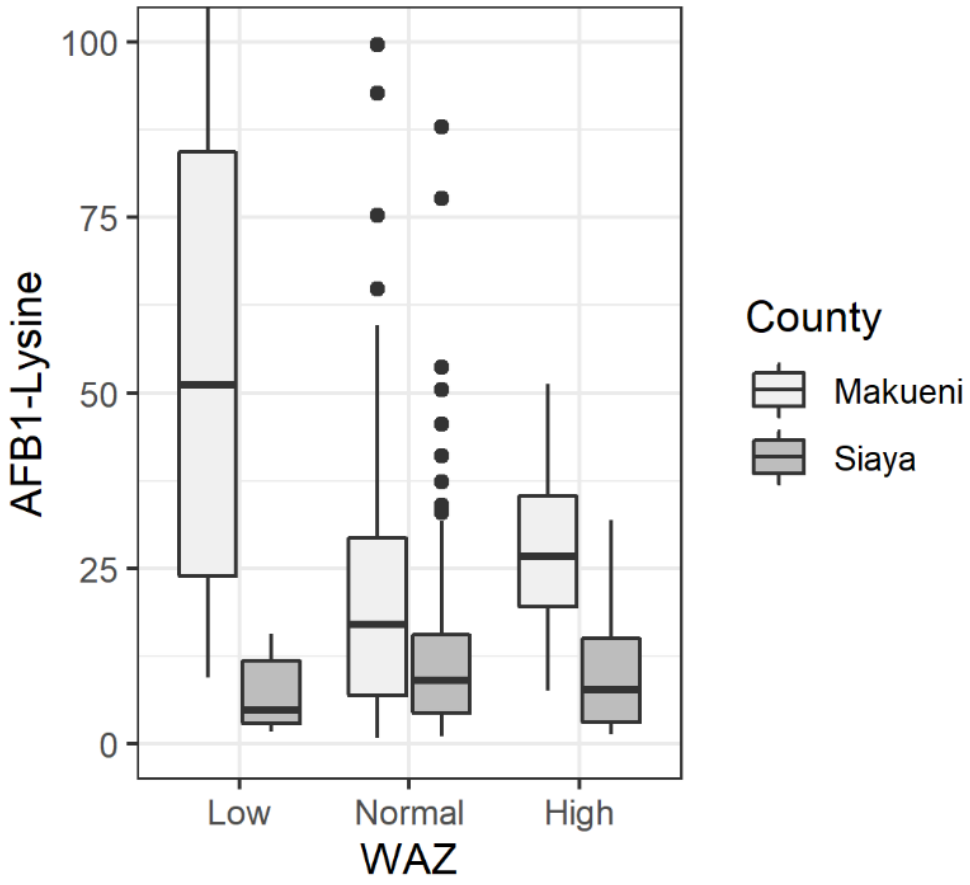

Figure 6.5. AFB_1-lysine adducts and Weight for Age Z-score(WAZ)

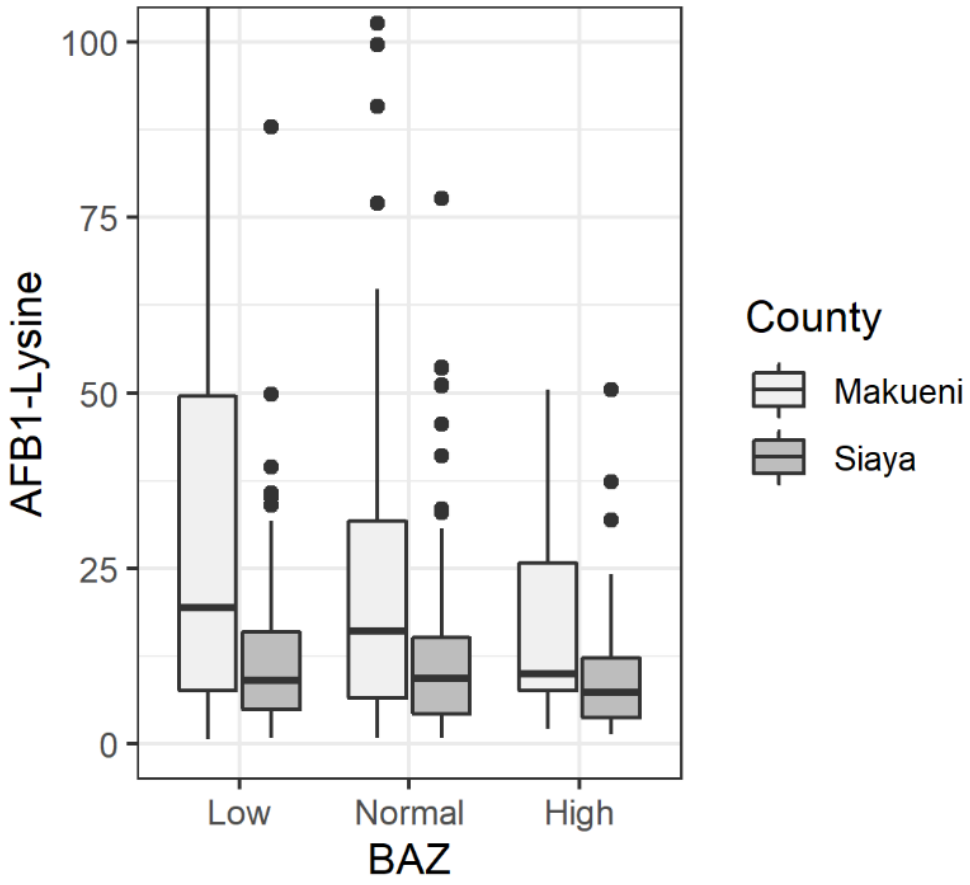

Figure 6.6. AFB_1-lysine adducts and BMI- for Age Z-score (BAZ)

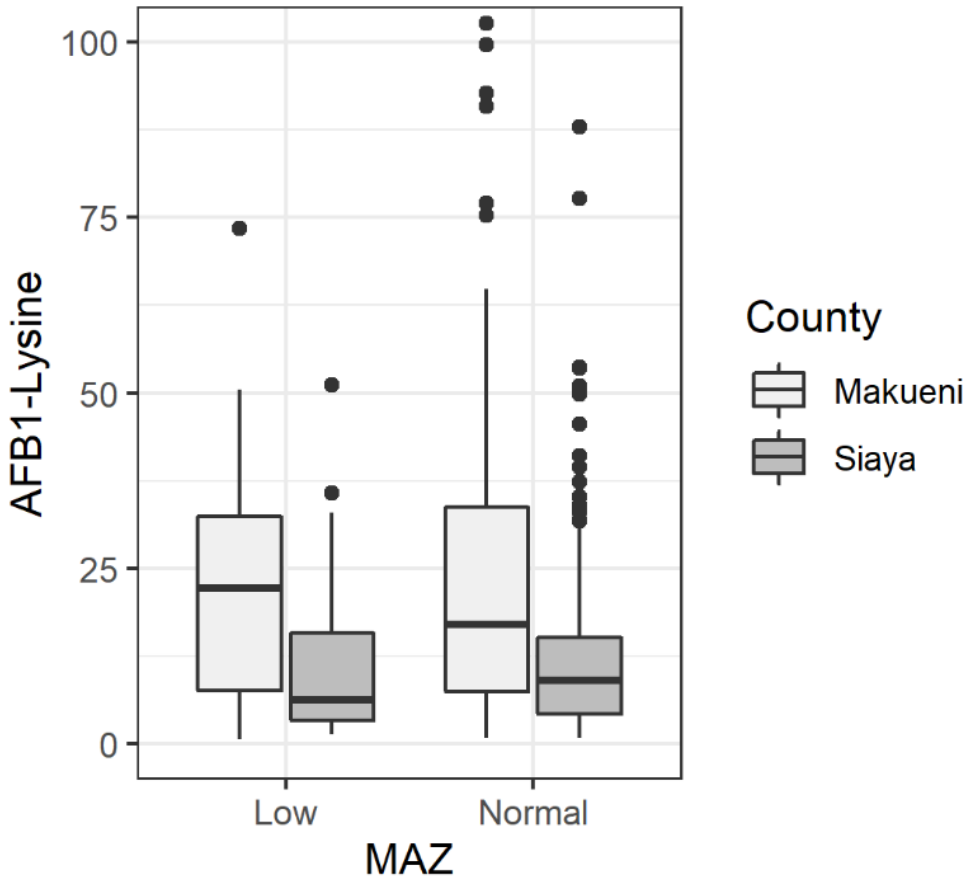

Figure 6.7. AFB_1-lysine by Mid-Upper-Arm-Circumference-for-Age-Z-score (MAZ).

Chapter 7

VALIDATION OF SPHINGOLIPIDS METABOLITES AS BIOMARKER OF EFFECT FOR FUMONISINS EXPOSURE IN KENYAN CHILDREN[5].

[5] Wangia, R. N., D. P. Githanga, K. S. Xue, L. Tang, O. A. Anzala, and J. S. Wang. 2019. Validation of urinary sphingolipid metabolites as biomarker of effect for fumonisins exposure in Kenyan children', *Biomarkers*, 24:379-88. This is an **Accepted Manuscript** of an article published by Taylor & Francis in ***Biomarkers*** on **March 20, 2019**, available online at the Taylor & Francis Ltd web site: www.tandfonline.com and **https://www.tandfonline.com/doi/full/10.1080/1354750X.2019.1587510. Reprinted here with permission of publisher.**

Abstract

Fumonisins (FNs), a group of mycotoxins produced mainly by Fusarium species, are ubiquitous food contaminants, especially for corn. Fumonisin B_1 (FB_1) caused severe toxicities in farm animals, induced kidney and liver tumors in rodents and is associated with many human adverse health effects, including esophageal cancer. International Agency for Research on Cancer (IARC) categorizes FB_1 as possible human carcinogen (Group 2B). Inhibition of ceramide synthesis and disruption of sphingolipids metabolism are well studied as the major mechanisms of FB_1-induced toxicity. Increases in sphinganine (Sa) and decrease in sphingosine (So) levels and their ratio are validated biomarkers of FB_1 effects. In this study we measured urinary levels of Sa, So and Sa/So in 284 children age 1 to 14 years who consume corn as a staple diet. Exfoliated cells from urine were processed and sphingolipids quantified by High Performance Liquid Chromatography. Sa and So were detectable in 95.07% and 98.94% of samples respectively. Creatinine adjusted mean levels and standard deviation of Sa, So, and Sa/So ratio were 1.23±2.18 nM, 4.99±8.3 nM, and 0.296±0.587 respectively. These results further confirmed findings in studies with human adults, i.e. urinary Sa, So levels, and Sa/So ratio are good biomarkers to assess FNs exposure in children.

Keywords: Biomarker, Fumonisins, Mycotoxins, Sphingolipids, Sphinganine, and Sphingosine.

Introduction

Fumonisins (FNs), a group of mycotoxins produced mainly by Fusarium species, are ubiquitous food contaminants, especially for corn (*Zea Mays*) and peanuts (*Arachis hypogaea*). FNs contamination poses a difficult challenge to food safety. FNs contamination is unavoidable and unpredictable even when good agricultural, storage and processing practices are implemented. Food regulatory agencies such as the European Codex Alimentarius recommends that corn based foods for infants and young children should not exceed 200µg/kg of total fumonisins. However, due to the ubiquitous nature of FNs contamination, it is normal to find levels that exceed 1000 µg/kg in corn and other food samples. The United States Food and Drug Administration (USFDA) recommends action levels between 2000 µg/kg - 4000 µg/kg (USFDA 2001). Up to 28 different types of FNs have been isolated and characterized. However, Fumonisin B_1 (FB_1) is the most biologically potent and therefore, widely studied (Cawood et al. 1991; MacKenzie et al. 1998). The International Agency for Research on Cancer (IARC) evaluated numerous toxicological studies on FB_1 toxicity and categorized FB_1 as group 2B, a possible human carcinogen (IARC 1993, 2004). The Joint Food and Agricultural Organization and World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) recommend 2µg/kg per body weight per day as the provisional maximum tolerable daily intake (PMTDI) of fumonisin (JECFA 2012).

Exposure to FNs through feed causes species specific diseases in farm animals. The most commonly described diseases are Equine leukoencephalomalacia (ELEM) and

Porcine Pulmonary Edema (PPE). ELEM is a neuro-degenerative disease that affects horses, mules, and donkeys. Incidences of ELEM have been widely reported in farm animals after consumption of fusarium-contaminated feed (Rosiles et al. 1998; Ross et al. 1990; Kellerman et al. 1990). Similarly, Porcine Pulmonary edema has also been documented in pigs fed diets contaminated with fumonisins (Haschek et al. 2001; Harrison et al. 1990). In addition, renal injury has been documented in rabbits and sheep. In toxicological laboratory assessments, exposure to FB_1 is reported to be carcinogenic, nephrotoxic, hepatotoxic and embryo-toxic in rodents (Gelineau-van Waes et al. 2005; Marasas et al. 2004; Wild and Gong 2010).

In human populations where corn is a dietary staple, FNs are associated with esophageal cancer, liver cancer and neural tube defects (Marasas et al. 2004; Missmer et al. 2006; Sun et al. 2007; Williams et al. 2010; Xu et al. 2010; Suarez et al. 2012). These observations are based on studies from Transkei in South Africa, China and Texas in the United States. FNs are also known strong tumor promoters with ability to accelerate the advancement from tumor initiation to mutagenesis in human cells and animal models (Gelderblom et al. 1988; Xue et al. 2015). Moreover, fumonisin has been shown to contribute to growth impairment in children who are weaned on corn-based complementary foods (Kimanya et al. 2010; Chen et al. 2018). The levels of FB_1 in food products for human consumption has been quantified and found to be heterogeneous and highly variable. Historically, FB_1 was quantified in corn or corn based foods, peanuts, sorghum, cowpeas seeds, beans and beer (Alshannaq and Yu 2017). More recently, FNs have been quantified in cassava, millet, farro grain, spices, black radish, medicinal plants, wine, coffee and tea (Scott 2012). In a review article, baby food and food intended for children

over 3 years old were found to be contaminated by multiple mycotoxins including fumonisins (Raiola et al. 2015). In Kenya, Alakonya et al reported FB_1 in corn at different times at harvest to range from 22 to 1348µg/kg (Alakonya, Monda, and Ajanga 2009).

Chronic exposure to fumonisin in children is widely studied and documented in neighboring Tanzania. Magoha et al reported that weaning flour used for infants below 6 months of age in Northern Tanzania had FNs in the range 48 - 1224 µg/kg and median of 124µg/kg (Magoha et al. 2016). Additionally, the researchers quantified FB_1 in 131 breast milk samples and 44.3% of the breast milk samples contained FB_1 at levels ranging from 6.57 to 471.05ng/ml (Magoha et al. 2014). In a different study, children's exposure to dietary fumonisin and aflatoxins was evaluated to determine the potential impacts on growth in 114 children less than 36 months of age in Haydom, Tanzania. The study reports that exposure to fumonisin was strongly associated with growth impairment in children (Chen et al. 2018). In a similar study, children with high levels of urinary FB_1 registered a strong negative correlation with Length-for-Age Z-score, an indicator of growth impairment (Shirima et al. 2013; Shirima et al. 2015). In another study to assess fumonisin contamination in corn based complementary foods for children, @RISK analysis software is used to model FNs exposure for infants. Up to 89% of infants were exposed to FNs above the provisional maximum daily intake of 2 µg/kg /bw (Kimanya et al. 2009).Taken together, these studies provided the risk of early exposure to fumonisins among children.

Mechanism of Fumonisin B_1 Toxicity

The mechanism of FB_1 toxicity is attributed to its structural similarity to sphingoid bases, which consists of twenty-carbon backbone as shown in Figure 7.1 (Wang et al.

1991). Due to its structural similarity to sphingoid bases, fumonisin B_1 acts as a competitive inhibitor of ceramide synthase (Riley et al. 2012; Marasas et al. 2004). The *de novo* pathway of sphingolipid synthesis is highly dependent on the action of ceramide synthase. The process begins with the condensation of serine and palmitoyl-CoA. This condensation is catalyzed by serine palmitoyl transferase to generate 3-ketodihydrosphingosine which is subsequently reduced to sphinganine. Sphinganine is then N-acylated by ceramide synthase to produce dihydroceramide (dhc) that through the action of dhc-desaturase is reduced to ceramide. In the presence of fumonisin exposure, FB_1 inhibits the N-acylation process by blocking ceramide synthase (Turner, Nikiema, and Wild 1999; Voss et al. 2002; Voss et al. 2006; Zitomer et al. 2009). Ultimately, this inhibition results in accumulation of intracellular sphinganine that can eventually lead to oxidatively generated DNA damage, sphingomyelin depletion and altered function of sphingolipids as second messengers. The use of biomarkers to assess exposure to fumonisin is paramount for early detection and prevention of diseases. In human populations, levels of free FB_1 have been quantified in urine and validated as biomarkers of fumonisin exposure (Ahn et al. 2010; Solfrizzo, Gambacorta, and Visconti 2014). Urinary sphinganine and sphingosine levels have also been quantified in human ecological studies and proposed as biomarkers of fumonisin exposure. FB_1, being structurally similar to sphingoid bases is known to disrupt sphingolipid metabolism by inhibiting ceramide synthase, and thus altering the sphinganine (Sa) to sphingosine ratios (So). Early studies on sphingoid bases as possible biomarkers of effect confirmed that the altered Sphinganine to Sphingosine ratios (Sa/So ratio) is specific to only FB_1 exposure (Riley 1994; Castegnaro et al. 1996). Despite widespread application of Sa/So ratio in both animal and human studies in the early 1990s,

the biomarker was not validated until a decade later. Sensitivity and specificity studies that validated use of urinary sphinganine to sphingosine ratios as plausible biomarkers of exposure were done in our laboratory (Cai, Tang, and Wang 2007). The sphinganine to sphingosine ratio (Sa/So ratio) was confirmed to be highly sensitive and specific to fumonisin exposure. In this study, 284 urine samples were collected from children living in Makueni County, Kenya, a region of high mycotoxins exposure. Levels of sphinganine and sphingosine bases were quantified using High Performance Liquid Chromatography (HPLC) with Fluorescence Detection (FD). The specific aim of this study is to assess any possible alterations in sphinganine to sphingosine ratio, a validated biomarker for fumonisin exposure among children consuming mycotoxins contaminated diets.

Materials and Methods

Sampling

This study is part of a larger study with the overall objective to assess aflatoxin and fumonisin exposure on vaccine uptake and various immune function in children. Participants were recruited from Makueni County, Kenya, a drought prone area with documented evidence of high prevalence to mycotoxins exposure. The study design is cross-sectional, where, 930 households were randomly sampled in Ilatu village, Nguumo location, Makindu division, Kibwezi Constituency in Makueni County of Kenya. Two Stage Sampling design was used where clusters of households were selected from sixteen Enumeration Areas created by 2009 Kenya Population and Housing Census. 500 households with children were eligible for enrolment in the study. Inclusion criteria included healthy children of both sexes, ages 1 to 14 years, availability of vaccination

records and the parents or guardian must be able to provide informed consent. Recruitment of study participants is summarized in flow diagram in Figure 7.2. The Joint Ethics Committee of the University of Nairobi and Kenyatta National Hospital in Kenya approved the research protocol and procedure for study referenced P601/10/2014. Using the local dialect, the study investigators explained in detail the purpose of study to community members before recruitment. Parents or guardians who agreed to study procedures provided written consent and completed a brief questionnaire on socio-economic indicators. Approximately 15ml of urine, 5ml of blood and 10g of stool specimens were collected from children in the presence of parents or guardians who had provided informed consent. This study only reports results from analysis of 284 urine samples.

While the estimated sample size was 425 children, only 284 children were able to provide more than the 10 ml minimum volume of urine required for analysis. Apart from the 284 children, the rest provided small volumes of less than 5ml urine which were mostly dark yellow in color. The low collection rate was largely contributed by small volumes of urine collected from children living in a semi-arid region with minimal water intake and high rates of dehydration. Urine color and volume are validated biomarkers for dehydration in children and adults (Guelinckx et al. 2015; Kavouras et al. 2016). Nonetheless, this study is the first to report sphingolipid biomarkers using the largest sample size of 284. Most studies previously done have a small sample size of less than 200 individuals as shown in Table 7.5. One-time collection of urine was done during the day where children who were old enough to take instructions provided urine in sterile urine cups. For younger children, a urine bag was strapped across the genitalia and urine collected on cue. Collected samples were placed in dry ice and shipped to KAVI Institute of Clinical Research at the University

of Nairobi. Samples were stored in -80 degrees Celsius freezer awaiting shipment on dry ice to University of Georgia in Athens. The samples were immediately stored in Thermo Scientific -80 degrees Celsius freezer at the University of Georgia until analysis.

Assessment of urinary sphingolipids levels to evaluate fumonisin toxicity in human populations has been widely studied based on altered levels of Sa/So ratio. The scope of this paper, therefore, is restricted to the assessment of sphinganine and sphingosine levels and the associated Sa/So ratio, which is a sensitive and specific biomarker for fumonisin exposure. To the best of our knowledge, this is the first validation study to use Sphinganine to Sphingosine ratio to evaluate possible bio-cellular effects in children between the ages of 1 to 14 years, who are presumably exposed to fumonisin. The household dietary structure was evaluated using food frequency questionnaires administered to caregivers and or parents of the enrolled children. The questionnaire collected information on the child's feeding habits, food consumed in the past 24 hours and yes/no questions aimed at assessing food availability and consumption of 16 different food groups in each household. The food groups were classified into cereals, roots and tubers, vitamin A rich vegetables and tubers, other vegetables, Vitamin A rich fruits, Other fruits, offal, meats, eggs, fish and sea food, legumes, nuts and seeds, milk and milk products, oils and fats, sweets and lastly, spices, condiments, and beverages. Dietary diversity score was then calculated based on the United Nations Food and Agricultural Organization recommendations (Food and Agriculture Organization (FAO) 2011) and United States Agency for International Development's Food and Nutrition Technical Assistance Guidelines.

Chemical Reagents and Standard Solutions

All chemical reagents used were HPLC grade. 5mM triethylammonium formate (TEAF) buffer solution was prepared using 1M TEAF buffer and formic acid, both purchased from FLUKA Analytical (St. Louis, MO). O-phthaldialdehyde (OPA) reagent, used for online derivatization was prepared every 7 days by dissolving 10mg of OPA and 30ul of 2-mercaptoethanol in 250ul of Methanol (MeOH). The solution was mixed with 4.75ml of 3% Boric Acid buffer (pH 10.5) and stored in amber glass tubes at 4°C to avoid light before use.

HPLC Analysis with Fluorescence Detection

The fluorescent derivatives of Sphingosine (So), Sphinganine (Sa) and D-erythro-C20-sphingosine (C20So) are resolved on an Agilent 1200 liquid chromatography system (Agilent Technologies, Wilmington, DE, USA). Chromatographic separations are performed on a Zorbax Eclipse XDB-C18 column (5 µm particle size, 250 × 4.6 mm, Agilent Technologies). C20So is used as an internal standard. The mobile phase consists of a linear gradient starting from 5 mM Triethylammonium Formate (TEAF) (pH 4.3)-methanol-acetonitrile (15/45/40, v/v/v) to methanol-acetonitrile (60/40, v/v) over 32 min at a flow rate of 1.0 ml/min. Excitation and Emission wavelengths are 340 nm and 455 nm, respectively. The peaks of Sa, So and C20So are identified by comparison with retention times of Sa So and C20So standards. Concentration of Sa and So is determined using external calibration curves generated from a serial of pure 80% methanol spiked with Sa, So and C20So standards at concentrations between 3.125nM and 50 nM.

Creatinine Adjustments

The levels of urinary Sa and So are adjusted for creatinine levels. Creatinine in urine is analyzed using the 96-well Creatinine Assay Kit from Cayman Chemicals (Ann Arbor, MI, USA). The assay was carried out according to manufacturer's instructions, and absorbance of each sample was measured using an ELx808 Absorbance Microplate Reader from BioTek Instruments, Inc. (Winooski, VT, USA). The analysis is based on a modified Jaffe colorimetric method that measures the difference in absorbance (495 nm) of the creatinine-picrate complex before and after acidification.

Statistical Analysis

All statistical analysis was done in Microsoft Excel Office 365 and the statistical software STATA (StataCorp. 2017. Release 15. College Station, TX: StataCorp LLC). The calibration curve used to quantify sphingolipid metabolites was obtained using linear least squares regression model in Microsoft Excel. Peak areas were plotted against 5 different concentrations of the standards. Age categorizations were done in accordance with a position paper available at the World Health Organization Website (Knoppert et al. 2007). Student t-tests were performed to assess significance levels and chi-square tests for independence between the samples. Significance level was defined as $p<0.05$. Wealth index is used as an indicator of Socio-Economic status, which is a proxy for household wealth and long-term standards of living. The Wealth index is generated using household asset data from questionnaires and calculated using the principal component analysis. Once the index is computed, national level wealth quintiles are obtained by assigning household scores with the lowest being categorized as poor, followed by lower middle class, then

middle class, upper middle class and the highest scores are considered rich households. The Wealth index has been shown to be consistent with expenditure and income measures (The DHS Program 2014). Box and Whisker Plots are used to graphically describe the distribution of the urinary sphingolipid metabolites.

Results

The mean age of children sampled is 8.07±3.54 years with a mean height 121.99±24.73cm and Mean Weight 23.71±8.79kg. 48% of the children were male and 44% female. 41% of the children sampled were between the ages of 6 – 12 years, 29% were young children between the ages of 3 – 6 years and 12% were adolescents. Only a total of 15 children aged 0 – 2 years were sampled. The mean age of parent or guardian who provided consent is 37.32±10.78 years. The mean dietary diversity score among children enrolled in the study is 3.88+/-0.09. In terms of Socio-Economic status, about 52% were considered middle class, 18% poor and 17% rich according to the national wealth index. Detailed data is shown in Table 7.1. The correlation co-efficient (r^2) of Sphinganine (Sa), Sphingosine (So) and D-erythro-C20-sphingosine (C20So) are 0.998, 0.999 and 0.999 respectively. The Average Retention times of Sa, So and C20So of all samples are 14.87, 16.84 and 18.70 minutes respectively. A sample chromatogram with respective retention times and areas used to determine levels of sphinganine, sphingosine and their ratio is shown in Figure 7.3. Sphinganine (Sa) was detected in 270 out of 284 samples which translates to a detection rate of 95.07% while Sphingosine (So) was detected in 281 out of 284 samples which reflects a detection rate of 98.94% of all samples. The overall total detection frequency of sphingoid bases is 97.00%.

The mean urinary sphinganine levels increases with increasing age. Children between the ages of 0 - 2 years registered mean urinary Sphinganine levels of 0.211±0.044 while children older than 12 years had a mean of 0.396±0.048. Detailed data are shown in Table 7.2. Conversely, mean urinary sphingosine levels are highly variable. Children between 0 to 2 years of age had mean levels of 0.029, suddenly increases for young children aged 3 – 6 years (0.363±0.029), then drops steadily for children between 7 to 12 years (0.351±0.024) and adolescents (0.321±0.037). Urinary sphingosine levels are highly variable across different age groups. Detailed data is shown in Table 7.3. In analysis of sphingoid bases, missing data were excluded for both age groups, and non-specified sex identities in statistical analyses. Thus, the sample size was invariably 252 and 262 respectively for tables 7.2 and 7.3 respectively. Urinary sphinganine and Sphingosine levels vary greatly.

On the contrary the Sa/So ratio is fairly stable and a preferred biomarker of effect of fumonisin exposure. Figures 7.4 and 7.5 represents graphical display of sphingolipid metabolites ratio according to socio-economic status and age respectively. Out of 284 urine samples, 125 were female, 137 male and 22 samples did not have specified sex identities. Non-specified sex identities represented 8% of the samples with mean Sa/So ratio of 0.21 and a standard deviation of 0.22. Comparing females to males, the Sa/So ratio ranges between 0.00nM – 1.66nM for female children and 0.00nM – 7.25nM for males. In addition, the computed Sa/So ratio means were 0.25±0.23 for females and 0.35±0.81 for males. Total Sphinganine (Sa) concentrations were found to range between 0.00nM – 19.49nM, with a mean of 1.23±2.18 whereas, Total Sphingosine (So) concentrations range between 0.17nM – 63.95nM and a mean of 4.84±8.17. The range of the Sa/So ratio is

0.00nM – 7.25, with a mean value of 0.30±0.59. The detection limit for sphingoid bases was 0.01nM. The overall results are shown in Table 7.4.

Discussion

The study population is dependent on corn-based foods as staple diets. Availability, access, utilization and sustainability of different food groups define household food security and are used to determine the dietary diversity scores (Steyn et al. 2006). Dietary diversity scores are the sum of a number of food groups consumed over a reference period of 24 hours or 7 days (Food and Agriculture Organization (FAO) 2011; Kennedy 2009). The International Food Policy Research Institute proposed the following thresholds; (< 4.5 low dietary diversity, 4.5 – 6 medium dietary diversity and >6 as high and good dietary diversity (Vhurumuku 2014). In this study, the mean dietary diversity score is 3.88+/-0.09, which is less than the minimum dietary diversity score. In a different study in Makueni and Machakos counties of Kenya, baseline findings of dietary diversity among children revealed low diversity with close to 80% of children in the study population consuming less than 4 food groups in a day (Bukania et al. 2014) which corroborates overreliance of corn based foods. Consuming a variety of foods from several food groups is a recommended approach to achieve necessary nutritional requirements. Previous ecological human studies have been done in adult subjects. The mean, range and or standard deviations of sphingosine, sphinganine and Sa/So ratios are more commonly reported compared to other measures of dispersion. Past Studies in Burkina Faso, South Africa, Bomet Kenya, Central Argentina and the Rural Zone in Portugal reported mean Sa/So ratios of 0.44 (0.01 – 7.18), 0.41 (0.01 – 5.75), 0.34 (0.03 – 0.74), 0.39±0.04 and 0.43±0.22 respectively (Nikiema et al. 2008; van der Westhuizen et al. 1999; Silva, Lino, and Pena

2009; Solfrizzo et al. 2004). A summary of similar ecological studies in human populations that assessed urinary Sa/So ratios in adult populations is shown in Table 7.5. The mean Sa/So ratio in this study was determined as 0.30±0.59. Compared to previous application studies to human populations, the mean sphinganine to sphingosine ratio is significantly lower in this study. The lower Sa/So ratio in this study could be due to the fact that the study subjects were children with a mean age of 8.07±3.54 years.

In addition, some studies assess the variation of sphingoid bases in subjects according to sex. This study is the first to report higher mean of urinary Sa/So ratio of males (0.35±0.8) than in female subjects (0.25±0.23). By contrast, most studies report higher urinary Sa/So ratios in females compared to males. For example, the study in Henan, China reported Mean urinary Sa/So ratios of 0.20 in females and 0.11 in males (Qiu and Liu 2001). Similarly, in Croatia, the female urinary Sa/So ratio of female control group was 0.25±0.08 compared to 0.18±0.12 in males (Ribar, Mesaric, and Bauman 2001). Other studies, one in France and another in Italy reported non-detectable urinary Sa/So ratios in male subjects (Solfrizzo, Avantaggiato, and Visconti 1997; Castegnaro et al. 1996). While male subjects tend to have a lower number of exfoliated cells in urine, these studies were done with earlier methodologies which have much been modified and improved to ensure high degree of sample sensitivity. The current study is the first to report a higher urinary Sa/So ratio in male subjects. A t-test to evaluate the influence of age distribution and socio-economic status (as categorized in table 7.1) yielded non-significant results (data not shown).

While female subjects tend to have higher urinary cells compared to males, this study reports a higher ratio in males. Our study population are mainly children between the

ages of 6 and 12 years. Previous studies reported higher ratios in females because study participants are female adults. Thus, higher ratios in female adults is attributed to the presence of sphingolipids in exfoliated residual cells available in the cell membranes from the monthly menstrual cycles. On the contrary, our study population was children which are not comparable to the adult population. Moreover, studies previously done in our laboratory showed that the difference in urinary Sa/So ratios even though significant between males and females, were not significant in either blood plasma or serum samples (Qingsong 2007), an indication of the contributory role of residual cells in urine. More studies on children are warranted for comparison purposes and to establish the biological plausibility of the difference in urinary Sa/So ratios. Other studies have reported Sa/So ratio in serum, tissues and buccal cells alongside urinary sphingoid bases. However, use of urinary Sa/So ratio is the only validated methodology. Studies done in Kenya, 2 regions in South Africa and Burkina Faso reported mean and range of Sa/So ratios in serum levels as 0.28(0.17 – 0.50), 0.34 (0.01 – 2.97), 0.43(0.12 – 0.88) and 0.64(0.13-0.31) respectively (van der Westhuizen et al. 1999; Nikiema et al. 2008). The mean Sa/So ratios in serum are higher than urinary Sa/So ratios. Nonetheless, urinary sphinganine to sphingosine ratio together with their sphingoid base analogs Sphinganine-1-Phosphate and Sphingosine-1-Phosphate are validated biomarkers of fumonisin exposure due to their specificity and sensitivity to fumonisin exposure (Cai, Tang, and Wang 2007). Conversion of sphingoid bases to their Sphinganine-1-Phosphate and Sphingosine-1-Phosphate, is a cellular response to adjust for altered Sa/So ratio thereby preventing cytotoxic effects of accumulated sphingoid bases which are more likely to be cytotoxic (Schwarz and Futerman 1998). Exfoliated cells in urine are the primary source of sphingolipids, an indication of

accumulation of sphingoid bases in the kidney. Fumonisin have been shown to be toxic to many mammalian organs, however, the liver and kidney are major targets for toxicity. FN induced disruption of sphingolipid metabolism has been demonstrated in many independent studies (Silva, Lino, and Pena 2009; Cano-Sancho et al. 2011; van der Westhuizen et al. 2008). The balance between intracellular concentrations of sphingolipid metabolites that prevent or induce apoptosis is altered when the rate of increase of sphingolipid metabolites exceed the cells' ability to convert them to their sphingoid base-1-phosphate thus accumulating to toxic levels (Merrill et al. 2001). This is important because the balance is a critical determinant of fumonisin induced hepato- and nephrotoxicity (Enongene et al. 2002; Rumora et al. 2007).

In a study to evaluate sensitivity of kidney and liver to fumonisin toxicity, researchers at USFDA fed male Sprague-Dawley rats with rodent chow containing *Fusarium verticillioides*. A rapid decrease in free sphingoid bases was observed in the liver while the kidneys accumulated the sphingolipids for a longer period (Riley and Voss 2006). Similarly, experimental studies in rats have shown persistent ratios in kidney tissues compared to liver tissues (Enongene et al. 2002). Overall, the kidneys are the most sensitive to FB_1 induced toxicity (Rumora et al. 2007; Riley 1994; Riley and Voss 2006). Fewer human studies have sought to establish a dose response relationship between Fumonisin B_1 exposure and altered sphinganine to sphingosine ratio. Some studies however have estimated Fumonisin intakes to be 0.55, 0.57, 0.81, 3.8, and 184 µg/kg bw/day (Qiu and Liu 2001; Solfrizzo, Avantaggiato, and Visconti 1997). Higher estimations of fumonisin intakes do not necessarily translate to higher Sa/So ratios and thus, human population

studies that aim to assess dose response are urgently needed to determine levels at which FB_1 triggers alteration in sphingoid base levels.

Conclusions

Owing to their structural similarity to sphingoid bases, FB_1 provokes the inhibition of sphingolipid biosynthesis. This leads to the accumulation of free Sphinganine and an increase in the ratio of free sphinganine to free sphingosine in biological fluids. The ratio between Sphinganine and Sphingosine becomes elevated after exposure to adequate amounts of fumonisin. While different studies have reported different ratios, further studies on dose-response especially in human populations is warranted.

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Table 7.1. Baseline Characteristics of the Children in the Study

Variables N = 284	Frequency (%)
Sex	
Male Children	137 (48)
Female Children	125 (44)
Non-Specified	22 (7)
Age (Years)	8.07±3.54
Age Grouping	
0 – 2 (Infants)	13 (4.58)
3 - 6 (Young Children)	82 (28.87)
6 – 12 (Child)	116 (40.87)
>12 (Adolescent)	34 (11.97)
N.S	39 (13.73)
Height (cm)	121.99±24.73

Weight (kg)	23.71±8.79
*Guardian Age	37.32±10.78
Socio-Economic Status	
Poor	50 (17.61)
Middle Class	149 (52.46)
Rich	48 (16.90)
N.S	37 (13.03)

Normally distributed variables are expressed as means ± SD and categorical variables are frequency (percentages). Age Groups is based on position paper by Knoppert et al (2007). N.S – Non-Specified *Age of parent or guardian who provided consent

Table 7.2. Urinary Sphinganine Metabolites according to Age Groups

Age Groups (Years)	Infants (0-2)	Young Children (3-6)	Children (7- 12)	Adolescents >12 years	All Children (0 – 14)
n	15	71	129	37	252
Mean± SE	0.211±0.044	0.267±0.029	0.333±0.025	0.396±0.048	0.316±0.017
GM	0.130	∞	0.188	0.267	∞
Median	0.140	0.171	0.208	0.298	0.214
SD	0.169	0.244	0.289	0.289	0.274
Minimum	0.011	-0.001	0.000	0.012	-0.001
Maximum	0.485	0.953	0.998	0.904	0.998

Table 7.3. Urinary Sphingosine Metabolites according to Age Groups

	Infants (0-2years)	Young Children (3-6 years)	Children (7- 12years)	Adolescents >12 years	All Children (0 – 14 years)
n	15	74	133	40	262
Mean± SE	0.029±0.006	0.363±0.029	0.351±0.024	0.321±0.037	0.346±0.016
GM	0.020	0.253	0.233	0.222	0.234
Median	0.022	0.313	0.271	0.251	0.274
SD	0.025	0.249	0.272	0.236	0.258
Minimum	0.003	0.003	0.002	0.003	0.002
Maximum	0.092	0.990	0.991	0.870	0.991

Table 7.4. Frequency, range and mean concentrations of Sa, So and Sa:So Ratio in Children

Gender		Range			Mean ±SD		
Frequency (%)		**Sa**	**So**	**Sa/So**	**Sa**	**So**	**Sa/So**
Female	125(44)	0.00 – 19.49	0.22 – 63.95	0.00 – 1.66	1.71±2.74	7.18±11.28	0.25±0.23
Male	137(48)	0.00 – 10.66	0.17 – 18.90	0.00 – 7.25	0.86±1.49	2.94±3.09	0.35±0.81
Total	262(92)	0.00 – 19.49	0.17 – 63.95	0.00 – 7.25	1.23±2.19	4.84±8.17	0.30±0.59

Table 7.5. Urinary sphingolipids metabolites ratios reported in other human studies.

Study Area	Sample Size	Sa/So Ratio	Reference
Burkina Faso	87	0.44 (0.01 – 7.18)	Nikiema et al. 2008
Henan, China **Pre-Exposure** **Post- Exposure**	 28 28	 †0.159(0.028– 0.240) †0.196(0.037-0.870)	Qui and Liu, 2001
Transkei, South Africa **Bomet, Kenya**	153 27	†0.41 (0.01 – 5.75) †0.34 (0.03 – 0.74)	van der Westhuizen et al. 1999
North Argentina **South Brazil** **Central Argentina** **South Italy**	74 116 20 66	ǂ0.69±12 ǂ1.57±0.49 ǂ0.39±0.04 ǂ0.35±0.02	Solfrizzo et al. 2004
Catalonia, Spain **Low & Non-Consumers** **Grain Consumers**	 43 35	 *0.55±0.47 (0.40) *0.62±0.47 (0.56)	Cano-Sancho et al. 2011
Central Zone, Portugal **Urban** **Rural**	 38 30	 ǂ0.42±0.17 ǂ0.43±0.22	Silva, Lino, and Pena 2009
Makueni, Kenya	284	ǂ0.30±0.59	This study (Wangia et al 2018)

†Reported Mean and Range *Reported median values and Range, mean in parenthesis, ǂReported Mean values and Standard Deviations (SD)

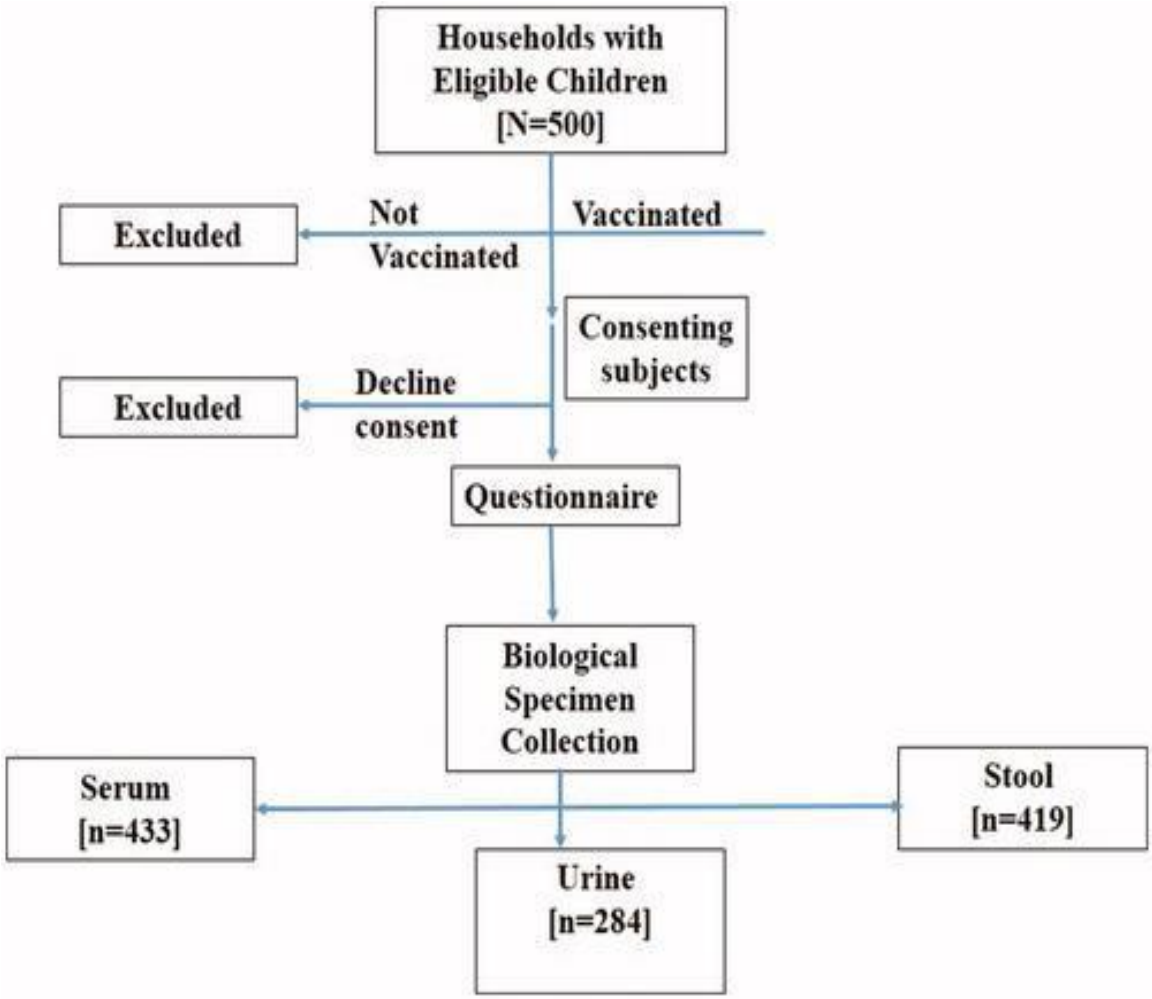

Figure 7.1. Recruitment of Study Participants

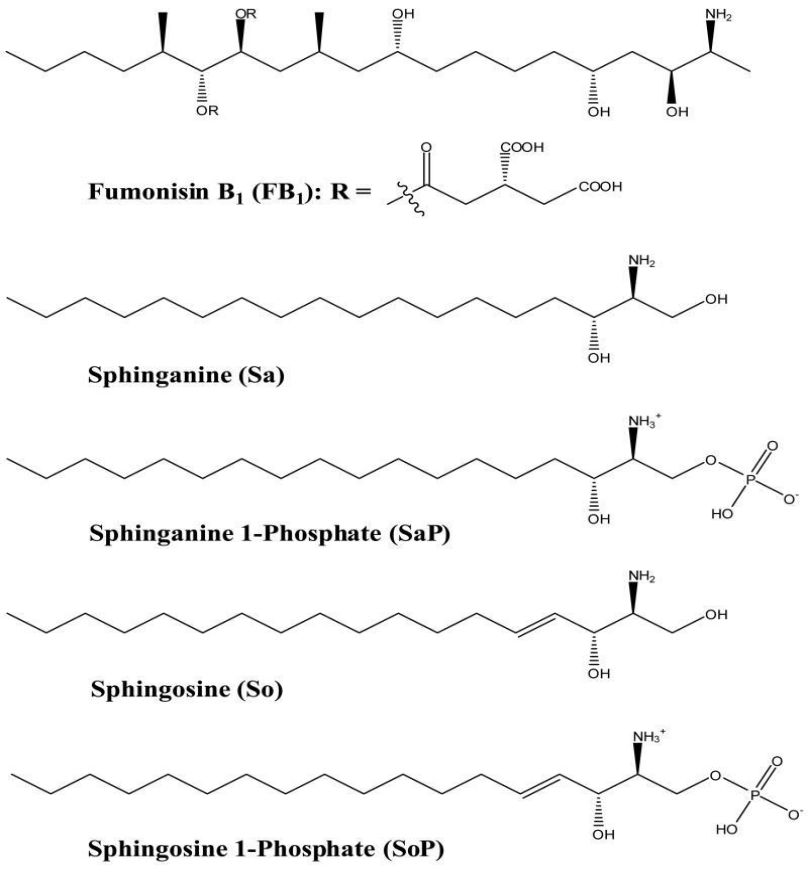

Figure 7.2. 2D Chemical Structures of Fumonisin B_1, Sphingosine, and Sphinganine

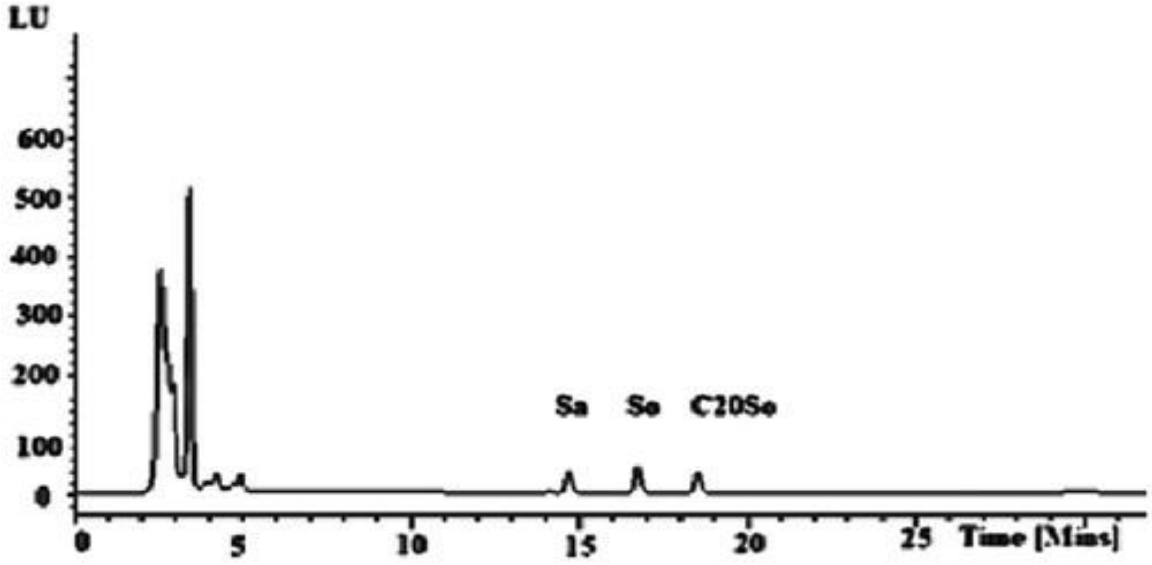

Figure 7.3. Chromatograph with Respective Retention Times and Areas

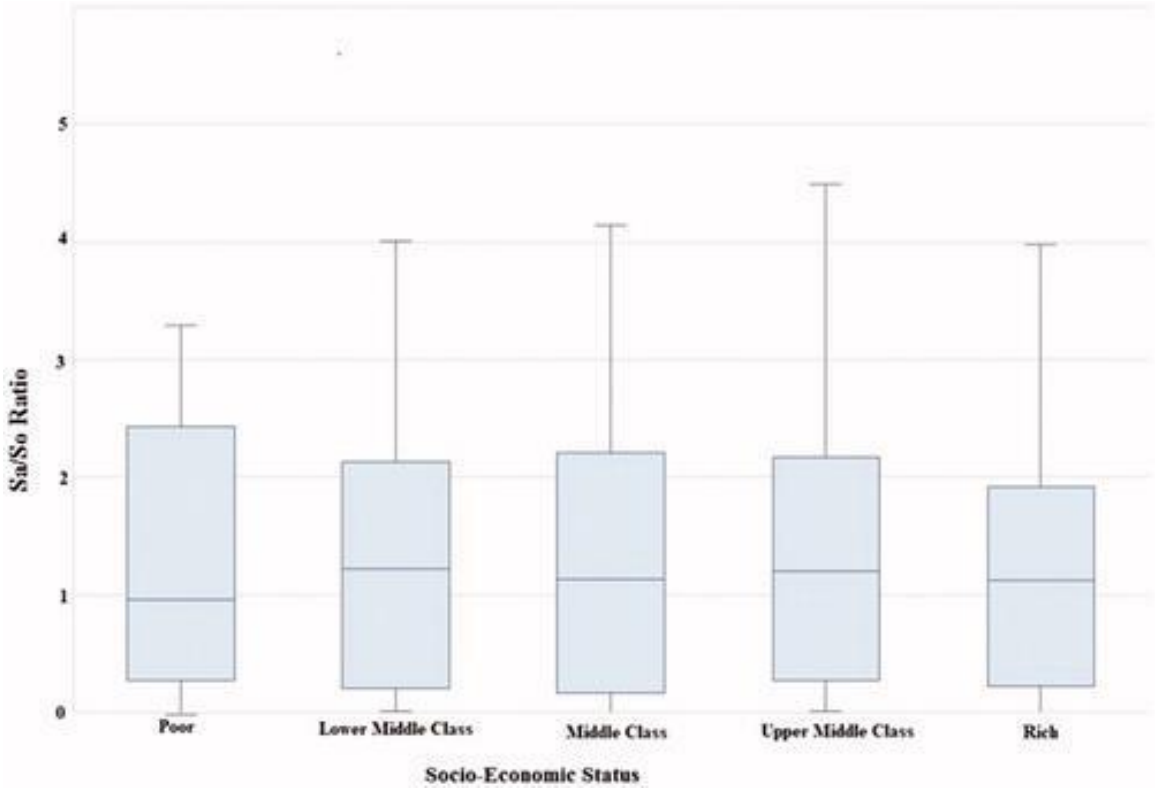

Figure 7.4. Variation of Sphingolipid Metabolites Ratio by Socio-Economic Status

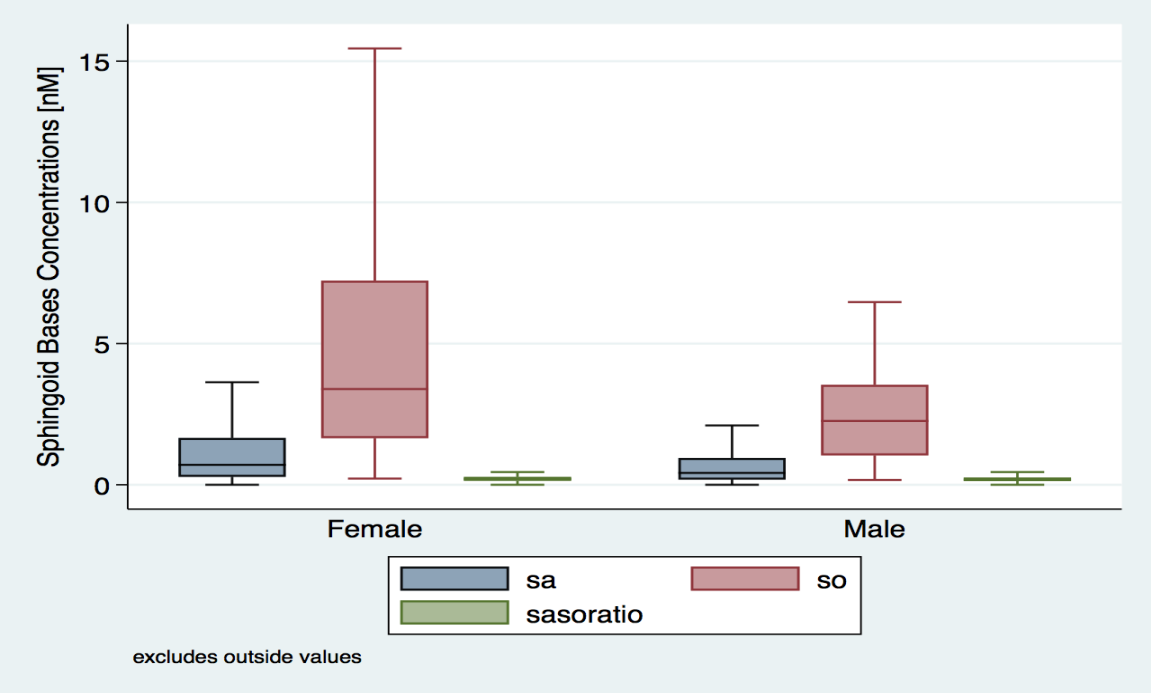

Figure 7.5. Variation of Sphingolipid Metabolites Ratio by Sex

Chapter 8

ASSESSMENT OF FUMONISIN EXPOSURE AMONG CHILDREN IN KENYA USING URINARY FUMONISINS B_1[6]

[6] Ruth Nabwire Wangia-Dixon, Brennan Carter, Marshae Castleberry, Kathy Siyu Xue and Jia-Sheng Wang.To be submitted to World Mycotoxin Journal.

Abstract

Fumonisins are a family of mycotoxins produced by the *Fusarium* fungi. Among the 28 different types of fumonisins, Fumonisin B_1 [FB_1] is classified to group 2B, a possible human carcinogen by the International Agency on Research Cancer. Exposure to Fumonisins results in species and organ specific toxicities including neurological disorders for equids, pulmonary edema in swine, esophageal cancer in humans and both kidney and liver related toxicities in rodents. Urinary free FB_1, sphingolipids and their ratio are used as specific biomarkers of fumonisin exposure. In this study, we assess fumonisin exposure using urinary free FB_1 in children between the ages of 6 – 12 years from Siaya and Makueni Counties of Kenya. When adjusted for creatinine levels, final geometric means of FB_1 ng/mg creatinine were 0.07 ng/mg and 0.08 ng/mg for Siaya and Makueni Counties respectively, p-value > 0.05. Children with high aflatoxin B_1-lysine adducts in their sera exceeding 20 pg/mg of albumin also had high urinary FB_1 compared to children with low aflatoxin B_1-lysine adducts less than 10pg/mg albumin. The geometric mean of urinary FB_1 was 0.13 ng/mg creatinine (95% CI 0.11, 0.15) and 0.04 ng/mg creatinine (95% CI 0.03, 0.05) among children with high and low aflatoxin B_1-lysine adducts respectively. It appears that the exposure to fumonisins is associated with growth retardation. Among children with low height-for-age z-score, the geometric mean of urinary FB_1 levels were 0.13 ng/mg creatinine (95% CI 0.10, 0.19), and 0.11 ng/mg creatinine (95% CI 0.06, 0.22) for low weight-for-age score. These exposure levels are significantly higher when compared to exposure levels among children without growth impairments. Overall, children with the worst growth outcomes had higher urinary FB_1. Reducing aflatoxin and fumonisin

contamination of corn and dietary diversification can prevent infants and the public, in general, from exposure to the mycotoxins.

Keywords: Fumonisins, Urinary FB_1, Children, Growth Impairments

Introduction

Fumonisins are naturally occurring mycotoxins produced by *Fusarium moniliforme* and other related *Fusarium* species (Bezuidenhout et al. 1988; Gelderblom et al. 1988). *Fusarium moniliforme* is a common contaminant of corn everywhere in the world (Shephard et al. 2002; Come et al. 2019; KuiperGoodman et al. 1996; Paterson et al. 2018). Fumonisins were identified and characterized in the late 80s and early 90s shortly after their discovery in 1988 in South Africa by researchers at Programme on Mycotoxins and Experimental Carcinogenesis. To date, 28 different fumonisins groups have been characterized and classified into A, B, C and P groups (Rheeder, Marasas, and Vismer 2002). Fumonisins in the B group are the most toxic, among which fumonisin B_1 [FB_1] is the most potent and predominant isomer accounting for more than 60% of the fumonisins contamination in food products.

The primary mechanism of fumonisins toxicity is established to be disruption of sphingolipid synthesis de novo by way of FB_1's inhibitive action of the enzyme ceramide synthase (Merrill Jr et al. 2001; Riley and Merrill Jr 2019; Voss, Smith, and Haschek 2007). Toxicity, and perhaps the carcinogenicity of fumonisins is attributed to the accumulation of bioactive sphingolipids as well as the depletion of complex sphingolipids (Merrill et al. 1996). Fumonisins toxicity is species-specific. Equids are highly susceptible to Fumonisins toxicity with rapid development of the neurological disorder Equine Leuko-Encephalomalacia (ELEM) shortly after exposure. Equine leucoencephalomalacia

[ELEM] is an acute, non-infectious and highly fatal neurological disease of equids previously reported in the United States, South America, China, Greece, Egypt, South Africa and Germany, mainly in horses fed fumonisin contaminated feed (Haliburton and Buck 1986; Marasas et al. 1988). Swine are also sensitive to fumonisin toxicity and likely to develop Porcine Pulmonary Edema after consumption of fumonisin contaminated feed (Smith et al. 1999; Haschek et al. 2001; Voss, Smith, and Haschek 2007). In humans, epidemiological studies have linked higher incidence of esophageal cancer to consumption of fumonisin contaminated corn (Dlamini and Bhoola 2005; Khan, Phulukdaree, and Chuturgoon 2018; Nowrozi et al. 2018; Come et al. 2019). Therefore, regulatory agencies such as FDA have utilized guideline levels for animal feed not only to limit animal diseases but also to protect public health.

The US FDA recommends that foods for human consumption should have total fumonisins levels between 2 to 4 mg/kg (USFDA 2001). Corn and/or corn products, a major staple diet to more than 5 billion of the world's population are the most susceptible to contamination (Yoshizawa, Yamashita, and Luo 1994; Wang et al. 2000; de Castro et al. 2004). Based on a large survey with various corn samples collected from Argentina, Benin, Croatia, Poland, Portugal, Italy, Romania and Zambia's fumonisin contamination levels; it was established that fumonisins accumulation in corn is highly dependent on environmental conditions in the specific area (Visconti 1996). Fumonisins may be found in sound whole kernel corn at levels at or below 1.0 mu g/g. By contrast animal disease problems begin to occur at fumonisin levels above 5.0 to 10.0 mu g/g. Corn based food products that have the most frequent and highest fumonisin levels, besides whole kernel corn, are corn meal, corn flour and corn grits (Bullerman 1996). Analytical methods have

been developed for fumonisin analysis in food products. Nonetheless, improvements are needed for more accurate, less expensive, and more rapid assays of food and feedstuffs can be done. The validity and significance of reported fumonisin levels depend on several factors such as the specificity detection limit, accuracy and reproducibility of the analytical method as well as on the sampling procedure used, and the integrity and purity of the analytical standards. Given that urinary FB_1 are validated biomarkers for fumonisins, the analytical methods of urinary fumonisin B_1 used in the current study is based on methodologies developed by (Cai, Tang, and Wang 2007; Xu et al. 2010).

Natural contaminants such as fumonisins present in animal feed can also enter the human food supply as residues present in animal tissues and other animal derived products (Miller, Honstead, and Lovell 1996; Troxell 1996). Moreover, weaning foods have been found to be contaminated by fumonisins (Kumi et al. 2014; Magoha et al. 2016). The FBs are heat stable, resistant to ammoniation, and unlike most mycotoxins, are water-soluble (Murphy et al. 1996). Ammoniation, a procedure used for decontamination of aflatoxins, yielded a 79% reduction in FB_1 levels in naturally contaminated corn (Park et al. 1996). Once ingested, FB_1 is poorly absorbed, rapidly excreted, and persists in small amounts in the liver and kidney (Norred et al. 1996). Therefore, fumonisins have been established to be acute renal and liver toxins in rats, and have tumor promoting activity. Culture material of this F. moniliforme isolate has also been shown to exhibit cancer-promoting activity in a short-term cancer initiation-promotion bioassay with diethyl nitrosamine-initiated rats and the induction of gamma-glutamyl-transpeptidase-positive [GGT+] foci as an endpoint after 4 weeks of promotion (Gelderblom et al. 1988). All FB_1-treated rats that died or were killed from 18 months onwards suffered from a micro- and macro nodular cirrhosis and

had large expansive nodules of cholangio fibrosis at the hilus of the liver (Gelderblom et al. 1991). The fumonisins are carcinogenic in laboratory rats and cause acute toxicity of domestic animals that mimics field cases of disease attributed to contamination of feed by F. moniliforme. Fumonisin B_1 [FB_1] is classified to group 2B, a possible human carcinogen by the International Agency on Research Cancer.

In the past, the basis for exposure assessment was based on surveys of foods as well as a knowledge of consumption patterns (Lombard et al. 2014; Esposito et al. 2016). Nonetheless, use of biomarkers have gained traction due to ability to evaluate disease risk (van der Westhuizen et al. 2011).This is the first study to assess fumonisin exposure in human population in Kenya using urinary fumonisin B_1 biomarkers.

Methods

10ml aliquot of urine samples, previously stored in -80 C was used in analysis. The samples were centrifuged at 1500 rpm for 5 minutes. Sample extractions uses FumoniTest column which contain antibodies specific to fumonisins where they bind during extraction processes. FumoniTest columns were set up by slicing the cap to create adaptors for 10ml glass syringes. All FumoniTest columns were filled up with 1xPBS to avoid bubbles prior to connecting the FumoniTest columns to the 10ml glass syringes. Using 1xPBS, the FumoniTest columns are washed to get rid of any impurities and prior contamination. The 1xPBS solution is drained off by use of vacuum to aid drainage but maintaining the flow rate at less than 2 drops per second. When liquid level is close to 0.5ml mark, stop flow by turning off valve then pour urine supernatant directly and let the liquid drain until 0.5mL mark of syringe. Wash again with 1xPBS and drain the liquid all the way. While waiting,

precondition Oasis HLB cartridges with 3x1ML MeOH and 3x1ml HPLC H2O. Transfer the conditioned cartridge to corresponding spots in the manifold insert rack and place into the manifold glass tank. Elute contents from FumoniTest with 3x1 ml 10mM HCL 20% methanol and allow it to collect into HLB cartridges. Once all contents are drained from the FumoniTest columns, empty the cartridges of all liquid using a bulb and then transfer the HLB cartridge onto the manifolds. Wash HLB with 3x1mL HPLC H2O and 3x1mL 25% MeOH and then dry the cartridges. Elute with 3x0.5mL 2% formic acid. Set up a bead bath temperature control turning on High setting for 10 minutes, and then switch to Low for the remainder of the time. Dry samples at 35 degrees Celsius under pure Nitrogen gas optimally for 20 minutes each. Once the samples are dry, reconstitute with 200ul 50%MeOH, mixing thoroughly for 10 minutes then centrifuge at 1500rpm for 5 minutes. Load 150ul into HPLC vials. Agilent 1100 LC system is used in HPLC analysis with the analytical column 250 X 4.6mm Luna 5u C18 (2), maintained at 40 degrees Celsius. The mobile phase A is comprised of 100mM Sodium Phosphate Monobasic, pH 3.35 by H3 PO4, and eluant B is 100% methanol. The gradient is set at 65% B from 0 to 5 minutes, linearly increased to 95% B in 13 minutes, hold at 3 minutes before back 65% B in 5 minutes. Flow rate maintained at 1.0mL/min. All urinary FB_1 was adjusted for individual creatinine levels in urine. Creatinine in urine is analyzed using the 96-well Creatinine Assay Kit from Cayman Chemicals (Ann Arbor, MI, USA). The assay was carried out according to manufacturer's instructions, and absorbance of each sample was measured using an ELx808 Absorbance Microplate Reader from BioTek Instruments, Inc. (Winooski, VT, USA). The analysis is based on a modified Jaffe colorimetric method that measures the difference in absorbance (495 nm) of the creatinine-picrate complex before

and after acidification. STATA v15 (College Station, TX) and R version 3.6.1 (Vienna, Austria) statistical tools were used for data cleanup and analyses.

Results

Overall, 53.3 % [439/811] of children enrolled in the study were female and 45.87% [372/811] were male. The sex distribution was slightly different per county where 49.8% children enrolled from Makueni County were male while for Siaya, 43.2% enrolled were male meaning more girls were enrolled compared to males. The median age of children enrolled in the study is 9 years, Interquartile Range [8, 11] years. Overall, the number of children below 9 years were 50.12% [404/806] and the number of children above 9 years were 49.88% [402/806]. The frequency of children with non-specified ages are 17. The median weight of children enrolled in the study was 28 kg, IQR [25, 35]; 44.12% [330/748] of the children weighed less than 28 kg while up to 55.88% [418/748] of the children weighed more than the median weight. The median height of children in our study is 135 cm and the proportion of children shorter than 135 cm is 48.66% [364/748] while 51.34% [384/748] were taller than 135cm. About 10.09 % [75/743] children are considered stunted due to their low height-for-age z-score. Among the children with low height for age z-score, 15/327 [4.6%] were enrolled from Siaya while 60/484 [12.4%] were from Makueni County. Detailed data is shown in Table 8.1.

In terms of urinary samples 242 samples were available from Siaya and 286 from Makueni County for analysis of free Fumonisins B_1. Total geometric mean of FB_1 (ng) in 200uL reconstituted extracts of 10mL urine was 0.44ng and 0.66 ng for Siaya and Makueni Counties respectively. When adjusted for creatinine levels, final urinary FB_1 were 0.07

ng/mg and 0.08 ng/mg for Siaya and Makueni Counties respectively. While higher exposures were observed in urinary samples from Makueni County, the difference was not statistically significant, p-value > 0.05. Detailed summary data is shown in Table 8.2.

Overall, boys had higher urinary FB_1 compared to girls across all age groups. The geometric mean of urinary FB_1 among boys was 0.08 ng/mg creatinine (95% CI 0.07, 0.10) while girls' GM was 0.07 ng/mg creatinine (95% CI 0.05, 0.08), p-value <0.01. The highest variation in exposure levels between boys and girls was observed among children 8 years old with the geometric mean reported for boys as 0.09 ng/mg creatinine (95% CI 0.05, 0.17) and a significantly lower geometric mean of 0.04 ng/mg creatinine (95% CI 0.03, 0.05) among girls (Table 8.3).

In terms of weaning, children weaned before the recommended six months had higher urinary FB_1 compared to children weaned after 6 months (Figure 8.1). The mothers reported using a myriad of different flours for weaning with children weaned on maize-based weaning foods had the highest urinary FB_1. Children weaned on commercially available baby porridge had urinary FB_1 levels 0.06 ng/mg creatinine (95% CI 0.04, 0.08) cassava-based weaning flour, 0.07 ng/mg creatinine (95% CI 0.06, 0.10), slightly higher urinary FB_1 in children weaned on maize-based weaning flours 0.08 ng/mg creatinine (95% CI 0.06, 0.10). All children were exposed notwithstanding the choice of weaning flours, which further indicates that mycotoxin contamination is widespread ad a persistent challenge.

Children with high aflatoxin B_1-lysine adducts in their sera exceeding 20 pg/mg of albumin also had high urinary FB_1 (Figure 8.3).The geometric mean was 0.13 ng/mg creatinine (95% CI 0.11, 0.15) compared to urinary FB_1 of 0.04 ng/mg creatinine (95% CI

0.03, 0.05) among children with low aflatoxin B_1-lysine adducts less than 10pg/mg of albumin. Moreover, Children with the worst growth outcomes had higher urinary FB_1 when compared to children without growth deficits (Table 8.3). For instance, among children with low height-for-age z-score, urinary FB_1 levels were 0.13 ng/mg creatinine (95% CI 0.10, 0.19), low weight-for-age score 0.11 ng/mg creatinine (95% CI 0.06, 0.22) both of which are significantly higher when compared to exposure levels among normal children.

Finally, in terms of socioeconomic status, children from high income households had lower levels of urinary FB_1 0.05 ng/mg creatinine (95% CI 0.03, 0.09) and children from low income households had the highest urinary FB_1. Compared to children from middle and high income households, the GM were 0.08 ng/mg creatinine (95% CI 0.06, 0.11) and 0.07 ng/mg creatinine (95% CI 0.06, 0.08) in high- and middle- income households respectively (Figure 8.2). There was no statistical difference between exposure levels among farming and non-farming households. Additionally, no statistically significant differences between urinary exposure among household across different education levels strata.

Discussion

Urinary free FB_1 was detected in 100% of urine samples collected from Siaya and in 96.85% of the samples from Makueni. In the past, urinary fumonisins were detectable in 96% of children recruited from different villages of Tanzania (Shirima et al. 2013). When adjusted for creatinine levels, final FB_1 ng/mg creatinine were 0.07 ng/mg and 0.08 ng/mg for Siaya and Makueni Counties respectively in the current study. While higher exposures were observed in urinary samples from Makueni County, the difference was not

statistically significant, p-value > 0.05. Similar exposure levels have been reported in different villages in Ghana, Tanzania, Guatemala, and Mexico (Kimanya et al. 2010; Riley et al. 2012; Torres et al. 2014; Shirima et al. 2015). The use of urinary FB_1 provides a reliable biomarker for fumonisins exposure. Urinary creatinine is used to adjust for fumonisins concentrations and to control for inter-individual variation in urine concentration.

Fumonisin intake is generally high in areas where corn is a dietary staple, and in the current study, corn is a dietary staple with the study participants reporting limited dietary diversity of less than 4 food groups (Wangia et al. 2019). In a study in Guatemala, 10 participants consumed 206g of tortillas, and the detection rate of urinary FB_1 was 61% (Riley et al. 2012). Similarly, FB_1 in corn and urine was significantly higher in tortilla consuming villages of Guatemala where fumonisins intake paralleled urinary fumonisin B_1 in a dose-dependent manner. Nonetheless, urinary fumonisin B_1 was present in much higher levels than urinary fumonisin B_2 or urinary fumonisin B_3 (Torres et al. 2014). In a previous study, Gong et al reported urinary FB_1 0.04 ng/mg creatinine (95% CI 0.02, 0.08) among low corn consumption households and 0.13 ng/mg creatinine (95% CI 0.08, 0.22) among high corn consumption areas (Gong et al. 2008). In different villages of Tanzania, the geometric mean of urinary fumonisin B_1 was 327.2 (95% CI 217.1- 493.0) pg/mL in Kigwa, 211.7 (95% CI 161.1- 278.1) pg/mL in Nyabula , and 82.8 (95% CI 58.3 -117.7) pg/mL in Kikelelwa (Shirima et al. 2013). In a different study also done in Tanzania reported that geometric means of urinary fumonisin B_1 among children at recruitment were 313.9 (95% CI 257.4, 382.9) pg/mL creatinine and increased significantly to 569.5 (95% CI 464.5, 698.2) pg/mL creatinine, by month 12 when the study was completed (Shirima

et al. 2015). Taken together, results from these studies support the use of urinary FB_1 to assess ongoing fumonisin exposure in population-based studies.

In the current study, weaning children before the recommended 6 months was a risk factor for higher urinary FB_1 compared to children weaned after 6 months. A study done in Tanzania also reported higher levels of urinary FB_1 in children who had been fully weaned before the recommended 6 months ($p < 0.01$) (Shirima et al. 2013). Similarly, children who were fully weaned had 3-fold higher urinary DON than those on partial breastfeeding (P=0.002) (Gong et al. 2015). It appears that the exposure to fumonisins is associated with growth retardation. Children with the worst growth outcomes had higher urinary FB_1. For instance, among children with low height-for-age z-score, urinary FB_1 levels were 0.13 ng/mg creatinine (95% CI 0.10, 0.19), low weight-for-age score 0.11 ng/mg creatinine (95% CI 0.06, 0.22) both of which are significantly higher when compared to exposure levels among normal children.

Co-exposure of mycotoxins is highly prevalent and has been associated with adverse health outcomes including vaccine interference and growth impairments in children (Kimanya et al. 2010; Shirima et al. 2015). In the current study, children with high urinary FB_1 concurrently had high aflatoxin B_1 lysine adducts in their sera with 100% co-exposure in Siaya County and 97% in Makueni County. Studies in Tanzania reported that up to 82% of all the children in the study were exposed to both aflatoxins and fumonisins (Shirima et al. 2013; Geary et al. 2016). Co-exposure with DON has also been previously reported where both DON and FB_1, were detected in urine samples in more than >96% of the children recruited into the study with urinary DON geometric means of 15.4, ng/ml and the urinary FB_1 were 0.62, ng/ml, respectively (Gong et al. 2015).

Exposure to fumonisins through dietary means has been shown to be a contributing factor to growth deficits in children exposed in early life. For instance, in an earlier study done in Tanzania, children exposed to fumonisins intakes above the provisional maximum tolerable daily intake of 2 μg/kg bodyweight were significantly shorter by 1.3 cm and 328g lighter (Kimanya et al. 2010). Similarly, another study in Tanzania reported an association between fumonisins exposures and growth retardation. Specifically, urinary fumonisin B_1 concentrations at recruitment were negatively associated with length-for-age z-scores (LAZ) at 6 months ($p = 0.016$) and at 12 months from recruitment ($p = 0.014$) (Magoha et al. 2016). Given the public health implications associated with fumonisin exposure, control and intervention strategies are urgently needed.

Conclusions

Fusarium moniliforme contamination of corn consumed by humans in certain areas of the world is associated with higher than average incidence of esophageal cancer and fumonisins may be responsible for growth impairments in children. Reducing aflatoxin and fumonisin contamination of corn and dietary diversification can prevent infants and the public, in general, from exposure to the toxins. Urinary fumonisin B_1 reflects individual fumonisins exposure and thus represents a valuable biomarker for future fumonisin risk assessment. Nonetheless, further studies are required to assess the risk to disease development based on exposure to fumonisins. Furthermore, it is imperative to develop additional biomarkers for human biomonitoring studies not only for common occurring aflatoxins and fumonisins, but also all mycotoxins because it has been established that co-occurrence is commonplace.

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Table 8.1. Characteristics of children in the study population

VARIABLES	FREQUENCY (%)
Sex	
Female	439 (53.3)
Male	372 (45.2)
Non-specified	12 (1.5)
Children's Age {median [IQR†]}	9.00 [8.00, 11.00]
Age of the Child Dichotomized	
<=9 Years	404 (49.1)
>9 Years	402 (48.8)
Missing entries	17 (2.1)
Weight in Kg(median [IQR])	28.00 [25.00, 35.00]
Height in cm(median [IQR])	135.60 [124.88, 153.10]
MUAC in cm(median [IQR])	18.00 [17.00, 20.00]
Height-for-Age-Z score [HAZ]	
Low	75 (9.1)
Normal	546 (66.3)
High	122 (14.8)
Missing entries	80 (9.7)
Weight-for-Age-Z score [WAZ]	
Low	21 (2.6)
Normal	428 (52.0)
High	33 (4.0)
Missing entries	341 (41.4)
BMI-for-Age-Z score [BAZ]	
Low	201 (24.4)
Normal	456 (55.4)
High	37 (4.5)
Missing entries	129 (15.7)
BMI kg/m^2(median [IQR])	15.58 [13.54, 17.86]

Table 8.2. Summary Data of Urinary Fumonisins B_1 among Children 6 – 12 years

	Geomean	Median	Mean ±SD	Range
Siaya (n=242)				
Total FB_1 (ng) in reconstituted extracts(200uL) of 10mL urine	0.44	0.45	0.58±0.43	0.07- 2.42
Creatinine (mg) in 10mL urine	6.05	7.41	8.65±6.74	0.15- 37.02
Final FB_1 ng/mg createnine	0.07	0.07	0.14±0.31	0.002-4.33
Makueni (n=286)				
Total FB_1 (ng) in reconstituted extracts(200uL) of 10mL urine	0.66	0.61	0.99±1.39	0.07-15.39
Creatinine (mg) in 10mL urine	8.06	8.98	10.04±7.07	31.58
Final FB_1 ng/mg createnine	0.08	0.07	0.59±7.06	0.01-8.06

Table 8.3 Variation of Urinary FB_1 (ng/mg creatinine) by age and sex

Categorization by Age	**Boy - Urinary FB_1 (ng/mg creatinine) Geomean (95% CI)**	**Girl - Urinary FB_1 (ng/mg creatinine) Geomean (95% CI)**
6 years	0.10 (0.06,0.19)	0.06 (0.03,0.13)
7 years	0.08 (0.04,0.14)	0.07 (0.04,0.12)
8 years	0.09 (0.05,0.17)	0.04 (0.03,0.05)
9 years	0.06 (0.04,0.09)	0.08 (0.05,0.12)
10 years	0.10 (0.06,0.17)	0.08 (0.06,0.11)
11 years	0.07 (0.03,0.15)	0.09 (0.05,0.15)
12 years	0.03 (0.02,0.11)	0.04 (0.03,0.15)
Overall	0.08 (0.07 0.10)	0.07 (0.05 0.08)

Table 8.4. Variation of Urinary FB_1 (ng/mg creatinine) and Growth Parameters

Growth Parameters	Low Geomean (95% CI)	Normal Geomean (95% CI)	High Geomean (95% CI)
Height-for-Age-Z score [HAZ]	0.13 (0.10,0.19)	0.06 (0.05,0.07)	0.08 (0.05, 0.12)
Weight-for-Age-Zscore [WAZ]	0.11 (0.06,0.22)	0.07 (0.06,0.08)	0.06 (0.03,0.09)
BMI-for-Age-Z score [BAZ]	0.07 (0.05,0.11)	0.07 (0.06,0.08)	0.08 (0.05,0.13)

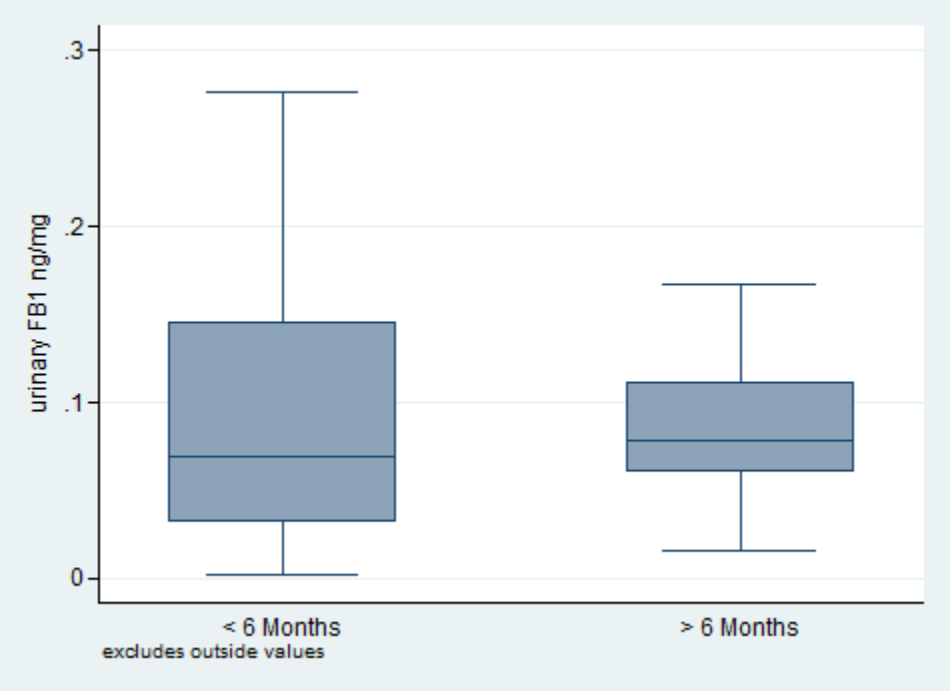


Figure 8.1. Urinary Fumonisins B_1 by Weaning Status

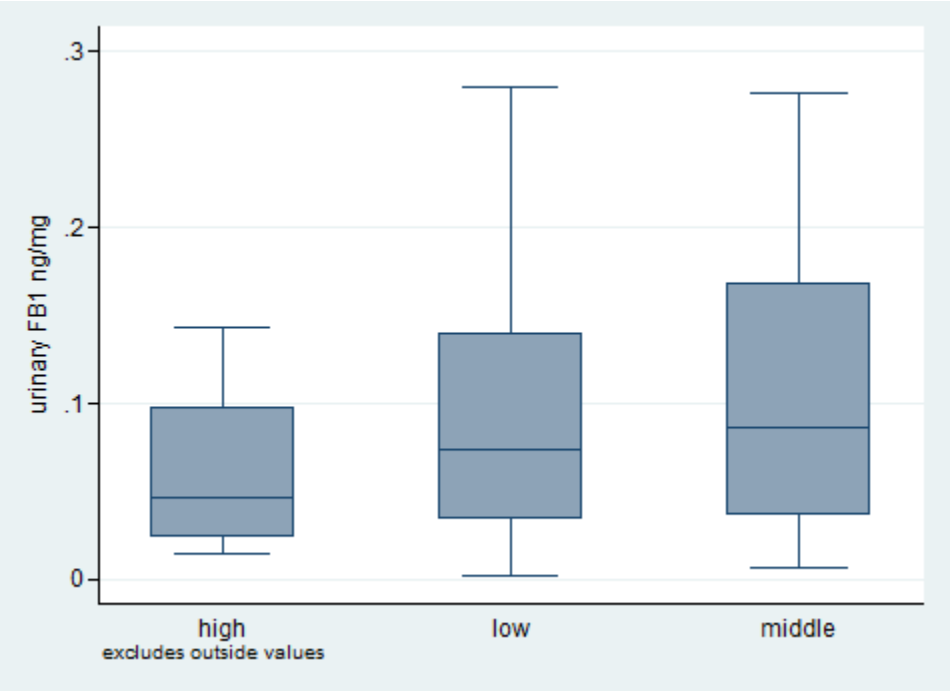
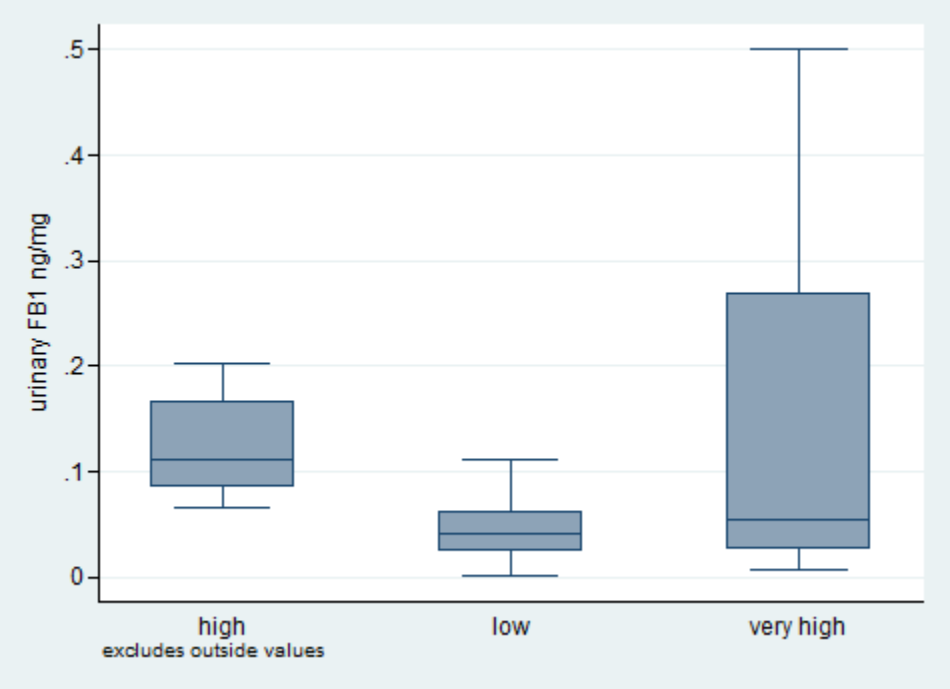


Figure 8.2. Urinary Fumonisins B_1 by Socio-Economic Status



AFB_1-lysine

Figure 8.3. Urinary Fumonisins B_1 by Aflatoxin B_1-lysine adducts

Chapter 9

CONCLUSIONS

This dissertation study set out to assess the extent of aflatoxins and fumonisin exposure levels among children between the ages of six and twelve years using the biomarkers approach. The choice of Makueni and Siaya Counties as study locations provided control for variability in ethnicity and genetic susceptibility, differences in weather conditions, and variation in mycotoxins exposure and poverty levels. Validated biomarkers for aflatoxins and fumonisins exposure including AFB_1-lysine adducts, urinary free FB_1 and sphingolipids were quantified using High Performance Liquid Chromatography (HPLC). Total aflatoxins in household grain was also quantified and risk factors associated with higher mycotoxin exposure levels in households was evaluated. Finally, we evaluated how mycotoxins exposures in early life via dietary contamination affect nutrition and growth outcomes.

The main findings of this dissertation study are that all children were exposed to both aflatoxins and fumonisins. All children had detectable levels of aflatoxins in serum, range 0.65 - 518.9 pg/mg albumin and geometric mean 10.5 pg/mg albumin 95%CI (9.4 – 11.7). Among children recruited from Makueni county, the geometric mean of AFB_1-lysine adducts was 14.0 pg/mg albumin (95%CI 12.5, 15.7) and is significantly higher than Siaya's, at 8.2 pg/mg albumin (95%CI 7.6, 8.8), $p<0.001$. Children with high aflatoxin B_1-lysine adducts in their sera exceeding 20 pg/mg of albumin con-currently had high urinary FB_1.The geometric mean was 0.13 ng/mg creatinine (95% CI 0.11, 0.15) compared to

urinary FB_1 of 0.04 ng/mg creatinine (95% CI 0.03, 0.05) among children with low aflatoxin B_1-lysine adducts less than 10pg/mg of albumin. Urinary free fumonisins B_1 and sphingolipid based biomarkers are validated for fumonisins exposure assessment. When adjusted for creatinine levels, final geometric means of urinary FB_1 ng/mg creatinine were 0.07 ng/mg and 0.08 ng/mg for Siaya and Makueni Counties respectively. While higher exposures were observed in urinary samples from Makueni County, the difference was not statistically significant, p-value > 0.05. Sphinganine and Sphingosine were detectable in 95.07% and 98.94% of samples respectively. Creatinine adjusted mean levels and standard deviation of Sphinganine, and Sphingosine were 1.23±2.18nM, and 4.99±8.3nM respectively while the Sphinganine to Sphingosine ratio were 0.296±0.587. Taken together, AFB_1-lysine adducts, urinary FB_1 and sphingolipid metabolites consistently confirmed dietary exposure to mycotoxins among young children living in low- and middle- income countries.

Another major finding is that food samples collected from Makueni County had higher levels of total aflatoxins compared to Siaya. The geometric mean of total aflatoxins in household food samples from Makueni County is 62.5 μg/kg with (95% CI: 53.7, 71.4) while in Siaya County is 52.8 μg/kg with (95% CI: 44.0, 61.7). These contamination levels are 5 to 6 times more than the recommended limit of 10 μg/kg. In the current study, the presence of AFs in food products in households indicate an elevated risk of dietary exposure to populations in Siaya and Makueni Counties.

This research project has also shown that the main determinants of mycotoxins exposure at individual levels include socio-economic status, farming practices, food production processes and food security, and environmental factors particularly climate

conditions. Moreover, number of children and family size are also determinants of household mycotoxins exposure as more children and bigger families are more likely to be of lower socio-economic strata. Surprisingly, being knowledgeable about mycotoxins is not significantly associated with exposure levels at an individual level which indicates that knowledge does not necessary translate to expertise valuable in preventing and/or limiting dietary exposure to mycotoxins.

One of the most significant findings to emerge from this study is that nutrition and growth outcomes of children is largely affected by mycotoxins exposure. After adjusting for demographic information, socio-economic indicators, farming practices, and household dietary patterns, the Height for age Z-score of children recruited from Makueni County decreases by 0.08 with (95% CI -0.21, - 0.04), the BMI for age z-score decreases by 1.17 with (95% CI -1.97, -0.36), Weight for age Z-score [WAZ] decreases by 0.82 with (95% CI -1.14,-0.50), and WHZ decreases by 1.66 with (95% CI -2.53,-0.78) compared to children recruited from Siaya County, $p<0.05$ with every unit increase in AFB_1-lysine adducts. Furthermore, reported results also show that exposure to fumonisins is associated with growth retardation. Among children with low height-for-age z-score, the geometric mean of urinary FB_1 levels were 0.13 ng/mg creatinine (95% CI 0.10, 0.19), and 0.11 ng/mg creatinine (95% CI 0.06, 0.22) for low weight-for-age score. These exposure levels are significantly higher when compared to exposure levels among children without growth impairments.

The findings of this research project complement those of earlier studies that children exposed to higher levels of mycotoxins which pose significant health risks to the pediatric population is widespread. While the main mechanism by which exposure to

mycotoxins contribute to growth impairment is currently unknown, there are proposed mechanisms of action. For example, aflatoxin associated immune suppression, enteropathy and aflatoxin related toxic damage on the intestinal epithelium severely limits nutrient uptake. Liver toxicity is exacerbated by exposure to aflatoxins and other mycotoxins may damage production of insulin like growth factor pathway proteins resulting in impaired child growth. The scope of this study is limited due to its cross sectional nature. The study administrators are not ethically allowed to contact participants in the future and thus, study results cannot be used to analyze behavior change, or determine cause and effect. The strength of the study is its multifaceted approach that involved molecular epidemiology in assessing health effects in children exposed to mycotoxins through the diet. This study is the first to provide baseline data on both aflatoxins and fumonisins exposure in children between the ages of 6 and 12 years recruited from high and low mycotoxin exposure regions of Kenya.

Chapter 10

FUTURE DIRECTIONS

This dissertation contributes significantly to the association between both aflatoxins and fumonisins and growth impairments. Based on results from this study, a prospective cohort study will be valuable in further studies aimed at explaining adverse health effects associated with mycotoxins exposures in early life. Moreover, future research needs to establish the threshold dose of not only aflatoxins and fumonisins but also other mycotoxins that would distinctively contribute to impaired child growth. Further studies should seek to establish the distinct mechanism of action. Moreover, robust longitudinal research with adequate sample size, ability to control for confounding factors, and use of validated biomarkers to assess risk of adverse health outcomes are urgently needed.

More broadly, research is needed to determine the potential synergistic effects of different mycotoxins on child health. While all mycotoxins as it relates to children's health were evaluated, published literature focused on aflatoxins and fumonisins. There are studies that have explored the effect of co-occurrence of mycotoxins such as aflatoxins, fumonisins, ochratoxins, deoxynivalenol, zearalenone and trichothecenes. Nonetheless, the greatest effort has been dedicated to aflatoxins and fumonisins with limited evaluation of all mycotoxins with public health relevance. Overall, there is still limited information on multi-contamination and associated health risks. Future studies should focus on better understanding the mechanistic pathways of all mycotoxin toxicity as it relates to public

health where it is not fully understood. Carefully designed longitudinal and/or prospective cohort studies are much needed to further evaluate mechanistic pathways.

Aflatoxins and other mycotoxins are either confirmed carcinogens, mutagens, and immune toxicants known to interfere with vaccine efficiency and a major contributor to growth impairment in children. Mycotoxin associated immune suppression has been shown to increase susceptibility to infectious diseases. The few studies that have assessed the role of mycotoxins on immune suppression used various immune markers including lymphocytes, cytokines and immunoglobulin level. Considerably, more work will need to be done to develop uniform parameters of assessment to allow comparability of studies.

This research project described the first school-based randomized cross-sectional study aimed at assessing health effects associated with dietary exposure to mycotoxins in early life. A further study could assess the long term effects on micronutrient deficiency, immune suppression and growth deficits related to mycotoxins exposures in early life. Studies aimed at early exposure are imperative to inform policy makers on early interventions to limit adverse health outcomes in the long term. Despite ongoing economic challenges in low- and middle- income nations, mitigation efforts to reduce mycotoxin contamination are urgently needed to limit exposure levels, promote food security, achieve economic benefits and protect public health from risk of developing adverse health outcomes.