ROLES OF DIETARY AFLATOXIN EXPOSURE IN THE ACQUISITION AND PROGRESSION OF INFECTIOUS DISEASES AND AN INTERVENTION STUDY WITH ACCS100

MINSU KANG

(Under the Direction of Jia-Sheng Wang)

ABSTRACT

Aflatoxin B_1 (AFB_1) is a known human carcinogen (Group1), and the mechanism of carcinogenicity has been extensively studied. However, other toxic effects of AFB_1, such as immunotoxicity, reproductive and developmental toxicity, have not fully understood. This dissertation presents research involving cohort study populations in rural Uganda and south-central Texas. AFB_1 exposure was predominant in rural Ugandan cohorts and higher odds of contracting human immunodeficiency virus (HIV) were observed in highly exposed individuals, which implies that chronic AFB_1 exposure at higher levels is associated with HIV acquisition. Additionally, a faster progression from HIV acquisition to the development of acquired immunodeficiency syndrome (AIDS) was observed among the HIV-infected individuals with a hazard ratio of 3.11 per ln (AFB_1-lysine). In rural Uganda, the level of AFB_1-lysine adduct was

significantly associated with abnormal liver enzyme levels, implying that AFB_1 exposure is associated with liver disease in the areas. AFB_1-lysine adduct in serum was detectable in 84.0 % of the residents of Bexar County, Texas. The geomean of 2.33 in Bexar County, Texas is comparable to the geomean in southwestern Uganda. Consumption of corn-based products and Mexican food items was associated with the higher level of AFB_1-lysine adduct in the study participants, Bexar County.

An intervention trial with a clay-based enterosorbent (ACCS 100) was carried out to reduce the risk of mycotoxin exposure. A reduction rate of 36% at 1 month after the intervention and 33% at 3 months after the intervention was observed in the low dose (1.5 g/day) treatment group. However, the reduction was not dose-dependent with regards to ACCS ingested. Through these data, we concluded that AFB_1 exposure in humans is not only a threat in the developing world but also among the southern U.S. populations; reducing chronic exposure to AFB_1 may benefit prevention of potential adverse health effects in these populations.

INDEX WORDS: Aflatoxin B_1, infectious disease, HIV, pathogenesis, intervention trial

ROLES OF DIETARY AFLATOXIN EXPOSURE IN THE ACQUISITION AND PROGRESSION OF INFECTIOUS DISEASES AND AN INTERVENTION STUDY WITH ACCS100

by

MINSU KANG

BS, Kyungpook National University, South Korea, 2007

MS, Gwangju Institute of Science and Technology, South Korea, 2009

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2016

ROLES OF DIETARY AFLATOXIN EXPOSURE IN THE ACQUISITION AND PROGRESSION OF INFECTIOUS DISEASES AN INTERVENTION STUDY WITH ACCS100

by

MINSU KANG

Major Professor:	Jia-Sheng Wang
Committee:	Ronald T. Riley
	Mary Alice Smith
	Luke P. Naeher
	Xiao Song

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
May 2016

DEDICATION

This dissertation is dedicated to people living underprivileged conditions and study participants and to those who believed in me and encouraged to keep doing this meaningful work (my beloved parents, sister, and brother, Royale, my true friends, and my God).

ACKNOWLEDGEMENTS

This dissertation work would not have been possible without considerable help from individuals and organizations. Major advisor, Dr. Jia-Sheng Wang, and dissertation committee (Dr. Ronald T. Riley, Dr. Luke P. Naeher, Dr. Mary Alice Smith, and Dr. Xiao Song) guided me and equipped me with the necessary toxicological knowledge and research skills. Dr. Wang introduced me the aflatoxin research and provided an environment to encounter many scholars and senior students. Dr. Riley offered revision suggestion several times, and his questions provoked me to broaden my perspectives in my research. Dr. Song's guidance and in-depth knowledge of math and statistic not only helped me find the right statistical methods for each research question. University of Georgia's Interdisciplinary Toxicology Program, Graduate School and Joanne supported me with assistantships and other services. The division of academic enhancement tremendously helped in this writing process.

I appreciate the members of our lab and scholars in UGA (Dr. Tang, Dr. Qian, Franklin Wang, Yan Wang, Shuhan Lin, Kathy Xue, Jincheng Wang, Hellen Noh, Kabiru Sunmola, Xiangyi Zhang, Amanda Seawright, Hongyuang Zhou, Jun Zhou, Wenji Cai, Dr. Su, Dr. Feng, Dr. Ding, Dr. Cai, Dr Tai Guo, and Dr. Christopher Whalen). Additionally, I appreciate many people with whom I have worked: Peter Nkurunzizab, Richard Muwanika, Dr. Janet Seeley, Dr. Tom Lutalo, Dr. Joanathan Williams, and so many others at the Ugandan sites; Dr. Timothy D. Phillips, Dr. Bradley Pollock, Dr. Fernando Guerra, Dr. Amelia Romoser, Sarah Elmore, Marisa Rodriguez, Nicole Jaime, Holly G. Hayes, and Michael Carden for the collation in the projects in Bexar County, Texas; Dr. Sabran Mohd-Redzwan and Rosita Jamaluddin for the study in Malaysia.

I am sincerely thankful to special people that I have met in Athens. Royale Walker always waits for me to give me a ride to home whenever I worked late nights. Dean Meyer helped me to settle in Athens and edit my writing in many occasions. Other friends made me feel welcomed in Athens: Mama Betty, uncle Larry, Scotty, Jennifer, C.J., Alison, Courtney, Joo-Young, Xiangyi, Rahat and Samir, Lori, Joe, Natalie, the members of Korean Prebysterian Church and the Church of the nation, and EHS students (Jason, Bela, Xiaoming, Anna, Femmi, Bei, David, John, and Jessy).

I thank my supportive family (Hee-Seok Kang, Soon-Bun Nam, Min-young Kang, Myeong-Goo Kang, and many relatives). Their presence motivated me and gave me strength. I have received enormous love from my family which I cannot repay, but I hope that I can support them as they supported me with the knowledge that I have gained. Also, I express my appreciation to Eun-Young who supported me with her prayer and love

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Problem statement

Chronic and low levels of exposure to chemical toxicants such as aflatoxin B_1 (AFB_1) differ in their effects on humans from acute and high levels of exposure. First, the adverse effects of chronic and low levels of exposure originate from altered pathways and the small degree of cellular adaptation (Currie et al. 2014). This is significantly different from the effects of acute and high dose exposure to AFB_1, which are a result of acute cell injury or cell death at the time of exposure. In addition, the adverse effects of chronic exposure to AFB_1 are cumulative in the case of repeated doses. In other words, chronic exposure to AFB_1 will elicit adverse effects even below the threshold exposure in the long run. Acute exposure and high levels of AFB_1 cause abdominal pain, nausea, jaundice, bleeding in the gastrointestinal (GI) tract, and death from aflatoxicosis in humans and animals (Azziz-Baumgartner et al. 2005; Ghosh et al. 1990; Otim et al. 2005; Pier 1986; Wild and Gong 2010; Williams et al. 2004b). Chronic exposure and low levels of AFB_1 cause primary liver cancer, immunotoxicity, reproductive toxicity, and an antinutritional effect. This dissertation project focused on the effects of chronic exposure to AFB_1.

Aflatoxin studies in humans are complex because aflatoxin exposure co-occurs with other chemical toxicants (such as fumonisins or deoxynivalenol) or infectious agents (such as the hepatitis B virus (HBV), the hepatitis C virus (HCV), or the human

immunodeficiency virus (HIV)). This co-occurrence may lead to confounding, where the effects of one exposure may be attributed to another exposure. Confounding may be one of major causes of the discrepancies between studies in humans and studies in laboratory settings (both *in vivo* and *in vitro*). AFB_1 exposure is problematic in the area between the latitudes of 40° N and 40° S(Williams et al. 2004b). Majority of developing countries, especially sub-Saharan Africa, reside in the region, and the prevalence of infectious disease such as HIV and tuberculosis is relatively high. Therefore, the interaction between those infectious agents and AFB_1 is plausible.

In this dissertation research, various epidemiological study designs were applied to study the effects of AFB_1 in humans, with the benefit of using biological samples obtained from pre-existing cohorts in Uganda and Bexar County, Texas.

Dissertation hypothesis

The working hypothesis of this dissertation is that AFB_1 exposure, represented by the levels of AFB_1-lysine (AFB_1-Lys) adduct in human serum, will alter immunity and increase the acquisition of infectious agents and progression of disease. The enterosorbent or classified calcium silicate (ACCS100) will reduce bioavailability of aflatoxin in human gut and lead to reduction of AFB_1-Lys adduct levels in AFB_1 exposed human participants in the Bexar County cohort.

The specific aims of this study include:

Aim 1. To assess aflatoxin exposure and temporal variations among different socio-demographic groups in the southwest Ugandan populations using AFB_1-Lys adduct as the indicator.

Aim 2. To test the hypothesis that AFB_1 exposure can affect the acquisition and progression of HIV in the Ugandan population, and to identify a possible mechanism behind the phenomenon.

Aim 3. Determined the relationship between aflatoxin exposure in a special Ugandan populations with abnormal liver function and risk behaviors.

Aim 4. Assess aflatoxin exposure in Bexar County residents after the severe 2012-2013 drought, and determine the relationship between aflatoxin exposure and selected risk factors, such as socio-economic status and ingestion of corn-based foods.

Aim 5. Assess the efficacy of a phase II chemoprevention trial with the enterosorbent ACCS100 in reduction of aflatoxin exposure in one of theBexar County, Texas populations known to be at high risk for hepatocellular carcinoma (HCC)

This dissertation research is focused on elucidating the immunomodulatory effects of AFB_1 and its contribution to infectious diseases, such as HIV, in Uganda cohort human populations, and exploring possible roles of aflatoxin exposure affecting subsequent disease infection and progression. Though a few epidemiological studies in Africa have addressed the topic, their study designs were mainly cross-sectional, and could not avoid temporal variations. We found that HIV-infected individuals who are chronically exposed to aflatoxin display faster pathogenic progression of HIV to AIDS.

The odds of HIV infection were significant among the participants chronically exposed to aflatoxin.

In addition, this dissertation undertook to test the efficacy of the AFB enterosorbant ACCS100 to reduce AFB exposure in humans recruited from populations at high risk of acquiring HCC in the USA. Though a previous trial in Ghana implied safety and efficacy, the clay-based enterosorbent has not been formally evaluated in a U.S. populations.

CHAPTER 2

LITERATURE REVIEW

Aflatoxins (AFs) are a group of secondary fungal metabolites produced by *Aspergillus* species; 14 different types of AFs AFB_1, AFB_2, AFG_1, and AFG_2 are major AFs which occur naturally in grains and oilseed crops, and AFM_1 and AFM_2 are metabolic products of AFs found in milk and dairy products (Figure 2.1)(IARC 2002). AFs are classified based on their fluorescence color and source: AFB_1 and AFB_2 (blue), AFG_1 (green), AFG_2 (green-blue), and AFM_1 and AFM_2 (milk, blue-violet fluorescence). AFs are heat-stable compounds and do not degrade easily by heating.

Among various *Aspergillus* species, *A. flavus*, *A. parasiticus*, *A. nomius*, and *A. pseudotamari* are the four that mainly contribute to the production of AFs. *A. flavus* and *A. parasiticus* critically affect the aflatoxin contamination the U.S. and African regions, whereas *A. nomius* and *A. pseudotamari* are common in the soils of Thailand and Japan(Varga et al. 2011). In the U.S., *A. flavus* is the most dominant species, and the optimal environment for *A. flavus* to grow and produce AFB_1 is a hot and humid climate. When host crops are contaminated with AFB_1, consumption of those contaminated crops is associated with aflatoxicosis (acute high dose exposure), liver cancer, immune modulation, and growth impairment (chronic low doses exposure) in humans and animals.

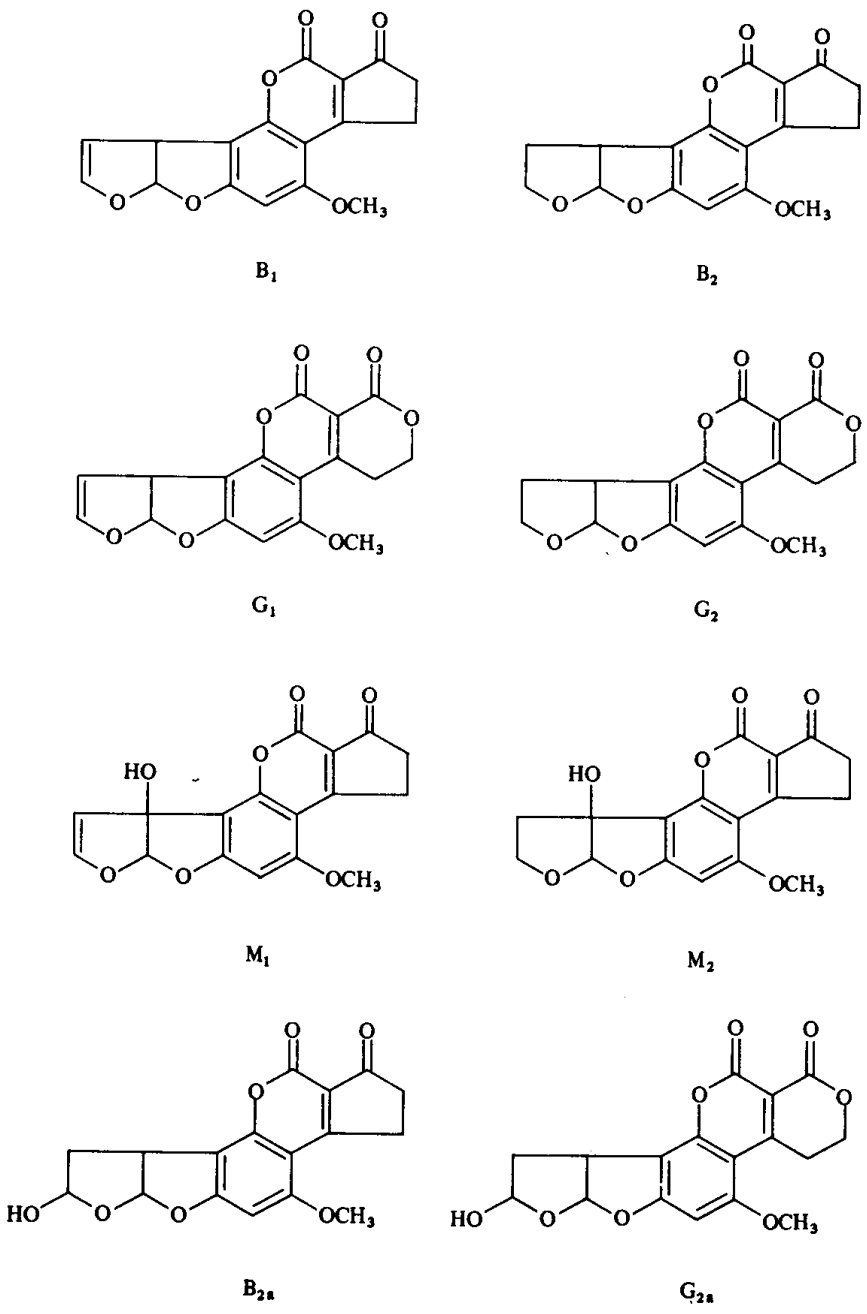


Figure 2.1. Structural formulas of the major aflatoxin derivatives

2.1. Toxicity of aflatoxins

AFs are the most extensively characterized mycotoxins. They occur without a distinguishing color or smell. AFs were firstly identified as causal agents in Turkey X disease in England in the early 1960s (Nesbitt et al. 1962). AFs contamination in foods and human AF exposure are still major problems in the developing world, causing many adverse health effects, including hepatocellular carcinoma (HCC) (Liu and Wu 2010; Williams et al. 2004a). Recent climate changes, including the increased frequency of extreme weather events and warmer average temperature, may have affected the prevalence of aflatoxingenic fungi growth , considering that aflatoxin contamination frequently occurs after extreme weather (Gillam and Ingwersen 2013; Li 2013; Piva et al. 2006; Wood 1992). Aflatoxin B_1 is the most potent toxicant among AFs because it triggers cytochrome P450 enzymes to form highly reactive epoxide metabolites, which readily react with DNA and protein. This epoxide is the key to AFB_1 carcinogenesis. The mechanism of AFB_1 carcinogenesis and its toxicokinetics have been studied extensively in rats and rainbow trout. However, other toxicity endpoints related to chronic AF exposure need to be explored, such as developmental, immunotoxicological, and anti-nutritional effects.

2.1.1. Toxicokinetics of aflatoxin B_1

Absorption

In humans, two exposure routes are possible: A dietary exposure through contaminated foods, and an inhalation exposure through working around AF-contaminated grains. However, the dietary exposure is the dominant route in humans. AFB_1 is a small lipophilic compound, is widely absorbed in the gastrointestinal system, and mainly in the small intestine (Ramos and Hernandez 1996; Steyn et al. 1971; Wogan et al. 1967a). The level of AFB_1 in plasma decreases in first order kinetics (Kumagai 1989; Qian et al. 2013).

Distribution

The distribution of toxicants depends on vascular permeability, regional blood flow, cardiac output, tissue perfusion rate, ability to bind plasma proteins, and the chemical's lipid solubility (Klaassen 2013). Early studies of AFB_1 found that the liver and kidney contained the majority of radio-labeled AFB_1 after a single dose to male Fischer rats, establishing the highest levels of AFB_1 at 30 minutes after administration (Wogan et al. 1967b). Experimentally administered AFB_1 was deposited in the liver of experimental animals such as goats (Helferich et al. 1986), ducks, hens, and quail (Bintvihok et al. 2002), rats (Wogan et al. 1967b), mink (Chou and Marth 1976), and cows (Trucksess et al. 1983). Stubblefield et al. (1983) observed total AF residues in cow tissues after oral doses of 0.35 mg of AFB_1 /kg body weight/day for three consecutive days. At one day after the final dose, AFB_1 and AFM_1 were found in a wide range of

tissue types, including the brain, gallbladder, heart, intestine, kidney, liver, lung, mammary gland, skeletal muscle, spleen, supra mammary lymph nodes, and tongue. Kidney had the highest concentrations of total AFs (57.9 ng/g), followed by mammary gland (25.1 ng/g) and liver (13.2 ng/g). AFM_1 was the predominant form in kidney, compared with AFB_1 (~ 40: 1). However, seven days after the last dose, a majority of AFs had been excreted, and only trace amounts (0.2 to 0.11 ng/g) were detectable in several tissues, including kidney, liver, and intestine. Although there are no experimental data to support this, deposition of AFB_1 and AFM_1 in fat tissues is highly possible due to their lipophilicity, which is consistent with our unpublished data that the level of AFB1 is constantly high in obese populations. After ceasing an aflatoxin exposure, the aflatoxins in fat tissue could be redistributed to other tissues, elevating their levels.

Membrane permeability can explain the higher susceptibility to AFB_1 in hepatocytes than mesenchymal cells (Terao et al. 1972). Microsomes exhibit higher concentrations of AFB_1 compared with other subcellular fractions (Ewaskiewicz et al. 1991). More than 85% of AFB_1 are associated with chromatin in nuclear fractions (Ewaskiewicz et al. 1991; Groopman et al. 1980), and 80% of which is bound to DNA (Groopman et al. 1980), displaying a preference for the internucleosomal (linker) DNA over the nucleosomal core DNA (G. S. Bailey et al. 1980). Histone H_1 is the major protein target among all the chromatin proteins for AFB_1 binding (Groopman et al. 1980).

Metabolism

Metabolic activation and detoxification of aflatoxins involve Phase I and II enzymes. AFB_1 converts to AFB_1-8,9-epoxide by cytochrome P450, mainly by CYP1A2

and CYP3A4 (Eaton and Gallagher 1994; Oda et al. 2001; Ueng et al. 1997) as well as by CYP2A6, CYP3A5, and CYP2A13 (Crespi et al. 1991; He et al. 2006; Pelkonen et al. 2000; Wojnowski et al. 2004; Zhu et al. 2006). The formed epoxide is highly nucleophilic, and thus, can bind tightly to macromolecules such as DNA, RNA, and protein. The hydroxylation and demethylation of AFB_1 are considered detoxification processes, producing less toxic metabolites than the parent AFB_1, including AFM_1, AFQ_1, and AFP_1 (Van Vleet et al. 2001). AFM_1 is the most prevalent detoxification product and is widely detected in bulk milk produced by cows consuming AFB_1-contaminated feeds (Atanda et al. 2007; Zinedine et al. 2007).

Liver cytosolic glutathione S-transferase (GST) plays a critical role in the detoxification of AFB_1-8,9-epoxide, which is then excreted in the urine (Bagshawe et al.). Additionally, AFB_1 and its metabolites may also be detoxified through conjugation with sulfates or glucuronic acid (WF Busby, Jr., and GN Wogan 1984). The individual- or species-specific rate of epoxidation and by the ability to conjugate AFB_1 epoxides to GSH primarily determine the susceptibility to AFB_1 (Kuilman et al. 2000).

Excretion

AFB_1 and its metabolites are excreted in urine and feces (Coulombe and Sharma 1985; Wild and Turner 2002). After a single dose of isotope-labeled AFB_1 administered intraperitoneally to male Fischer rats, over 70% of the total dose was excreted in the first 24 hours, nearly 60% of it in the feces, and 20% in urine (Wogan et al. 1967b). In male Sprague-Dawley rats administered AFB_1 intratracheally and orally, by day 23, urinary

excretion of AFB_1 accounted for 16.4% and 15.0% of the dose, respectively, and fecal excretion for 56.0% and 54.6% (Coulombe and Sharma 1985).

2.1.2. Mechanism of Action (MOA)

Mechanism of action describes the process by which toxicant functions to produce toxicological effects. A mechanism of action usually includes the specific molecular targets to which the toxicant binds, such as an enzyme or receptor.

DNA damage and sequence specific binding to DNA

Exposure to AFs, which was estimated by either food consumption surveys or measuring biomarkers of AF exposure, has been shown to have a correlation with the incidence of various cancers (liver, kidney, lung). AFB_1 reacts *in vivo* with the DNA in target cells to produce primarily trans-8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 (Essigmann et al. 1977; Lin et al. 1977; Martin and Garner 1977). Studies on the reaction of synthesized AFB_1-8,9-epoxide (Baertschi et al. 1988) with DNA *in vitro* strongly suggest that adduct formation *in vivo* proceeds via a precovalent intercalation complex between double-stranded DNA and the highly electrophilic, unstable AFB_1-exo-8,9-epoxide isomer (Gopalakrishnan et al. 1990). The presence of a positive charge on the imidazole portion of the initial N^7-guanyl adduct gives rise to a ring-opened formamidopyrimidine (FAPY) derivative with distinct chromatographic behavior (Croy and Wogan 1981). Accumulation of this derivative is time dependent, nonenzymatic, and of some biological importance because it resides in a minor portion of the AFB_1-exo-8,9-

epoxide isomer form adducts with adenine and cytosine (Iyer et al. 1994; Yu et al. 1991). However, the importance of those adducts in carcinogenesis is unknown.

The level of AFB_1-DNA adducts explained the organ and species specificity of AF toxicity. The Liver is the major target organ of AFB_1, and the level of AFB_1-DNA adducts is significantly higher in the liver than in other organs. A study with F344 rats exposed to AFB_1 showed the patterns of AFB_1 acid hydrolysis products from DNA, mainly 2,3-dihydro-3-hydroxy-(N^7-guanyl)AFB_1. The Liver had a 10-fold higher level of the hydrolysis product than the kidneys. This study also showed that the levels of liver DNA adducts per unit AFB_1 dosage correlate with species susceptibility by comparing CD-1 Swiss mice and F344 rats. The CD-1 Swiss mouse is relatively resistant to AFB_1-induced hepatotoxicity compared to the F344 rat. In the study, the level of AFB_1-DNA in CD-1 Swiss mice was 2% of that of F344 rats when the rats were exposed to 12 fold higher levels of AFB_1. A similar tendency was observed in another study with rainbow trout and coho salmon. The trout exposed for four weeks to 20 ppb AFB_1 had a 62% tumor response after 12 months, whereas salmon exposed to 40 ppb AFB_1 for four weeks failed to develop tumors (Bailey et al. 1988).

The formation of AFB-DNA adducts is significantly affected by chromatin structure as well as DNA sequence. Guanyl sites residing within internucleosomal DNA are 5-fold more likely to form AFB_1-DNA adducts than guanyl sites within nucleosomal DNA(George S Bailey et al. 1980). Similarly the transcriptionally active ribosomal RNA genes in rat livers are 4- to 5-fold more susceptible to AFB_1 adduct formation than is the total nuclear DNA (Irvin and Wogan 1984). Proteins and small molecular weight ligands

interact with a major or minor groove of a specific sequence of DNA using hydrogen bonding, hydrophobic interactions, and electrostatic interaction. Many investigators have tried to understand the "sequence context" of AFB_1 like DNA-reactive drugs. Key findings are 1) the rate of alkylation of particular guanines is higher than the rate of others in double-stranded DNA, 2) sequence selectivity among 190 guanine residues is in this order: 5'GG*G>5'GG*T>5'CG*G(Warpehoski and Hurley 1988).

Damage to chromatin and gene structure temporarily or permanently alters the chromatin configuration of Ras, p53, and other genes that are important in carcinogenesis and would modify the potential of these genes to be damaged by AFB_1. Recently, studies have shown the sequence specificity of mutational hotspots in liver tumors from the area where AF is frequently consumed was different from the mutation spectrum in the liver tumors from where AF exposure is rare. This association provides evidence the mutational hotspots in the liver tumors from high-risk population of AF exposure probably linked to AFB_1 exposure (Hussain et al. 2007). The predominant mutational hotspot found in human hepatocellular carcinoma is a GC→TA transversion in the third position of codon 249 of the p53 gene (5'AG<u>G</u>3'). The mutation of p53 can lead to the alteration of controlled growth of cells because p53 is a tumor suppressor gene. Also, the activation of the ras gene by single-base mutation is a key step in the transformation of normal cells to a malignant cell. Ras proteins belong to the small GTPase class and are involved in transmitting signals (cell growth, differentiation, and survival) within a cell.

The sequence and base specificity of AFB_1 induced DNA mutation possibly related to DNA repair system. Evidence support that mutational hotspot in p53 associated

to AF exposure was not matched to theoretical mutational hotspot sequences based on the mechanistic possibility of mutation(Aguilar et al. 1993; Levy et al. 1992). This can be explained by the involvement of DNA repair system. It is possible that the DNA secondary structure of the mutational hotspots makes DNA repair system less or not accessible. As a result, the mutation in the sequence will not be checked and remain as mutational hotspots.

Reactive oxygen species-mediated toxicity

Reactive oxygen species (ROS) have utilized for eliminating pathogens by immune cells and for intracellular signaling and regulation of cell function, including apoptosis (Circu and Aw 2010). ROS is a collective term that broadly describes O_2-derived free radicals such as superoxide anions, hydroxyl radicals, peroxyl, alkoxyl, as well as O_2-derived non-radical species such as hydrogen peroxide. However, the levels of ROS exceeds the ability of the antioxidant defenses, and ROS will react and degenerate biomacromolecules such as DNA and proteins.

Chickens fed AF-contaminated diets showed a significant increase in serum malondialdehyde (MDA) content when compared to the untreated control group ($p<0.05$) (Chen et al. 2013; Essiz et al. 2006). In cultured rat hepatocytes, exposure to 1,000 nM AFB_1 for up to 72 hr significantly elevated levels of MDA and lactate dehydrogenase (LDH) (Shen et al. 1995). The same authors detected ROS by the formation of highly fluorescent dichlorofluorescein (DCF). The fluorescent DCF will be generated only if ROS is present in the system. The fluorescence was observed in a dose-

dependent manner in a range of 10 nM to 1,000 nM under the presence of a fluorescence probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA), and the AFB_1 (Shen et al. 1996). Towner et al found that rats treated with 3 mg/kg i.p. AFB_1 had a radical adduct of 4-POBN in rat bile and a methyl adduct of 4-POBN from the reaction of hydroxyl radicals (Towner et al. 2003).

Receptor-mediated toxicity

Nuclear receptors are a class of proteins found within cells that are responsible for sensing steroid and thyroid hormones and certain other molecules. In response, these receptors work with other proteins to regulate the expression of specific genes, thereby controlling the development, homeostasis, and metabolism of the organism. Nuclear receptors have the ability to directly bind to DNA and regulate the expression of adjacent genes, hence there receptors are classified as transcription factors. The regulation of gene expression by nuclear receptors generally only happens when a ligand, a molecule that affects the receptor's behavior, is present. More specifically, ligand binding to a nuclear receptor results in a conformational change in the receptor, which in turn activates the receptor, resulting in up- or down-regulation of gene expression. Nuclear receptors bound to hormone response elements recruit a significant number of other proteins such as coactivators and corepressors that facilitate or inhibit the transcription of the associated target gene into mRNA. Nuclear receptors share transcriptional targets and serve as transcriptional inducers of one another; therefore, the specificity of nuclear receptors is not significant.

Ayed-Boussema et al observed the interaction between AFB_1 and nuclear receptors (PXR, CAR, and AhR) which are the important nuclear receptors involved in the induction of CYP3A, CYP2B, and CYP1A (Ayed-Boussema et al. 2012). AFB_1 exposed hepatocytes showed the induction of receptor mRNA molecules. PXR gene was induced from 2.78 folds to 11 folds after AF exposure. CAR is also induced but to a less extent. The AhR gene was significantly ($p<0.05$) induced in all tested livers. PXR and CAR constitute important members of the NR1I nuclear receptor family. They were originally defined as xenobiotic receptors and are master regulators of phase I and II drug-metabolizing enzymes as well as of drug uptake and export systems preventing the accumulation of toxic chemicals within the body. Aryl hydrocarbon receptor (AhRE) is a member of the basic helix-loop-helix/PER-aryl hydrocarbon nuclear translocator (ARNT)-SIM family of DNA-binding proteins. The AhR activation has been suggested to control the expression of xenobic-metabolizing enzymes, particularly, CYP1A1 and CYP1A2 enzymes. Consequently, a significant up-regulation of cytochrome P2B6 (CYP2B6), CYP3A5, and to a lesser extent CYP3A4 and CYP2C9 was observed; the up-regulation of phase I enzymes increases the production of metabolically active molecule (AFB_1-lysine).

Fumonisin B_1 (FB_1) and AF impact on the activation and expression of CYP 1A and its transcription factor AhRE in hepatoma cell and spleen mononuclear cells of rats either alone or combined (Mary et al. 2015). AFB_1 and FB_1 induced the expression of CRP 1A and AhRE gene in spleen cells. AFB_1 induced an increase in CYP 1A activity, CRP 1A transcription, associated with an enhanced AhR activity.

AFB1 has the structural similarity with vitamin D (Vit D), which is responsible for enhancing intestinal absorption of calcium, iron, magnesium, phosphates, and zinc. Costanzo et al observed the down-regulation of Vit D receptors in AF-exposed osteosarcoma cell line SAOS-2 (Costanzo et al. 2015). Fifty-eight to 86 % of the receptors were decreased when the cells were exposed to 5 ng/ml to 50 0ng/mL of AFB_1. Vit D receptors (also known as NR1I1) interact with various components of the body, regulating the expression of more than 900 genes with a wide array of physiological functions including t cell function (Kongsbak et al. 2013). The exposure to 1 ppm AFs reduced plasma 25-hydroxyvitamin D [25 OH)D] and 1, 25-dihydroxyvitamin D [1,25(OH)2D] concentration after 5 d of treatment (Glahn et al. 1991). Also, the biological active form of Vit D (1,25(OH)2D), which is a hydroxylated Vit D in liver, was decreased in a group of male broiler chicks with 2.5 ppm AFB_1 feeding for four weeks (Britton and Wyatt 1978).

Epigenetics

Epigenetics means a heritable change in gene expression without altering DNA sequences. In other words, it refers to a change in phenotype without a change in genotype. Major examples of epigenetic modification of gene expression are DNA methylation and histone modification. In animals, majority of methylation happens in CpG islands and the large portion of CpG islands resides in promoter region of genome. DNA methylation either silenced genes through hypermethylation or activated genes through hypomethylation. Histone modification alters chromatin structure, in turn, it impacts gene expression.

An early study showed AFB_1 interacts with histones in rat liver in vivo (Groopman et al. 1980), and approximately 5 to 10 % of the total nuclear-bound AF residues were associated with histone. Activity measurements revealed that histone H1 was the major protein target for]AFB_1, binding to the AFB_1 adduct 3 to 4 times higher than that for any other histone. A group studying Taiwanese HCC patients showed that no methylation was found in normal liver control tissues for both RASSF1A and p16 whereas methylation was detected in one of 10 and seven of 10 adjacent nontumor tissue samples for p16 and RASSF1A(Zhang et al. 2002). Significant correlation that AFB_1-albumin adduct and a tumor suppressor gene p16 methylation was observed in Taiwan (Zhang et al. 2006). Wu et al demonstrated LINE-1 and Sat2 methylation was associated with the levels of the aflatoxin-albumin adduct in white blood cells (Wu et al. 2013).

Gut microbiome

Recent study found that increasing AFB_1 doses decreased diversity but increased evenness of microbial communities in rat fecal samples using 16S rRNA sequence analysis (Wang et al. 2016)

2.1.3. Carcinogenicity

Cancer is a disease that involves uncontrolled cell proliferation, and the affected cells have the ability to invade or spread to other parts of the body. Cancer cells have six essential alternations: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replication potential,

sustained angiogenesis, tissue invasion, and metastasis. Significant attempts have been made to understand the causes of cancer in the last century. As a result of these attempts, five representative models were made, each focusing on one of the following causes: mutation by chemical carcinogens, familiarity genomic instability, clonal expansion/epigenetics, clonal expansion/cell selection, and micro-environment morphostats. Specifically, the first carcinogenesis model, the mutational model, describes the case in which mutations in an oncogene and a proto-oncogene may cause unregulated cell growth and survival; also, a mutation in a tumor-suppressor gene can reduce the control of cell growth, leading to cancer. The genetic instability model presents the development of cancer as being caused by prior DNA damage that results in an inaccurate translation process; a DNA damage tolerance process allows the DNA replication machinery to replicate past DNA lesions, leaving the damage or errors unrepaired. Also, endogenous sources frequently cause genetic mutation at a rate of 10^{-10} mutations per nucleotide per cell per generation (Jackson and Loeb 2001). Therefore, the reduction of DNA repair genes due to epigenetic and mutational changes may increase the chance of cancers. The clonal expansion/epigenetic model focuses on the non-genotoxic effect, which refers to the several important modulators of cancer risk, such as diet, obesity, hormones, etc., that can cause epigenetic changes (DNA methylation, Histone modification, noncoding functional RNAs, etc) and increase clonal expansions. The clonal expansion/cell selection model is based on the concept that cancer is commonly initiated, but promotion to full malignancy is rare. Thus, the promotion factor (environmental factor) is more important than initial mutation with an emphasis on the role of environment in selecting cells that have some acquired advantage(Farber 1984).

AFB_1 follows a typical chemical mutagen model 1 (model 1). After the aflatoxin B_1-8,9 epoxide is formed, it readily binds to DNA and proteins in the cells. The liver, intestines, and kidneys are major target organs of AFB_1 because CYP450 is largely distributed in those organs. Also, within a cell, CYP450 is located mainly in the membrane of the endoplasmic reticulum(ER) and partially in mitochondria. Thus, those two cell compartments are expected to be damaged by the activation by AFB_1. The damage to the ER causes liver malfunction (Koo et al.) (Explaining reasons). Also, AFB_1 affects the epigenetic changes that are critical in causing cancer later preferential binding to methylated lysine or modified histones leading to DNA damage (Herceg 2007). Studies show that the methylation status in RASSF1 (Ras association domain-containing protein 1), p16 (cyclin-dependent kinase inhibitor 2A), and MGMT (O-6-methylguanine-DNA-methyltransferase) was decreased after AFB_1 exposure (Zhang et al. 2003; Zhang et al. 2006). Those genes are closely related to cancer. RASSF1 is associated with inhibition of the accumulation of cyclin D1 and the induction of cell cycle arrest. P16 inhibits the cyclin dependent kinases such as CDK4 and CDK6, and it plays an important role in cell cycle regulation by decelerating cell progression from the G1 to the S phase. MGMT is crucial for genome stability because it repairs the naturally occurring mutagenic DNA lesions. Down-regulation of any of these genes is related to an increased risk of cancer. Although the precise mechanism by which AFB_1 alters epigenetic states is unclear, AFB_1 may bind preferentially to methylated CpG sites and/or specific structures in chromatin, inducing damage to DNA and histones.

Epidemiologic and experimental evidence support the hypothesis that carcinogenesis is the result of a multistage process (Pitot 1993). In experimental systems,

a latent period occurs between exposure to a carcinogen and the development of cancers. The latency period is comprised of a series of stages that can be characterized both in experimental protocols and in humans: initiation, promotion, and progression. Initiation has been described as irreversible genetic damage that results from exposure to a carcinogen. Several studies have suggested that initiation is induced most effectively during the G1-S and early S phases (Maguire and Rabes 1987). When coupled with a proliferative stimuli, carcinogen exposure could result in formation of an initiated cell population with an increased susceptibility to further neoplastic development. The clonal proliferative growth of the initiated cells initially constitutes the succeeding stage of promotion and accounts for part of the time necessary for the development of human and experimental neoplasms. Thus, during the promotion stage, an acceleration of the growth is accompanied by the development of the characteristics of aggressive malignant growth. Thus, in experimental models, carcinogenesis has been divided into the stages of initiation, promotion, and progression. Agents with a potential carcinogenic risk can be classified as acting at any one or a combination of these stages.

AFB_1 is a procarcinogen that must be activated metabolically to 8,9-epoxide, the putative ultimate carcinogen. The mutagenicity of aflatoxin also has been well characterized (WF Busby and GN Wogan 1984), and AFB_1 is classified as a complete carcinogen. Initiation starts with the primary nucleic acid adduct resulting from AFB_1 administration, the N^7-guanine derivative 8,9-dihydro-8-(N7-guanyl)-9-hydroxyAFB_1 (Essigmann et al. 1977; Muench et al. 1983). Other minor N^7-guanly adducts can arise through enzymatic oxidation of AFP_1, M_1, and other AFB_1 phase I metabolites unsaturated in the 8,9 position (Croy and Wogan 1979; Croy and Wogan 1981). These

adducts have been found primarily in GC-rich region of DNA. The resultant primary DNA mutation (>90%) in at least one *in vitro* model is a GC→ TA transversion (Foster et al. 1983). Moreover, the genomic damage by AFs have shown non-random pattern. Internucleosomal DNA and ribosomal RNA gene were more susceptible to mutation than nucleosomal core DNA (George S Bailey et al. 1980). AFB_1 shows sequence selectivity toward DNA (Misra et al. 1983; Muench et al. 1983). Studies revealed that p53 and ras mutation at specific sites were observed among the AFB_1 exposed groups (Aguilar et al. 1993; Bressac et al. 1991; Hsu et al. 1991; Hussain et al. 2007; McMahon et al. 1990; Shen and Ong 1996). The mutation of codon 249 in p53 tumor suppressor gene was significantly common among the hepatocellular carcinoma (HCC) cases where AFB_1 exposure is common. P53 mutations indicate that the sites and features of DNA base changes differ among the various human tumor types. In human HCC, the mutational spectrum has provided a strong molecular link between carcinogen exposure and cancer. In geographical areas of AFB_1 dietary exposure and chronic viral hepatitis are common, a point mutation at the third position of codon 249 ser resulting in a G:C to T:A transversion was common in HCC (Bressac et al. 1991). Two ways can explain this specific mutational spectrum: one is that the higher relative abundance of the 249^{Ser} mutant liver cells may be due to the high mutability of the third base at codon 249 to AFB_1. Another is that 249^{Ser} mutant p53 protein may provide a special growth and/survival advantage to these liver cells. Through the epigenetic changes previously mentioned, the defected cells will expand and make the cancers.

The chronic infection with HBV in the major cause of HCC in human but the precise mechanism of carcinogenesis at molecular level is still indefinable. However,

overall evidence supports the direct and in-direct effects by HBV combines with host response develop tumor. HBV possibly initiates tumor by oncogene induction or genomic instability either by viral DNA integration or by activity of X protein. However, considering the long latency of HCC development after initial HBV infection, chronic inflammation and subsequent genetic alternations by HBV infection are probably superior cause to induce cancer than oncogene induction by HBV or genomic instability. Evidence have accumulated that co-exposure of AFB_1 and HBV synergistically increase the chance of hepatocellular carcinoma (Yeh et al. 1989).

2.1.4. Anti-nutritional effects.

Micronutrient plays a critical role in maintaining proper functioning of the immune system and developmental system. Vitamin A (a fat-soluble micronutrient that is essential for immunity, cellular differentiation, maintaining epithelial surfaces, growth, reproduction, and vision), Vitamin E (a lipid-soluble antioxidant that decreases free radical-induced damages to cellular membranes). Their levels were influenced by AFB_1 exposure. The multivariable analysis in Ghanaians demonstrated that high AFB_1-albumin concentration was associated 2.6 fold increased risk of low vitamin A concentration. Those with high AFB_1-albumin were 2.4 times more likely to have low vitamin E than (Obuseh et al. 2010)

2.1.5. Growth retardation

Pregnant women in Ghana with the highest quartile of AF-albumin were twice as likely to have low birthweight infants as women in the lowest quartile(Shuaib et al. 2010).

Studies in West Africa showed the Children with stunting or who were underweight had higher mean AF-albumin concentrations (Gong et al. 2004; Gong et al. 2002) . a. The unit for the animal experiments (Single dose, ug AFB_1/kg; Chronic dose ug AFB_1/day) and for human study (ug AFB_1/day). A recent study in Kenya showed the inverse association between the level of AF marker and IGF1 and IGFBP3 level(Castelino et al. 2015).

2.2.Aflatoxin exposure in humans

2.2.1. Biomarkers used in the human studies

The major source of AF exposure is through a consumption of AF-contaminated foods. To estimate exposure to AFs various methods were applied. In earlier studies, questionnaires about the consumption of AF-contaminated food items were used to assess AF exposure in human. One study carefully measured AF intake based on plate foods in a village in Gambia (Hudson et al. 1992). They found that the intake was less than those estimated from AF–albumin and urinary adduct levels in the same individuals. This is possibly due to the accumulation of AF adducts in biological specimens before the assessment of the markers of AF exposure. Additionally, the assessment of exposure to AFs through major staples in human population possibly neglects the exposure through "snack food", and other sources such as soy souce and herbal tea, whereas the measurements of AF markers will integrate the exposure to AFs by different food sources. Current major epidemiological studies have adapted biomarkers of exposure because the use of biomarkers prevents any bias in questionnaires.

After ingestion, AF is metabolized by cytochrome P450 enzymes, predominantly in the hepatocyte. The epoxide generated binds covalently to nucleic acids and proteins. Rigorous quantitative comparisons of dietary intakes and AF metabolites in body fluids were conducted in animal models and humans (WF Busby and GN Wogan 1984; Wong and Hsieh 1976).

AFM_1 concentrations in urine and human milk have been correlated with dietary AF intake. However, studies of human exposure have yielded quantitatively very different correlations between AF concentrations in foods and either AF–protein or AF–DNA adducts in urine and sera (Hall & Wild, 1994). There is individual variability in the rate of activation of AF, including between children and adults, which may be material to the pharmacokinetics (Ramsdell & Eaton, 1990; Wild et al., 1990). The pharmacokinetics of AFs in humans is still not clearly known.

As seen in figure 2.2, the development of molecular biomarkers for AFs is based on knowledge of the metabolism and essential genetic macromolecular adduct formation of these compounds and of possible target sites. Unmetabolized AFs (AFB_1, AFG_1), hydroxylated metabolites (AFM_1, AFQ_1), and demethylated metabolites (AFP_1) have been measured in urine. More emphasis has been placed on the nucleic acid adduct AF-N^7-guanine, because this metabolite reflects DNA damage in the presumed target cell for AF carcinogenesis and possibly other AF-related risks.

Linear regression analysis was used to determine the functional relationsihp between metabolite level and dose described by straight line shown in Figure 2.3. Those AFB_1 metabolites were correlated with AFB_1-alb adducts, urinary AFM_1, and urinary AFB_1-N^7-Gua in the molecular epidemiology of AF carcinogenesis. Gan et al found that

1.4-2.3 % of ingested AFB_1 become covalently bound to serum albumin, a value was very similar to that observed when rats are administered AFB_1(Gan et al. 1988). The AFB_1-alb adduct is 3-5 times greater or 30 times greter under the chronic exposure than produced by a 1day exposure because the adduct will be accumulated in the albumin. The half-life for albumin turnover are 20 days and 2.6 days for human and rats, respectively. The diet of 20 individuals with ages ranging from 15 to 56 years were monitored for 1 week in Gambia (Wild et al. 1992). AF intake levels were determined for each day. A correlation between AFB_1-alb adduct and dietary intake was 0.55. To assess the validity of AF-biomarkers in the calculating dosage, several studies were conducted and listed in table 2.1.

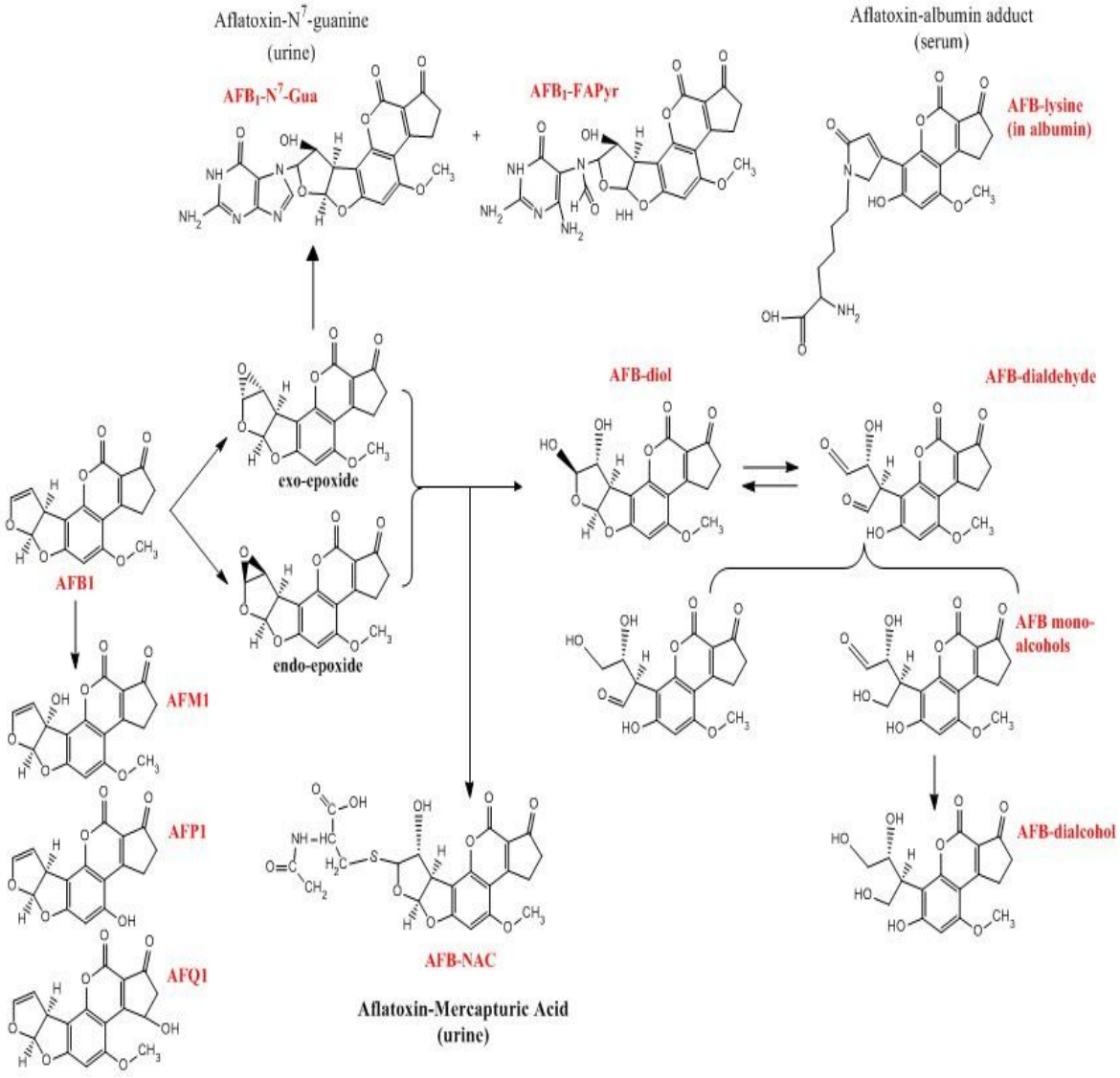


Figure 2.2. Biotransformation pathways for AFB_1. Products are measured in biofluids for use as biomarkers in epidemiological and intervention studies. Adapted from (Kensler et al. 2011)

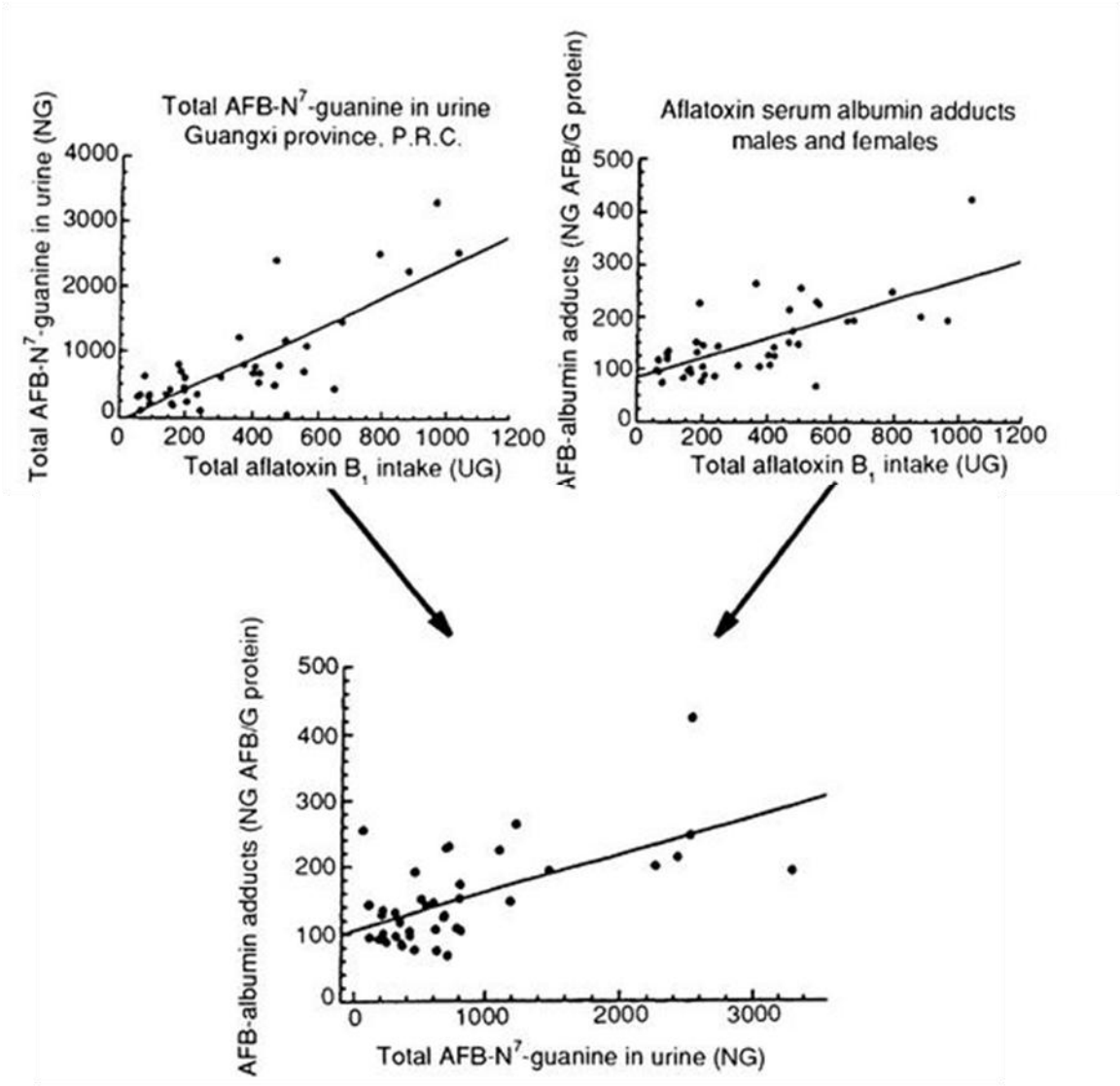


Figure 2.3. Human data for AFB-N^7-guanine in urine and AFB-albumin adduct formation in the same individuals. (Source: David L. Eaton and John D. Groopman, "The Toxicology of Aflatoxins", p271)

Table 2.1. DNA and protein adducts formed by aflatoxin B_1 for reverse dosimetry using urinary metabolites.

AFB_1 intake[a]	Metabolites	Strain/Ethnic[b]	Interval[c]	conversion ratio[d]	reference
animal models					
3.5-200 AFB_1/kg,	AFB_1-alb	Wistar rat	24 hr after dose	0.98%-2.15% of administered dose	(Wild et al. 1986)
0.5µg AFB_1/day	AFB_1-alb	Wistar rat	24 days	3-fold higher than single dose	(Wild et al. 1986)
50 ug AFB_1/kg, a single dose	AFB_1-alb	Male F344 rats	2 hr after dosing	3.68% of administered dose	(Qian et al. 2013)
250 ug AFB_1/kg	AFB1-alb	Male F344 rats	2 hr after dosing	3.90 % of administered dose	(Qian et al. 2013)
1000 ug AFB_1/kg	AFB_1-Alb	Male F344 rats	2 hr after dosing	3.00 % of administered dose	(Qian et al. 2013)
Humans					
average daily intake of 48.4 µg AFB_1/day (men) 77.4 µg AFB_1/day (women)	AFB1-N^7-Guanine	30 male, 12 woman 25-64 years Fusui county, Guangxi province, China	12 hr after AFB1 contaminated food intake	0.2% of the AFB1 intake correlation with AFB_1 intake r=0.65	(John D. Groopman et al. 1992)
average daily intake of 48.4 µg AFB_1/day (men) 77.4 µg AFB_1/day (women)	AFM_1	30 male, 12 woman 25-64 years Fusui county, Guangxi province, China	12 hr after AFB_1 contaminated food intake	approximately 1.1% correlation with AFB_1 intake r= 0.55	(John D. Groopman et al. 1992)
average daily intake of 48.4 µg AFB_1/day	AFP_1	30 male, 12 woman 25-64 years Fusui county,	12 hr after AFB_1 contaminated food intake	no correlation	(John D. Groopman et al. 1992)

(men) 77.4 µg AFB_1/day (women)		Guangxi province, China			
average daily intake of 43 µg AFB_1/day (men) 95.8 µg AFB_1/day (women)	AFM_1	30 male and 12 female adults normal population in Beijing, China 24 hr collection .	next morning (about 12 hr) after the dietary survey	**Male** 1.23-2.18 % AFM_1 /total consumed AFB_1 r=0.66 **female** 1.30-1.78% AFM_1 /total consumed AFB_1 r=0.57	(Zhu et al. 1987)
10 -170 µg AFB_1/day	AFB_1-ALB	42 residents of Guangxi Province, China 25-64 years of age	1 week dietary survey day 5(blood collection)	1.4-2.3 % of the daily intake r=0.69	(Gan et al. 1988)
10-170µg AFB_1/day	AFM_1	42 residents of Guangxi Province, China	1 week survey 3days of urine collection	r=0.64	(Gan et al. 1988)
Average daily intake of 1.4µg AFB_1/day	AFB_1-ALB	20 residents (10 male, 10 female) of Keneba, West Kiang, The Gambia	1 weeks of survey peripheral blood collection at day 1 & 7	r = 0.5	(Wild et al. 1992)

a. The unit for the animal experiments (Single dose, ug AFB_1/kg; Chronic dose ug AFB_1/day) and for human study (ug AFB_1/day)

b. The strain of animals in a study or ethnic group studied.

c. For animal experiments(single dose), time interval from last dose to sampling; For animal experiments(chronic dose), time interval from last dose to sampling;
Time interval from last aflatoxin-contaminated food consumption to sample collection in human study.

d. conversion ratio (excreted metabolites/AF intake)

2.2.2. Assessment of aflatoxin exposure in various human populations

Humans are exposed to AFs by consuming foods contaminated with the mycotoxin. Mostly affected populations are located within the latitude of thirty degree where weather conditions are apt for *Aspergillus* species to produce AFs. Malaysia, the consumption of AF-contaminated noodles caused acute hepatic encephalopathy in thirteen children, as up to 3 mg of AF was suspected to be present in a single serving of the contaminated noodles (Lye et al. 1995). Acute exposure to AFs linked to aflatoxicosis in humans, and chronic exposure to AFs linked to HCC, immune suppression, and growth retardation (Azziz-Baumgartner et al. 2005). However, a few studies provided daily exposure to AFs during non-outbreak periods (Wild et al. 1990). Accumulation of chronic exposure data would allow for a better understanding and quantification of the health effects associated with chronic exposure and for a better estimate of the level of AF exposure necessary to trigger an outbreak.

2.3.Immunotoxic effects of aflatoxins

Constant observations of domestic animals fed AF-contaminated feed have shown that those domestic animals have an increased vulnerability to pathogens. Repeated low-dose administration of AFB_1 is now known to modulate several parameters of innate and adaptive immune systems in avian and mammalian species. However, the study of AF immunotoxicity is still in its infancy compared to the study of AF carcinogenesis. An extensive number of immunotoxicity studies have been conducted in bird models to provide evidence that the chance of infection by the Newcastle virus has increased among birds exposed to AFs due to the depressed immunities in AF-exposed birds.

The immune system is comprised of numerous lymphoid organs and numerous, different cellular populations with a variety of functions. The bone marrow and thymus are referred to as primary lymphoid organs because they contain the microenvironments capable of supporting the production of mature B- and T- cell, respectively. Mature, naïve, or virgin lymphocytes are first bought into contact with exogenously derived antigens within the highly organized microenvironment of the spleen and lymphnodes, otherwise known as the secondary lymphoid organs. The spleen serves as a filter for the blood, removing both foreign antigens and any circulating dead lymphatic veins that filter antigens from the fluid surrounding the tissues of the body. To assess immunologic integrity after AF exposure, early studies measured a relative size of lymphoid organs and leukocyte counts (Ghosh et al. 1991; Reddy and Sharma 1989; Thaxton et al. 1974). The relative sizes of the bursa of Fabricius and thymus were reduced by 30% and 55 % in the group of chickens given diets containing ten μg AF/g feed (Thaxton et al. 1974). Ghosh showed there was a decrease in spleen/body weight ratio in the groups exposed to AFB_1 at 0.3 and one ug/g.

The primary determinant in either type of immune response is the ability of the immune system components to recognize nonself. In one recent review, the definition of nonself is anything other than that encoded in one's own germ line genome (Nathan, 2006). A nonself substance that can be recognized by the immune system is called an antigen. Antigens are about 10 kDa or larger in size. Smaller antigens are called "haptens" and must be conjugated with carrier molecules (larger antigens) in order to elicit a specific response.

Molecular weights of AFs range from 312 g/mol (AFB_1) to 330 g/mol (AFM_2); an anthropogenic molecule lower than 10 kDa (≈10 kg/mol) probably elicits toxicity by forming an agent-protein complex (hepten). It seems that aflatoxins and protein complexs may elicit an innate immune response by PRRs. Also, research shows that AFs elicit ROS production, although the causes of ROS production are not entirely understood. Also, secondary effects from liver damage due to aflatoxin exposure can produce immunotoxic effects. There is evidence showing that the liver is an important immunological organ (Gao et al. 2008; Racanelli and Rehermann 2006). About 70% of liver cells are hepatocytes while the rest are endothelial cells, stellate cells, Kupffer cells, and lymphocytes. Therefore, liver damage due to AFs can elicit immunotoxicity.

AFs are known to have an immunosuppressive effect in animal models. However, growing evidence has shown that aflatoxins have immunomodulatory effects, eliciting both immunostimulation and immunosuppression, in human and animal models. A classical immunotoxicant produces toxicity either by increasing the chance of infection/cancer (immunosuppressive agent) or by autoimmunity/hypersensitivity (immunostimulatory agent). AFs can produce both effects, probably due to the types of AFs, dose, timing, and duration of exposure. However, these issues have not been addressed in previous studies. This section will focus on the components of immune systems impaired by AF exposure.

Innate immunity

Innate immunity is the first line of host defense against pathogens and operates by recognizing the pathogens and expressing pathogen-specific antigens on cell surfaces.

It is assumed that innate immunity operates non-specifically against pathogens; however, the understanding of pattern recognition receptors (PRRs), which recognize pathogens-associated molecular patterns (PAMPs) that are essential for the survival of pathogens, has made it clear that innate immunity is not completely non-specific. Toll-like receptors are well-characterized PRRs; TLRs are involved in cross-talk with other molecules and cell signaling, resulting in inflammatory responses and subsequent cellular activation (Akira et al. 2006; Janeway and Medzhitov 2002). The activation of TLR provokes an innate immune response through the activation of Toll/interleukin-1 receptor (TIR) homology domain-containing adaptors, such as myeloid differentiation primary response gene 88 (MyD88) and TIR domain-containing adapter protein (TIRAP). MyD88 is a key downstream adapter for most TLRs and interleukin-1 receptors (IL-1Rs). The MyD88 knock-out mice suffered from life-threatening and recurring bacterial infection; however, the redundant host defense mechanisms of MyD88-dependent TLRs are present in humans (von Bernuth et al. 2008).

Recent research on AF immunotoxicity supports the idea that AFs increase susceptibility to infections by altering the expression of TLRs (Bahari et al. 2014; Bruneau et al. 2012; Malvandi et al. 2013; Mehrzad et al. 2013; Mohammadi et al. 2014). The mRNA expression of TLR2 and TLR4, followed by inflammatory cytokines (IL-1beta and IL-6) in human dendritic cells, was significantly upregulated after 2hr exposure to 10 ng/ml of AFB_1 (Mohammadi et al. 2014). Similar results were found (Mehrzad et al. 2013): mRNA expression of TLR4 was up-regulated at post exposure 2 and 5 hr in a group exposed to an AF mixture (1.0 ng AFB_1/ml, 0.5 ng AFB_2/ml, 0.25 ng AFG_1/ml, 0.25 ng AFG_2/ml) in a study using bovine peripheral blood mononuclear cells (PBMCs).

Additionally, mRNA expression of MyD88, TLR4, and CD14 was also increased in human PBMCs after exposure to a mixture of environmentally relevant levels of AFB_1 (0.5 ng/ml), AFB_2 (0.25 ng/ml), AFG_1 (0.125 ng/ml), and AFG_2 (0.125 ng/ml) (Malvandi et al. 2013). The engagement of TLR2 and TLR4 triggers the activation of signaling cascades, leading to the induction of genes involved in antimicrobial host defense, especially inflammation. Their unnecessary activations due to AFB_1 exposure may increase these adverse outcomes.

Antigen presentation by Antigen presenting cells (APCs), a group of cells that display a foreign antigen with major histocompatibility complexes (MHCs) on their surfaces, plays a significant role in T cell activation by using its interaction with T cell receptors (TCRs). Antigen presentation is achieved through a successive process of antigen capture, processing, and presentation by APCs, such as dendritic cells, macrophages, certain B cells, certain epithelial cells, and non-professional APCs expressing MHC I. Alteration of any component of the process can cause disruption of the proper presentation of antigens to T cells and alteration of cell-mediated immune responses. Recent studies have shown that AF exposure alters antigen presentation ability in animals by changing phagocytic activity (and/or receptor-mediated endocytosis) and the presentation of MHC II and costimulatory molecules of MHC II. A depression of phagocytic efficiency was seen in animals fed AFB_1. Low dose AFB_1 (10 ng/mL) exposure to porcine monocyte-driven dendritic cells transiently reduced phagocytic capacity and CD40 (co-stimulatory molecule of MHC II) expression, but the phagocytic capacity, the expression of CD40, and CD25 increased after 24 hr exposure. However, the expression of MHC II seems to be downregulated after 24 hr, and the T cell

proliferation was down-regulated (Mehrzad et al. 2014). The mixture of AFs (AFB_1(2.0 ng/ml), AFB_2(1.0 ng/ml), AFG_1(0.5 ng/ml), and AFG_2(0.5 ng/ml)) do not impair the phagocytic capacity of porcine myeloid driven dendritic cells but enhances the expression of activation markers by dendritic cells (Mehrzad et al. 2015). AFG_1 expression increased MHC-II expression, enhancing Treg (Triggering regulatory T cells) activity in AFG_1-induced inflammatory microenvironments (Shen et al. 2015).

The complement system plays an important role in innate immunity, consisting of several plasma proteins that are activated by microbes and promote destruction of the microbes and inflammation. Recently, the activation of adaptive immunity by the complement system has also been elucidated (Iwasaki and Medzhitov 2015). The complement system is now understood to serve as a functional bridge between innate and adaptive immune responses, which allows an integrated host defense to pathogenic challenges. As such, a study of its functions allows insight into the molecular underpinnings of host-pathogen interactions as well as the organization and orchestration of the host immune response. Broiler chickens from one day to 42 days of age that were fed rations containing 1.25 and 2.5 μg total AF/g of feed had significantly lower complement titers compared to the control group at the 14, 28, 42 days of age (Stewart et al. 1985). Treatment groups were segregated according to sex because males and females had significantly different complement titers.

Cell-mediated immunity

Early studies set the research direction to cover which components of the immune systems are impaired by AF exposure (mode of action). Total lymphocytes and T

lymphocytes counts were significantly lower in the chickens fed 1ppm AFB_1 (Ghosh et al. 1991). The reduction of total T lymphocytes initiated from 7 days of exposure in AFB_1 fed group, and the gap between AFB_1 fed group and control were significantly broaden by the end of 42 days of feeding with 1ug AFB_1 /g feed. The contact hypersensitivity tested by either an or the 2,4- dinitrofluorobenzene (DNFB) skin sensitivity test was observed in chicks fed ug AFB_1/g feed. Also, deterioration of cellular immunity was tested using graft-versus-host reaction (GVHR), and the test result showed that GVHR was tempered in 0.3 ugs AFB_1/g feed. GVHR was a more sensitive indicator of cellular immunity than the DNFB skin test.

Recent studies have shown that AF exposure alters antigen presentation ability in animals by altering phagocytic activity (and/or receptor mediated endocytosis) and the presentation of MHC II and costimulatory molecules of MHC II. A depression of phagocytic efficiency was seen in animals fed AFB_1 contaminated diets. Low dose AFB_1 (10 ng/mL) exposure to porcine monocyte-driven dendritic cells transiently reduced phagocytic capacity and CD40 (co-stimulatory molecule of MHC II) expression, but the phagocytic capacity, the expression of CD40, and CD25 increased after 24 hr exposure. However, the expression of MHC II seems to be down regulated after 24 hr, and the T cell proliferation was down-regulated (Mehrzad et al. 2014). The mixture of AFs [AFB_1 (2.0 ng/ml), AFB_2 (1.0 ng/ml), AFG_1 (0.5 ng/ml), and AFG_2 (0.5 ng/ml)] do not impair the phagocytic capacity of porcine myeloid driven dendritic cells but enhances the expression of activation markers by dendritic cells (Mehrzad et al. 2015). This might be due to the combinative effects of AFB_1 and AFG_1. AFG_1 expression increased MHC-II

expression, enhancing Treg (Triggering regulatory T cells) activity in AFG_1-induced inflammatory microenvironments (Shen et al. 2015).

Antigen recognition – antibodies (antigen receptors, the effector molecules of B lymphocytes). Antigen recogniction by T cells, a central role in all immune response to protein antigens. Genetics and biochemistry of major histocompatibility complex (MHC), whose products are integral components of the ligands that T cells specifically recognize. AFG_1 reduces the molecular expression of HLA-I, TAP-1 and LMP-2 of adult esophageal epithelial cells *in vitro* (Li et al. 2010)

Hormonal immunity

Most T lymphocytes recognize only peptides, whereas B cells can recognize peptides, proteins, nucleic acids, polysaccharides, lipids, and small chemicals. As a result, T cell-mediated immune response are induced only by protein antigens whereas humoral immune response are seen with protein and nonprotein antigens.

Leroy found that neither immunoglobuin G(IgG), immunoglobulin M(IgM), nor complement 3(C3), complement4 (C4) serum concentrations were affected by AFB_1 in chickens, suggesting that a lower dose AFB1 may not affect the humoral immune response(Leroy et al. 2014). However, a study of Gambian children with higher levels of aflatoxin-albumin adducts had lower levels of secretory IgA in saliva (Turner et al. 2003)

Lymphocyte proliferation is a fundamental characteristic of the response of lymphocytes to antigenic stimulation in which lymphocytes begin to synthesize DNA after cross-linking of their antigen receptor either following recognition of an antigen or stimulation by a polyclonal activator. Contact between a lymphocyte and an antigen-

presenting cell (APC) forms an immunological synapse, in which MHC, T-cell receptor (TCR), and antigen costimulatory signals initiates proliferation. These events result in an increase in the cytoplasmic concentration of NFκB. Consequently, the complex will translocate to the nucleus and initiate transcription of genes for IL-2 and its receptor (CD25). The increased production of interleukin-2, through an autocrine process, further stimulates proliferation. While cross-linking of the antigen receptor is a critical event in lymphocyte proliferation, other factors determine the outcome of the stimulatory process, in particular the signals delivered by different types of antigen-presenting cells. Only antigen-presentation by professional antigen-presenting cells, such as dendritic cells, is capable of providing the accessory signals needed to drive proliferation of T cells, leading to an effective immune response to an antigen. The Absence of some of those signals will lead instead to anergy or clonal deletion. The result of cell stimulation by an antigen or a mitogen is a shift of the cell from the G0 phase of the cell cycle to the G1 phase. At this particular phase of the cycle, regulation of progression through the cycle is governed by proteins which are involved in cell-cycle regulation in all cells, so at this point control is no longer specific to cells of the immune system. Following activation and entry into G1, the lymphocyte will progress through the subsequent phases of the cycle-S phase, in which DNA synthesis takes place, G2 and M phases, where cell division actually takes place, before returning to G1. Failure at any of these steps diminishes the proliferation of lymphocytes.

Interaction with pathogens

The overall picture from studies of immunosuppressive effects of AFs in animals is of increased susceptibility to bacterial and parasitic infections and an adverse effect on acquired immunity, as evidenced by experimental challenge with infectious agents after vaccination (reviewed by Denning, 1987). In contrast to the evidence of the immunosuppressive action of aflatoxins in animal studies, evidence in humans comes only from in-vitro experiments. Extremely low doses of AFB_1 (0.5–1.0 pg/mL) in cultures of human monocytes *in vitro* were shown to decrease phagocytosis and microbicidal activity against Candida albicans (Cusumano et al., 1996). Concentrations as low as 0.05 pg/mL were shown to reduce the release of interleukins 1 and 6 and TNFα (Rossano et al., 1999). Mycotoxin-induced immune disruption may influence susceptibility to childhood infections, but may also increase later susceptibility to hepatocellular carcinoma through the child's reduced immune response to hepatitis B virus (HBV) and risk of subsequent development of chronic HBV-carrier status.

Pier et al. 1972 showed the deterioration of vaccine efficiency in AFB_1 exposed turkeys. Thirty-five turkeys were exposed to aflatoxin B1 (0.25-0.5 ug AFB_1/g feed) from 7 days after hatching to 28 days after hatching and vaccinated with the fowl cholera vaccine on the 9^{th} day(Pier et al. 1972). On the 28^{th} day after hatching, the group of turkeys were challenged with Pasteurella multocida (fowl cholera). Nine out of thirty-five turkeys (26%) died after the challenge. Another group of 18 turkeys were administered the same regimen of AFB_1 and vaccination, but on the 26^{th} day after hatching, this group was supplemented with the immune plasma from a group of turkeys that received normal feed and were vaccinated as the AF exposed group. None of the turkeys with the immune

plasma supplementation died due to fowl cholera challenge. However, another study conducted by the same author showed that hormonal immunity that was represented by the levels of γ-globulin was not impaired when turkeys were exposed to AFs prior to vaccination (Pier and Heddleston 1970). The author attributed the difference to the timing of exposure which, in one study, AFB_1 exposure occurred during the vaccination, whereas in another study exposure to AFs occurred before the vaccination and was completely stopped before vaccination. However, γ-globulin used as the indicator of hormonal immunity against fowl cholera in one of the studies, an it may not a good indicator of established hormonal immunity. Also, the restorative effect of supplementation with immune plasma in aflatoxin-exposed turkeys may be due to complement plasma proteins in the supplementing plasma, not only antibodies.

Moreover, Reddy et al. (Reddy et al. 1987) reported that using the CD-1 mice model, the antibody production was decreased upon 2 week exposure to AFB_1 (0.03-0.07 ug AFB_1/g feed) after being challenged with a T-dependent antigen (sheep red blood cells), but not after a challenge with a T-independent antigen LPS). They also observed the suppression of a delayed hypersensitivity reaction to keyhole limpet hemocyanin in a group exposed to 0.145 and 0.7 ug/g AFB_1.

AFB_1 in feed causes immunotoxicity and interferes with vaccination efficacy in piglets. (Meissonnier et al. 2008; Meissonnier et al. 2009). For 28 days, pigs fed a control diet or a diet contaminated with 385, 867, or 1807 ug pure AFB_1/kg feed. At days 5 and 15, pigs were vaccinated with ovalbumin. Twenty 3-week-old weaned castrated male pigs. Five pigs were then allocated to each experimental group on the basis of body weights among groups. (0, 385, 867, or 1807 ug/kg).

Effect of AFB_1-contaminated diet intake on the mitogenic and antigenic responses of blood lymphocytes. The cellular immune response was evaluated by measuring the blood cell proliferative response following antigenic (OVA) stimulations. AFB_1-fed piglets did not show lymphocyte proliferation upon OVA stimulation after the first and second immunization whereas piglets fed control diet showed proliferative reaction. The proliferative reaction reached 6.3 fold increase in control pigs at the day 21 of experiment (second immunization with OVA).

Low doses of aflatoxins (140 and 280 ppb, 140 ug AFB_1/kg feed or 280 ug AFB_1/kg feed) were included in a corn-soybean diet provided for ad libitum consumption to 36 weanling piglets for 4 weeks(Marin et al. 2002). AF increased the concentration of gamma-globulin in the serum and reduced immune response by Mycoplasma agalactiae in the 280 ppb treated group.

2.4.Aflatoxins and human infectious diseases

In this disertation, the interaction between human immunodeficiey virus and aflatoxin exposure was examined. Conturies resides in sub-tropical regions are at risk of various human infectious agents and aflatoxin eposure. The relationships between infection and disease are frequently dynamic in nature. They center on the “balance” that can be achieved between the resistance mechanism of the host and the infectivity and virulence of the agent under the proper environment. Classical epidemiological triad explains agent, host, and environment influence disease transmission. Considerting aflatoxins' immunomodulatory effects in the host such as humans, transmission of the infectious

agents and pathogenesis due to the infection may altered in the human populations exposed aflatoxins.

Within the framework of epidemics, controlling the transmission of infectious agents is critical , generally represented by basic reproductive number ($R_0=\beta Nd$; R_0, number of secondary cases; β, transmission parameter; N, population size of susceptibles; d, duration of infectiousness). The basic reproductive number higher than 1 means the increased number of infected cases. In this part, the possible effects of aflatoxin exposure on the transmission of infectious agents and pathogenesis of diseases caused by the infectious agents.

2. 4. 1. Human Immunodeficiency Virus (HIV)

Like other lentiviruses, HIV has the long terminal repeats (LTR) which play critical roles in the integration of proviral DNA into host genome, providing transcription factor binding sites, and possibly affecting the long incubation time of HIV. During the incubation period, the virus can deliver a significant amount of viral RNA into the DNA of the host cell, even non-dividing cells. As the virus binds and enters to cells. It reverse transcribes the RNA genome, integrates the resulting proviral DNA into the host genome, expresses a viral gene, assemble the virus, bud the progeny, and the progeny will mature. Various transcription factors binding sites within the LTR of HIV were found (Ghosh et al. 1993; Jones and Peterlin 1994; Lu et al. 1990). Those transcription factors either enhance or repress the transcription of the viral DNA. The plethora of the binding sites allows the virus to be affected by various cellular and viral transcription factors within the context of specific cell type, cell cycle regulation, cellular differentiation, and cellular

activation. The transcriptional regulation becomes more complicated when we consider the activity of viral regulatory proteins within HIV genome such as Tat and Vpr.

Toxicants such as 2,3,7,8-tetrahlorodibenzo-p-dioxin (TCDD) and AFB_1 increased the viral production (Pokrovsky et al. 1991; Tsyrlov and Pokrovsky 1993), possibly by interaction with the LTR sequence (Yao et al. 1995). Yao et al. showed the effect TCDD exposure on the expression of an a chloramphenicol acetyltransferase (cat) reporter gene linked to the promoter sequences in the LTR of HIV-1 (Yao et al. 1995). The expression of cat report was significantly increased when the hepa-1 cells carrying pHIVLTCAT are exposed to TCDD. The linker-scanning mutational analysis of LTR of HIV-1 revealed that NFkB and an adjacent aromatic hydrocarbon receptor element (AhRE) are associated with TCDD-dependent CAT expression. Also, the induction of a functional CYP1A1 monooxygenase by TCDD stimulates a pathway that generates reactive oxygen intermediates which are responsible for the TCDD-dependent activation of genes linked to the LTR. The same authors showed the increased expression of cat reporter with AFB_1 exposure. AFB_1 is known to interact with NFκB and AhRE as well as produce the reactive oxygen species (Ref). It is possible that AFB_1 increases the transcription of LTR linked CAT reporter through the interaction between those two elements with the LTR of HIV-1.

Micronutrient levels were altered in a group of people chronically exposed to AFs and animal models treated with AFs. Vitamins are critical constituents of our diet, influencing our immune system. Chronic exposure to AFs altered the levels of the vitamin in humans and animal models (vitamin A, C, D, and E). Feeding female broiler chicks with AFs in a range from 0 to 2000 ppb decreased the level of liver vitamin A

(Reddy et al. 1989). A study of Ghanaian people, the plasma level of vitamin A and E was negatively associated with the levels of AFB_1-alb adduct (Obuseh et al. 2010; Tang et al. 2009). A study of Gambian children revealed that the level of vitamin C was negatively correlated to the level AFB_1-albumin adduct (Turner et al. 2003). Besides, AFs have shown a negative relation with the level of vitamin D and suppressed vitamin D receptors (Britton and Wyatt 1978; Costanzo et al. 2015; Glahn et al. 1991). Moreover, selenium concentrations in a group of the Chinese population were inversely correlated to AF-albumin adducts concentration (Chen et al. 2000). The similar phenomenon was shown in chick model, and the author linked to suppressed immunity (Hegazy and Adachi 2000). The reduction of those micronutrients due to aflatoxin exposure may weaken the immunity and increase the pathogenesis of infectious agents.

The level of micronutrients has shown the association with the progression of HIV. Selected nutritional interventions with vitamins B, C, D, E, and selenium have been found to decrease the risk of HIV disease progression and AIDS-related deaths(Cirelli et al. 1991; Fawzi et al. 1998). The beneficial effect of vitamin supplements on health and survival outcomes among HIV-infected persons is likely to be mediated by enhancements in specific aspects of immunity that include increases in CD4+ T cell counts, reduced viral loads. In a study of 884 HIV-infected pregnant women in Tanzania, low vitamin D status (serum 25-hydroxyvitamin D <32 ng/mL) was significantly associated with progression to WHO HIV disease stage III or greater in multivariate models (Mehta et al. 2010). The women with low vitamin D status had 46% higher risk of developing severe anemia during follow.

Studies of 64 Ghana showed the cellular immune status in relation to levels of AFB_1-alb adduct in plasma (Jiang et al. 2005). Higher level of AFB_1-alb adduct has a strong negative correlation with activated T cells (CD3+CD69+) and B cells (CD19+CD69+) as well as lower percentages of CD8+ T cells that contained cytolytic mediators, perforin or both perforin and granzyme A. Another study in 166 HIV positive and 80 aged-matched HIV negative Ghanaians showed that AFB_1-alb concentrations in plasma were correlated to lower perforin expression on CD8+ T cells and the reduced percentages of CD4+ T regulatory cells (Tregs), naive CD4+ T cells, B-cells (Jiang et al. 2008). However, in a study of HIV-positive and HIV-negative Ghanaians, Jolly et al found no statistically significant difference of CD4+ T-cells counts between low AF and high AF in HIV positives(Jolly et al. 2011). The same study showed significantly increased odds of having higher HIV viral loads and higher direct bilirubin levels among HIV-positive participants in the high AFB_1-alb.

2.4.2. Tuberculosis (TB)

Infection with *M. tuberculosis* is prevalent in the developing countries, and tuberculosis affected locations have prevalent aflatoxin exposure as well.
T cells play an important role in the immune response against M. tuberculosis. IFN-gamma production by CD4+T cells was crucial to the control of the infection. A study of Ghanaians found that the higher AF-albumin adduct concentration in plasma was associated to the higher hazard of symptomatic tuberculosis (HRs; 0.43-3.39)(Keenan et al. 2011). However, in the study, the prevalence of tuberculosis infection was not varied across the groups with different levels of AF exposure.

2.4.3. Hepatitis B Virus (HBV)

Current knowledge of the molecular mechanisms of AF-induced carcinogenesis contributes to understand the nature of the biological interaction between hepatitis B virus (HBV) and AFs in determining the risk for hepatocellular carcinoma. Evidence have shown that HBV and AFB_1 work synergistically in an AF-induced carcinogenesis. Among the various theories, inflammation due to HBV and AFB_1 is well-accepted theory. In Asia and Africa, HBV-induced chronic active hepatitis and cirrhosis constitute major risk factors for liver cancer.Infection with HBV may increase AF metabolism. In HBV-infected children in The Gambia there was a higher level of AF–albumin adducts than in non-infected children, an observation consistent with altered AF metabolism (Allen et al. 1992; Turner et al. 2000). However, similar studies in adults did not show such differences (J. D. Groopman et al. 1992; Wild et al. 2000). Glutathione S-transferase activity is reduced in human liver in the presence of HBV infection (Zhou et al. 1997).

2.4.4. Influenza

Pathogenesis and infectivity of H1N9 avian influenza virus was more significant in AF-fed birds (El Miniawy et al. 2014; Umar et al. 2015a). In a study of 80 non-vaccinated turkeys, Umar et al found that role of AFB_1 toxicity on transmissibility and pathogenicity of H9N2 avian influenza virus in turkeys (Umar et al. 2015b). AFB_1 exposed turkeys had more severe clinical signs in turkeys of the containing 0.5 ppm AFB_1+H9N2; 35% mortality in the group was observed whereas other group had no mortality. Significant lower antibody titres against H9N2 AIV were observed in birds fed AFB_1-treated diet as well as poor seroconversion (vaccine-induced seroconversion)

2. 4.5. Malaria

Allen et al found that AFB_1-alb levels were higher in children with Plasmodium falciparum parasitaemia than controls in rural Gambian children (Allen et al. 1992), but pathogenesis of malaria and malaria-specific antibody responses were not associated with AFB_1-alb level.

2.5. Summary and Perspectives

AFs induce toxicity in humans and animals by the mutation of critical genes, oxidative stress by forming ROS, DNA methylation, receptor-mediated toxicity, and perturbation of gut microbiome. The role of mutagenesis in aflatoxin-induced hepatotoxicity studied extensively after AFB_1-N^7-guanine adduct in urine. However, the mechanisms of action by other pathways have not fully explored. More research is needed to see how AFs affect immunity, development, and nutritional status.

Acute and chronic exposure to AFs deteriorate innate, cell-mediated, and hormonal immunity in birds, pigs, mice, and humans. Exposure to AFs is common in dietary foods in the area between the latitude of 35ºN and 35ºS where infectious pathogens are rich. Therefore, the interaction between AFs and infectious agents is possible. Evidence are compelling that pathogenesis of disease due to infections was accelerated in the host exposed to AFs; however, whether or not the infectivity of infectious agents increases under the chronic exposure to AFs need more studies. This dissertation project tried to use samples collected from existing cohort studies in Uganda to explore roles of AF exposure in human HIV acquisition and progression. Moreover,

currently human exposure to AFs in US populations was studied. An intervention with a refined enterosorbent to reduce AF exposure was also evaluated in a US population.

Reference

Aguilar F, Hussain SP, Cerutti P. 1993. Aflatoxin b1 induces the transversion of g-->t in codon 249 of the p53 tumor suppressor gene in human hepatocytes. Proc Natl Acad Sci U S A 90:8586-8590.

Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. Cell 124:783-801.

Allen SJ, Wild CP, Wheeler JG, Riley EM, Montesano R, Bennett S, et al. 1992. Aflatoxin exposure, malaria and hepatitis b infection in rural gambian children. Trans R Soc Trop Med Hyg 86:426-430.

Atanda O, Oguntubo A, Adejumo O, Ikeorah J, Akpan I. 2007. Aflatoxin m1 contamination of milk and ice cream in abeokuta and odeda local governments of ogun state, nigeria. Chemosphere 68:1455-1458.

Ayed-Boussema I, Pascussi JM, Maurel P, Bacha H, Hassen W. 2012. Effect of aflatoxin b1 on nuclear receptors pxr, car, and ahr and their target cytochromes p450 mrna expression in primary cultures of human hepatocytes. Int J Toxicol 31:86-93.

Azziz-Baumgartner E, Lindblade K, Gieseker K, Rogers HS, Kieszak S, Njapau H, et al. 2005. Case-control study of an acute aflatoxicosis outbreak, kenya, 2004. Environ Health Perspect 113:1779-1783.

Bagshawe AF, Gacengi DM, Cameron CH, Dorman J, Dane DS. 1975. Hepatitis bs antigen and liver cancer: A population based study in kenya. Br J Cancer 31:581-584.

Bahari A, Mehrzad J, Mahmoudi M, Bassami MR, Dehghani H. 2014. Cytochrome p450 isoforms are differently up-regulated in aflatoxin b(1)-exposed human lymphocytes and monocytes. Immunopharmacol Immunotoxicol 36:1-10.

Bailey GS, Nixon JE, Hendricks JD, Sinnhuber RO, Van Holde KE. 1980. Carcinogen aflatoxin b1 is located preferentially in internucleosomal deoxyribonucleic acid following exposure in vivo in rainbow trout. Biochemistry 19:5836-5842.

Bailey GS, Nixon JE, Hendricks JD, Sinnhuber RO, Van Holde KE. 1980. Carcinogen aflatoxin b1 is located preferentially in internucleosomal deoxyribonucleic acid following exposure in vivo in rainbow trout. Biochemistry 19:5836-5842.

Bailey GS, Williams DE, Wilcox JS, Loveland PM, Coulombe RA, Hendricks JD. 1988. Aflatoxin b1 carcinogenesis and its relation to DNA adduct formation and adduct persistence in sensitive and resistant salmonid fish. Carcinogenesis 9:1919-1926.

Bintvihok A, Thiengnin S, Doi K, Kumagai S. 2002. Residues of aflatoxins in the liver, muscle and eggs of domestic fowls. J Vet Med Sci 64:1037-1039.

Bressac B, Puisieux A, Kew M, Volkmann M, Bozcall S, Mura JB, et al. 1991. P53 mutation in hepatocellular carcinoma after aflatoxin exposure. The Lancet 338:1356-1359.

Britton WM, Wyatt RD. 1978. Effect of dietary aflatoxin on vitamin d3 metabolism in chicks. Poultry science 57:163-165.

Bruneau JC, Stack E, O'Kennedy R, Loscher CE. 2012. Aflatoxins b(1), b(2) and g(1) modulate cytokine secretion and cell surface marker expression in j774a.1 murine macrophages. Toxicol In Vitro 26:686-693.

Busby WF, Wogan GN. Aflatoxins, in chemical carcinogens. In: Proceedings of the Chemical Carcinogens, 1984. Washington, DC, American Chemical Society.

Busby WF, Jr.,, Wogan GN. 1984. Aflatoxins. In: Chemical carcinogens, (Searle CE, ed). Washington, DC:American Chemical Society, 945-1136.

Castelino JM, Routledge MN, Wilson S, Dunne DW, Mwatha JK, Gachuhi K, et al. 2015. Aflatoxin exposure is inversely associated with igf1 and igfbp3 levels in vitro and in kenyan schoolchildren. Mol Nutr Food Res 59:574-581.

Chen J, Chen K, Yuan S, Peng X, Fang J, Wang F, et al. 2013. Effects of aflatoxin b1 on oxidative stress markers and apoptosis of spleens in broilers. Toxicology and industrial health:0748233713500819.

Chen SY, Chen CJ, Tsai WY, Ahsan H, Liu TY, Lin JT, et al. 2000. Associations of plasma aflatoxin b1-albumin adduct level with plasma selenium level and genetic polymorphisms of glutathione s-transferase m1 and t1. Nutr Cancer 38:179-185.

Chou CC, Marth EH. 1976. Distribution of aflatoxin b1 in tissues of mink (mustela vison). Toxicology 5:351-358.

Circu ML, Aw TY. 2010. Reactive oxygen species, cellular redox systems, and apoptosis. Free radical biology & medicine 48:749-762.

Cirelli A, Ciardi M, de Simone C, Sorice F, Giordano R, Ciaralli L, et al. 1991. Serum selenium concentration and disease progress in patients with hiv infection. Clinical biochemistry 24:211-214.

Costanzo P, Santini A, Fattore L, Novellino E, Ritieni A. 2015. Toxicity of aflatoxin b1 towards the vitamin d receptor (vdr). Food Chem Toxicol 76:77-79.

Coulombe RA, Jr., Sharma RP. 1985. Clearance and excretion of intratracheally and orally administered aflatoxin b1 in the rat. Food Chem Toxicol 23:827-830.

Crespi CL, Penman BW, Steimel DT, Gelboin HV, Gonzalez FJ. 1991. The development of a human cell line stably expressing human cyp3a4: Role in the metabolic activation of aflatoxin b1 and comparison to cyp1a2 and cyp2a3. Carcinogenesis 12:355-359.

Croy R, Wogan G. Identification of an aflatoxin-p1-DNA adduct formed invivo in rat-liver. In: Proceedings of the PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, 1979, Vol. 20AMER ASSOC CANCER RESEARCH 615 CHESTNUT ST, 17TH FLOOR, PHILADELPHIA, PA 19106-4404 USA, 182-182.

Croy RG, Wogan GN. 1981. Temporal patterns of covalent DNA adducts in rat liver after single and multiple doses of aflatoxin b1. Cancer Res 41:197-203.

Currie HN, Vrana JA, Han AA, Scardoni G, Boggs N, Boyd JW. 2014. An approach to investigate intracellular protein network responses. Chem Res Toxicol 27:17-26.

Eaton DL, Gallagher EP. 1994. Mechanisms of aflatoxin carcinogenesis. Annu Rev Pharmacol Toxicol 34:135-172.

El Miniawy HM, Ahmed KA, El-Sanousi AA, Khattab MMS. 2014. Effect of aflatoxin induced immunosuppression on pathogenesis of h9n2 avian influenza virus. Pak Vet J 34:234-238.

Essigmann JM, Croy RG, Nadzan AM, Busby WF, Jr., Reinhold VN, Buchi G, et al. 1977. Structural identification of the major DNA adduct formed by aflatoxin b1 in vitro. Proc Natl Acad Sci U S A 74:1870-1874.

Essiz D, Altintas L, Das YK. 2006. Effects of aflatoxin and various adsorbents on plasma malondialdehyde levels in quails. BULLETIN-VETERINARY INSTITUTE IN PULAWY 50:585.

Ewaskiewicz JI, Devlin TM, Ch'ih JJ. 1991. The in vivo disposition of aflatoxin b1 in rat liver. Biochem Biophys Res Commun 179:1095-1100.

Farber E. 1984. The multistep nature of cancer development. Cancer Res 44:4217-4223.

Fawzi WW, Msamanga GI, Spiegelman D, Urassa EJ, McGrath N, Mwakagile D, et al. 1998. Randomised trial of effects of vitamin supplements on pregnancy outcomes and t cell counts in hiv-1-infected women in tanzania. The Lancet 351:1477-1482.

Foster PL, Eisenstadt E, Miller JH. 1983. Base substitution mutations induced by metabolically activated aflatoxin b1. Proc Natl Acad Sci U S A 80:2695-2698.

Gan LS, Skipper PL, Peng XC, Groopman JD, Chen JS, Wogan GN, et al. 1988. Serum albumin adducts in the molecular epidemiology of aflatoxin carcinogenesis: Correlation with aflatoxin b1 intake and urinary excretion of aflatoxin m1. Carcinogenesis 9:1323-1325.

Gao B, Jeong WI, Tian Z. 2008. Liver: An organ with predominant innate immunity. Hepatology 47:729-736.

Ghosh RC, Chauhan HV, Roy S. 1990. Immunosuppression in broilers under experimental aflatoxicosis. Br Vet J 146:457-462.

Ghosh RC, Chauhan HV, Jha GJ. 1991. Suppression of cell-mediated immunity by purified aflatoxin b1 in broiler chicks. Vet Immunol Immunopathol 28:165-172.

Ghosh S, Selby MJ, Peterlin BM. 1993. Synergism between tat and vp16 in trans-activation of hiv-1 ltr. Journal of molecular biology 234:610-619.

Gillam C, Ingwersen J. 2013. Dog food recall underscores toxic danger in drought-hit u.S. Corn. Reuters Feb 25, 2013.

Glahn RP, Beers KW, Bottje WG, Wideman RF, Jr., Huff WE, Thomas W. 1991. Aflatoxicosis alters avian renal function, calcium, and vitamin d metabolism. J Toxicol Environ Health 34:309-321.

Gong Y, Hounsa A, Egal S, Turner PC, Sutcliffe AE, Hall AJ, et al. 2004. Postweaning exposure to aflatoxin results in impaired child growth: A longitudinal study in benin, west africa. Environ Health Perspect 112:1334-1338.

Gong YY, Cardwell K, Hounsa A, Egal S, Turner PC, Hall AJ, et al. 2002. Dietary aflatoxin exposure and impaired growth in young children from benin and togo: Cross sectional study. Bmj 325:20-21.

Groopman JD, Busby WF, Jr., Wogan GN. 1980. Nuclear distribution of aflatoxin b1 and its interaction with histones in rat liver in vivo. Cancer Res 40:4343-4351.

Groopman JD, Hall AJ, Whittle H, Hudson GJ, Wogan GN, Montesano R, et al. 1992. Molecular dosimetry of aflatoxin-n7-guanine in human urine obtained in the gambia, west africa. Cancer Epidem Biomar 1:221-227.

Groopman JD, Jiaqi Z, Donahue PR, Pikul A, Lisheng Z, Jun-shi C, et al. 1992. Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in guangxi autonomous region, people's republic of china. Cancer Res 52:45-52.

He XY, Tang L, Wang SL, Cai QS, Wang JS, Hong JY. 2006. Efficient activation of aflatoxin b1 by cytochrome p450 2a13, an enzyme predominantly expressed in human respiratory tract. Int J Cancer 118:2665-2671.

Hegazy SM, Adachi Y. 2000. Comparison of the effects of dietary selenium, zinc, and selenium and zinc supplementation on growth and immune response between chick groups that were inoculated with salmonella and aflatoxin or salmonella. Poultry science 79:331-335.

Helferich WG, Baldwin RL, Hsieh DP. 1986. [14c]-aflatoxin b1 metabolism in lactating goats and rats. J Anim Sci 62:697-705.

Herceg Z. 2007. Epigenetics and cancer: Towards an evaluation of the impact of environmental and dietary factors. Mutagenesis 22:91-103.

Hsu I, Metcalf R, Sun T, Welsh J, Wang N, Harris C. 1991. Mutational hot spot in the p53 gene in human hepatocellular carcinomas.

Hudson GJ, Wild CP, Zarba A, Groopman JD. 1992. Aflatoxins isolated by immunoaffinity chromatography from foods consumed in the gambia, west africa. Nat Toxins 1:100-105.

Hussain S, Schwank J, Staib F, Wang X, Harris C. 2007. Tp53 mutations and hepatocellular carcinoma: Insights into the etiology and pathogenesis of liver cancer. Oncogene 26:2166-2176.

IARC. 2002. Some traditional herbal medicines, some mycotoxins, naphthalene, and styrene. In: IARC Monograph on the Carcinogenic Risks to Humans, Vol. 82, 171-300.

Irvin TR, Wogan GN. 1984. Quantitation of aflatoxin b1 adduction within the ribosomal rna gene sequences of rat liver DNA. Proc Natl Acad Sci U S A 81:664-668.

Iwasaki A, Medzhitov R. 2015. Control of adaptive immunity by the innate immune system. Nat Immunol 16:343-353.

Iyer RS, Voehler MW, Harris TM. 1994. Adenine adduct of aflatoxin b1 epoxide. Journal of the American Chemical Society 116:8863-8869.

Jackson AL, Loeb LA. 2001. The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 477:7-21.

Janeway CA, Jr., Medzhitov R. 2002. Innate immune recognition. Annu Rev Immunol 20:197-216.

Jiang Y, Jolly PE, Ellis WO, Wang JS, Phillips TD, Williams JH. 2005. Aflatoxin b1 albumin adduct levels and cellular immune status in ghanaians. International immunology 17:807-814.

Jiang Y, Jolly PE, Preko P, Wang JS, Ellis WO, Phillips TD, et al. 2008. Aflatoxin-related immune dysfunction in health and in human immunodeficiency virus disease. Clinical & developmental immunology 2008:790309.

Jolly PE, Shuaib FM, Jiang Y, Preko P, Baidoo J, Stiles JK, et al. 2011. Association of high viral load and abnormal liver function with high aflatoxin b1-albumin adduct levels in hiv-positive ghanaians: Preliminary observations. Food additives & contaminants Part A, Chemistry, analysis, control, exposure & risk assessment 28:1224-1234.

Jones KA, Peterlin M. 1994. Control of rna initiation and elongation at the hiv-1 promoter. Annual review of biochemistry 63:717-743.

Keenan J, Jolly P, Preko P, Baidoo J, Jia-Sheng W, Phillips TD, et al. 2011. Association between aflatoxin b1 albumin adduct levels and tuberculosis infection among hiv+ ghanaians. Archives of Clinical microbiology 2.

Kensler TW, Roebuck BD, Wogan GN, Groopman JD. 2011. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. Toxicological Sciences 120:S28-S48.

Klaassen CD. 2013. Casarett and doull's toxicology: The basic science of poisons:McGraw-Hill New York (NY).

Kongsbak M, Levring TB, Geisler C, von Essen MR. 2013. The vitamin d receptor and t cell function. Frontiers in immunology 4:148.

Koo JH, Lee HJ, Kim W, Kim SG. Endoplasmic reticulum stress in hepatic stellate cells promotes liver fibrosis via perk-mediated degradation of hnrnpa1 and upregulation of smad2. Gastroenterology.

Kuilman ME, Maas RF, Fink-Gremmels J. 2000. Cytochrome p450-mediated metabolism and cytotoxicity of aflatoxin b(1) in bovine hepatocytes. Toxicol In Vitro 14:321-327.

Kumagai S. 1989. Intestinal absorption and excretion of aflatoxin in rats. Toxicology and applied pharmacology 97:88-97.

Leroy V, Sturm N, Faure P, Trocme C, Marlu A, Hilleret M-N, et al. 2014. Prospective evaluation of fibrotest®, fibrometer®, and hepascore® for staging liver fibrosis in chronic hepatitis b: Comparison with hepatitis c. J Hepatol 61:28-34.

Levy DD, Groopman JD, Lim SE, Seidman MM, Kraemer KH. 1992. Sequence specificity of aflatoxin b1-induced mutations in a plasmid replicated in xeroderma pigmentosum and DNA repair proficient human cells. Cancer Res 52:5668-5673.

Li W. 2013. Aspergillus flavus in corn ears from indiana under the drought conditions in 2012. Phytopathology 103:80-80.

Li Z, Cui J, Zhang X, Kang W. 2010. Aflatoxin g1 reduces the molecular expression of hla-i, tap-1 and lmp-2 of adult esophageal epithelial cells in vitro. Toxicol Lett 195:169-173.

Liu Y, Wu F. 2010. Global burden of aflatoxin-induced hepatocellular carcinoma: A risk assessment. Environ Health Perspect 118:818-824.

Lu Y, Touzjian N, Stenzel M, Dorfman T, Sodroski JG, Haseltine WA. 1990. Identification of cis-acting repressive sequences within the negative regulatory element of human immunodeficiency virus type 1. Journal of virology 64:5226-5229.

Lye MS, Ghazali AA, Mohan J, Alwin N, Nair RC. 1995. An outbreak of acute hepatic encephalopathy due to severe aflatoxicosis in malaysia. The American journal of tropical medicine and hygiene 53:68-72.

Maguire S, Rabes HM. 1987. Transformation sensitivity in early s-phase and clonogenic potential are target-cell characteristics in liver carcinogenesis by n-methyl-n-nitrosourea. Int J Cancer 39:385-389.

Malvandi AM, Mehrzad J, Saleh-moghaddam M. 2013. Biologically relevant doses of mixed aflatoxins b and g up-regulate myd88, tlr2, tlr4 and cd14 transcripts in human pbmcs. Immunopharmacology and Immunotoxicology 35:528-532.

Marin DE, Taranu I, Bunaciu RP, Pascale F, Tudor DS, Avram N, et al. 2002. Changes in performance, blood parameters, humoral and cellular immune responses in weanling piglets exposed to low doses of aflatoxin. Journal of animal science 80:1250-1257.

Mary VS, Valdehita A, Navas JM, Rubinstein HR, Fernandez-Cruz ML. 2015. Effects of aflatoxin b(1), fumonisin b(1) and their mixture on the aryl hydrocarbon receptor and cytochrome p450 1a induction. Food Chem Toxicol 75:104-111.

McMahon G, Davis EF, Huber LJ, Kim Y, Wogan GN. 1990. Characterization of c-ki-ras and n-ras oncogenes in aflatoxin b1-induced rat liver tumors. Proceedings of the National Academy of Sciences 87:1104-1108.

Mehrzad J, Milani M, Mahmoudi M. 2013. Naturally occurring level of mixed aflatoxins b and g stimulate toll-like receptor-4 in bovine mononuclear cells. Vet Q 33:186-190.

Mehrzad J, Devriendt B, Baert K, Cox E. 2014. Aflatoxin b(1) interferes with the antigen-presenting capacity of porcine dendritic cells. Toxicol In Vitro 28:531-537.

Mehrzad J, Devriendt B, Baert K, Cox E. 2015. Aflatoxins of type b and g affect porcine dendritic cell maturation in vitro. J Immunotoxicol 12:174-180.

Mehta S, Giovannucci E, Mugusi FM, Spiegelman D, Aboud S, Hertzmark E, et al. 2010. Vitamin d status of hiv-infected women and its association with hiv disease progression, anemia, and mortality. PloS one 5:e8770.

Meissonnier GM, Pinton P, Laffitte J, Cossalter AM, Gong YY, Wild CP, et al. 2008. Immunotoxicity of aflatoxin b1: Impairment of the cell-mediated response to vaccine antigen and modulation of cytokine expression. Toxicology and applied pharmacology 231:142-149.

Meissonnier GM, Raymond I, Laffitte J, Cossalter AM, Pinton P, Benoit E, et al. 2009. Dietary glucomannan improves the vaccinal response in pigs exposed to aflatoxin b-1 or t-2 toxin. World Mycotoxin J 2:161-172.

Misra RP, Muench KF, Humayun MZ. 1983. Covalent and noncovalent interactions of aflatoxin with defined deoxyribonucleic acid sequences. Biochemistry 22:3351-3359.

Mohammadi A, Mehrzad J, Mahmoudi M, Schneider M. 2014. Environmentally relevant level of aflatoxin b1 dysregulates human dendritic cells through signaling on key toll-like receptors. Int J Toxicol 33:175-186.

Muench KF, Misra RP, Humayun MZ. 1983. Sequence specificity in aflatoxin b1--DNA interactions. Proceedings of the National Academy of Sciences 80:6-10.

Nesbitt BF, O'Kelly J, Sargeant K, Sheridan ANN. 1962. Aspergillus flavus and turkey x disease: Toxic metabolites of aspergillus flavus. Nature 195:1062-1063.

Obuseh FA, Jolly PE, Jiang Y, Shuaib FMB, Waterbor J, Ellis WO, et al. 2010. Aflatoxin b-1 albumin adducts in plasma and aflatoxin m-1 in urine are associated with plasma concentrations of vitamins a and e. International Journal for Vitamin and Nutrition Research 80:355-368.

Oda Y, Aryal P, Terashita T, Gillam EM, Guengerich FP, Shimada T. 2001. Metabolic activation of heterocyclic amines and other procarcinogens in salmonella typhimurium umu tester strains expressing human cytochrome p4501a1, 1a2, 1b1, 2c9, 2d6, 2e1, and 3a4 and human nadph-p450 reductase and bacterial o-acetyltransferase. Mutat Res 492:81-90.

Otim MO, Mukiibi-Muka G, Christensen H, Bisgaard M. 2005. Aflatoxicosis, infectious bursal disease and immune response to newcastle disease vaccination in rural chickens. Avian Pathology 34:319-323.

Pelkonen O, Rautio A, Raunio H, Pasanen M. 2000. Cyp2a6: A human coumarin 7-hydroxylase. Toxicology 144:139-147.

Pier A, Heddleston K, Cysewski S, Patterson J. 1972. Effect of aflatoxin on immunity in turkeys. Ii. Reversal of impaired resistance to bacterial infection by passive transfer of plasma. Avian diseases:381-387.

Pier A. 1986. Immunomodulation in aflatoxicosis. In: Diagnosis of mycotoxicoses:Springer, 143-148.

Pier AC, Heddleston KL. 1970. The effect of aflatoxin on immunity in turkeys. I. Impairment of actively acquired resistance to bacterial challenge. Avian Diseases 14:797-809.

Pitot HC. 1993. The molecular biology of carcinogenesis. Cancer 72:962-970.

Piva G, Battilani P, Pietri A. 2006. Emerging issues in southern europe: Aflatoxins in italy.Wageningen Academic Publishers: Wageningen, The Netherlands, 139-153.

Pokrovsky AG, Cherykh AI, Yastrebova ON, Tsyrlov IB. 1991. 2,3,7,8-tetrachlorodibenzo-p-dioxin as a possible activator of hiv infection. Biochem Biophys Res Commun 179:46-51.

Qian G, Tang L, Wang F, Guo X, Massey ME, Williams JH, et al. 2013. Physiologically based toxicokinetics of serum aflatoxin b1-lysine adduct in f344 rats. Toxicology 303:147-151.

Racanelli V, Rehermann B. 2006. The liver as an immunological organ. Hepatology 43:S54-S62.

Ramos A, Hernandez E. 1996. In situ absorption of aflatoxins in rat small intestine. Mycopathologia 134:27-30.

Reddy KV, Rao PV, Reddy VR. 1989. Effect of aflatoxin on the performance of broiler chicks fed diets supplemented with vitamin-a. Indian J Anim Sci 59:140-144.

Reddy RV, Taylor MJ, Sharma RP. 1987. Studies of immune function of cd-1 mice exposed to aflatoxin b1. Toxicology 43:123-132.

Reddy RV, Sharma RP. 1989. Effects of aflatoxin b1 on murine lymphocytic functions. Toxicology 54:31-44.

Shen H-M, Ong C-N, Shi C-Y. 1995. Involvement of reactive oxygen species in aflatoxin b1-induced cell injury in cultured rat hepatocytes. Toxicology 99:115-123.

Shen H-M, Ong C-N. 1996. Mutations of the p53 tumor suppressor gene and ras oncogenes in aflatoxin hepatocarcinogenesis. Mutation Research/Reviews in Genetic Toxicology 366:23-44.

Shen H-M, Shi C-Y, Shen Y, Ong C-N. 1996. Detection of elevated reactive oxygen species level in cultured rat hepatocytes treated with aflatoxin b1. Free Radical Biology and Medicine 21:139-146.

Shen H, Liu C, Shao P, Yi L, Wang Y, Mills Ko E, et al. 2015. Enhanced phenotypic alterations of alveolar type ii cells in response to aflatoxin g1 -induced lung inflammation. J Cell Physiol 230:1199-1211.

Shuaib FMB, Jolly PE, Ehiri JE, Jiang Y, Ellis WO, Stiles JK, et al. 2010. Association between anemia and aflatoxin b-1 biomarker levels among pregnant women in

kumasi, ghana. American Journal of Tropical Medicine and Hygiene 83:1077-1083.

Stewart RG, Skeeles JK, Wyatt RD, Brown J, Page RK, Russell ID, et al. 1985. The effect of aflatoxin on complement activity in broiler chickens. Poultry science 64:616-619.

Steyn M, Pitout MJ, Purchase IFH. 1971. A comparative study on aflatoxin b(1) metabolism in mice and rats. British Journal of Cancer 25:291-297.

Tang L, Xu L, Afriyie-Gyawu E, Liu W, Wang P, Tang Y, et al. 2009. Aflatoxin-albumin adducts and correlation with decreased serum levels of vitamins a and e in an adult ghanaian population. Food additives & contaminants Part A, Chemistry, analysis, control, exposure & risk assessment 26:108-118.

Terao K, Yamazaki M, Miyaki K. 1972. The distribution of tritium-labeled aflatoxin b1, dihydro-aflatoxin b1, and tetrahydrodesoxoaflatoxin b1 in liver cells cultured in vitro from the chicken embryo. Z Krebsforsch Klin Onkol Cancer Res Clin Oncol 78:303-309.

Thaxton JP, Tung HT, Hamilton PB. 1974. Immunosuppression in chickens by aflatoxin. Poultry science 53:721-725.

Towner RA, Qian SY, Kadiiska MB, Mason RP. 2003. In vivo identification of aflatoxin-induced free radicals in rat bile. Free radical biology & medicine 35:1330-1340.

Trucksess MW, Richard JL, Stoloff L, McDonald JS, Brumley WC. 1983. Absorption and distribution patterns of aflatoxicol and aflatoxins b1 and m1 in blood and milk of cows given aflatoxin b1. Am J Vet Res 44:1753-1756.

Tsyrlov IB, Pokrovsky A. 1993. Stimulatory effect of the cyp1a1 inducer 2,3,7,8-tetrachlorodibenzo-p-dioxin on the reproduction of hiv-1 in human lymphoid cell culture. Xenobiotica; the fate of foreign compounds in biological systems 23:457-467.

Turner PC, Mendy M, Whittle H, Fortuin M, Hall AJ, Wild CP. 2000. Hepatitis b infection and aflatoxin biomarker levels in gambian children. Trop Med Int Health 5:837-841.

Turner PC, Moore SE, Hall AJ, Prentice AM, Wild CP. 2003. Modification of immune function through exposure to dietary aflatoxin in gambian children. Environmental Health Perspectives 111:217-220.

Ueng YF, Kuwabara T, Chun YJ, Guengerich FP. 1997. Cooperativity in oxidations catalyzed by cytochrome p450 3a4. Biochemistry 36:370-381.

Umar S, Younus M, Muti Ur R, Aslam A, Shah MA, Munir MT, et al. 2015a. Role of aflatoxin toxicity on transmissibility and pathogenicity of h9n2 avian influenza virus in turkeys. Avian Pathol 44:305-310.

Umar S, Younus M, Rehman MU, Aslam A, Shah MA, Munir MT, et al. 2015b. Role of aflatoxin toxicity on transmissibility and pathogenicity of h9n2 avian influenza virus in turkeys. Avian Pathol:1-22.

Van Vleet TR, Klein PJ, Coulombe RA, Jr. 2001. Metabolism of aflatoxin b1 by normal human bronchial epithelial cells. J Toxicol Environ Health A 63:525-540.

Varga J, Frisvad JC, Samson RA. 2011. Two new aflatoxin producing species, and an overview of aspergillus section flavi. Studies in Mycology 69:57-80.

von Bernuth H, Picard C, Jin Z, Pankla R, Xiao H, Ku C-L, et al. 2008. Pyogenic bacterial infections in humans with myd88 deficiency. Science 321:691-696.

Wang J, Tang L, Glenn TC, Wang J-S. 2016. Aflatoxin b1 induced compositional changes in gut microbial communities of male f344 rats. Toxicological Sciences 150:54-63.

Warpehoski MA, Hurley LH. 1988. Sequence selectivity of DNA covalent modification. Chem Res Toxicol 1:315-333.

Wild CP, Garner RC, Montesano R, Tursi F. 1986. Aflatoxin b1 binding to plasma albumin and liver DNA upon chronic administration to rats. Carcinogenesis 7:853-858.

Wild CP, Jiang YZ, Allen SJ, Jansen LA, Hall AJ, Montesano R. 1990. Aflatoxin-albumin adducts in human sera from different regions of the world. Carcinogenesis 11:2271-2274.

Wild CP, Hudson GJ, Sabbioni G, Chapot B, Hall AJ, Wogan GN, et al. 1992. Dietary intake of aflatoxins and the level of albumin-bound aflatoxin in peripheral blood in the gambia, west africa. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 1:229-234.

Wild CP, Yin F, Turner PC, Chemin I, Chapot B, Mendy M, et al. 2000. Environmental and genetic determinants of aflatoxin-albumin adducts in the gambia. Int J Cancer 86:1-7.

Wild CP, Turner PC. 2002. The toxicology of aflatoxins as a basis for public health decisions. Mutagenesis 17:471-481.

Wild CP, Gong YY. 2010. Mycotoxins and human disease: A largely ignored global health issue. Carcinogenesis 31:71-82.

Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. 2004a. Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr 80:1106-1122.

Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. 2004b. Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr 80:1106-1122.

Wogan GN, Edwards GS, Shank RC. 1967a. Excretion and tissue distribution of radioactivity from aflatoxin b1-14c in rats. Cancer Res 27:1729-&.

Wogan GN, Edwards GS, Shank RC. 1967b. Excretion and tissue distribution of radioactivity from aflatoxin b1-14-c in rats. Cancer Res 27:1729-1736.

Wojnowski L, Turner PC, Pedersen B, Hustert E, Brockmoller J, Mendy M, et al. 2004. Increased levels of aflatoxin-albumin adducts are associated with cyp3a5 polymorphisms in the gambia, west africa. Pharmacogenetics 14:691-700.

Wong JJ, Hsieh DP. 1976. Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential. Proceedings of the National Academy of Sciences 73:2241-2244.

Wood GE. 1992. Mycotoxins in foods and feeds in the united states. Journal of animal science 70:3941-3949.

Wu H-C, Wang Q, Yang H-I, Tsai W-Y, Chen C-J, Santella RM. 2013. Global DNA methylation in a population with aflatoxin b1 exposure. Epigenetics 8:962-969.

Yao Y, Hoffer A, Chang C-y, Puga A. 1995. Dioxin activates hiv-1 gene expression by an oxidative stress pathway requiring a functional cytochrome p450 cyp1a1 enzyme. Environmental Health Perspectives 103:366-371.

Yeh F-S, Mimi CY, Mo C-C, Luo S, Tong MJ, Henderson BE. 1989. Hepatitis b virus, aflatoxins, and hepatocellular carcinoma in southern guangxi, china. Cancer Res 49:2506-2509.

Yu F-L, Huang J-X, Bender W, Wu Z, Chang JC. 1991. Evidence for the covalent binding of aflatoxin b1-dichloride to cytosine in DNA. Carcinogenesis 12:997-1002.

Zhang YJ, Ahsan H, Chen Y, Lunn RM, Wang LY, Chen SY, et al. 2002. High frequency of promoter hypermethylation of rassf1a and p16 and its relationship to aflatoxin b1–DNA adduct levels in human hepatocellular carcinoma. Molecular carcinogenesis 35:85-92.

Zhang YJ, Chen Y, Ahsan H, Lunn RM, Lee PH, Chen CJ, et al. 2003. Inactivation of the DNA repair gene o6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relationship to aflatoxin b1-DNA adducts and p53 mutation in hepatocellular carcinoma. Int J Cancer 103:440-444.

Zhang YJ, Rossner P, Chen Y, Agrawal M, Wang Q, Wang L, et al. 2006. Aflatoxin b1 and polycyclic aromatic hydrocarbon adducts, p53 mutations and p16 methylation in liver tissue and plasma of hepatocellular carcinoma patients. Int J Cancer 119:985-991.

Zhou T, Evans AA, London WT, Xia X, Zou H, Shen F-m, et al. 1997. Glutathione s-transferase expression in hepatitis b virus-associated human hepatocellular carcinogenesis. Cancer Res 57:2749-2753.

Zhu J-q, Zhang L-s, Hu X, Xiao Y, Chen J-s, Xu Y-c, et al. 1987. Correlation of dietary aflatoxin b1 levels with excretion of aflatoxin m1 in human urine. Cancer Res 47:1848-1852.

Zhu LR, Thomas PE, Lu G, Reuhl KR, Yang GY, Wang LD, et al. 2006. Cyp2a13 in human respiratory tissues and lung cancers: An immunohistochemical study with a new peptide-specific antibody. Drug Metab Dispos 34:1672-1676.

Zinedine A, Gonzalez-Osnaya L, Soriano JM, Molto JC, Idrissi L, Manes J. 2007. Presence of aflatoxin m1 in pasteurized milk from morocco. Int J Food Microbiol 114:25-29.

CHAPTER 3

LONGITUDINAL EVALUATION OF AFLATOXIN EXPOSURE IN TWO COHORTS IN SOUTHWESTERN UGANDA

Min-Su Kang, Peter Nkurunziza, Richard Muwanika, Guoqing Qian, Lili Tang, Xiao Song, Kathy Xue, Allan Nkwata, John Ssempbewa, Tom Lutalo, David Serwadda, Janet Seeley, Pontiano Kaleebu, Fred Nalugoda, Jonathan. H. William and Jia-Sheng Wang,
2015, Food Addit Contam Part A, In print. Reprinted here with permission of the publisher

Abstract

Aflatoxins (AF) are a group of mycotoxins. AF exposure causes acute and chronic adverse health effects such as aflatoxicosis and hepatocellular carcinoma in human populations, especially in the developing world. In this study AF exposure was evaluated using archived serum samples from HIV seronegative participants from two cohort studies in southwestern Uganda. AFB_1-lysine (AFB-Lys) adduct levels were determined via HPLC-fluorescence in a total of 713 serum samples from the General Population Cohort (GPC), covering eight time periods between 1989 to 2010. Overall, 90% (642/713) of the samples were positive for AFB-Lys and the median level was 1.58 pg mg^{-1} albumin (range: 0.40-168 pg mg^{-1} albumin). AFB-Lys adduct levels were also measured in a total of 374 serum samples from the Rakai Community Cohort Study (RCCS), across four time periods between 1999 and 2003 . The averaged detection rate was 92.5% (346/374) and the median level was 1.18 pg mg^{-1} albumin (range: 0.40-122.5 pg mg^{-1} albumin). In the GPC study, there were no statistically significant differences between demographic parameters, such as age, sex and level of education, and levels of serum AFB-Lys adduct., In the RCCS study, longitudinal analysis using generalized estimating equations revealed significant differences between the adduct levels and residential areas (P=0.05) and occupations (P=0.02). This study indicates that AF exposure in people in two populations in southwestern Uganda is persistent and has not significantly changed over time. Data from one study, but not the other indicated that agriculture workers and rural area residents had more AF exposure than those non-agricultural workers and non-rural area residents. These results suggest the need for

further study of AF-induced human adverse health effects, especially the predominant diseases in the region.

Keywords:

Aflatoxins; aflatoxin B_1-lysine adduct; human exposure; Uganda; cohort studies.

Introduction

Aflatoxins (AF), mainly produced by *Aspergillus flavus* and *A. parasiticus*, represent a group of naturally occurring fungal metabolites (mycotoxins) that have long been recognized as hazardous contaminants of food, especially in peanuts, corn/maize and dried cassava (Busby and Wogan 1984; Wang et al. 1998; Williams et al. 2004). Aflatoxin B_1 (AFB_1) is a potent hepatotoxic and genotoxic agent (Essigmann et al. 1977; Wang and Groopman 1999) and has been evaluated as a Group I human carcinogen (sufficient evidence of carcinogenicity to humans) by the International Agency for Research on Cancer (IARC), World Health Organization (WHO) (IARC 1993; International Agency for Research on Cancer. 2002). Exposure to high levels of AFB_1 via the diet causes acute hepatotoxicity (aflatoxicosis) and death in humans, as demonstrated by recent outbreaks in Kenya, which were responsible for the deaths of more than 150 people (Azziz-Baumgartner et al. 2005; Lewis et al. 2005). Chronic exposure to low levels of AFB_1 is a risk factor in the etiology of human hepatocellular carcinoma (HCC) in several regions of Africa and Southeast Asia, particularly in conjunction with hepatitis B virus infection (IARC 1993; International Agency for Research on Cancer. 2002; Wild and Hall 2000; Wogan et al. 2012). Importantly, AFB_1 has also been shown to be an anti-nutritional agent that reduces concentrations of vitamins and proteins in animals and humans (Gong et al. 2012; Tang et al. 2009; Wild 2007; Williams et al. 2004). Further, AFB_1 is a potent immunotoxic agent in animals and also changes T cell phenotypes in human, which may aggravate the burden of infectious diseases in the developing world (Jiang et al. 2005; Wild 2007; Williams et al. 2004) . AF Contamination is an important problem in relation to food quality/food safety in Africa, as a result of poor

production and storage practices for major staples of the African diet(Hell et al. 2000; Lewis et al. 2005; Williams et al. 2004; Williams et al. 2010). Studies in Uganda indicate that AF exposure is broadly similar to that reported from other African countries (Kaaya et al. 2005). The environmental conditions in parts of Uganda are ideal for *Aspergillus* growth during crop cultivation and storage in the subsequent post-harvest period, due to improper drying and inadequate storage conditions. AF contamination has been reported in maize, cassava, groundnuts, and locally manufactured baby foods including baby soya and rice porridge (Kaaya and Eboku 2010; Kitya et al. 2010; Williams et al. 2004; Williams et al. 2010).

Human exposure to AF and its linkage to adverse health effects in various human populations have been well studied in some West African countries (Williams et al. 2004; Williams et al. 2010), as well as in Kenya (Azziz-Baumgartner et al. 2005; Lewis et al. 2005; Yard et al. 2013), a neighboring country of Uganda. However, no human exposure studies have ever been assessed in Uganda with the exception of an earlier pilot investigation in small numbers of children and a recent pilot study (Asiki et al. 2014; Wild et al. 1990a; Wild et al. 1990b), although a link between AF and hepatoma was suggested decades ago in Uganda (Alpert et al. 1968; Alpert et al. 1971; Serck-Hanssen 1970). In order to further explore relationships between AF exposure, archived serum samples previously collected from two existing cohort studies in the central and southwest regions of Uganda were analysed for AFB_1-lysine adduct (AFB-Lys) levels to evaluate AF exposure in these human populations.

Materials and Methods

Study Populations and Study Samples

The geographic locations of both cohort study sites are indicated on the map of Uganda (Figure 3.1). The General Population Cohort (GPC) was established by the UK Medical Research Council/Uganda Virus Research Institute (MRC/UVRI) in 1989 and sampling was initially conducted in 15 rural villages (expanded to 25 villages in 2000) in a sub-county of Masaka district located in south western Uganda (Asiki et al. 2014). This is an open cohort study, with new births, deaths, inward and outward migration reported at each round of follow-up. The total population covered is about 22,000 people, and there is no age limit. Every year since its inception, data were collected on annual household censuses of the resident population covering age, sex, education, and relationship to household head. Medical sero-survey was conducted in participants aged 13 years and above (although some rounds also include all children), including collection of blood specimens for HIV testing and a brief behavioural questionnaire.

The Rakai Community Cohort Study (RCCS), initiated in 1987 in the Rakai District of south-western Uganda, is a collaborative study conducted by the Ministry of Health through the Uganda Virus Research Institute (UVRI), Makerere University, and Johns Hopkins University. The current cohort, which consists of some of the adult residents in 50 villages, was established in 1994-1995 (Gray et al. 1998; Sewankambo et al. 1994; Wawer et al. 1991). All participants are followed annually in their homes, at which time they provide personal information (demographics, education, current and past sexual behaviours and sexually transmitted infection history) through surveys, and blood samples were collected for detection of human immunodeficiency virus (HIV), sexually

transmitted diseases (STDs) and other infections. This is an open cohort of adults aged 15-49 years, which enrols new immigrants and age-eligible residents at each survey visit annually. The numbers of participants, who are under surveillance, are maintained between the ranges of 12,000 to 16,000 annually. The main objectives of these two cohort studies are similar: to study the dynamics of HIV infection; to identify major risk factors; to measure impacts of HIV infection on mortality and fertility. However, there are two important differences between the GPC and the RCCS. Firstly, the GPC is concentrated in one sub-county of Masaka District, working in contiguous villages, while the RCCS study communities were selected to represent different parts of the District, so are scattered across the area. Secondly, the GPC includes all age groups, from infants to the very old, and the RCCS focuses on collecting data in age groups between 15-49 years.

Archived serum samples were randomly selected from GPC samples collected in 1989, 1992, 1995, 1998, 2001, 2004, 2007 and 2010 and from the RCCS samples collected in 2000, 2001, 2002, and 2003, respectively. A total of 713 and 374 archived sample were thawed and aliquots were separated, stored on dry ice, and shipped via air to analyse AFB-Lys adduct at the College of Public Health, University of Georgia (UGA), Athens, Georgia, USA with the import permit issued by the US Center for Diseases Control and Prevention. All samples selected for this analysis were HIV negative.

Materials for Laboratory Analysis

AFB1 (>98% purity), albumin determination reagent, bromocreosol purple, and normal human serum were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). Protein assay dye reagent concentrate and protein standards were purchased from Bio-

Rad Laboratories Inc. (Hercules, CA). Pronase (25kU, Nuclease-free) was purchased from Calbiochem (La Jolla, CA). Mixed mode solid phase extraction (SPE) cartridges were purchased from the Waters Corp. (Milford, MA). Authentic AFB-Lys was synthesized as previously described (Sabbioni et al. 1987). The authentic AFB-Lys standard was purified and characterized by UV absorption and mass spectrometry (Sabbioni et al. 1987; Wang et al. 2001a). All other chemicals and solvents used were of the highest grade commercially available.

Measurement of Serum AFB-Lys Adduct

Serum samples were coded separately and analyzed with a newly developed HPLC-fluorescence method (Qian 2012). Briefly, thawed human serum samples were heated to 56°C for 30 min to inactivate infectious microbial agents. Total protein and albumin concentrations were determined using a modification of a previously described method (JS Wang et al. 1996). A portion of each serum sample (150 μL) were digested by pronase (pronase:total protein, 1:4, w:w) at 37 °C for 3 h to release AFB-Lys adduct. The serum sample digests were subjected to solid phase extraction (SPE) clean-up using Waters MAX SPE cartridges, which had been conditioned with methanol and equilibrated with water prior to loading with the AFB-Lys containing digests. The loaded cartridges were sequentially washed by water, 70% methanol, and 1% ammonium hydroxide in methanol at a flow rate of 1 ml/min. Purified AFB-Lys was eluted with 2% formic acid in methanol. The eluent was vacuum-dried with a Labconco Centrivap concentrator (Kansas City, MO) and reconstituted for HPLC-fluorescence detection. The analysis of AFB-Lys adduct was conducted in an Agilent 1200 HPLC-fluorescence

system (Santa Clara, CA). The mobile phases consist of buffer A (20 mM $NH_4H_2PO_4$, pH 7.2) and buffer B (100% methanol). The Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 x 250 mm) equipped with a guard column was used. Column temperature was maintained at 25°C during analysis, and a volume of 100 μL was injected into HPLC. The flow rate was kept at 1 mL/min. A gradient was generated to separate the AFB-Lys adduct within 25 min of injection. AFB-Lys adduct was detected by fluorescence at maximum excitation and emission wavelengths of 405 nm and 470 nm, respectively. Calibration curves of authentic standard was generated weekly, and the standard AFB-Lys was eluted at approximately 13.1 min. Quality assurance and quality control procedures were conducted during analyses, which include analysis of one authentic standard and a quality control sample daily in same sequnce. The limit of detection was 0.4 pg mg^{-1} albumin. The average recovery rate with various spiked concentrations of AFB-Lys adduct standard was 90%. The serum AFB-Lys concentration was normalized toalbumin content for statistical analysis and report. The technicians who perform the analyses had no information about sample sources, which guarantees the confidentiality.

Statistical Analysis

An Excel database was established to include AFB-Lys adduct level and other demographic information. Data analysis was performed using the SAS software version 9.1 (SAS Institute, Cary, NC, USA). Levels of AFB-Lys adduct in the groups with different demographic parameters such as age, gender, area, occupation, and education were compared. Since participants in both cohorts provided serum samples multiple times,

longitudinal analysis for repeated measure was used. Generalized estimating equations were used to handle the inconsistent participation of some participants and non-normal distribution of AFB-Lys adduct in serum. We separately analyzed the data from the MRC site and the Rakai site, due to the discrepancy of baseline demographic factors in both cohorts, which was tested by χ^2 and t test. Monotonic trend of AFB-Lys across rounds (time) in the participants was estimated by Wilcoxon-type test for trend and generalized estimating equations (Cuzick 1985). Temporal variation of AFB-Lys was analyzed using Kruskal-Wallis test. Box plot analysis was done through SigmaPlot 10.0 (San Jose, CA). A p-value of less than 0.05 was considered statistically significant.

Ethics

Ethical approval for this study was granted by the Uganda Virus Research Institute Science Ethics Committee and the Uganda National Council of Science and Technology. The laboratory study protocol of serum sample analysis for AFB-Lys adduct was approved by the Institutional Review Board for Human Subject Protection at the University of Georgia.

Results

Demographic information for study participants at both cohort sites is described in Table 3.1. As shown in the table, there were significant differences between the two cohort study sites on basic demographic parameters in terms of age, sex, area of residency, occupation, and education level. Thus, the statistical analysis was done separately.

AFB-Lys adduct levels in a total of 713 archived GPC serum samples were determined at eight separate time points between the years 1989-2010. Longitudinal temporal variations of AFB-Lys adduct levels in these serum samples was shown in Table 3.2 and Figure 3.2. The averaged detection rate was 90% (642/713) and the median level was 1.58 pg mg^{-1} albumin (range: 0.40-168 pg mg^{-1} albumin) over all analyzed cohort samples. There was statistically significant variation across the various rounds ($p<0.0001$), showing the great increase of AFB-Lys adduct for rounds 9- 21, representing 1998-2010 , However, there was no monotonic increase in detection rates and the median level of serum AFB-Lys adduct among 8 rounds ($P_{trend}=0.073$), Further longitudinal analysis using generalized estimating equations for AFB-Lys adduct did not show significant differences between demographic parameters, such as age, gender, residence, occupation, and level of education (Table 3.4).

AFB-Lys adduct levels in a total of 374 archived Rakai RCCS serum samples were determined according to 4 consecutive rounds of sample collections, representing years 2000-2003. Longitudinal temporal variations of AFB-Lys adduct level in these serum samples were shown in Table 3.3 and Figure 3.3. The averaged detection rate was 92.5% (346/374) and the median level was 1.18 pg mg^{-1} albumin (range: 0.40-122.5 pg

mg^{-1} albumin) over all analyzed cohort samples. There was a significant variation across 4 round (p=0.0006), showing increasing AFB-Lys adduct in 2001 -2003, but there was no statistical significance in increasing trend of the AF exposure in RCCS (Fig. 3, Pt_{rend} =0.061). . Although there were no significant differences between demographic parameters, such as age, gender and level of education with sorted AFB-Lys adduct levels (Table 3.4), analysis using generalized estimating equations revealed significant differences between levels of AFB-Lys adduct and residential areas (P=0.045) and occupations (P=0.016) in this cohort samples, as demonstrated in Table 3.4 and Figure 3.4, respectively.

Discussion

This study showed that AF exposure is almost ubiquitous in study participants from both cohorts, as evidenced by the detection rate above 90% and the constant detection of AFB-Lys adduct in these cohort serum samples throughout different times. These results also suggested the universal contamination of AF in food supply in southwestern regions of Uganda. AF contamination in food has been a long-term problem in Uganda (Alpert et al. 1971; Kaaya et al. 2005; Kitya et al. 2010).

While contamination by the AF-producing *Aspergillus* fungi may be universal within a given geographical area, the levels or final concentration of AF in the grain product can vary from < 1 μg/kg (1 ppb) to > 12,000 μg/kg (12 ppm) (Gan et al. 1988). For this reason, the measurement of human consumption of AF by sampling foodstuffs is imprecise. Further, obvious contamination of a commodity with the fungi does not necessarily demonstrate the presence of AF, and the appearance of a sound, uninfected sample of commodity does not preclude the existence of significant quantities of AFs (IARC 2002; Kaaya et al. 2006). Therefore, accurate assessment of human AF exposure using the biomarker approach has been highly recommended in the past 20 years. AFB-Lys adduct in serum albumin has been proven to be the most reliable exposure biomarker (Qian et al. 2013; Sabbioni et al. 1987). As a result of its longer *in vivo* half-life as compared to other urinary AFB_1 metabolites, such as AFM_1 and AFB-N^7-Guanine, AFB-Lys adduct can reflect integrated exposures over 2-3 months. The long-term stability (up to 20 years) in frozen serum or plasma made this adduct a first choice of biomarkers to study human AF exposure and its linkage to diseases (Scholl and Groopman 2008; JS Wang et al. 1996; Wang et al. 2001b). Highly significant associations between AFB-Lys

adduct level and dietary AFB_1 exposure has been found in human populations from several regions of the world. In addition, AFB-Lys adduct has been used as a biological response indicator of acute and chronic human diseases, such as aflatoxicosis in Africa, risks of HCC in Taiwan, China, and Africa, and infectious disease linked immune suppression (Azziz-Baumgartner et al. 2005; Lewis et al. 2005; Turner et al. 2003; Wang et al. 1998; Wang et al. 2001b; LY Wang et al. 1996). Moreover, AFB-Lys adduct has been used as the primary biomarker to assess efficacy of several human chemoprevention and intervention trials (Wang et al. 2001a; JS Wang et al. 2008; P Wang et al. 2008; Wild et al. 2000). Therefore, application of AFB-Lys adduct is a significant tool as an optimal biomarker for studying global public health concern on AF-associated human adverse health effects and evaluating efficacies of various intervention or prevention strategies, especially in developing world.

The present data are consistent with the previous reported cross-sectional pilot study in GPC samples collected in 2011 (Asiki et al. 2014). The geometric means of AFB-Lys in our GPC study was 1.62 pg mg^{-1} albumin and 1.66 pg mg^{-1} albumin in RCCS, which is different from the geometric mean of 11.5 pg mg^{-1} albumin in that report (Asiki et al. 2014). The difference possibly originated from different analytical methods for AFB-Lys measurement: HPLC-fluorescence detection was used in our study, and ELISA method was used in that study. Another explanation would be the difference of dietary pattern changes, study designs, and source of samples. In contrast to the pilot study using cross-sectional design for samples only collected in 2011, which may reflect recent dietary pattern changes in GPC study participants, our longitudinal study design reflects temporal pattern of the AFB-Lys adduct levels from 1989 to 2010. Additionally,

our study used archived serum samples in both cohorts. Even though AFB-Lys adduct has been shown to have a long term stability over 20 years under the proper storage condition (Scholl and Groopman, 2008; Wang et al. 1996, 2001a), the repeated thaw and freeze cycle of these samples may degrade the adduct level in the original samples. Nevertheless, by using longitudinal study design and more accurate detection method for AFB-Lys measurement, our study shows the constant AF exposure over 21 years and provides better estimates of AF exposure in central and southwestern Ugandan populations.

Compared to other African countries, the averaged AFB-Lys level found in this study was among those lower levels reported.Yard et al (2013) suggested that the estimated AFB-Lys adduct values from ELISA method is typically 4.6 times higher than that from HPLC-fluorescence measurement. If taking their proposed 4.6 conversion, AFB-Lys adduct level in our study using GPC study samples is similar to that of the study of Asiki et al (2014). The averaged adduct level is actually higher than the levels found in children from Kikelelwa, Tanzania (0.78 pg mg^{-1} albumin after conversion by division of 4.6) (Shirima et al. 2013; Yard et al. 2013). However, the adduct level is still lower than averaged levels found from neighboring countries, such as Kenya, Tanzania, and the west Africa countries, such as Ghana, Gambia, Benin and Togo (Gong et al. 2002, 2012; Ofori-Adjei 2012; Shirima et al. 2013). This may be again due to different diet patterns in study participants or different AF exposure levels, or the degradation of AFB-Lys adducts in our serum samples analyzed. Also, direct comparisons between countries may not be valid because of different study designs, various analytical methods used, and seasonal variations of sample collections. However, considering the consistency in our

data and the data from Asiki et al (Asiki et al. 2014), it is no doubt that human populations residing in southwestern Ugandans have been constantly exposed to AF via their diets.

Exposure to AF has been documented in Uganda since 1967 when AF contamination was found in peanuts sold for human consumption (Lopez and Crawford, 1967). Approximately 30% of the sampled human food items (n=480) were positive for AF contamination and 7.7% (37/480) had concentrations of total AF between 100-1,000 ppb (Alpert et al. 1971; Kaaya and Warren, 2005). About 60-88% of the corn samples collected from three agro-ecological zones contained detectable levels of AF contamination with AFB_1 as the dominant type found in 86-100% of corn samples cross zones (Kaaya and Kyamuhangire, 2006). A more recent study found AF contamination in common processed food samples collected from southwestern Uganda, covering ground nuts, cassava, millet, sorghum flour and Eshabwe sauce, which is a traditional food in this region made from milk and cheese (Kitya et al. 2010). The mean of AF levels in these food items (n=90) was 15.7 ppb (range: 0 to 55) with 82.2% (74/90) exceeding the regulatory levels of the European Union (4 ppb for processed food).

In this study we also found that the serum samples from participants of rural areas had a significantly higher AFB-Lys adduct levels than samples from semi-urban residents in the RCCS cohort, whereas no such a difference can be examined in GPC samples, because all study participants were recruited from rural areas. Food resource of rural residents is usually limited, dependent on local productions, and highly reliant on natural environmental conditions (Huang and Bouls 1996; Mukwaya et al. 2012; Romanik 2008). Their food production in rural Uganda is vulnerable to toxigenic *Aspergillus* growth

during crop cultivation, and higher AF contamination may occur due to inadequate post-harvest drying and improper storage practices. Thus, rural populations are likely to be exposed to higher levels of AF. On the other hand, residents in semi-urban areas usually have better economic status and have better chance to access diverse food items, such as meat, fish, and fruit, rather than just cereal or other staples such as bananas (Huang and Bouls 1996; Mukwaya et al. 2012). According to a survey in Malawi, 90% of food consumed by household in urban area is purchased, whereas only 30 % of food consumed in rural area is purchased in market (Romanik 2008). The diverse food items other than AF contaminated grains will protect the residents in semi-urban area from high AF exposure. Also, rural populations suffer the poverty more frequently than urban population in Uganda (Mukwaya et al. 2012). Association of AF exposure with poverty has been well proven (Adejumo et al. 2013; Shuaib et al. 2010).

In summary, our study showed that AF exposure has been a constant problem over decades in the central and southwestern Ugandan. It has been well documented that AF exposure is correlated to an increased risk of HCC, immune-modulations, and anti-nutrition in human populations (Wild 2007; Williams et al. 2004). How AF exposure impacts human health in these regions needs to be further explored, especially for its linkage to aggrevation of infectious diseases, such as HIV, and disturbance of growth and development in children.

Acknowledgements:

This work was partially supported by the research contract, ECG-A-00-07-00001-00, from the United States Agency for International Development via Peanut

CRSP at University of Georgia, and by the planning grant, 1R24TW009489, from the National Institute of Health Fogarty International Center. Acknowledge cohort funding – from various sources for RCCS and for GPC joint funding by the UK Medical Research Council (MRC) and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement.

Table 3.1. Demographic information for participants in two study sites

	MRC Site	Rakai Site	p-value
Sex			
Male	183	151	0.0034
Female	174	223	
Age (mean ± sd)	44.3 ± 18.6	30.2 ± 9.76	<0.001
Area of residency			
Rural	357	314	<0.0001
Semi-urban	-	60	
Occupation			
Agriculture	323	233	<0.0001
Others	34	141	
Level of education			
Never	54	23	
Primary	261	227	<.0001
Secondary and above	42	124	
HIV Status			
Negative	357	374	
Positive	-	-	

* P-value obtained by χ^2 test(sex, area, occupation, education) and t test(age).

Table 3.2. AFB-Lys adduct levels in the GPC study participants over time.

AFB-Lys adduct level (pg mg^{-1} albumin) in MRC GPC sample									
	Round (year)								
	R1 (1989)	R3 (1992)	R6 (1995)	R9 (1998)	R12 (2001)	R15 (2004)	R18 (2007)	R21 (2010)	**Total**
Number	87	11	86	49	80	128	141	131	713
Mean	3.09	2.71	5.62	1.06	1.51	1.89	3.00	3.12	2.85
SD	3.70	1.43	22.46	1.05	1.70	1.62	4.07	3.69	8.37
Median	1.82	2.44	1.50	0.77	0.92	1.46	2.03	1.93	1.58
Minimum	0.40	1.02	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Maximum	22.5	5.16	168	5.18	11.6	7.7	32.3	24.08	168.0
Prevalence of detectable AFB-Lys (%)	95.4 (83/87)	100 (11/11)	95.3 (82/86)	83.7 (41/49)	81.3 (65/80)	85.2 (109/128)	91.5 (129/141)	93.9 (123/131)	90.0 (642/713)

Table 3.3. AFB-Lys adduct levels in Rakai RCCS study participants over time

	AFB-Lys adduct level (pg mg^{-1} albumin) in Rakai RCCS Sample Round (year)				
	R6 (2000)	R7 (2001)	R8 (2002)	R9 (2003)	Total
Number	84	146	128	14	374
Mean	5.71	2.35	2.95	6.55	3.46
SD	11.8	4.22	11.2	8.82	9.25
Median	1.86	1.11	1.12	2.31	1.18
Minimum	0.40	0.40	0.40	0.40	0.4
Maximum	94.8	26.0	122.5	30.9	122.5
Prevalence of detectable AFB-Lys (%)	95.2 (80/84)	94.5 (138/146)	89.8 (115/128)	78.6 (11/14)	92.5 (346/374)

Table 3.4. AFB-Lys adduct levels in all participants according to selected demographic characteristics

	Number	Detection Rate (%)	Geometric Mean	Median (95 % CI)	25th Percentile	75th Percentile	90th Percentile	P-value[1]
MRC GPC Site								
Sex								
Male	183	87.4	1.63	1.56 (0.4-7.25)	0.81	3.09	7.25	0.367
Female	174	90.8	1.75	1.84 (0.40-8.20)	0.93	2.79	4.54	
Residential Area								
Rural	357	89.1	1.69	1.71 (0.4-7.63)	0.87	3.07	5.5	-
Occupation								
Agriculture	323	88.9	1.63	1.66 (0.4-7.16)	0.86	2.85	5.26	0.464
Others	34	91.2	2.19	2.41 (0.4-11.33)	1.06	4.54	7.7	
Education								
None	54	90.7	1.9	1.97 (0.4-11.33)	0.95	3.52	7.47	0.386
Primary	261	88.5	1.66	1.62 (0.4-7.02)	0.87	2.87	5.26	
Secondary or higher	42	90.5	1.69	1.78 (0.4-6.05)	0.79	2.99	5.16	
Age								
<20	24	79.2	1.2	1.05 (0.4-4.1)	0.66	2.34	3.96	0.348
20-39	56	92.9	1.96	1.75 (0.4-15.34)	1.03	3.13	6.79	
40-59	194	89.7	1.68	1.7 (0.4-7.47)	0.89	2.71	5.89	
>60	83	88.0	1.64	1.8 (0.4-7)	0.69	3.39	5.29	
Rakai RCCS Site								
Sex								
Male	151	92.1	1.49	1.11 (0.4-14.2)	0.74	2.48	6.32	0.975
Female	223	92.8	1.53	1.25 (0.4-13)	0.68	2.63	7.39	
Residential Area								
Rural	314	93.3	1.58	1.25 (0.4-18.2)	0.72	2.63	6.84	0.045
Semi-Urban	60	88.3	1.21	0.95 (0.4-8.34)	0.67	1.87	4.85	
Occupation								
Agriculture	233	91.4	1.59	1.19 (0.4-20.92)	0.68	2.66	9.93	0.016
Others	141	94.3	1.39	1.17 (0.4-6.32)	0.75	2.43	5.08	
Education								
None	23	91.3	2.06	1.05 (0.4-5.93)	0.71	1.82	3.14	0.493
Primary	227	93.4	1.46	1.3 (0.4-15.6)	0.73	2.85	8.14	
Secondary or higher	124	91.9	1.52	1.07 (0.4-8.14)	0.69	2.26	5.13	
Age								
<20	42	90.5	1.33	0.99 (0.4-5.83)	0.58	1.98	4.57	0.404
20-39	257	91.4	1.59	1.30 (0.4-15.59)	0.72	2.95	7.39	
40-59	75	97.3	1.37	1.04 (0.44-20.01)	0.74	2.01	5.39	

[1]longitudinal analysis using GEE (generalized estimating equations).

Figure 3.1. A map of Uganda and geographic distribution of GPC and RCCS study sites

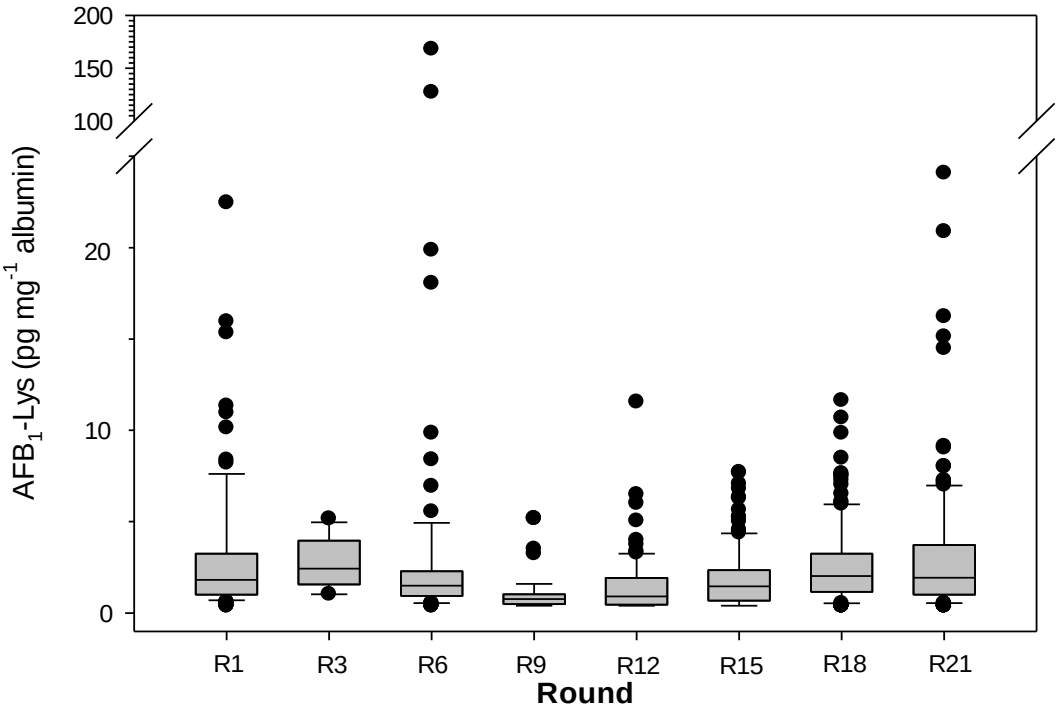


Figure 3.2. Temporal variations of AFB-Lys adduct level in MRC study participants.

The box plots shows distribution of serum AFB-Lys adducts levels in each round. The box values ranged from 25 to 75 percentile of the total samples, the line within it indicating the median value. The bars on both sides of a box represent values ranging from5 to 25 percentiles and from75 to 95 percentile, respectively.

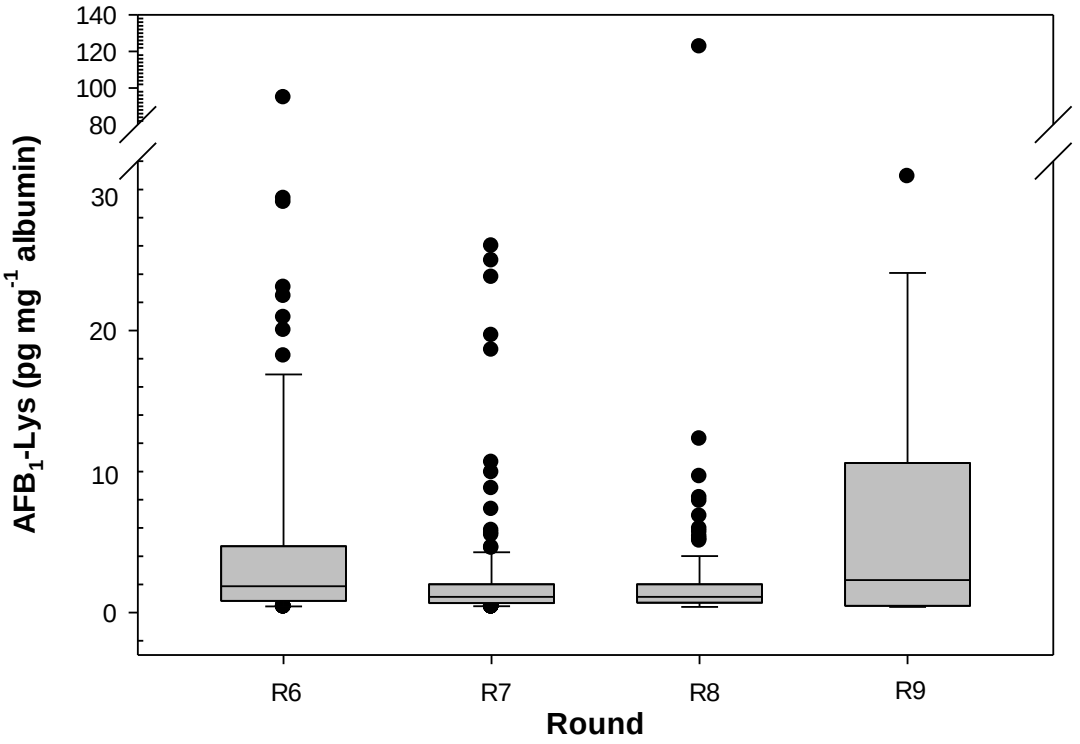


Figure 3.3. Temporal variations of AFB-Lys adduct levels in Rakai RCCS study participants.

The box plots shows distribution of serum AFB-Lys adducts levels in each round. The box values ranged from 25 to 75 percentile of the total samples, the line within it indicating the median value. The bars on both sides of a box represent values ranging from5 to 25 percentiles and from75 to 95 percentile, respectively.

Reference

Adejumo O, Atanda O, Raiola A, Somorin Y, Bandyopadhyay R, Ritieni A. 2013. Correlation between aflatoxin m 1 content of breast milk, dietary exposure to aflatoxin b 1 and socioeconomic status of lactating mothers in ogun state, nigeria. Food Chem Toxicol 56:171-177.

Alpert M, Uriel J, De Nechaud B. 1968. Alpha1 fetoglobulin in the diagnosis of human hepatoma. New England Journal of Medicine 278:984-986.

Alpert M, Hutt MSR, Wogan G, Davidson C. 1971. Association between aflatoxin content of food and hepatoma frequency in uganda. Cancer 28:253-260.

Asiki G, Seeley J, Srey C, Baisley K, Lightfoot T, Archileo K, et al. 2014. A pilot study to evaluate aflatoxin exposure in a rural ugandan population. Trop Med Int Health 19:592-599.

Azziz-Baumgartner E, Lindblade K, Gieseker K, Rogers HS, Kieszak S, Njapau H, et al. 2005. Case-control study of an acute aflatoxicosis outbreak, kenya, 2004. Environ Health Perspect 113:1779-1783.

Busby WF, Jr.,, Wogan GN. 1984. Aflatoxins. In: Chemical carcinogens, (Searle CE, ed). Washington, DC:American Chemical Society, 945-1136.

Cuzick J. 1985. A wilcoxon-type test for trend. Stat Med 4:87-90.

Essigmann JM, Croy RG, Nadzan AM, Busby WF, Jr., Reinhold VN, Buchi G, et al. 1977. Structural identification of the major DNA adduct formed by aflatoxin b1 in vitro. Proc Natl Acad Sci U S A 74:1870-1874.

Gan LS, Skipper PL, Peng XC, Groopman JD, Chen JS, Wogan GN, et al. 1988. Serum albumin adducts in the molecular epidemiology of aflatoxin carcinogenesis:

Correlation with aflatoxin b1 intake and urinary excretion of aflatoxin m1. Carcinogenesis 9:1323-1325.

Gong YY, Wilson S, Mwatha JK, Routledge MN, Castelino JM, Zhao B, et al. 2012. Aflatoxin exposure may contribute to chronic hepatomegaly in kenyan school children. Environmental Health Perspectives 120:893-896.

Gray RH, Wawer MJ, Serwadda D, Sewankambo N, Li CJ, Wabwire-Mangen F, et al. 1998. Population-based study of fertility in women with hiv-1 infection in uganda. Lancet 351:98-103.

Hell K, Cardwell KF, Setamou M, Poehling H. 2000. The influence of storage practices on aflatoxin contamination in maize in four agroecological zones of benin, west africa. Journal of stored products research 36:365-382.

Huang J, Bouls H. 1996. Structural changes in the demand for food in asia. Washington, D.C.:International Food Policy Research Institute (IFPRI).

IARC. 1993. Iarc monographs on the evaluation of carcinogenic risks to humans. In: IARC Monograph on the Carcinogenic Risks to Humans, Vol. 56, 245-396.

IARC. 2002. Some traditional herbal medicines, some mycotoxins, naphthalene, and styrene. In: IARC Monograph on the Carcinogenic Risks to Humans, Vol. 82, 171-300.

International Agency for Research on Cancer. 2002. Some traditional herbal medicines, some mycotoxins, naphthalene, and styrene. In: IARC Monograph on the Carcinogenic Risks to Humans, Vol. 82, 171-.

Jiang Y, Jolly PE, Ellis WO, Wang JS, Phillips TD, Williams JH. 2005. Aflatoxin b1 albumin adduct levels and cellular immune status in ghanaians. International immunology 17:807-814.

Kaaya AN, Warren HL, Kyamanywa S, Kyamuhangire W. 2005. The effect of delayed harvest on moisture content, insect damage, moulds and aflatoxin contamination of maize in mayuge district of uganda. Journal of the science of food and agriculture 85:2595-2599.

Kaaya AN, Kyamuhangire W, Kyamanywa S. 2006. Factors affecting aflatoxin contamination of harvested maize in the three agroecological zones of uganda. Journal of Applied Sciences 6:2401-2407.

Kaaya AN, Eboku D. 2010. Mould and aflatoxin contamination of dried cassava chips in eastern uganda: Association with traditional processing and storage practices. Journal of Biological Sciences 10:718-729.

Kitya D, Bbosa G, Mulogo E. 2010. Aflatoxin levels in common foods of south western uganda: A risk factor to hepatocellular carcinoma. European journal of cancer care 19:516-521.

Lewis L, Onsongo M, Njapau H, Schurz-Rogers H, Luber G, Kieszak S, et al. 2005. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central kenya. Environ Health Perspect 113:1763-1767.

Mukwaya P, Bamutaze Y, Mugarura S, Benson T. 2012. Rural-urban transformation in uganda. Kampala, Uganda.

Qian G. 2012. Integrative toxicological evaluation of aflatoxin b_1 exposure in fischer 344 rats and biomarker based exposure assessment of aflatoxin b_1 in human populations. [electronic resource]:2012.

Qian G, Tang L, Wang F, Guo X, Massey ME, Williams JH, et al. 2013. Physiologically based toxicokinetics of serum aflatoxin b1-lysine adduct in f344 rats. Toxicology 303:147-151.

Romanik CT. 2008. An urban-rural focus on food markets in africa. (Institute TU, ed):Washington, DC.

Sabbioni G, Skipper PL, Buchi G, Tannenbaum SR. 1987. Isolation and characterization of the major serum albumin adduct formed by aflatoxin b1 in vivo in rats. Carcinogenesis 8:819-824.

Scholl PF, Groopman JD. 2008. Long-term stability of human aflatoxin b-1 albumin adducts assessed by isotope dilution mass spectrometry and high-performance liquid chromatography-fluorescence. Cancer Epidem Biomar 17:1436-1439.

Serck-Hanssen A. 1970. Aflatoxin-induced fatal hepatitis? Archives of Environmental Health: An International Journal 20:729-731.

Sewankambo NK, Wawer MJ, Gray RH, Serwadda D, Li C, Stallings RY, et al. 1994. Demographic impact of hiv infection in rural rakai district, uganda: Results of a population-based cohort study. Aids 8:1707-1713.

Shuaib FMB, Jolly PE, Ehiri JE, Yatich N, Jiang Y, Funkhouser E, et al. 2010. Association between birth outcomes and aflatoxin b-1 biomarker blood levels in pregnant women in kumasi, ghana. Trop Med Int Health 15:160-167.

Tang L, Tang Y, Huang T, Wang J-S. 2009. Long-term green tea polyphenol intervention decreases aflatoxin biomarker levels in a high-risk population for liver cancer. Proceedings of the American Association for Cancer Research Annual Meeting 50:944-945.

Turner PC, Moore SE, Hall AJ, Prentice AM, Wild CP. 2003. Modification of immune function through exposure to dietary aflatoxin in gambian children. Environmental Health Perspectives 111:217-220.

Wang JS, Qian GS, Zarba A, He X, Zhu YR, Zhang BC, et al. 1996. Temporal patterns of aflatoxin-albumin adducts in hepatitis b surface antigen-positive and antigen-negative residents of daxin, qidong county, people's republic of china. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 5:253-261.

Wang JS, Kensler TW, Groopman J. 1998. Toxicants in food: Fungal contaminants. In: Cu.

Wang JS, Groopman JD. 1999. DNA damage by mycotoxins. Mutat Res-Fund Mol M 424:167-181.

Wang JS, Abubaker S, He X, Sun G, Strickland PT, Groopman JD. 2001a. Development of aflatoxin b(1)-lysine adduct monoclonal antibody for human exposure studies. Applied and environmental microbiology 67:2712-2717.

Wang JS, Huang T, Su J, Liang F, Wei Z, Liang Y, et al. 2001b. Hepatocellular carcinoma and aflatoxin exposure in zhuqing village, fusui county, people's republic of china. Cancer epidemiology, biomarkers & prevention : a publication

of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 10:143-146.

Wang JS, Luo H, Wang P, Tang L, Yu J, Huang T, et al. 2008. Validation of green tea polyphenol biomarkers in a phase ii human intervention trial. Food Chem Toxicol 46:232-240.

Wang LY, Hatch M, Chen CJ, Levin B, You SL, Lu SN, et al. 1996. Aflatoxin exposure and risk of hepatocellular carcinoma in taiwan. Int J Cancer 67:620-625.

Wang P, Afriyie-Gyawu E, Tang Y, Johnson NM, Xu L, Tang L, et al. 2008. Novasil clay intervention in ghanaians at high risk for aflatoxicosis: Ii. Reduction in biomarkers of aflatoxin exposure in blood and urine. Food Addit Contam 25:622-634.

Wawer MJ, Serwadda D, Musgrave SD, Kondelule JK, Musagara M, Sewankambo NK. 1991. Dynamics of spread of hiv-i infection in a rural district of uganda. Brit Med J 303:1303-1306.

Wild CP, Jiang YZ, Allen SJ, Jansen LA, Hall AJ, Montesano R. 1990a. Aflatoxin-albumin adducts in human sera from different regions of the world. Carcinogenesis 11:2271-2274.

Wild CP, Jiang YZ, Sabbioni G, Chapot B, Montesano R. 1990b. Evaluation of methods for quantitation of aflatoxin-albumin adducts and their application to human exposure assessment. Cancer Res 50:245-251.

Wild CP, Hall AJ. 2000. Primary prevention of hepatocellular carcinoma in developing countries. Mutation research 462:381-393.

Wild CP, Yin F, Turner PC, Chemin I, Chapot B, Mendy M, et al. 2000. Environmental and genetic determinants of aflatoxin-albumin adducts in the gambia. Int J Cancer 86:1-7.

Wild CP. 2007. Aflatoxin exposure in developing countries: The critical interface of agriculture and health. Food Nutr Bull 28:S372-S380.

Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. 2004. Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr 80:1106-1122.

Williams JH, Grubb JA, Davis JW, Wang JS, Jolly PE, Ankrah NA, et al. 2010. Hiv and hepatocellular and esophageal carcinomas related to consumption of mycotoxin-prone foods in sub-saharan africa. Am J Clin Nutr 92:154-160.

Wogan GN, Kensler TW, Groopman JD. 2012. Present and future directions of translational research on aflatoxin and hepatocellular carcinoma. A review. Food Addit Contam A 29:249-257.

Yard EE, Daniel JH, Lewis LS, Rybak ME, Paliakov EM, Kim AA, et al. 2013. Human aflatoxin exposure in kenya, 2007: A cross-sectional study. Food additives & contaminants Part A, Chemistry, analysis, control, exposure & risk assessment 30:1322-1331.

CHAPTER 4

EFFECTS OF LONG-TERM AFLATOXIN EXPOSURE ON THE ACQUISITION AND PROGRESSION OF HIV IN RAKAI, UGANDA

Min-Su Kang, Tom Lutalo, David Serwadda, Lili Tang, Richard Muwanika, Peter Nkurunziza, Guoqing Qian, Joseph Sekasanvu, Fred Nalugoda, Xiao Song, Janet Seeley, Jonathan. H. Williams, and Jia-Sheng Wang, To be submitted to Lancet Global Health

Abstract

Background

Aflatoxin B_1 (AFB_1) is not only a class 1 carcinogen, but chronic exposure to AFB_1 also negatively affects the immune system by chronic inflammation and by altering cell-mediated immunity. In previous study, rural Ugandans were found predominantly exposed to AFB_1. Considering the high prevalence of HIV in the region, an interaction between HIV and AFs is plausible. This study examined the effects of chronic exposure to AFB_1 on the acquisition and pathogenesis of HIV.

Methods

Three different rounds of serum samples collected in 259 HIV cases and 150 area-age matched controls in Rakai, Uganda were repeatedly measured for AFB_1-Lysine adducts to provide long-term AF exposure status of subjects. Logistic regression was used to calculate the odds ratio. In HIV-positive participants, AFB_1-lysine adduct levels were compared to HIV stages in each round. The incubation time of AIDS was collated by the serum levels of the adduct. Hazard ratio was obtained using the Cox proportional hazard model, and the proportional hazard assumption was tested using the Schoenfeld residual plot (testing proportional hazard), functional form of covariates

Findings

The odds ratio of having HIV was 5.11 (95% CI, 2.99 – 8.74) among rural Ugandan participants who frequently had higher than the median level of AFB_1-lysine adducts. Among 259 HIV participants, the mean AFB_1-Lys adduct level of was significantly higher in the AIDS group than e other groups (p= 0.0013) in the third round. The incubation time from HIVinfection to progress to AIDS status was significantly different between high and low AF exposure groups. Along with baseline viral load and CD4+ T

cell counts, chronic exposure to high levels of AFB_1 was related to a higher risk of progression to AIDS among HIV participants.

Interpretations

Chronic exposure to AFs may increase both the acquisition and pathogenesis of HIV. This study indicates that reduction of AF exposure is beneficial to HIV patients, especially in regions where AF exposure are common.

Keywords: aflatoxin B1, HIV progression, HIV acquisition

Introduction

Aflatoxin B_1 (AFB_1) is a group 1 carcinogen (carcinogenic to humans) as evaluated by the International Agency for Research on Cancer (IARC 2002). AF contamination in food is an ongoing food security/safety problem in tropical regions (Africa and Southeast Asia) where AF-producing fungal species grow well due to favorable weather conditions. Previous studies have shown that AF exposure in Rakai, Uganda is widespread (Asiki et al. 2014; Kang et al. 2015).

In animal models, AFB_1 alters cellular/innate immunity and suppresses the immune response to opportunistic infections in the host. Recent study showed an increased shedding of H9N2 avian influenza virus in AF-fed turkey (Umar et al. 2015). AFB_1 exposure has been associated with altered cell-mediated immunity in humans. For example, the reduced expression of effector-type CD8+ T cells, the percentage of the CD4+ T_{reg} cells, and the number of naïve CD4+ T cells (Jiang et al. 2005; Jiang et al. 2008) have been correlated with increased AFB_1 exposure. Thus, chronic exposure to AFs may suppress the immune response in human. In addition, in animal models AF exposure is associated with inflammation that can activate the immune system (Dvorackova 1976; Hinton et al. 2003; Meissonnier et al. 2008; Qian et al. 2014). Since continuous activation of the immune system has been shown to accelerate HIV progression by expansion of infected cells (Fahey et al. 1998; Hunt 2012), AF-induced immunotoxic effects and inflammation may contribute to the progression of HIV. Sub-Saharan Africa accounts for over 60% of the current cases of HIV infection and for 75% of the world's HIV-infected women and children (HIV/AIDS 2012). Incidence and progression of HIV seems to be more prevalent and severe in Sub-Saharan Africa than in

the developed world (CASCADE 2000; Hendrickse and Maxwell 1988). At the population level, HIV transmission is affected by major factors, including host-related factors, environment, and the HIV virus (Royce et al. 1997). Host-related biological factors can either increase or decrease the chance of transmission per coital act. The presence of genital ulcers, immune activation, local infection, immunosuppression, and inflammation have been shown to increase the chance of HIV acquisition (Royce et al. 1997).

Considering the prevalent HIV infection and AFB_1 exposure in sub-Saharan African populations, rural Uganda is a suitable region to study the interaction between these two significant health-risk agents. A few studies have examined the effect of AFB_1 exposure as a risk factor to increase the transmission of HIV; however, none of them have been longitudinal in design (Jolly et al. 2011; Obuseh et al. 2011). In this follow-up study we hypothesize that chronic AF exposure will increase the risk of pathogenic infections such as HIV as well as accelerate progression to AIDS. We tested this hypothesis using samples and information from the pre-existing Rakai Community Cohort (RCCS) via repeatedly measurement of serum AFB_1-lysine adduct levels of study participants to get a better representation of their AFB_1 exposure and HIV acquisition and progression outcomes.

Methods

Cohort design

The Rakai Community Cohort Study (RCCS), initiated in 1987 in the Rakai District of south-western Uganda, is a collaborative study conducted by the Ministry of Health through the Uganda Virus Research Institute (UVRI), Makerere University, and Johns Hopkins University. The current cohort, which consists of some of the adult residents in 50 villages, was established in 1994-1995 (Gray et al. 1998; Sewankambo et al. 1994; Wawer et al. 1991). All participants are followed annually in their homes, at which time they provide personal information (demographics, education, current and past sexual behaviours and sexually transmitted infection history) through surveys, and blood samples were collected for detection of human immunodeficiency virus (HIV), sexually transmitted diseases (STDs) and other infections. This is an open cohort of adults aged 15-49 years, which enrols new immigrants and age-eligible residents at each survey visit annually. The numbers of participants, who are under surveillance, are maintained between the ranges of 12,000 to 16,000 annually. The main objectives of RCCS is to study the dynamics of HIV infection, to identify major risk factors, and to measure impacts of HIV infection on mortality and fertility. HIV care has been provided to the participants enrolled in a community based HIV care program within RCCS since June 2004 through funding from the President's Emergency Plan for AIDS Relief (PEPFAR) (Nakigozi et al. 2011; PEPFAR 2008). The program offers health and nutritional education, counselling on living with HIV, prophylaxis of opportunistic infections, bed nets, and clean water vessels.

Specific study strategy

Two different study strategies in RCCS were taken to meet our aims. The first is a case-control study on 258 HIV cases and 151 controls among the participants of RCCS. The main goal of this analysis is to test if there is an association between HIV infection and AF exposure. To measure AFB1 exposure status, the serum samples were collected for three consecutive rounds; an interval was 12-15 months between rounds. Second study is a survival analysis that was conducted to test the effect of AF exposure on the progression of HIV to AIDS among the 258 HIV cases. The participants were followed from 1999 to 2012 (from study enrolment to the last CD4 visit). All subjects were free of hepatocellular carcinoma (HCC) at the beginning of the study. If a subject tested positive for HCC during follow-up, the subject was removed from further study. All participants included in the study were alive and free of ART until the last follow-up (April 2012).

Laboratory measurements

Seroconversion was defined as when a patient had HIV positive results from two ELISA assays (Vironostika HIV-1, Organon Teknika, Charlotte, North Carolina, and Cambridge Biotech, Worcester, Massachusetts) confirmed by western blot test (HIV-1 Western Blot, Bio-Merieux-Vitek, St. Louis, Missouri). CD4 cell counts were performed with FACSCount (BD Biosciences, San Jose, California).

Serum samples were coded separately and analyzed for AFB_1-Lysine adducts using a newly-developed HPLC-fluorescence method(Qian 2012). Briefly, thawed human serum samples were deactivated for viral/bacterial infection, and measured for albumin and total protein concentrations using procedures modified as previously described. A portion of each serum sample (150 μL) was digested by pronase (pronase:total protein,

1:4, w:w) at 37°C for 3 h to release AFB_1-Lys adduct. AFB_1-Lys adducts in digests were further extracted and purified by passing through Oasis MAX SPE cartridges (Waters Corporation, Milford, Massachusetts). The eluate was vacuum-dried with a CentriVap concentrator (Labconco, Kansas City, Missouri) and reconstituted for HPLC-fluorescence detection. The analysis of AFB_1-Lys adduct was conducted in a 1200 HPLC-fluorescence system (Agilent, Santa Clara, California). The mobile phases consisted of buffer A (20 mM $NH_4H_2PO_4$, pH 7.2) and buffer B (100% methanol). A Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 x 250 mm) equipped with a guard column was used. Column temperature was maintained at 25°C during analysis, and a volume of 100 μL was injected into the HPLC instrument. The flow rate was kept at 1 mL/min. A gradient was generated to separate the AFB_1-Lys adduct within 25 min of injection. The lysine adduct was detected by fluorescence at maximum excitation and emission wavelengths of 405 nm and 470 nm, respectively. Calibration curves of authentic standard were generated weekly, and the standard AFB_1-Lys was eluted at approximately 13.1 min. Quality assurance and quality control procedures were taken during analyses, which included simultaneous analysis of one authentic standard and a quality control sample daily. The method for producing the AFB-Lys standard and the procedure for maintaining quality control are described elsewhere in detail(Qian et al. 2010). Briefly, the serum from rats dosed with AFB_1 (Sigma, St Louis, MO) was purified using Oasis MAX SPE cartridges; The AFB_1-Lys adduct isolated from the serum was concentrated by the Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 x 250 mm). For quality control, the AFB_1-Lys standard prepared from the rat serum was processed and analyzed with the human serum samples simultaneously. The limit of detection was 0.4

pg/mg albumin. The technicians conducting AFB1-Lys analysis were blinded for participants' HIV status and other clinical/demographic factors.

To identify the specific cytokines involved in an inflammatory or immune response, we analyzed 29 cytokines and chemokines using the Human Cytokine/Chemokine Panel (EMD Millipore, Billerica, Massachusetts) from 15 HIV negative and 44 HIV-positive participants: VEGF, EGF, Eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, and TNF-β. Median fluorescence intensity, calculated from duplicates for each sample, was collected using Luminex MAGPIX. xPONENT (Luminex, Austin, Texas) was used to calculate sample cytokine concentrations, incorporating a weighted five-parameter logistic curve-fitting method. Lower values were covered by dilution.

Statistical analysis

We repeatedly measured participant's AFB_1-Lys adduct levels in serum for three consecutive rounds. Based on the half-life of albumin, the adduct levels reflect AF exposure in previous 2-3 months (Montesano et al. 1997). An individual's chronic AF exposure was represented by the geometric means of AFB_1-Lys adduct in three consecutive rounds. Due to the skewed distribution of AFB_1-Lys adduct level in the participants, the natural log-transformation was applied. For the analysis of AFB_1-Lys adduct, three consecutive serum samples were provided from HIV cases and controls. Among the 258 HIV positive participants, 241, 251, and 235 of serum samples were analyzed for detecting AFB_1-Lys adduct in each consecutive round. In the 151 HIV

negative participants, 104, 144, and 132 of serum samples were tested for AFB_1-Lys adduct consecutively. The missing analysis in each round was due to the lack of sample volume for quantification of AFB_1-Lys adduct. The missing was at random and not relevant to health. For each participant, the average of the log AFB_1-Lys was used in the analysis.

Baseline characteristics of HIV cases and controls were compared by χ^2 (categorical variables) and Wilcoxon rank-sum test (non-normally distributed continuous variables) such as age, gender, education, occupation, marital status, and baseline CD4 counts. To assess if the AFB_1-Lys adduct levels change over time, we adopted the GEE approach on the longitudinally measured log-transformed AFB_1-Lys adduct. The logistic regression model was used to assess the effect of AFB_1-Lys adduct level on the probability of HIV status, adjusting for the risk factors. The risk factors were determined by the backward selection which keeps the covariates with p-values less than 0.1 at each step.

Incubation time of AIDS was obtained by calculating the length of time between HIV infection and development of AIDS. The date of HIV infection was based on the enrollment date to HIV care program or the date of baseline HIV serum collection, whichever occurs first. For survival analysis, time to diagnosis of AIDS is the outcome. The patient is defined as having AIDS if his or her CD4 counts fall below 200. Participants were removed from the study due to outmigration, refusal to participate, absence, incapacitation, etc. In our study, we were able to obtain the CD4 counts from all HIV infected participants at initiation of the HIV care program (2003-2004); these values were used as the baseline CD4 counts.

To assess the effect AFB_1-Lys adduct level on the time to AIDS, , the Cox proportional hazard model (Cox PH model) was used adjusting for the risk factors which were determined by the backward selection which keeps the covariates with p-values less than 0.1 at each step. The proportional hazard assumption was tested by cumulative sums of martingale residuals (LIN et al. 1993) We compared the survival time of the subjects with geometric mean of AFB_1-Lys adduct level greater than or not exceeding the median using the logrank test.

All analyses were done using the SAS 9.3 software package (Cary, North Carolina) and graphs were generated through SigmaPlot 10.0 (San Jose, California). Two-sided *p*-values lower than 0.05 were considered statistically significant.

Ethics statement

Ethical approval for this study was granted by the Uganda Virus Research Institute Science Ethics Committee and the Uganda National Council of Science and Technology. The laboratory study protocol of analysis for AFB_1-Lys adduct in serum samples was approved by the Institutional Review Board for Human Subjects Protection at the University of Georgia.

Conflict of Interest

The authors declare no conflict of interest.

R**esults**

A subset of participants (408) enrolled in the Rakai Community Cohorts Study (RCCS) between 1999 and 2006 were selected for this study. Among these study participants, , 258 participants were diagnosed with HIV infection, and 150 participants who were negative for HIV infection by December 2009. There was no significant difference between HIV-infected cases and negative controls with respect to age (p= 0.191) and whether they resided in an urban or rural area (p=0.392) in RCCS participants (Table 4.1).

To correctly represent the participants' long-term aflatoxin exposure status, we repeatedly measured AFB_1-Lys adduct levels in serum samples collected from both infected cases and negative controls in three different rounds (years) (Figure 4.1). The level of AFB_1-Lys adduct was significantly different between HIV-infected cases and negative controls (p = 0.002) (Figure 4.2). The odds ratio of being HIV positive of 3.82 (2.62, 5.56) per an increment of log (AFB_1-Lys). The median AFB_1-Lys adduct level in HIV positive cases was 3.94 pg/mg albumin, whereas the median of the adduct levels in the negative controls was 1.61 pg/mg albumin.

Clinically diagnosed AIDS patients had significantly higher AFB_1-Lys adduct levels (median=4.88) than HIV-infected participants (median=2.64), p=0.002 (Figure 3.3). The relationship between AFB_1-Lys adduct levels and HIV progression stages was further tested in each round according to the classification on CD4 + T Cell counts (Figure 4): high progression = AIDS patient (CD4+ T Cell counts < 200), Medium progression = 200 $\leq$ CD4+ T Cell counts < 500), and low progression (CD4+ T Cell

counts $\geq$ 500). The AFB_1-Lys adduct level in the AIDS group was significantly different from other groups, and negatively correlated with CD4+T cell counts (Table 4.4).

We further divided 258 HIV-infected cases into low AF exposure group and high AF exposure group using the same rationale. Demographic and clinical characteristics of these 258 HIV-infectedcases were compared according to AF exposure status (Table 4.3). Except for the area of residency, they were similar in terms of gender, age, education, tribe, marital status, VLs, and CD4 counts at baseline. Therefore, the incubation time of AIDS development was modeled (Table 4.4). Multiple Cox PH models showed that the repeated high AF exposure was associated with the higher hazard of AIDS. The hazard of progression was significantly increased in the HIV-infected cases with low CD4+ T Cell counts (<500 cells/mm^3) at baseline. After stratification by area, the multivariate Cox PH model also presented the same finding, that the hazard to progress to AIDS was significantly higher in HIV-infected people with high AF exposure and low CD4 counts. A proportional hazard assumption in the Cox PH model was tested using the Schoenfeld residuals for covariates in the model, and all the variables satisfied the assumption.

A survival analysis model was applied to monitor HIV progression to AIDS among 258 HIV-infected cases who were classified as high AF and low AF exposure groups based on the median of log (AFB_1-Lys). The incubation time of AIDS development varied from 1.09 to 5.88 years and follow-up time varied from 0.47 to 8.97 years. The log rank test showed that the incubation time of AIDS development was significantly different between high and lowAF exposure groups (p=0.048) (Figure 4.4). After 8 years of monitoring, only 8% of HIV-infected cases progressed to AIDS (CD4+

T Cell counts $\leq$ 200) in the low AF exposure group, whereas 18% of HIV-infected cases progressed to AIDS in the high AF exposure group.

HIVviral setpoints were compared between low AF and high AF exposure groups. The means of log VLs in these two groups were 4.18 (3.88, 4.48) and 4.20 (3.90, 4.50), respectively. Multivariate and univariate linear regression showed that viral setpoints were not significantly affected by AF exposure after adjusting for CD4 count and VLs at baseline and age at sero-conversion of the study.

Serum cytokine/chemokine levels in accordance with AFB-Lys adduct levels were measured in the subset of RCCS participant s(Table 4 & Figure 5): 44 HIV positive and 15 HIV negative age-gender matched participants. In the serums of 44 HIV-infected cases, higher AFB1-Lys adduct levels were significantly or margin significantly associated with inflammatory markers such as MIP-1α (p=0.036), IL8 (p=0.059), and VEGF (p=0.009) and a marker of bacterial infection IP10 (p=0.064). However, no such associations were found among the 15 HIV-negative participants. Pathway enrichment analysis revealed that increased induction of the T cell activation marker, CD40L were marginally associated with high AF exposure (p=0.068) in HIV negative participants. Whereas CD4 T cell differentiation (p=0.040) and B cell activation (p=0.052) were significantly down-regulated in the HIV negative participants with high AF exposure. In the HIV-infected cases, highAF exposure marginally associated with induced cell fraction, a disruption and lysis of cells (p=0.078).

Discussion

In this study, we found that chronic AF exposure in southwestern Ugandan populations increased the odds of HIV infection, as well as shortened the incubation time of AIDS development within HIV-infected cases. establishment that environmental AF exposure may contribute to the acquisition of HIV and the progression of HIV to AIDS provides a possible answer to the question of why HIV cases in African are more prevalent and severe than would be expected in western countries, where exposure to AFs is relatively rare (Hendrickse and Maxwell 1988; van der Helm et al. 2014).

Studies in Ghana and Cameroon support our finding that AF exposure was significantly higher in HIV-positive participants than HIV-negative populations (Abia et al. 2013; Hendrickse and Maxwell 1988; Jolly et al. 2011; Obuseh et al. 2011; Williams et al. 2010), although there is an exception in a recent Ugandan study due to a small HIV sample size (n=10) (Asiki et al. 2014). Most of participants in our study were followed for about 10 years, therefore, it enables us to study the progression of disease in different AF exposure groups, and this study is the first study to test the possible effect of AF exposure on HIV progression.

The results of our study show that there was higher AFB_1-Lys adduct levels in HIV-infected cases as compared to the controls. One explanation for how AFB exposure might contribute to HIV pathogenesis and progression is through increased host susceptibility due to immune-suppression by continuous exposure to AFs. Immunosuppression in animals due to AFs has been seen in other studies (Ghosh et al. 1990; Giambrone et al. 1985; Liu et al. 2002; Meissonnier et al. 2008); it is further hypothesized that a susceptibility to infection is higher in AF-exposed animals and

humans (Hendrickse 1991; Pier and Heddleston 1970; Raisuddin et al. 1993; Turner et al. 2003; Umar et al. 2015). Another way to explain this result is that AFs damage the liver, which clears pathogens in circulating blood by innate immunity. Liver damage could reduce the ability of the liver to efficiently defend against the pathogens (Gao et al. 2008). However, liver disease is prevalent among the HIV-positive living in Uganda as well as other developed countries (Gandhi 2007; Morris et al. 2012; Ocama et al. 2008; Pol et al. 2004; Stabinski et al. 2011); HIV infection is associated with decreased levels of glutathione and it is possible that low levels of glutathione might result in slow elimination of the active AFB_1-epoxide as well as increase the amount of AF that binds to a single albumin molecule, though no research currently exists regarding this possibility. However, to account for this possible confounding due to liver damage, we excluded the participants with severe liver damage in the beginning of study and during the follow-up.

High AF exposure was associated with reduced incubation time of AIDS development among the HIV-infected cases, which is the interval between the HIV infection and the onset of AIDS. The depletion of CD4+ T-cells is a central process for pathogenesis and progression of HIV to AIDS, and the rate of CD4+ T-cells depletion is varies among different individuals. The difference is attributed to the presence of chemokine receptors, age at seroconversion, viral setpoints, immune activation as well as the subtype of HIV (CASCADE 2000; Hunt 2012; Kaleebu et al. 2002; Kiwanuka et al. 2008; Lavreys et al. 2006). A mechanism is proposed that AF exposure accelerates the progression of HIV by increasing viral setpoints, a balancing point between the virulence of virus and the strength of an individual's immune system (Jolly 2014; Quinn et al. 2000). However, the viral setpoints of 113 HIV positive participants were not

significantly different by AF exposure status in this study. This may be because VLs were not measured until the initiation of HIV care program; the participants of this study were enrolled typically 5 years before the initiation of the HIV care program. Another explanation is that chronic AF exposure accelerates the pathogenesis by constant immune activation (Hinton et al. 2003; Meissonnier et al. 2008; Qian et al. 2013; Whalen et al. 2000), supported by our pathway enrichment analysis; the activation of T cells helps HIV virus complete its viral replication in the viral reservoir of HIV—the resting T cells.

Thirdly, a reduction of cell membrane integrity may accelerate the HIV progression. From the pathway enrichment analysis, the increased cytokines/chemokines that induce cell-fractioning were found in the HIV-infected cases with higher AF exposure, which means membrane damage in cells and results in faster pathogenesis of HIV. Alternatively, the mutagenic activity AFs could increase the viral diversities within an individual highly exposed to AFs as has been shown with dioxin (Yao et al. 1995) and as shown in other studies where higher viral diversities led to faster progression in the HIV-infected cases (Shankarappa et al. 1999; Troyer et al. 2005).

Two animal studies connected the multifaceted effects of AFs on the immune system, which suppresses and activates (Hinton et al. 2003; Meissonnier et al. 2008). Intermittent feeding of AFB_1 to rats increased production of inflammatory cytokines (Il-1 and IL-6), but the inflammatory response was suppressed after 12 weeks of dosing by a decrease of T lymphocytes in the periarterioalar lymphocyte sheaths (Hinton et al. 2003). Another study using swine showed that the expression of inflammatory cytokines such as IL-6, IL-10, IFN-γ were increased in a dose-dependent manner by AF exposure (Meissonnier et al. 2008); however, antigen specific lymphocyte proliferation in swine

cells was diminished when the inflammatory cytokines were increased. These studies in animals show that chronic inflammation resulting from AF exposure can induce a suppression of T cell mediated immunity so that the host becomes more vulnerable to infection.

Other studies in sub-Saharan Africa revealed that AF exposure can affect both T-cell and B-cell mediated immunity (Jiang et al. 2005; Jiang et al. 2008; Turner et al. 2003). The enriched pathway in HIV negative participants with higher AF exposure supported these reported resultss: reduction of CD4 T cell differentiation and B cell activation. As a result, the immune system of a person chronically exposed to AFB_1 would be less diverse and susceptible to infections.

Dietary AF exposure varies across seasons, weather, socio-economic status, and age (Jolly et al. 2009; Wild et al. 2000). Therefore, the variation of an individual's AF exposure is significant. To avoid the influence of outliers in the calculation of AFB-Lys adduct average, a participant was classified by how frequently his or her AFB_1-Lys adduct level was higher than the median in each round. In order to compare the incubation time of AIDS development using the Kaplan-Meier estimator, it was necessary to divide the HIV participants into two groups—low AF exposure and high AF exposure. Sensitivity analysis was conducted to exclude the influence of inconsistent AF exposure; for instance, a participant who was classified as low AF group in one round and as high AF group in others was excluded. In sensitivity analysis, the survival curves were even more significantly different in terms of AF exposure status in the HIV-infected cases after accounting for inconsistent AF exposure.

Cox PH model was used to estimate hazard to AIDS development; multiple Cox PH model was used to adjust effect of confounding variables. HIV-positive participants with high AF exposure, low CD4+ T-cell counts, and high HIV-1 RNA copies had increased hazard for HIV progression to AIDS as compared to HIV-positive participants with low AF exposure, high CD4+ T-cell counts, and high HIV-1 RNA copies. The demographic factors such as gender, age, occupation, education, marital status, tribe, and residential area did not significantly affect the HIV progression. After stratification by area, which was differently distributed by the AFB_1 exposure status, AF exposure status more highly influenced on the incubation time of AIDS in the stratified Cox PH model compared to the model without stratification. Although studies in other developed countries showed that the age of seroconversion was significantly correlated to incubation time of AIDS (CASCADE 2000; Phillips et al. 1991), we could not find any effect by age group. This difference is possibly due to the small age gap (15-44 years) among the HIV-positive participants in Ugandan compared to those in developed countries.

In our study, we restricted our AIDS definition to CD4 lymphocyte counts of less than 200. In a resource-limited setting, AIDS diagnosis is typically defined by WHO clinical stages III and IV, along with CD4+ T cell counts lower than 200 (WHO 2007). However, the WHO clinical staging includes a fair number of HIV patients whose CD4 lymphocyte counts are greater than 200. Thus, the diagnosis based on CD4 lymphocyte counts alone possibly reduces human error and increases the specificity of diagnosis (Kagaayi et al. 2007; Teck et al. 2005).

Possible confounders of the results of this study include: 1) AF exposure was ot randomly assigned. The AF exposure associated to social status and area; therefore, the known confounders were controlled by logistic regression model as well as Cox PH model. 2) the length of incubation time of AIDS development in this study may be underestimated because 88% of participants already had HIV infection in the beginning of study. However, this will not affect the conclusion that AF exposure shortens the incubation time of AIDS development because the AF exposure status was similar in the participants regardless of seroconversion date.

This study is important because it shows the possible effect of an environmental toxicant on infectivity and pathogenesis of HIV/AIDS in a human population. Other immunosuppressive toxicants such as TCDD, PCB, and UV may have similar effects on the human immunity. However, the lack of reliable biomarkers for those toxicants prevents easy assessment of chronic exposure; as a result, it is difficult to find an association between an environmental exposure and human health. Also, this study has greater power compared to previous cross-sectional studies [15,16, 46] and provides a plausible explanation for the possible interaction between HIV and AF exposure (Jolly et al. 2006; Jolly et al. 2011; Obuseh et al. 2011). Considering the uncertainty inherent in human studies, longitudinal assessment using reliable biomarkers is critical for revealing possible interactions between infectious agents and environmental toxicants.

This study suggests that chronic AF exposure increased the odds of HIV infection as well as HIV/AIDS pathogenesis in a rural Ugandan population. Reduction of AF exposure in Uganda where HIV-1 subtype A and D are dominant and the AF exposure is continuous, could benefit HIV/AIDS patients.

Table 4.1. Descriptive statistics of HIV cases and controls

	HIV + **N (%)**	**HIV -** **N (%)**	**p value**
Number of participants	258	151	
Gender			
Male	54 (47.0)	61 (53.0)	<0.0001
Female	204 (69.4)	90 (30.6)	
Education			
None	17 (65.4)	9 (34.6)	
Primary	186 (66.9)	92 (33.1)	0.031
Secondary or above	55 (52.4)	50 (47.6)	
Occupation			
Agriculture	159 (62.6)	95 (37.4)	0.796
Others	99 (63.9)	56 (36.1)	
Marital Status			
Never Married	39 (48.2)	42 (51.8)	
Currently Married	157 (61.1)	100 (38.9)	<0.0001
Divorced/Separated/Widowed	62 (87.3)	9 (12.7)	
Tribe			
Muganda	201 (63.8)	114 (36.2)	0.576
Others	57 (60.6)	37 (39.4)	
Residential Area			
Rural	208 (62.5)	125 (37.5)	0.588
Urban	50 (65.8)	26 (34.2)	

Table 4.2. Factors associated with HIV prevalence in Rakai community cohort

Variables	N	OR (95% CI)
ln(AFB_1-lysine)[a]	408	3.82 (2.62, 5.56)
age[b]	408	1.06 (1.03, 1.10)
Sex		
Male	115	1
Female	294	3.03 (1.72, 5.36)
Occupation		
Agriculture	254	1
Others	155	1.64 (0.95, 2.84)
Marital status		
Never Married	81	1
Currently married	257	1.31 (0.67, 2.56)
Divorced/Separated	71	9.63 (3.02, 30.74)

Odds ratio for HIV acquisition was obtained through multiple logistic regression after adjusting for other risk factors.

[a.] RCCS participants tested the level of AFB_1-lys adduct three times; a natural log transformation of the geometric mean of AFB_1-lys adduct in each participant was used for estimating odds ratio of HIV acquisition.

[b.] Age at the first serum samples were collected for the assessment of aflatoxin exposure was compared to obtain odds ratio of HIV acquisition.

Table 4.3. Baseline characteristics of participants in the HIV progression study with their AFs exposure status

ln (AFB_1-Lys Adduct(pg/mg alb))	*Low (< 0.994)	*High (≥ 0.994)	p value
Total number of participants	129	129	
ln (CD4 cell counts (mean ± SD cells/µL))	6.39 ± 0.40	6.39 ± 0.43	0.889
Gender			
Male	29 (53.7)	25 (46.3)	0.54
Female	100 (49.0)	104 (51.0)	
Age at seroconversion	32.5 ± 6.5	31.8 ± 6.8	0.423
Education			
Never	11 (64.7)	6 (35.3)	
Primary	87 (46.8)	99 (53.2)	0.209
Secondary or above	31 (56.4)	24 (43.6)	
Marital Status			
Never Married	13 (33.3)	26 (66.7)	
Currently Married	82 (52.2)	75 (47.8)	0.073
Divorced/Seperated/Widowed	34 (54.8)	28 (45.2)	
Tribe			
Muganda	101 (50.2)	100 (49.8)	0.881
Others	28 (49.1)	29 (50.9)	
Residential Area			
Rural	116 (55.8)	92 (44.2)	0.0002
Urban	13 (26.0)	37 (74.0)	

*The 258 HIV positive participants were divided into two groups with respect to the geometric mean of AFB_1-lys addcut in serums to test the effect of AFB_1 exposure on HIV pathogenesis. The natural log of the median of the geometric means of AFB_1-lys adduct in 258 HIV positive participants is 0.994.

Table 4.4. Multiple Cox hazards regression models with aflatoxin exposue for HIV progression to AIDS

	Time to AIDS	
	Cox PH model Total (n=258)	
	Hazard ratio (95% C.I.)	P-value
ln (AFB_1-Lys)[a]	3.11 (1.76, 5.52)	0.0001
ln (CD4 cell counts (cells/µL))	0.02 (0.01, 0.07)	<0.0001
Occupation		
Agriculture	1.00	0.0013
Others	4.68 (1.82, 12.04)	
Marital status		
Never married	1.00	
Currently married	4.84 (1.06, 11.18)	0.0624
Divorced/Widowed/Separated	1.56 (0.25, 9.66)	

The natural logs of the geometric means of AFB_1-Lys and baseline CD4+ T cell counts, occupation, and marital status were significant factors associated with the progression to AID after adjusting for other factors (age at seroconversion, sex, residential area, education, and tribe).

Table 4.5. Enriched biological processes and pathways associated with aflatoxin B_1 exposure

Enriched processes/pathways	Changed	Cytokines/Chemokines/Growth factors in this pathwaay	P-value
HIV$^-$ negative			
Up-regulated			
immunosurveillance(CD40L)		IL12p70, MIP-1α, IL-1α, IL10, IL8	**0.0676**
Down-regulated			
CD4 T Cell Differentiation		IFNγ, IL13, IL4	**0.0395**
Positive regulation of B cell activation		IFNγ, IL13, IL4	**0.0517**
Dendritic cells in regulating Th1 and Th2 development		IFNγ, IL13, IL4	**0.0798**
Th1 and Th2 cells		IFNγ, IL13, IL4	**0.083**
HIV positive			
Up-regulated			
Cell fraction		IL13, IL5, MIP-1α, EGF, IL15	**0.0775**

Pathway enrichment analysis reveals specific immune and inflammatory response pathways with respects to AFB_1 exposure in both HIV negative and HIV positive participants.

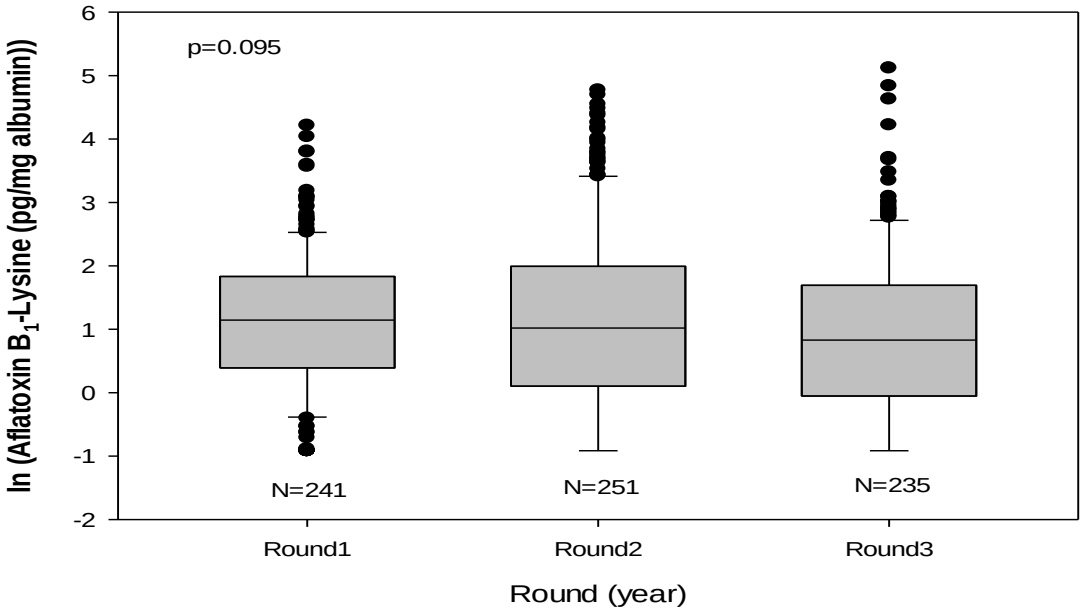
[a.] The mean fold changes of the detected markers were calculated between the low aflatoxin exposure and the high aflatoxin exposure groups. Cytokines/chemokines showing 1.5-fold changes were used for pathway enrichment analysis.

[b.] Fifteen samples of 150 HIV negative participants in the last round were randomly selected to test their cytokine/chemokine levels; the 15 serums were classified based on AFB_1-Lys adduct levels: 8 low AFB_1 exposed participants (lower than median levels of AFB_1-Lys adduct) and 7 high AFB_1 exposed participants (higher than the median of AFB_1-Lys adduct groups). In high AFB_1 exposed participants, the serum levels of

eotaxin, IL10, IL12p70, IL1α, IL8, IP10, and MIP1α were above 1.5 fold increased, and the levels of IFNγ, IL13, IL1α, and IL4 were decreased among the 29 Human Cytokine/Chemokine Panel.

[c.] The serums of 44 age/gender-matched participants out of 259 HIV-positive participants in the last round were randomly selected to measure the cytokine/chemokine concentration in their plasma. The 44 HIV positive participants were divided into two groups based AFB_1-Lys levels: 22 low AFB_1 exposed group (lower than the median of AFB1-lys among the 44 HIV positive participants) and 22 high AFB_1 exposed group (higher than the median of AFB1-lys among the 44 HIV positive participants). EGF, GM-CSF, INFα2, IL13, IL15, IL1rα, IL1α, IL2, IL5, IL8, MIP1α, TNFβ, and VEGF were increased and IL12p40 was decreased in 22 high AFB_1 exposure group compared to the 22 low AFB_1 exposure group. [d.] Enriched pathways and their corresponding cytokine/chemokine/growth factors were listed above. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was carried out using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resources tool version 6.7.

a. HIV positive participants



b. HIV negative participants

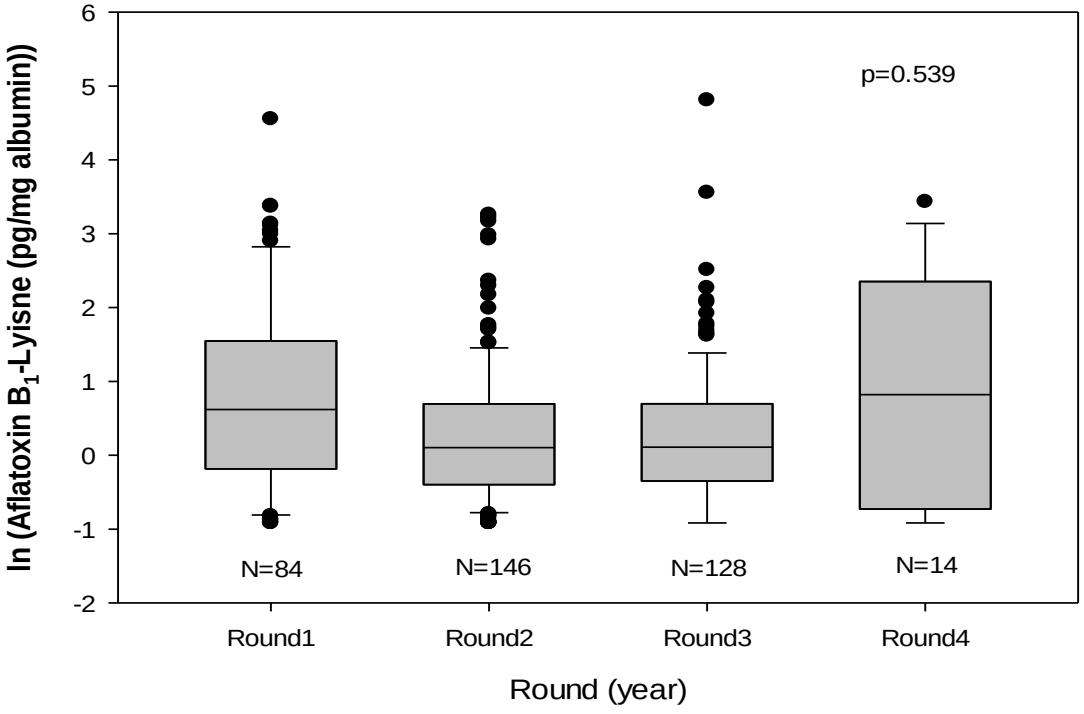


Figure 4.1. Temporal variation of AFB1-Lys level in Rakai community cohort participants

Box plots showing the median (solid line within bars), 25^{th} and 75^{th} percentile (upper and lower limits of the box), 10^{th} and 90^{th} percentile (error bars) and outliers (solid circles). Inset is the p-value for generalized estimating equations with respect to three repeated measurements of AFB_1-Lys adduct within each individual across rounds.

Missing measurements were occurred due to lack of serum samples. Therefore, the missing was completely at random and not relevant to disease status.

The box plot for HIV negative participants was adapted from a published article (Kang et al. 2015).

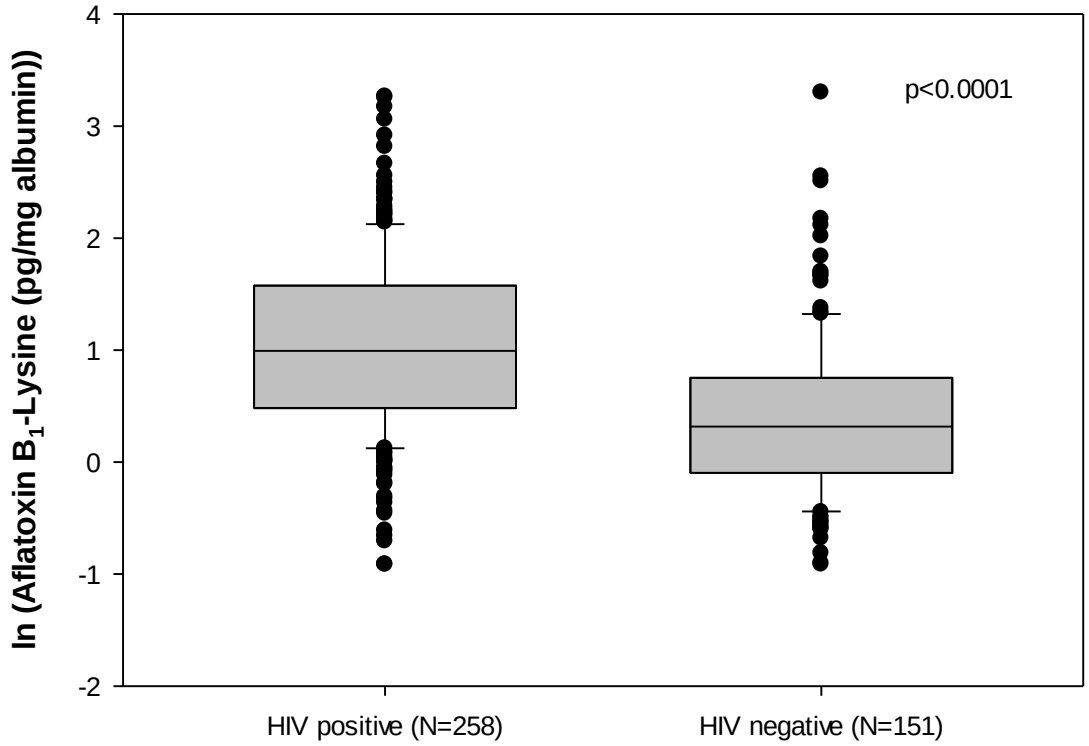


Figure 4.2 Geometric means of AFB_1-Lys adducts in HIV-infected cases and healthy control.

Each participants provided three serum samples for the analysis of AFB_1-Lys adduct. The natural log-transformed geometric means of AFB_1-Lys adduct were compared.

Box plots showing the median (solid line within bars), 25^{th} and 75^{th} percentile (upper and lower limits of the box), 10^{th} and 90^{th} percentile (error bars) and outliers (solid circles). Inset is the p-value for the Wilcoxon rank sum test of HIV-infected cases and controls.

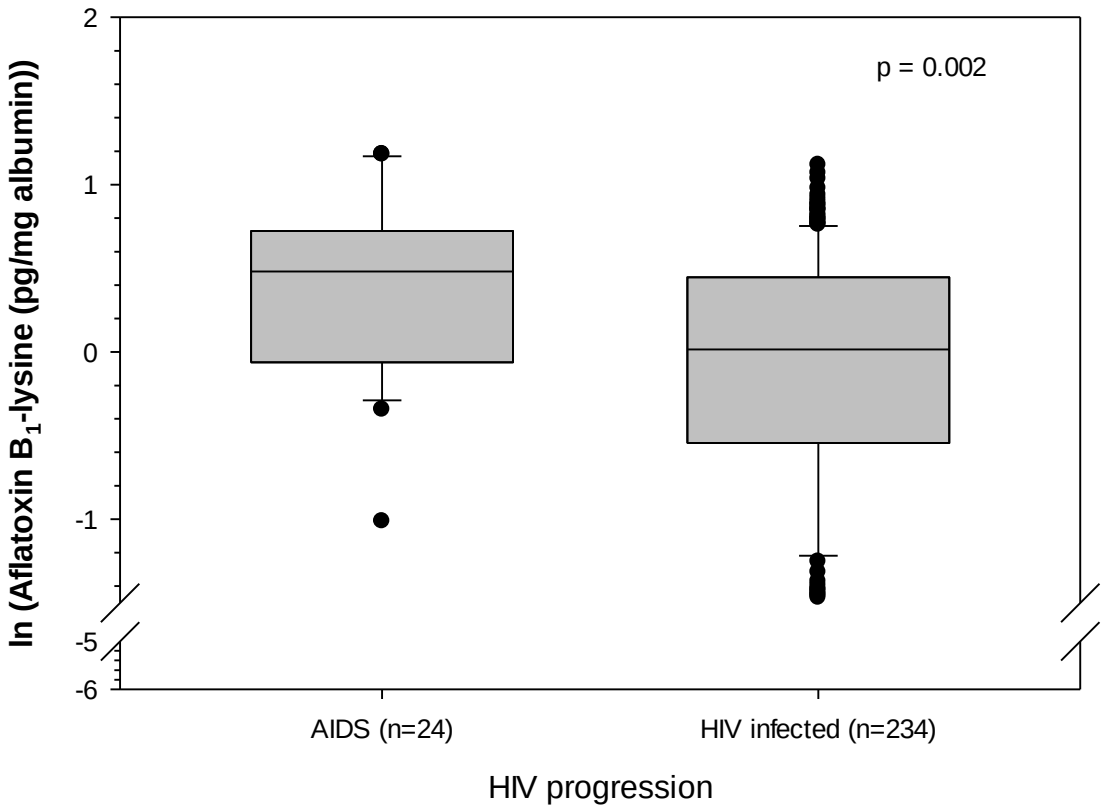


Figure 4.3. Log AFB_1-Lys adducts in AIDS patients and HIVinfected cases.

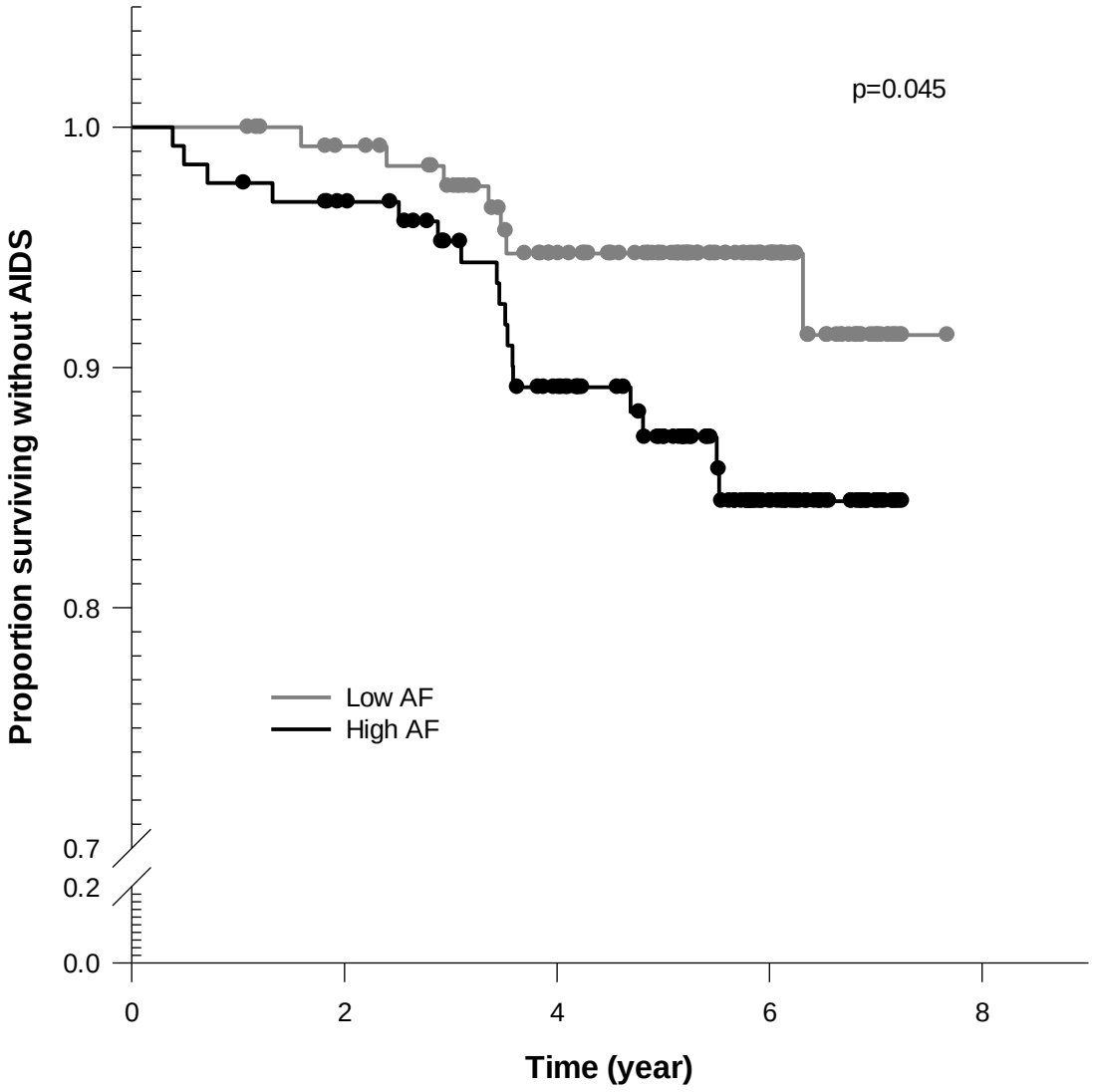


Figure 4.4. . Kaplan-Meier survival analysis for AF exposure in HIV to AIDS progression

The Kaplan–Meier curves for progression-free survival, defined as the time from HIV seroconversion to the date of the first AIDS diagnosis (CD4<200), were drawn in both of the low AF and the high AF groups. The dots on curves represent the censored participants due to the end of study.

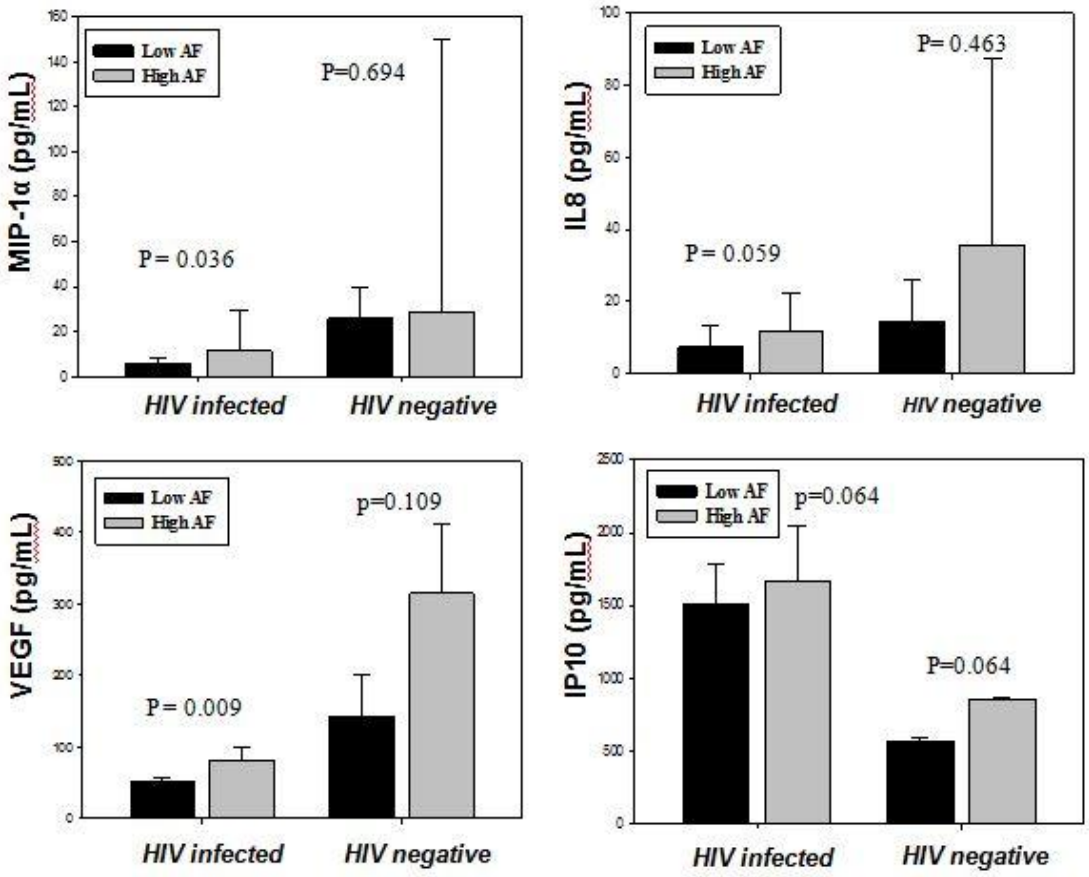


Figure 4.5. Effects of AFB_1 exposure on the serum cytokine/chemokine levels

Reference

Abia WA, Warth B, Sulyok M, Krska R, Tchana A, Njobeh PB, et al. 2013. Bio-monitoring of mycotoxin exposure in cameroon using a urinary multi-biomarker approach. Food Chem Toxicol 62:927-934.

Asiki G, Seeley J, Srey C, Baisley K, Lightfoot T, Archileo K, et al. 2014. A pilot study to evaluate aflatoxin exposure in a rural ugandan population. Trop Med Int Health 19:592-599.

CASCADE. 2000. Time from hiv-1 seroconversion to aids and death before widespread use of highly-active antiretroviral therapy: A collaborative re-analysis. The Lancet 355:1131-1137.

Dvorackova I. 1976. Aflatoxin inhalation and alveolar cell carcinoma. Br Med J 1:691.

Fahey JL, Taylor JM, Manna B, Nishanian P, Aziz N, Giorgi JV, et al. 1998. Prognostic significance of plasma markers of immune activation, hiv viral load and cd4 t-cell measurements. Aids 12:1581-1590.

Gandhi RT. 2007. Cirrhosis is associated with low cd4+ t cell counts: Implications for hiv-infected patients with liver disease. Clin Infect Dis 44:438-440.

Gao B, Jeong WI, Tian Z. 2008. Liver: An organ with predominant innate immunity. Hepatology 47:729-736.

Ghosh RC, Chauhan HV, Roy S. 1990. Immunosuppression in broilers under experimental aflatoxicosis. Br Vet J 146:457-462.

Giambrone JJ, Diener UL, Davis ND, Panangala VS, Hoerr FJ. 1985. Effect of purified aflatoxin on turkeys. Poultry science 64:859-865.

Gray RH, Wawer MJ, Serwadda D, Sewankambo N, Li CJ, Wabwire-Mangen F, et al. 1998. Population-based study of fertility in women with hiv-1 infection in uganda. Lancet 351:98-103.

Hendrickse RG, Maxwell SM. 1988. Heroin addicts, aids, and aflatoxins. British Medical Journal (Clinical research ed) 296:1257-1257.

Hendrickse RG. 1991. Clinical implications of food contaminated by aflatoxins. Ann Acad Med Singapore 20:84-90.

Hinton DM, Myers MJ, Raybourne RA, Francke-Carroll S, Sotomayor RE, Shaddock J, et al. 2003. Immunotoxicity of aflatoxin b1 in rats: Effects on lymphocytes and the inflammatory response in a chronic intermittent dosing study. Toxicol Sci 73:362-377.

HIV/AIDS JUNPo. 2012. Global report: Unaids report on the global aids epidemic: 2012:UNAIDS.

Hunt PW. 2012. Hiv and inflammation: Mechanisms and consequences. Curr HIV/AIDS Rep 9:139-147.

IARC. 2002. Some traditional herbal medicines, some mycotoxins, naphthalene, and styrene. In: IARC Monograph on the Carcinogenic Risks to Humans, Vol. 82, 171-300.

Jiang Y, Jolly PE, Ellis WO, Wang JS, Phillips TD, Williams JH. 2005. Aflatoxin b1 albumin adduct levels and cellular immune status in ghanaians. International immunology 17:807-814.

Jiang Y, Jolly PE, Preko P, Wang JS, Ellis WO, Phillips TD, et al. 2008. Aflatoxin-related immune dysfunction in health and in human immunodeficiency virus disease. Clinical & developmental immunology 2008:790309.

Jolly P, Jiang Y, Ellis W, Awuah R, Nnedu O, Phillips T, et al. 2006. Determinants of aflatoxin levels in ghanaians: Sociodemographic factors, knowledge of aflatoxin and food handling and consumption practices. Int J Hyg Environ Health 209:345-358.

Jolly P, Jiang Y, Preko P, Wang JS, Ellis W, Phillips T, et al. 2009. Socio-demographic, health and clinical predictors of high aflatoxin levels in hiv positive ghanaians. Epidemiology 20:S239-S239.

Jolly PE, Shuaib FM, Jiang Y, Preko P, Baidoo J, Stiles JK, et al. 2011. Association of high viral load and abnormal liver function with high aflatoxin b1-albumin adduct levels in hiv-positive ghanaians: Preliminary observations. Food additives & contaminants Part A, Chemistry, analysis, control, exposure & risk assessment 28:1224-1234.

Jolly PE. 2014. Aflatoxin: Does it contribute to an increase in hiv viral load? Future Microbiology 9:121-124.

Kagaayi J, Makumbi F, Nakigozi G, Wawer MJ, Gray RH, Serwadda D, et al. 2007. Who hiv clinical staging or cd4 cell counts for antiretroviral therapy eligibility assessment? An evaluation in rural rakai district, uganda. Aids 21:1208-1210.

Kaleebu P, French N, Mahe C, Yirrell D, Watera C, Lyagoba F, et al. 2002. Effect of human immunodeficiency virus (hiv) type 1 envelope subtypes a and d on disease

progression in a large cohort of hiv-1-positive persons in uganda. Journal of Infectious Diseases 185:1244 - 1250.

Kang MS, Nkurunziza P, Muwanika R, Qian G, Tang L, Song X, et al. 2015. Longitudinal evaluation of aflatoxin exposure in two cohorts in southwestern uganda. Food additives & contaminants Part A, Chemistry, analysis, control, exposure & risk assessment 32:1322-1330.

Kiwanuka N, Laeyendecker O, Robb M, Kigozi G, Arroyo M, McCutchan F, et al. 2008. Effect of human immunodeficiency virus type 1 (hiv-1) subtype on disease progression in persons from rakai, uganda, with incident hiv-1 infection. Journal of Infectious Diseases 197:707-713.

Lavreys L, Baeten JM, Chohan V, McClelland RS, Hassan WM, Richardson BA, et al. 2006. Higher set point plasma viral load and more-severe acute hiv type 1 (hiv-1) illness predict mortality among high-risk hiv-1–infected african women. Clinical Infectious Diseases 42:1333-1339.

LIN DY, WEI LJ, YING Z. 1993. Checking the cox model with cumulative sums of martingale-based residuals. Biometrika 80:557-572.

Liu BH, Yu FY, Chan MH, Yang YL. 2002. The effects of mycotoxins, fumonisin b1 and aflatoxin b1, on primary swine alveolar macrophages. Toxicology and applied pharmacology 180:197-204.

Meissonnier GM, Pinton P, Laffitte J, Cossalter AM, Gong YY, Wild CP, et al. 2008. Immunotoxicity of aflatoxin b1: Impairment of the cell-mediated response to vaccine antigen and modulation of cytokine expression. Toxicology and applied pharmacology 231:142-149.

Montesano R, Hainaut P, Wild CP. 1997. Hepatocellular carcinoma: From gene to public health. Journal of the National Cancer Institute 89:1844-1851.

Morris D, Guerra C, Donohue C, Oh H, Khurasany M, Venketaraman V. 2012. Unveiling the mechanisms for decreased glutathione in individuals with hiv infection. Clinical and Developmental Immunology 2012:10.

Nakigozi G, Makumbi F, Reynolds S, Galiwango R, Kagaayi J, Nalugoda F, et al. 2011. Non-enrollment for free community hiv care: Findings from a population-based study in rakai, uganda. Aids Care 23:764-770.

Obuseh FA, Jolly PE, Kulczycki A, Ehiri J, Waterbor J, Desmond RA, et al. 2011. Aflatoxin levels, plasma vitamins a and e concentrations, and their association with hiv and hepatitis b virus infections in ghanaians: A cross-sectional study. J Int Aids Soc 14.

Ocama P, Katwere M, Piloya T, Feld J, Opio KC, Kambugu A, et al. 2008. The spectrum of liver diseases in hiv infected individuals at an hiv treatment clinic in kampala, uganda. African Health Sciences 8:8-12.

PEPFAR. 2008. Fy 2007 pepfar country operational plans. Available: http://www.pepfar.gov/countries/cop/2007/index.htm.

Phillips AN, Lee CA, Elford J, Webster A, Janossy G, Timms A, et al. 1991. More rapid progression to aids in older hiv-infected people: The role of cd4+ t-cell counts. JAIDS Journal of Acquired Immune Deficiency Syndromes 4:970-975.

Pier AC, Heddleston KL. 1970. The effect of aflatoxin on immunity in turkeys. I. Impairment of actively acquired resistance to bacterial challenge. Avian Diseases 14:797-809.

Pol S, Lebra P, Vallet-Pichard A. 2004. Hiv infection and hepatic enzyme abnormalities: Intricacies of the pathogenic mechanisms. Clinical Infectious Diseases 38:S65-S72.

Qian G, Tang L, Liu W, Wang J-S. 2010. Development of a non-antibody method for rapid detection of serum aflatoxin b1-lysine adduct. In: Toxicologist, Vol. 114, 1163.

Qian G. 2012. Integrative toxicological evaluation of aflatoxin b_1 exposure in fischer 344 rats and biomarker based exposure assessment of aflatoxin b_1 in human populations. [electronic resource]:2012. https://getd.libs.uga.edu/pdfs/qian_guoqing_201212_phd.pdf

Qian G, Tang L, Guo X, Wang F, Massey ME, Su J, et al. 2013. Aflatoxin b modulates the expression of phenotypic markers and cytokines by splenic lymphocytes of male f344 rats. Journal of applied toxicology : JAT.

Qian G, Tang L, Guo X, Wang F, Massey ME, Su J, et al. 2014. Aflatoxin b-1 modulates the expression of phenotypic markers and cytokines by splenic lymphocytes of male f344 rats. Journal of Applied Toxicology 34:241-249.

Quinn TC, Wawer MJ, Sewankambo N, Serwadda D, Li C, Wabwire-Mangen F, et al. 2000. Viral load and heterosexual transmission of human immunodeficiency virus type 1. New England Journal of Medicine 342:921-929.

Raisuddin S, Singh K, Zaidi S, Paul B, Ray P. 1993. Immunosuppressive effects of aflatoxin in growing rats. Mycopathologia 124:189-194.

Royce RA, Seña A, Cates W, Cohen MS. 1997. Sexual transmission of hiv. New England Journal of Medicine 336:1072-1078.

Sewankambo NK, Wawer MJ, Gray RH, Serwadda D, Li C, Stallings RY, et al. 1994. Demographic impact of hiv infection in rural rakai district, uganda: Results of a population-based cohort study. Aids 8:1707-1713.

Shankarappa R, Margolick JB, Gange SJ, Rodrigo AG, Upchurch D, Farzadegan H, et al. 1999. Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. Journal of Virology 73:10489-10502.

Stabinski L, Reynolds SJ, Ocama P, Laeyendecker O, Ndyanabo A, Kiggundu V, et al. 2011. High prevalence of liver fibrosis associated with hiv infection: A study in rural rakai, uganda. Antiviral therapy 16:405-411.

Teck R, Ascurra O, Gomani R, Manzi M, Pasulani O, Kusamale J, et al. 2005. Who clinical staging of hiv infection and disease, tuberculosis and eligibility for antiretroviral treatment: Relationship to cd4 lymphocyte counts. Int J Tuberc Lung D 9:258-262.

Troyer RM, Collins KR, Abraha A, Fraundorf E, Moore DM, Krizan RW, et al. 2005. Changes in human immunodeficiency virus type 1 fitness and genetic diversity during disease progression. Journal of Virology 79:9006-9018.

Turner PC, Moore SE, Hall AJ, Prentice AM, Wild CP. 2003. Modification of immune function through exposure to dietary aflatoxin in gambian children. Environmental Health Perspectives 111:217-220.

Umar S, Younus M, Rehman MU, Aslam A, Shah MA, Munir MT, et al. 2015. Role of aflatoxin toxicity on transmissibility and pathogenicity of h9n2 avian influenza virus in turkeys. Avian Pathol:1-22.

van der Helm JJ, Geskus R, Lodi S, Meyer L, Schuitemaker H, Gunsenheimer-Bartmeyer B, et al. 2014. Characterisation of long-term non-progression of hiv-1 infection after seroconversion: A cohort study. The Lancet HIV 1:e41-e48.

Wawer MJ, Serwadda D, Musgrave SD, Kondelule JK, Musagara M, Sewankambo NK. 1991. Dynamics of spread of hiv-i infection in a rural district of uganda. Brit Med J 303:1303-1306.

Whalen CC, Nsubuga P, Okwera A, Johnson JL, Hom DL, Michael NL, et al. 2000. Impact of pulmonary tuberculosis on survival of hiv-infected adults: A prospective epidemiologic study in uganda. AIDS (London, England) 14:1219-1228.

WHO. 2007. Who case definitions of hiv for surveillance and revised clinical staging and immunological classification of hiv-related disease in adults and children.

Wild CP, Yin F, Turner PC, Chemin I, Chapot B, Mendy M, et al. 2000. Environmental and genetic determinants of aflatoxin-albumin adducts in the gambia. Int J Cancer 86:1-7.

Williams JH, Grubb JA, Davis JW, Wang J-S, Jolly PE, Ankrah N-A, et al. 2010. Hiv and hepatocellular and esophageal carcinomas related to consumption of mycotoxin-prone foods in sub-saharan africa. The American Journal of Clinical Nutrition 92:154-160.

Yao Y, Hoffer A, Chang C-y, Puga A. 1995. Dioxin activates hiv-1 gene expression by an oxidative stress pathway requiring a functional cytochrome p450 cyp1a1 enzyme. Environmental Health Perspectives 103:366-371.

CHAPTER 5

ASSOCIATION OF SOCIO-ECONOMIC FACTORS, ILLICIT DRUG AND ALCOHOL USE, AND ELEVATED ASPARTATE OR ALANINE TRANSAMINASE LEVELS WITH AFLATOXIN EXPOSURE IN UGANDA

Introduction

Aflatoxin B1, classified as a group 1 carcinogen, is a cause of hepatocelluar (liver) cancer in humans, and is produced by the fungi *Aspergillus flavus and A. parasiticus* and a few other species of fungi (International Agency for Research on Cancer. 2002). Uganda is a the Sub-Saharan African country that is located in the tropical temperature zone. The hot and humid environment of Uganda is conducive to aflatoxin production. . Studies indicate that about 60% of mortality in Sub-Saharan Africa will be caused by non-communicable diseases by 2030(Mathers and Loncar 2006). The incidence of primary liver cancer among Ugandan women has increased from 2.94 per 100,000 person-year in 1960-1980 to 5.33 per 100,000 person-year in 1991-2005 (Ocama et al. 2009). Assessment of aflatoxin exposure and factors related to the exposure in Uganda is important because it will improve our understanding of the potential of aflatoxin intake to contribute to increased incidence of non-communicable liver disease in Uganda.

Socioeconomic and behavioral factors have been shown to be associated with aflatoxin exposure in other studies conducted in Sub-Saharan countries (Egal et al. 2005; Jolly et al. 2006; Matumba et al. 2011; Shuaib et al. 2012). In these studies, the use of alcohol and

illicit drugs was assumed to be associated with exposure to aflatoxins because those substances were produced from fungal infected plants and grains (Efuntoye 1999; Hendrickse et al. 1989; Nikander et al. 1991). In our study, we have compared the level of AFB_1-Lys in serum with respect to alcohol and illicit drug use among high-risk women living in slum areas of Kampala, Uganda. Urban slum areas have risen throughout Kampala as a result of a massive influx of people into Uganda's capital city (Anesi 2012; UN-HABITAT 2006).

To examine if there is an association among socio-economic and behavioral factors and aflatoxin exposure, the concentration of aflatoxin B1-lysine adducts in the serums were compared between groups. Also, the burden of aflatoxin was estimated in a cohort of rural Ugandans with elevated levels of serum aspartate or alanine transaminase, indicators of possible abnormal liver function.

Materials and Methods

Study design and participants

We conducted a retrospective assessment of aflatoxin exposure with respect socio-economic and behavioral risk factors, and their relationship to indicators of liver function in each of the following prospective cohorts: the General Population Cohort (GPC) and Good Health for Women Project (GHWP) cohort. The primary goals of these cohorts are described in detail elsewhere (Asiki et al. 2013; Vandepitte et al. 2011).

The GPC cohort study was established by the British Medical Research Council/Uganda Virus Research Institute (MRC/UVRI) and was conducted in 15 rural villages (expanded to 25 villages in 2000) in a sub-county in the Masaka district of Uganda. Since 2000, the total rural cohort population of approximately 20,000 people have been followed. Participants of all age are enrolled. Because this is an open cohort study, in-migration and out-migration are allowed. A medical sero-survey is conducted for participants aged 13 and above, including collection of blood specimens for HIV testing and a brief behavioral questionnaire. Every year since its inception, the survey has collected annual household census information about the resident population, including age, sex, education, and relationship to household head. A total of 23 sampling rounds have been completed as of Dec 2014. At round 22 (2010-2011), the questionnaire and sero-survey were modified to obtain information about factors related to non-communicable diseases, including lifestyle (diet, tobacco and alcohol consumption), the presence of hepatitis B and C virus, liver function tests, and genetic factors (Asiki et al. 2014).

The GHWP study was established at Makingdye and Rubaga, two districts of southern Kampala, Uganda in 2008. British MRC established the cohort study to better understand the dynamics of HIV/STI (Sexually Transmitted Disease) epidemiology and to prepare HIV prevention trials among women involved in high-risk sexual behaviour in Uganda. Women were eligible for the cohort study if they were 18 years or over. After enrollment, participants responded to the questionnaire with help from representatives from a local NGO. The questionnaire covered socio-economic status, sexual risk behaviour, alcohol, and drug use. Blood and vaginal swab were tested for HIV/STI.

For this study, GPC participants who had at least one serum sample with elevated levels (level greater than the upper limits of normal) of serum aspartate or alanine transaminase were selected (n=132) in order to observe the burden of aflatoxin exposure in Ugandans with indicators of possible liver abnormalities. Also, samples from 300 HIV positive participants in GHWP were obtained to test the association of socio-economic status and alcohol and drug use with aflatoxin exposure. Socio-economic status of participants in GHWP was calculated as a continuous variable based on possession of house, primary source of water, type of bed, and number of people per sleeping room. The data on demographics, socio-economic status, and abnormal liver function results were last updated in February 2014.

Laboratory measurements

Serum samples were coded separately and analyzed for AFB_1-Lys adducts using a HPLC-fluorescence method (Qian 2012). Briefly, human serum samples were deactivated for viral/bacterial contamination and albumin and total protein concentrations were

determined using procedures modified as previously described. A portion of each serum sample (150 μL) was digested by pronase (pronase:total protein, 1:4, w:w) at 37°C for 3 h to release the AFB_1-Lys adduct. AFB_1-Lys adducts in digests were further extracted and purified by passing them through Oasis MAX SPE cartridges (Waters Corporation, Milford, Massachusetts). The eluate was vacuum-dried with a CentriVap concentrator (Labconco, Kansas City, Missouri) and reconstituted for HPLC-fluorescence detection. The analysis of the AFB_1-Lys adduct was conducted in a 1200 HPLC-fluorescence system (Agilent, Santa Clara, California). The mobile phases consisted of buffer A (20 mM $NH_4H_2PO_4$, pH 7.2) and buffer B (100% methanol). A Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 x 250 mm) equipped with a guard column was used. Column temperature was maintained at 25°C during analysis, and a volume of 100 μL was injected into the HPLC instrument. The flow rate was kept at 1 mL/min. A gradient was generated to separate the AFB_1-Lys adduct within 25 min of injection. The lysine adduct was detected by fluorescence at maximum excitation and emission wavelengths of 405 nm and 470 nm, respectively. Calibration curves of authentic AFB_1-Lys standard were generated weekly, and the standard AFB_1-Lys was eluted at approximately 13.1 min. Quality assurance and quality control procedures were performed during analyses, which included simultaneous analysis of one authentic standard and a quality control sample daily. The method for producing the AFB_1-Lys standard (Sabbioni et al., 1987) and the procedure for maintaining quality control are described elsewhere (Qian et al. 2010; Qian 2012). Briefly, serum collected from rats dosed with AFB1 (Sigma, St Louis, MO) was purified using Oasis MAX SPE cartridges; the AFB_1-Lys adduct in the serum was concentrated using the Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 x 250 mm). For

quality control, the AFB_1-Lys standard was processed and analyzed with other samples simultaneously. The limit of detection was 0.4 pg/mg albumin.

The abnormal liver function test was defined as aspartate transaminases (AST) or alanine transaminases (ALT) concentrations that were more than the upper limit of normal (ULN) for at least one measurement. The local reference interval (range) for normal was 17-55 IU/l for AST and 10-56 IU/l for ALT. Other liver enzymes were not assayed.

Statistical analysis

Descriptive statistics were gathered for both GPC and GHWP participants whose liver test was greater than the upper limits of normal as defined above. We performed χ^2 (categorical variables), Student's *t*-test (normally distributed continuous variables), and Wilcoxon rank-sum test (non-normally distributed continuous variables) for the following descriptive variables: Age, gender, education, occupation, marital status, alcohol and drug use. Data distribution (normality) was tested using Shapiro-Wilk.

All analyses were performed using the SAS 9.3 software package (Cary, North Carolina). Two-sided *p*-values lower than 0.05, based on the χ^2 test, Student's *t*-test or Fisher's exact test, were considered statistically significant.

Ethic statement.

Ethical approval for this study was granted by the Uganda Virus Research Institute Science Ethics Committee and the Uganda National Council of Science and Technology.

The laboratory study protocol of serum sample analysis for AFB_1-Lys adduct was approved by the Institutional Review Board for Human Subjects Protection at the University of Georgia.

Conflict of interest.

The authors declare no conflict of interest.

Result

Three hundred and fifty-seven healthy general participants and 132 age, gender, and area matched participants with at least one serum sample with either an AST or ALT level >ULN were selected to compare the aflatoxin exposure based on serum AFB1-Lys levels (Table 5.1). The >ULN group had a geometric mean of 3.78 pg/mg of AFB1-Lys, which was twice as high as that of group with <ULN: 1.67 pg/mg albumin ($p<0.001$) (Figure 5.1.). The levels of AFB adducts in the >ULN-group (n=132) were further compared across the following demographic factors (Table 5.2): age, sex, occupation, and education. Casual laborers and brewers had higher geometric means (4.61 and 3.93 pg/mg albumin) of the levels of AFB_1-Lys than agricultural or other occupational workers (1.05 and 1.28 pg/mg albumin), but the difference by occupation was not statistically significant. Also, the adduct levels did not significantly vary with regard to any of the

demographic factors. All of the individuals in the >ULN-group resided in areas considered "rural".

The AFB_1-Lys adduct levels of the 300 Good Health for Women Project (GHWP) participants in Kampala, Uganda were obtained to test the influence of socio-economic status, and drug and alcohol usage. The levels of AFB1-Lys adducts ranged from 0.4 to 149.28 pg/mg albumin. The AFB1-Lys adduct levels did not vary significantly when compared by the following demographic factors: age, education, marital status, and others as shown in Table 5.3. The difference in aflatoxin exposure with regard to economic status was not statistically significant. However, the geometric means for AFB1-Lys adduct levels were higher for the groupings having no electricity, using free water (from a public pipe water or well), having no bed or mattress (sleeping on the floor with a mat, sack or plastic bag), or living with more than 4 roommates (the Kampala standard family size is about 5 persons). Self-employed participants had higher levels of geometric mean and median of AFB1-Lys than those participants working for others.

AFB_1-lys levels were significantly different with regards to alcohol addiction and frequency of drug usage (Table 5.4). Participants' drinking habits, such as frequency and addictive behaviors, which were determined by CAGE questions, were associated with significantly higher geometric mean and median of AFB_1-Lys (Table 5.4). Participants' illicit drug usage was also significantly ($p<0.05$) associated with high levels of AFB_1-Lys. Participants who had experienced at least 2 weeks of daily drug usage during last three months had levels of AFB_1-Lys that were three times as high as participants who did not experience this frequency of drug usage($p=0.0334$).

Discussion

This study found that the levels of AFB_1-Lys were significantly higher in Ugandans who had serum ALT or AST levels higher than the ULN, a result suggestive of abnormal liver function. Also, the level of AFB_1-Lys adduct within the cohort of urban sex-workers was significantly higher than that of the general Ugandan population. Levels of AFB_1-Lys varied significantly with regard to selected indicators of a drug/alcohol addiction.

Our findings agree with previous studies that compared AFB_1-Lys adduct and abnormal liver function tests in different populations (Giannini et al. 1999; Kim et al. 2008; Nguyen et al. 1997; Nyblom et al. 2004; Verslype 2004). To our knowledge, this is the first study in Uganda to observe a significant increase of aflatoxin B1-lysine in a group of people exhibiting elevated levels of markers indicative of abnormal liver function. A few studies reported an elevated level of liver enzymes (AST, ALT) in a group of people with levels of AFB_1-Lys that were greater than the group average or median level of AFB_1-Lys (Jolly et al. 2007; Jolly et al. 2011; Mohd Redzwan et al. 2014; Tao et al. 2005). This elevation could be explained in several ways. First, the aflatoxin B1-8,9-epoxide, which is a metabolite of aflatoxin B1, will act as a reactive oxygen species within a hepatocytes, causing damage to the cell membrane and increased release of liver enzymes, such as aspartate transaminase (AST) and alanine transaminase (ALT). Secondly, it is also possible that the marker of aflatoxin exposure, aflatoxin B1-lysine/mg albumin, is higher in the abnormal liver function group because the production of albumin was reduced due to liver damage. Thirdly, the epoxide formation, which is necessary for adduct formation, may be increased in the group with evidence suggesting

abnormal liver function due to the induction of phase I enzyme by alcohol consumption(Boffetta and Hashibe 2006).

In Uganda, sorgum, cassava, maize, and millet are typically used to brew beer; those grains are known to be susceptible to aflatoxin contamination(Odhav and Naicker 2002). The association between drinking habits such as frequencies and how frequently drinking more than 6 cups per sitting and higher levels of AFB_1-Lys may be due to contamination of raw materials for brewing beer by aflatoxins. Although there was no statistical significance, those participants who consumed locally brewed beers, which are mainly made of locally grown grains such as millets, sorghum, and maize, had higher levels of aflatoxin B1-lysine, compared to those who consumed commercial beers which are brewed from barley(Mwesigye and Okurut 1995).

Socio-economic status and the level of AFB1- Lys have been statistically related, but the various studies in Africa do not report consistent findings with regard to the direction of the relationship (O. Adejumo et al. 2013; Egal et al. 2005; Jolly et al. 2006; Shirima et al. 2015). Some studies show that the AFB_1-Lys adduct was higher in groups with low socio-economic status, which is determined by possessions, income, and miscellaneous factors (Oloyede Adejumo et al. 2013; Jolly et al. 2006; Leroy et al. 2015; Shirima et al. 2015; Shuaib et al. 2012). Other studies have reported that populations with higher economic status have a higher risk of exposure to aflatoxins because those groups have more accessibility to groundnut-based food, which is one of the major sources of aflatoxins (Egal et al. 2005). Our study found that a higher median and mean of AFB_1-Lys was found in those with the indicators of low socio-economic status, although statistical significance was not found, corresponding with the findings of studies

showing an inverse relationship between the level of AFB_1-Lys and socio-economic status. It is possible that those with lower socio-economic status in Uganda have limited choices of food items due to their limited resources. Therefore, they are likely to consume foods contaminated with aflatoxin, and to be less informed about the danger of aflatoxin consumption due to limited education (O. Adejumo et al. 2013; Ezekiel et al. 2013).

Our work shows that aflatoxin exposure can be quite high in some Ugandans. There is also some evidence of an association between AFB_1 and socio-economic and behavioral factors. The use of plant-based illicit drugs, heavy alcohol use(based on number of drinks at one sitting), and relatively lower socio-economic status may be risk factors for aflatoxin B1 intake. Increased concentration of the enzymes (ALT, AST) in blood of the rural Ugandan groups was significantly associated with a higher concentration of AFB_1-Lys in serum. This finding is consistent with the fact that aflatoxin exposure is one of the risk factors for hepatocellular damage; moreover, this hepatic damage associated with aflatoxin exposure could increase the risk of progression to liver tumors and cancers. Our results supports the belief that aflatoxin exposure in Uganda is one of several risk factors for chronic liver disease cancers in the region.

Table 5.1. Statistical analysis and comparison of some demographic variables in the groups with ALT or AST serum enzyme levels greater than the upper limits of normal (>ULN) or less than the upper limits of normal (<ULN)*

	<ULN (Center for Disease Control and Prevention)	**≥ULN (Pope et al.)**	**p-value****
Variables			
Number of participants	357	132	
Age			
<20	24 (6.7)	7 (5.3)	
20-39	120 (33.6)	44 (33.3)	0.3
40-59	130 (36.4)	40 (30.3)	
≥ 60	83 (23.3)	41 (31.1)	
Sex			
Male	183 (51.3)	78 (59.1)	0.123
Female	174 (48.7)	54 (40.9)	
Residential Area			
Rural	357	132	-
Occupation			
Agriculture	322 (90.5)	78 (59.1)	<0.0001
Others	34 (9.5)	54 (40.9)	
Level of education			
Illiterate	54 (15.1)	32 (24.2)	
Primary	261 (73.1)	85 (64.4)	0.061
Secondary and above	42 (11.8)	15 (11.4)	

* A participant whose levels of either AST or ALT are higher than ULN at least one measurement was classified as '≥ULN (Pope et al.)'. ULN for AST and ALT in the population was 55 IU/l and 56 IU/l, respectively. ** The values in each column are n (%). Wilcoxon rank sum test was used for categorical variables (Age class, sex, Residential Area, Occupation, and Level of education).

Table 5.2. Comparison of aflatoxin B1-lysine levels (pg/mg albumin) by demographic factors in the >ULN group*

	n	GM	%>ULN [%]	Median	25th Pct	75th Pct	90th Pct	p-value
Age								
<20	7	0.7	42.86	0.4	0.2	0.99	4.01	0.575
20-39	44	1.52	56.81	1.4	0.4	4.19	16.03	
40-59	40	1.23	52.5	0.56	0.4	2.29	8.13	
>60	41	1.25	43.9	0.4	0.4	2.88	8.21	
Sex								
Male	78	1.45	53.85	0.74	0.4	3.62	20	0.257
Female	54	1.02	46.3	0.4	0.4	2.54	5.97	
Occupation								
Agriculture	78	1.05	44.87	0.55	0.4	3	8.23	0.113
Bar owner/Brewer	3	3.93	51.8	0.4	0.4	29.42	29.42	
Casual Labourer	7	4.61	71.43	4.29	0.4	33.83	153.06	
Others	44	1.28	56.81	1.06	0.4	2.77	8.23	
Education								
Illterate	32	1.28	46.88	0.4	0.4	2.67	20	0.263
Primary	84	1.12	47.62	0.4	0.4	3.13	7.62	
Secondary and above	16	2.11	75	1.69	0.63	4.25	29.42	

* The values in each column are 'pg AFB_1-Lys/mg albumin' otherwise stated. The Wilcoxon rank sum test was used for categorical variables (age class, sex, residential area, occupation, level of education).

Table 5.3. Aflatoxin B1-lysine levels (pg/mg albumin) by selected demographic factors and alcohol/drug consumption

Variable	n	GM	Detection Rate [%]	Median	25th Pct	75th Pct	90th Pct	p-value
Age								
<20	116	2.29	75	1.62	0.4	9.7	27.8	
20-39	156	2.82	78.85	2.27	0.47	12.81	32.36	0.548
>40	28	2.2	78.57	1.65	0.42	9.87	22.88	
Sex								
Female	300							
Education								
Illicit	40	2.43	77.5	1.85	0.4	11.39	30.58	
Primary	179	2.58	77.1	1.9	0.44	12.74	31.17	0.961
Secondary or above	81	2.53	77.78	1.89	0.4	12.56	28.92	
Marriage								
Currently Married	26	3.08	76.92	2.32	0.7	21.51	27.82	
Never Married	38	3.5	78.95	3.24	0.4	18.09	43.82	
Divorced/Separated	204	2.44	77.45	1.78	0.41	10.47	29.24	0.504
Widowed	32	1.94	75	1.44	0.42	6.21	25.08	
Single/Divorced/Widowed	274	2.5	77.37	1.79	0.41	11.35	31.17	
Age of main partner								
<20	53	2.13	75.61	1.52	0.4	6.75	25.7	
20-39	55	2	76.36	1.93	0.47	5.34	14.4	0.338
40-59	25	1.52	76	1.06	0.4	2.57	20.35	
>60	56	3.31	83.93	2.05	0.47	17.31	32.88	
House								
Own a house	18	3.9	94.44	2.67	1.56	15.4	27.82	
Renting a room	253	2.4	74.7	1.72	0.4	11.43	32.56	0.171
Free room	29	3.25	89.66	4.18	0.99	10.35	22.46	
Electricity								
Have electricity	140	2.25	76.43	1.89	0.4	8.21	26.61	0.257
No electricity	160	2.83	78.13	1.99	0.49	14.41	32.14	
Water								
Tap water in compound	33	2.369	72.73	1.934	0.4	10.02	20.35	
Well	38	4.039	73.68	4.727	0.4	20.47	32.36	
Vendor Water	224	2.356	76.34	1.59	0.43	10.25	31.17	0.481
Others (Well+Vendor, Tank, Pipe water)	5	3.922	60	3.219	0.4	36.32	49.64	
Bed								
Bed and mattress	242	2.419	73.55	1.855	0.4	10.585	28.553	
Mattress on the floor	53	2.908	81.13	1.797	0.554	14.415	31.167	0.248
Mat on the floor and others	5	7.362	100	10.154	2.763	25.679	43.819	
Number of Roommates								

≤ 4	285	2.497	74.73	1.888	0.4	11.35	28.919	0.34
> 4	15	3.67	86.67	2.442	0.916	14.402	33.847	
Employment								
Self-employed	182	2.821	75.13	2.175	0.4	13.857	31.167	0.33
Work for other	103	2.38	78.81	1.559	0.446	8.056	24.582	
Days at work								
Everyday	207	2.821	75.13	1.559	0.4	9.563	27.8	0.12
Others	78	2.38	78.81	2.913	0.4	15.593	32.877	
Drinking habits								
Ever used alcohol								
Yes	252	2.429	75.79	1.792	0.409	10.675	27.8	0.378
No	48	3.252	72.92	2.338	0.4	24.794	40.43	
Frequency								
Everyday	74	2.334	77.027	1.514	0.436	11.425	24.657	0.878
Other	178	2.47	75.28	1.81	0.408	10.351	28.553	
6 or more drinks at a time.								
Never	162	2.049	73.01	1.62	0.4	8.033	21.51	
Less than once a month	10	3.486	63.64	8.61	0.4	15.04	29.05	
Monthly	14	3.7	73.33	9.593	9.59	17.18	29.05	0.0462
Weekly	44	2.078	77.78	1.431	1.43	0.49	6.26	
Daily or almost daily	21	5.331	86.36	10.76	0.92	25.68	51.33	
not able to stop drinking								
Yes	77	2.823	83.11	2.147	0.594	11.425	32.359	0.239
No	175	2.274	72.57	1.786	0.4	10.142	25.679	
Illicit drug habit								
Ever had drug addition								
Yes	67	2.476	70.15	2.28	0.4	11.35	28.553	0.74
No	233	2.566	76.82	1.822	0.446	12.744	31.912	
At least 2 weeks daily drugs								
Yes	51	3.229	74.51	3.448	0.4	17.186	28.919	0.0334
No	16	1.062	56.25	0.519	0.4	2.722	6.357	
Types of drug								
Marihuana	12	1.158	58.33	0.794	0.4	2.361	11.425	
Khat	4	0.663	50	0.431	0.4	1.536	2.61	0.117
Other	45	3.346	75.56	2.899	0.489	17.186	28.919	
Several of above	6	2.853	66.67	3.65	0.4	4.805	52.709	

* The values in each column are 'pg AFB_1-Lys/mg albumin' otherwise stated. The Wilcoxon rank sum test was used for categorical variables

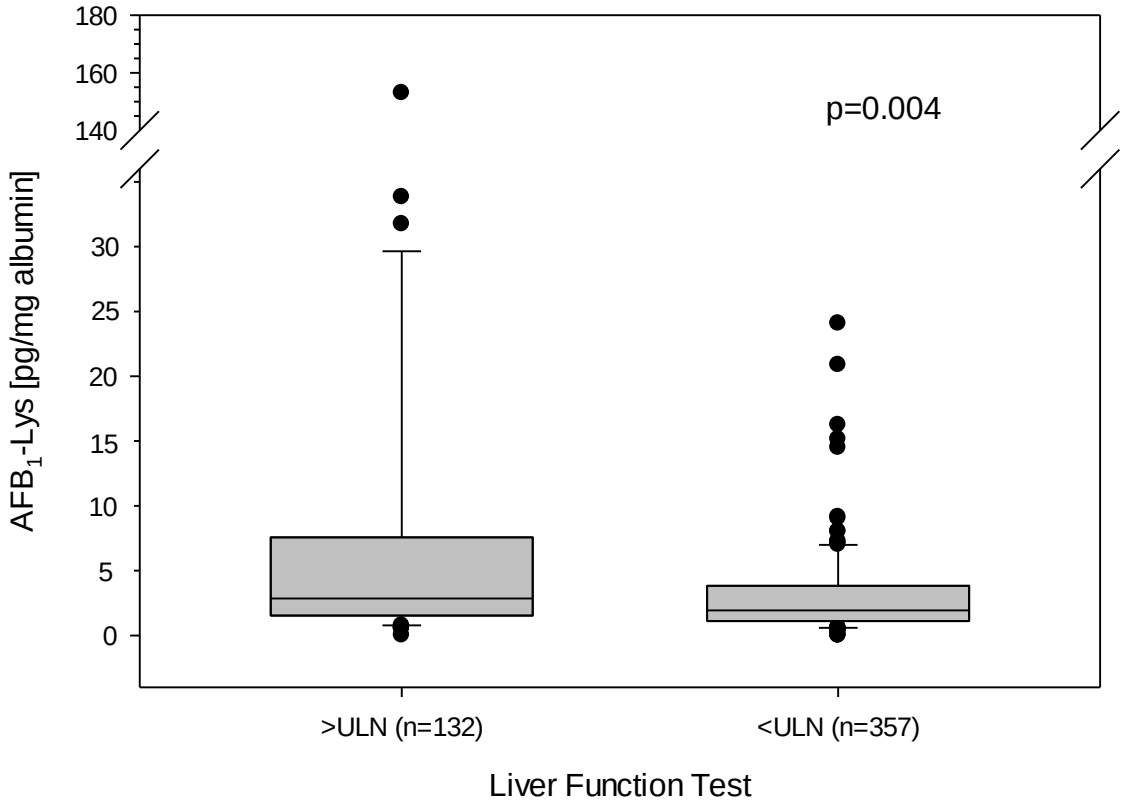


Figure 5.1. Box plot of aflatoxin B1-lysine levels comparing participants with AST or ALT levels greater than (>ULN) or less than (<ULN) the upper limits of normal.

Reference

Adejumo O, Atanda O, Raiola A, Somorin Y, Bandyopadhyay R, Ritieni A. 2013. Correlation between aflatoxin m1 content of breast milk, dietary exposure to aflatoxin b1 and socioeconomic status of lactating mothers in ogun state, nigeria. Food Chem Toxicol 56:171-177.

Adejumo O, Atanda O, Raiola A, Somorin Y, Bandyopadhyay R, Ritieni A. 2013. Correlation between aflatoxin m1 content of breast milk, dietary exposure to aflatoxin b1 and socioeconomic status of lactating mothers in ogun state, nigeria. Food Chem Toxicol 56:171-177.

Anesi V. 2012. Conflict intensity on socioeconomic welfare: A ugandan case study:University of Nottingham.

Asiki G, Murphy G, Nakiyingi-Miiro J, Seeley J, Nsubuga RN, Karabarinde A, et al. 2013. The general population cohort in rural southwestern
uganda: A platform for communicable
and non-communicable disease studies. International Journal of Epidemiology 42:129-141.

Asiki G, Seeley J, Srey C, Baisley K, Lightfoot T, Archileo K, et al. 2014. A pilot study to evaluate aflatoxin exposure in a rural ugandan population. Trop Med Int Health 19:592-599.

Boffetta P, Hashibe M. 2006. Alcohol and cancer. Lancet Oncology 7:149-156.

Center for Disease Control and Prevention.

Efuntoye MO. 1999. Mycotoxins of fungal strains from stored herbal plants and mycotoxin contents of nigerian crude herbal drugs. Mycopathologia 147:43-48.

Egal S, Hounsa A, Gong YY, Turner PC, Wild CP, Hall AJ, et al. 2005. Dietary exposure to aflatoxin from maize and groundnut in young children from benin and togo, west africa. Int J Food Microbiol 104:215-224.

Ezekiel CN, Sulyok M, Babalola DA, Warth B, Ezekiel VC, Krska R. 2013. Incidence and consumer awareness of toxigenic aspergillus section flavi and aflatoxin b1 in peanut cake from nigeria. Food Control 30:596-601.

Giannini E, Botta F, Fasoli A, Ceppa P, Risso D, Lantieri PB, et al. 1999. Progressive liver functional impairment is associated with an increase in ast alt ratio. Digestive diseases and sciences 44:1249-1253.

Hendrickse RG, Maxwell SM, Young R. 1989. Aflatoxins and heroin. BMJ : British Medical Journal 299:492-493.

International Agency for Research on Cancer. 2002. Some traditional herbal medicines, some mycotoxins, naphthalene, and styrene. In: IARC Monograph on the Carcinogenic Risks to Humans, Vol. 82, 171-.

Jolly P, Jiang Y, Ellis W, Awuah R, Nnedu O, Phillips T, et al. 2006. Determinants of aflatoxin levels in ghanaians: Sociodemographic factors, knowledge of aflatoxin and food handling and consumption practices. Int J Hyg Environ Health 209:345-358.

Jolly PE, Jiang Y, Ellis WO, Awuah RT, Appawu J, Nnedu O, et al. 2007. Association between aflatoxin exposure and health characteristics, liver function, hepatitis and malaria infections in ghanaians. Journal of Nutritional & Environmental Medicine 16:242-257.

Jolly PE, Shuaib FM, Jiang Y, Preko P, Baidoo J, Stiles JK, et al. 2011. Association of high viral load and abnormal liver function with high aflatoxin b1-albumin adduct levels in hiv-positive ghanaians: Preliminary observations. Food additives & contaminants Part A, Chemistry, analysis, control, exposure & risk assessment 28:1224-1234.

Kim WR, Flamm SL, Di Bisceglie AM, Bodenheimer HC, Public Policy Committee of the American Association for the Study of Liver D. 2008. Serum activity of alanine aminotransferase (alt) as an indicator of health and disease. Hepatology 47:1363-1370.

Leroy JL, Wang J-S, Jones K. 2015. Serum aflatoxin b1-lysine adduct level in adult women from eastern province in kenya depends on household socio-economic status: A cross sectional study. Social Science & Medicine 146:104-110.

Mathers CD, Loncar D. 2006. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Medicine 3:e442.

Matumba L, Monjerezi M, Khonga EB, Lakudzala DD. 2011. Aflatoxins in sorghum, sorghum malt and traditional opaque beer in southern malawi. Food Control 22:266-268.

Mohd Redzwan S, Rosita J, Mohd Sokhini AM, Nurul ‘Aqilah AR, Wang J-S, Kang M-S, et al. 2014. Detection of serum afb1-lysine adduct in malaysia and its association with liver and kidney functions. Int J Hyg Envir Heal 217:443-451.

Mwesigye PK, Okurut TO. 1995. A survey of the production and consumption of traditional alcoholic beverages in uganda. Process Biochem 30:497-501.

Nguyen TL, Nguyen TH, Tieu NT. 1997. The impact of dengue haemorrhagic fever on liver function. Res Virol 148:273-277.

Nikander P, Seppälä T, Kilonzo G, Huttunen P, Saarinen L, Kilima E, et al. 1991. Ingredients and contaminants of traditional alcoholic beverages in tanzania. Transactions of the Royal Society of Tropical Medicine and Hygiene 85:133-135.

Nyblom H, Berggren U, Balldin J, Olsson R. 2004. High ast/alt ratio may indicate advanced alcoholic liver disease rather than heavy drinking. Alcohol Alcoholism 39:336-339.

Ocama P, Nambooze S, Opio CK, Shiels MS, Wabinga HR, Kirk GD. 2009. Trends in the incidence of primary liver cancer in central uganda, 1960-1980 and 1991-2005. British Journal of Cancer 100:799-802.

Odhav B, Naicker V. 2002. Mycotoxins in south african traditionally brewed beers. Food Addit Contam 19:55-61.

Pope WH, Jacobs-Sera D, Russell DA, Peebles CL, Al-Atrache Z, Alcoser TA, et al. 2011. Expanding the diversity of mycobacteriophages: Insights into genome architecture and evolution. PloS one 6:e16329.

Qian G, Tang L, Liu W, Wang J-S. 2010. Development of a non-antibody method for rapid detection of serum aflatoxin b1-lysine adduct. In: Toxicologist, Vol. 114, 1163.

Qian G. 2012. Integrative toxicological evaluation of aflatoxin b_1 exposure in fischer 344 rats and biomarker based exposure assessment of aflatoxin b_1 in human populations. [electronic resource]:2012.

Shirima CP, Kimanya ME, Routledge MN, Srey C, Kinabo JL, Humpf HU, et al. 2015. A prospective study of growth and biomarkers of exposure to aflatoxin and fumonisin during early childhood in tanzania. Environmental Health Perspectives 123:173-178.

Shuaib FMB, Jolly PE, Ehiri JE, Ellis WO, Yatich NJ, Funkhouser E, et al. 2012. Socio-demographic determinants of aflatoxin b1-lysine adduct levels among pregnant women in kumasi, ghana. GHANA MEDICAL JOURNAL 46:179-188.

Tao P, Zhi-Ming L, Tang-Wei L, Le-Qun L, Min-Hao P, Xue Q, et al. 2005. Associated factors in modulating aflatoxin b1-albumin adduct level in three chinese populations. Digestive diseases and sciences 50:525-532.

UN-HABITAT. 2006. Situation analysis of informal settlements in kampala. (Cities without slums : Sub-Regional Programme for Eastern and Southern Africa). Nairobi, Kenya:United Nations Human Settlements Programme (UN-HABITAT).

Vandepitte J, Bukenya J, Weiss HA, Nakubulwa S, Francis SC, Hughes P, et al. 2011. Hiv and other sexually transmitted infections in a cohort of women involved in high risk sexual behaviour in kampala, uganda. Sexually transmitted diseases 38:316-323.

Verslype C. 2004. Evaluation of abnormal liver-enzyme results in asymptomatic patients. Acta Clin Belg 59:285-289.

CHAPTER 6

DIETARY AFLATOXIN EXPOSURE AMONG THE RESIDENTS OF BEXAR COUNTY, TEXAS AFTER THE 2012-2013 DROUGHT

Introduction

Aflatoxin B_1 (AFB_1) is a secondary fungal metabolite and is classified as a group 1 carcinogen by the IARC. AFB_1 is known to cause hepatotoxicity and has also been shown to be associated with growth retardation and modulation of immune response in humans. AFB_1 is predominantly produced by *Aspergillus flavus* and *A. parasiticus.* These aflatoxin producing fungi are prevalent in the soil in tropical and subtropical latitudes, and they infect oilseeds such as corn, cotton, peanuts, sorghum, and soybeans.

Weather events, such as drought and flooding, can affect the frequency of pathogen emergence in farm ecosystems (Anderson et al. 2004; Cotty and. Jaime-Garcia 2007; Fisher et al. 2012; Harvell et al. 2002). Climate change may affect both the fungal community and host crop susceptibility (Cotty and Jaime-Garcia 2007). The change towards warmer, and in some areas more humid or dry, climate may increase aflatoxin in crops because *A. flavus* and *A. parasiticus* optimally produce aflatoxin at 33 ºC and 0.99 water activity; these fungi grow optimally at 35 ºC and 0.95 water activity (Sanchis and

Magan 2004) but drought stress pre-harvest seasons has also been linked to the increasing chance of *A. flavus* infection in corn due to reduced integrity ("silk cut") and in peanuts due to reduced phytoalexin production (Odvody et al. 1997; Wotton and Strange 1987). During the planting seasons of 2012 and 2013, severe droughts affected the southern and mid-western United States (Kellner and Niyogi 2014). The areas mainly affected by the droughts produce corn, soybean, and sorghum; these grains are highly susceptible to infection with *Aspergillus species*. The impact of the severe drought in the midwestern region of U.S.,which includes the corn belt region, seems to have been related to the pet food recall in 2012 and *Aspergillus* ear rot in corn (Gillam and Ingwersen 2013; Hurburgh et al. 2012; Li 2013; Robertson 2013). It is possible that contamination of crops with AFs increased during the severe drought years, and subsequently, could have could have adversely affected human consumers.

In the U.S., the prevalence and the level of AFB_1-Lys in serum were higher among the Hispanic population, and it is possibly due to consumption of corn-based Hispanic diet (Batis 2011; Johnson et al. 2010; Plasencia 2004; Schleicher et al. 2013; Wood 1992). According to the census data, a significant proportion of the Bexar County (Texas) population (59.3%) is Hispanic, compared to the smaller proportion for Texas as a whole (38.6%). The liver cancer incidence between 2008 and 2012 was 13.8 (95% C.I.; 13.0-14.6) per 100,000 in Bexar County, Texas, which is significantly higher than 9.3 (95% C.I; 9.2-9.5) per 100,000 (Texas average) and 6.60 (95% C.I.; 6.56-6.64) per 100,000 (U.S. average)(cancer-rates.info 2014); the disparity in the disease rates might originate from the increased consumption of corn -based foods by the Hispanic population. Also, the risk for aflatoxin exposure (consumption of corn-based foods)

within the Hispanic population may be associated with education and socio-economic status.

To the best of our knowledge, this is the first study that has investigated the association between drought stress during the planting seasons of crops and the level of aflatoxin exposure biomarker in a population living in the drought-affected areas. We also tested other possible risk factors for aflatoxin B1 exposure, including diet, socio-economic status, ethnicity, age, sex, education, occupation

Materials and Methods

Participant recruitment and data collection.

Three-hundred and eighty participants were recruited from various residential areas and public places in Bexar County, Texas; the area covers most of the San Antonio metropolitan region. The recruitment phase was conducted from October 2012 to May 2014 after the severe drought in 2012.

All study subjects gave written informed consent. Anthropometric data such as age, sex, height were obtained during the medical visit. Questionnaires were provided in English and Spanish to obtain: 1) demographic factors such as ethnicity, marital status, number of children, and level of education; 2) economic information such as employment status, annual income, and the health insurance; 3) dietary risk factors such as consumption of maize-based and peanut-based foods (including both frequency and amount per meal); 4) lifestyle factors such as alcohol consumption and smoking status; and5) Medical history, including major surgery, pregnancy, and birth defects. Also, a complete blood panel, with differential and comprehensive metabolic profile (micronutrient, renal function, liver function), was conducted during the visit to the collaborating clinics within Bexar County, TX.

Weather data

Weather data, Palmer drought severity index (PDSI) and average temperature, were obtained from NOAA weather stations to test the effect of drought with the level of aflatoxin B_1-lysine within the population. PDSI is a measurement of dryness based on recent precipitation and temperature. It was assumed that grain, which residents consume, was in circulation from each summer's harvest to the next year's harvest season (from

September to August). PDSI values and average temperature during the harvest season each year were compared to the levels of aflatoxin B_1-lysine during follow-up year in the Bexar County residents. The PDSI values in the primary Cornbelt region and in south-central Texas (which includes Bexar County, TX) were compared from February 2007 to August 2014. The data were obtained from NOAA Historical Palmer Drought Indices(NOAA).

Blood sample collection

Blood was collected from 380 participants during screening at the various recruitment sites. Urine samples were collected from 233 participants for aflatoxin M1 measurement. A portion of the blood was centrifuged within 1h of collection to separate serum, and stored at - 80°C, then shipped to the University of Georgia for AFB1-lysine adduct analysis. Aliquots were also sent to LabCorp (San Antonio, Texas) for a complete blood count with differential and comprehensive metabolic profile (micronutrient, renal function, liver function).

Aflatoxin B_1-lysine measurement

Serum samples were coded separately and analyzed for AFB1-Lys adducts using a newly-developed HPLC-fluorescence method (Qian 2012). Briefly, human serum samples were deactivated for viral/bacterial infection and measured for albumin and total protein concentrations using procedures modified as previously described (Qian 2012). A portion of each serum sample (150 μL) was digested by pronase (pronase: total protein, 1:4, w:w) at 37°C for 3 hr to release the AFB1-Lys adduct. AFB1-Lys adducts in digests were further extracted and purified by passing them through Oasis MAX SPE cartridges (Waters Corporation, Milford, Massachusetts). The eluate was vacuum-dried with a

CentriVap concentrator (Labconco, Kansas City, Missouri) and reconstituted for HPLC-fluorescence detection. The analysis of the AFB1-Lys adduct was conducted in a 1200 HPLC-fluorescence system (Agilent, Santa Clara, California). The mobile phases consisted of buffer A (20 mM $NH_4H_2PO_4$, pH 7.2) and buffer B (100% methanol). A Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 x 250 mm) equipped with a guard column was used. Column temperature was maintained at 25°C during analysis, and a volume of 100 μL was injected into the HPLC instrument. The flow rate was kept at 1 mL/min. A gradient was generated to separate the AFB1-Lys adduct within 25 min of injection. The lysine adduct was detected by fluorescence at maximum excitation and emission wavelengths of 405 nm and 470 nm, respectively. Calibration curves of authentic standard were generated weekly, and the standard AFB1-Lys was eluted at approximately 13.1 min. Quality assurance and quality control procedures were performed during analyzes, which included simultaneous analysis of one authentic standard and a quality control sample daily. The method for producing the AFB-Lys standard and the procedure for maintaining quality control are described elsewhere in detail (Qian 2012). Briefly, rat serum dosed with AFB1 (Sigma, St Louis, Missouri) was purified using Oasis MAX SPE cartridges; the AFB1-Lys adduct in the serum was concentrated by the Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 x 250 mm). For quality control, the AFB1-Lys standard was processed and analyzed with other samples simultaneously. The limit of detection was 0.4 pg/mg albumin.

Statistical analysis

We performed the χ^2 test (categorical variables), Student's *t*-test (normally distributed continuous variables), and the Wilcoxon rank-sum test (non-normally distributed continuous variables) for the following descriptive variables: Age, gender, education, occupation, marital status, alcohol and drug use, and food intake. Data distribution (normality) was tested using the Shapiro-Wilk test. The distribution of AFB_1-lysine was compared by year using the Wilcoxon rank-sum test. Linear regression ANOVA of the geomeans of AFB_1-lysine was carried out with the average PDSI values in southcentral Texas and with the average PDSI values corn belt area, respectively. All analyses were performed using the SAS 9.3 software package (Cary, North Carolina), and graphs were generated using SigmaPlot 10.0 (San Jose, California). Two-sided *p*-values lower than 0.05, based on the χ^2 test, Student's *t*-test or Fisher's exact test, were considered statistically significant.

Ethical statement.

The study was approved by the institutional review boards at the University of Texas at Health Science Center in San Antonio (UTHSCSA) and Texas A&M University, and by the Protocol Review Committee of the Cancer Therapy and Research Center at UTHSCSA .

Conflict of interest.

The authors declare no conflict of interest.

Results

During the severe 2012-2013 drought, the aflatoxin B_1-lysine levels in 357 residents of Bexar County, TX were measured. The sampled group was 70% Hispanic, 25.3% non-Hispanic white, and 4.8% black and other ethnic groups. Along with ethnicity, demographic information, such as age, occupation, education, socio-economic status was collected (Table 6.1), because these factors have shown association with the level and prevalence of aflatoxin biomarkers in humans (Schleicher et al. 2013; Leroy et al. 2015; Viegas et al. 2015). Contrary to our expectations, this study revealed that the prevalence and level of aflatoxin B_1-lysine was not correlated with race and ethnicity among the participants. We did find that the magnitude of aflatoxin B1 adduct levels among the participants differed with respect to socio-economic status; the adduct level was highest among participants with an annual household income ranging from $10,000 to $14,999, and was lowest among participants with an annual household income between $50,000 and $74,999.

Food intake information for some of the 2012-2014 participants (n=233) was collected through a questionnaire because a corn-based diet is known as a risk factor for aflatoxin B1 exposure. As shown in Table 6.2, the frequency of Mexican food consumption was significantly associated with an increased level of aflatoxin B1-lysine in participants, whereas peanut and peanut butter consumption did not show a statistically significant association with aflatoxin B1-lysine. Also, the consumption of corn- and rice-based drinks was significantly associated with the level of AFB_1-lysine among the participants. To test the direction of interaction, the quantity of consumed foods per week was obtained by multiplying both frequency and amount of consumption per sitting. The

quantity of Mexican food, corn, and corn flour tortillas per week were positively correlated with aflatoxin B_1-lysine levels with statistical significance (Figure 6.1).

The positive sample rate and level of aflatoxin B_1-lysine adduct among the population of Bexar County increased in 2012-2014 compared with our previous study in 2007-2008 (Johnson et al. 2009). During the severe 2012-2013 drought in the Midwestern and Southern U.S., the aflatoxin B1-lysine adduct levels in the residents of south-central Texas (which includes Bexar County) were elevated, compared with the adduct levels during 2007-2008 in the region (Table 6.5 and Graph 6.2). The prevalence of positive samples and geometric mean of aflatoxin B1-lysine in the sampled population in 2007 to 2008 were 27% and 0.67 pg/mg albumin, respectively, and in the sampled population in 2012-2013 the prevalence and geometric mean was to 88.76 % and 2.57 pg/mg albumin, respectively. In Table 6.4, aflatoxin B1-lysine levels in Bexar County residents are compared with respect to the year of harvested oilseed and grain (corn, peanut, and sorghum) in circulation. It was assumed that grain, which residents consume, was in circulation from each summer's harvest to the next year's harvest season (from September to August). From here on, we refer to the sets of serum samples from Bexar County residents as follows: From October 2007 to May 2008 as the "2007 samples"; from October 2012 to August 2013 as the "2012 samples"; and from September 2013 to August 2014 as the "2014 samples." Seventy-nine percent of the residents had detectable level of aflatoxin B1-lysine adduct in their serum in the 2012 samples, and 100% had detectable levels in the 2013 samples, whereas only 27% had the aflatoxin B1 marker in the 2007 samples (Table 6.4). The geometric means of aflatoxin B1-lysine in Bexar County residents were 0.62, 2.48, and 2.67 pg/mg albumin in the 2007, 2012, and 2013

samples, respectively. Among the serum samples from Bexar County participants, the magnitude and prevalence of aflatoxin B1-lysine were similar for both the 2012 and 2013 samples; both had higher aflatoxin B1-lysine measurements than the 2007 samples.

It was expected that levels of aflatoxin B1 in the serum would correlate with the severity of drought during the corn pre-harvest season in the Corn Belt and in south-central Texas because of aflatoxin contamination in the corn. The magnitude of drought stress, indicated by PDSI in south-central Texas (which includes Bexar County) followed a similar pattern($p=0.0408$): The PDSI values in the area were similar in both the 2012 and 2013 pre-harvest seasons, but the PDSI values in the 2007 pre-harvest season were significantly higher than in 2012 and 2013 (meaning that there was more rainfall and less evaporation). The south-central Texas area produces both corn and peanuts; a contamination of these oilseed products during the drought might be the source of the increase in aflatoxin B-lysine. However, the PDSI values of the Corn Belt did not follow the expected pattern ($p=0.905$). The rank of PDSI values during the pre-harvest seasons in the Cornbelt region was in the order of 2012, 2007, and 2013 (highest to lowest).

Improper storage has been shown to be associated with aflatoxin contamination in grains. Studies in developing countries, such as China, Nepal, and Iran have revealed a seasonal effect in which the level of aflatoxin B1-lysine in humans increases during the time that crops are stored and decreases when newly harvested cereal is available (Tajkarimi et al. 2007). It is assumed that the prevalence and level of aflatoxin B1-lysine in Bexar County residents peaked before harvest seasons (July-August) and decreased after harvest (September-November). However, the levels of aflatoxin B1-lysine adduct among the residents of Bexar County did not follow this particular pattern. This

difference may also be due to the fact that residents of Bexar County do not completely rely on corn or oilseed crops, unlike the populations of developing nations. Also, storage systems of these oilseeds in U.S. are less prone to contamination than those in developing nations (Hell et al. 2000; Strosnider et al. 2006).

Discussion

The level of aflatoxin B1-lysine adducts in Bexar County residents had arisen during the severe drought 2012-2013. Also, the level of aflatoxin B1-lysine adduct did not show any seasonal trend. This reflects the food diversity in the U.S.—residents of Bexar County do not completely rely on corn or oilseed crops for their nutrition.

According to the Intergovernmental Panel on Climate Change (IPCC), the frequency of extreme weather events and average temperature over all habitable continents has risen beyond the range of natural variability (IPCC 2012, 2013; Solomon et al. 2007). The production of aflatoxins is affected by several environmental factors: Optimal temperatures for the growth of the *Aspergillus* species and aflatoxin production, water availability, and insect stress (Dorner et al. 1989; Mc Millian et al. 1985; Miraglia et al. 2009; Paterson and Lima 2011). But how climate factors affect the aflatoxin contamination of crops depends on the timing of the environmental factors that affect the crops: Drought during pre-harvest seasons and flooding from harvest to post-harvest seasons are associated with increased risk of aflatoxin contamination (Cotty and Jaime-Garcia 2007). Many aflatoxin-producing fungi— *A. flavus* in particular—are frequently found in warm and humid climate zones between the latitudes 26° and 35°, as opposed to

tropical climates zones between 23.5°S and 23.5°N . Typically, aflatoxin contamination has not been a problem in Europe and North America; however, recent global warmings—temperature increases in Europe ranging from 2.5 °C to 5.0 °C and frequent droughts and flooding in the U.S.—have created an environment favorable to *Aspergillus* species (Commission 2007). Because of these changes, crops growing in Europe and North America are now at increased risk for aflatoxin contamination. Seventy-five percent of tested foods in southern Italy (dried fruit and derivatives, spices and derivatives, cereals, herb, and olive oils) were contaminated with aflatoxin B1 with a mean of 4.4 μg/kg and a maximum of 154.5 μg/kg after the 2003 drought (Piva et al. 2006). After the severe 2012 drought in Serbia, 132 of 200 maize samples were contaminated with aflatoxins ranging from 1.01 μg/kg to 86.1 μg/kg, which is significant considering that zero out of 180 samples had aflatoxins from 2009 to 2011 (Kos et al. 2013). Additionally, aflatoxins were found in maize, milk, and animal feeds in other central European countries after the 2013 drought (Dobolyi et al. 2013; Iha et al. 2013; Kočube et al. 2013; Tóth et al. 2013). Flooding during the harvest seasons was associated with increased levels of aflatoxins in cottonseeds in southern Texas (Cotty and Jaime-Garcia 2007), and the increased geocarposphere temperatures, temperature of soil around the peanut, and drought levels during pre-harvest seasons were associated with increased aflatoxin contamination in peanuts and corn (Cole et al. 1985; Li 2013). Due to climate change, traditionally safe areas could in the future have higher levels of aflatoxin contamination in foods.

In addition, climate change will alter the fungal community, and this alteration may affect the quantity and types of aflatoxins produced. Although it is assumed that the

consequences of climate change will vary from one region to another (Cotty and Jaime-Garcia 2007; Singh et al. 2010), the changes in temperature and humidity may increase mycotoxin production by altering both the makeup of fungal communities and the rates of carbon conversion from precursors (i.e. carbon, nitrogen) to mycotoxins. The current increase in atmospheric carbon dioxide could temporarily increase the precursors of mycotoxins, and it is generally accepted that the increased carbon dioxide levels could alter the release of sugars, organic acids, and amino acids from plant roots (Bardgett et al. 2009). This could promote microbial growth in general and result in an increased rate of conversion of minerals to mycotoxins (Singh et al. 2010). *A. flavus* is the most dominant aflatoxin-producing species in the southern U.S., compared with *A. parasiticus, A. nomius, and A. tamarii* (Cardwell and Cotty 2002; Cotty 1997). Under high temperature and/or drought conditions, it is possible that the fungal population will tend towards more *Aspergillus flavus* that produce mainly aflatoxin B1, which is the most toxic among aflatoxins. In vitro, *A. flavus* grows well under higher atmospheric carbon dioxide pressure (Medina et al. 2014). Among various mycotoxin-producing fungi, *A. flavus* grows well in temperatures optimal for mycotoxin production (35 °C) and fungal growth (33 °C), which are much higher than the optimal temperatures for other mycotoxin-producing species, such as *Alternaria alternata, Fusarium verticillioides, F. proliferatum, Penicillium verrucosum*, and others. (Sanchis and Magan 2004). Therefore, it is possible that the prevalence of aflatoxins may increase in a region where other types of mycotoxins were prevalent due to rising temperatures.

The consumption of corn and corn-based foods is a known risk factors for aflatoxin exposure. However, Hispanic ethnicity does not affect the level of aflatoxin B1-

lysine adduct among the Bexar County residents, although the Hispanic population tends to eat more corn-based foods such as fresh corn , corn tortillas, and Mexican food than other populations in both frequency and amount per sitting (Table 6.3). Our previous study in Uganda (unpublished data) and other research studies indicated that humans are exposed to aflatoxins through various food items such as maize-based beer, tea, herbal drugs, and spices (Mohd-Redzwan et al. 2013; Park 2002; Romagnoli et al. 2007; Wagacha and Muthomi 2008). The cumulative exposure to maize may be one predominant cause of exposure to aflatoxins. However, surveillance of aflatoxins in various food items is lacking in the U.S. This knowledge gap should be addressed, to elucidate aflatoxin exposure across ethnic cultures, as well as to protect the U.S. population.

Socio-economic status (SES) has been shown to be associated with the level of aflatoxin B1-lysine in serum, meaning that aflatoxin adduct levels are lower in socio-economically well-offs groups (Jolly et al. 2006a; Jolly et al. 2006b; Shirima et al. 2015; Shuaib et al. 2012). In this study, education and household income were used as the indicators of SES instead of Multidimensional Poverty Index (MPI) or asset-index which are typically used in developing countries. It is possible that the marginal association between income and aflatoxin B1 adduct levels in the participants could be attributed to the ability to purchase high quality food items among the wealthy participants. Among the participants, higher education (college degree and above) was associated with avoidance of corn products, such as corn, corn tortillas and Mexican food ($p=0.012$). Although the magnitude of aflatoxin B1-lysine did not vary significantly with respects to education, the avoidance of the corn-based diets may seem like an educated behavior.

Studies in other countries have reported similar findings (Jolly et al. 2006a); for example, dietary aflatoxin M1 exposure was significantly lower among Nigerian infants whose parents had a higher educational status (Adejumo et al. 2013).

The incidence of hepatocellular carcinoma (HCC) has been rising in the U.S. since 1975 (Altekruse et al. 2014; Siegel et al. 2014). The possible causes of HCC involved multiple risk factors, such as hepatitis B virus (HCV), hepatitis C virus,(HCV) alcohol, obesity, metabolic syndrome, mycotoxins. Four risk factors, including HCV, obesity, metabolic syndrome, and mycotoxin exposure, seem to be on the increase during the same period (El-Serag 2002; Larsson and Wolk 2007; Paschos and Paletas 2009). Therefore, along with other factors, aflatoxin exposure could be contributing to the rise in HCC incidence in the U.S. Mycotoxin exposure in a Texan Hispanic population is a possible threat.

Table 6.1. Aflatoxin B1-lysine by demographic/socio-economic factors, October 2012 – August 2014

	N	%, >LOD	G.M.	Median (95% C.I.)	25%	75%	90%	p-value
Sex								
Male	86	92.9	3.64	3.51 (3.09, 4.27)	2.25	6.45	8.18	0.181
Female	264	87.9	3.14	3.41 (3.13, 3.65)	2.02	4.83	6.86	
Age group								
18-29	93	90.3	3.09	3.13 (2.76, 3.62)	1.97	4.66	6.68	
30-39	82	86.6	3.2	3.51 (3.05, 4.96)	2.19	4.96	6.2	
40-49	84	83.3	3.4	3.41 (3.14, 3.89)	2.48	5.54	8.14	0.435
50-59	64	93.8	3.64	3.84 (3.21, 4.65)	2.32	6.25	8.64	
>60	34	88.2	2.93	2.77 (1.67, 4.55)	1.58	6.92	8.78	
Ethnic Group								
Hispanic	249	88.4	3.19	3.4 (3.18, 3.65)	1.98	4.96	7.41	
Non-Hispanic White	92	88.0	3.5	3.61 (3.18, 3.65)	3.61	2.51	5.93	0.513
Non-Hispanic Black/Others	15	93.3	3.19	3.62 (2.45, 5.18)	3.62	2.45	4.06	
Education								
High school diploma or less	59	100	3.32	3.42 (2.9, 4.44)	2.17	5.95	7.52	
Associate's Degree or certificates	110	99.1	3.32	3.4 (3.09, 3.87)	2.24	4.67	6.63	0.913
Bachelor's degree and above	62	100	3.28	3.5 (3.14, 4.03)	2.33	4.82	7.03	
Employment								
Employed for wages	147	99.3	3.4	3.47 (3.18, 3.94)	2.41	4.89	7.11	
Self-employed/homemaker	29	100	3.13	3.71 (2.77, 4.26)	2.01	5.32	6.78	0.792
Unemployed/retired	33	100	3.26	3.33 (2.09, 4.39)	1.93	6.22	7.91	
Student	22	100	3.03	3.35 (2.45, 3.62)	2.33	3.62	4.97	
Income								
Less than $10,000	27	100	2.78	2.84 (2.48, 3.99)	1.85	4.44	6.61	
$10,000 - $14,999	21	100	4.33	4.67 (3.77, 6.34)	2.77	6.90	7.90	
$15,000 - $19,999	24	100	3.44	3.48 (3.18, 4.47)	2.53	6.03	9.92	
$20,000 - $24,999	16	100	3.14	2.97 (2.57, 4.47)	2.26	4.36	7.08	0.076
$25,000 - $34,999	38	100	3.48	3.63 (3.29, 4.74)	2.98	4.60	7.13	
$35,000 - $49,999	37	100	3.79	4.03 (3.71, 5.25)	3.38	6.32	8.14	
$50,000 - $74,999	25	100	2.75	2.93 (2.43, 4.48)	2.01	4.26	5.93	
More than $75,000	30	96.7	3.08	3.48 (2.60, 5.07)	1.95	4.39	5.27	

* The number of participants in each categorical variable is lower than the total number of participants screened for assessing the level of aflatoxin B_1-lysine (n=357), because of non-response to the survey questions about the variables.

** %> LOD (Limit of Detection) indicates the percentage of aflatoxin B1—lysine positive samples

G.M. stands for a geometric mean. Values are 'pg AFB1-Lys/mg albumin' otherwise stated.

*** Comparisons of AF-alb between classes were performed by the Kruskal–Wallis test. The p-value <0.05 is considered statistically significant.

Table 6.2. Aflatoxin B1-lysine in Bexar County residents with respect to food items

		Aflatoxin B1-lysine			
	N	**%, >LOD**	**G.M.**	**Median (95% C.I.)**	**p-value**
Mexican foods					
Consumption frequency					
≤ 1 time/ week	132	100	3.27	4.77 (2.99, 3.96)	
2-7 times/ week	90	96.67	3.18	3.39 (3.00, 3.59)	0.005
>7 times/week	9	100	5.84[#]	6.78 (2.90, 8.14)	
Consumption amount					
≤ 1 item /meal	16	100	2.97	2.88 (2.25, 4.83)	
1-2 items/meal	139	98.56	3.25	3.39 (2.98, 3.94)	0.222
>2 items/ meal	76	98.68	3.55	3.63 (3.34, 4.11)	
Corn					
Consumption frequency					
< 1item /week	138	98.55	3.31	3.40 (3.09, 3.70)	
1 time/week	58	98.28	3.03	315 (2.77, 3.73)	0.096
2-7 times/week	36	100	3.7[#]	3.88 (3.39, 4.67)	
Consumption amount					
< 1 ear/meal	82	100	3.15	3.13 (2.67, 3.76)	
1 ear/meal	99	100	3.24	3.45 (3.26, 3.73)	0.150
>1 ear/meal	30	100	3.81	4.16 (2.77, 4.98)	
Corn Tortillas					
Consumption frequency					
< 1 time/ weeks	116	99.14	3.32	3.45 (2.97, 3.94)	
1-7 times/ weeks	114	98.25	3.22	3.37 (3.05, 3.72)	0.025
>7 times/weeks	3	100	7.41[#]	6.78 (5.42, 11.06)	
Consumption amount					
≤ 1 tortillas/ meal	48	97.92	2.88	3.09 (2.41, 3.59)	
1-2 tortillas/meal	67	100	3.52	3.70 (3.35, 4.14)	0.415
> 2 tortillas/meal	117	98.26	3.27	3.35 (2.97, 3.70)	
Corn flour tortillas					
Consumption frequency					
< 1 time/ week	70	100	3.29	3.60 (2.99, 3.73)	
1-7 times/ week	155	100	3.24	3.35 (3.05, 3.76)	0.028
>7 times/week	6	100	5.75[#]	6.55 (1.73, 11.06)	
Consumption amount					
< 1 tortilla/ meal	40	100	2.98	3.60 (2.40, 3.96)	
1-2 tortilla/meal	125	100	3.35	3.41 (2.99, 3.94)	0.179
> 2 tortilla/meal	63	100	3.4	3.42 (3.05, 4.10)	
Peanut					
Consumption frequency					
< 1 time/ week	114	97.37	3.2	3.41 (2.98, 3.76)	
1-7 times/ week	81	100	3.44	3.45 (3.13, 4.03)	0.939
>7 times/week	34	100	3.41	3.48 (2.93, 4.21)	
Consumption amount					

< 1/4 cup	93	96.77	3.29	3.46 (3.00, 3.76)	
1/4 - 1/2 cups	101	100	3.14	3.23 (2.90, 3.73)	0.450
> 1/2 cup	38	100	3.73	3.93 (3.09, 5.06)	
Corn/rice-based drinks					
Consumption frequency					
Never	162	98.15	3.36	3.42 (3.13, 3.87)	
< 1 time/ week	62	100	3.14	3.43 (2.50, 4.05)	0.741
1 time/week	7	100	3.26	3.39 (1.49, 6.86)	
2 times/week	3	100	4.67	3.59 (3.48, 8.14)	
Consumption amount					
Never	160	98.12	3.34	3.42 (3.13, 3.87)	
< 1/4 cup	15	100	2.79	2.28 (1.95, 4.27)	0.042
1/4 cups-1/2 cup	35	100	2.95	3.39 (2.33, 3.66)	
>1/2 cup	20	100	4.21#	4.52 (3.59, 5.75)	

* Comparisons of AF-alb between categories were performed by the Duncan's multiple range test. The significant group was indicated in the geometric mean value (#).

** Values in the column are the level of aflatoxin B1-lysine (pg/mg albumin), otherwise stated.

Table 6.3. Food consumption patterns by ethnicity and education level

a. ethnicity

	Hispanic	Non-Hispanic White	Non-Hispanic Black/Others	p-value
Mexican food				
consumption frequency				
< 1item /week	79 (48.8)	46 (74.2)	7 (70.0)	
1 time/week	74 (45.7)	16 (25.8)	3 (30.0)	0.007
2-7 times/week	9 (5.6)	0 (0.0)	0 (0.0)	
Amount per sitting				
≤ 1 item /meal	8 (5.0)	5 (8.3)	3 (30.0)	
1-2 items/meal	94 (58.4)	40 (66.7)	5 (50.0)	0.019
>2 items/ meal	59 (36.7)	15 (25.0)	2 (20.0)	
corn				
Consumption frequency				
< 1item /week	88 (54.3)	44 (72.1)	6 (66.7)	
1 time/week	44 (27.2)	13 (21.3)	1 (11.1)	0.087
2-7 times/week	30 (18.5)	4 (6.6)	2 (22.2)	
Amount per sitting				
< 1 ear/meal	53 (35.8)	24 (42.9)	6 (66.7)	
1 ear/meal	69 (46.3)	29 (51.8)	3 (33.3)	0.058
>1 ear/meal	27 (18.1)	3 (5.4)	0 (0.0)	
corn tortillas				
Consumption frequency				
≤ 1 time/ week	66 (41.0)	44 (71.0)	6 (60.0)	
2-7 times/ week	92 (57.1)	18 (29.0)	4 (40.0)	0.002
>7 times/week	3 (1.9)	0 (0.0)	0 (0.0)	
Amount per sitting				
< 1 ear/meal	22 (13.7)	21 (34.4)	5 (50.0)	
1 ear/meal	81 (50.3)	32 (52.5)	4 (40.0)	0.0001
>1 ear/meal	58 (36.0)	8 (13.1)	1 (10.0)	
Corn/rice-based drinks				
Consumption frequency				
Never	97 (60.3)	54 (88.5)	9 (90.0)	
< 1 time/ week	57 (35.4)	5 (8.2)	1 (10.0)	0.003
1 time/week	5 (3.1)	2 (3.3)	0 (0.0)	

2 times/week	2 (1.2)	0 (0.0)	0 (0.0)	
Consumption amount				
Never	97 (61.0)	54 (88.5)	9 (90.0)	
< 1/4 cup	14 (8.8)	1 (1.6)	0 (0.0)	0.002
1/4 cups-1/2 cup	33 (20.8)	1 (1.6)	1 (10.0)	
>1/2 cup	15 (9.4)	5 (8.2)	0 (0.0)	

b. education

	High school or less	Associate degree or certificates	Bachelor's degree and above	p-value
Mexican foods				
Consumption frequency				
≤ 1 time/ week	23 (38.3)	68 (61.3)	41 (65.1)	
2-7 times/ week	31 (51.7)	40 (36.0)	22 (34.9)	0.003
>7 times/week	6 (10.0)	3 (2.7)	0 (0.0)	
Amount per sitting				
≤ 1 item /meal	1 (1.7)	9 (8.3)	6 (9.7)	
1-2 items/meal	34 (56.7)	70 (64.2)	35 (56.5)	0.178
>2 items/ meal	25 (41. 8)	30 (27.5)	21 (33.9)	
Corn				
Consumption frequency				
< 1item /week	26 (44.8)	58 (59.2)	37 (61.7)	
1 time/week	23 (39.7)	22 (22.5)	13 (21.7)	
2-7 times/week	9 (15.5)	18 (18.4)	10 (16.7)	0.144
Amount per sitting				
< 1 ear/meal	17 (30.4)	44 (54.9)	22 (36.7)	
1 ear/meal	25 (44.6)	44 (44.9)	32 (53.3)	
>1 ear/meal	14 (25.0)	10 (10.2)	6 (10.0)	0.056
Corn Tortillas				
Consumption frequency				
< 1 time/ weeks	47 (48.5)	31 (56.4)	12 (21.8)	
1-7 times/ weeks	49 (50.5)	24 (43.6)	41 (74.6)	0.002
>7 times/weeks	1 (1.0)	0 (0.0)	2 (3.6)	

Amount per sitting				
≤ 1 tortillas/ meal	3 (5.5)	12 (12.4)	8 (14.6)	
1-2 tortillas/meal	28 (50.9)	56 (57.7)	33 (60.0)	0.206
> 2 tortillas/meal	24 (43.6)	29 (29.9)	14 (25.5)	

*Data are n (%), otherwise stated. χ^2 test was performed for testing the interaction between education/ethnicity and food consumption pattern.

Table 6.4. Palmer Drought Severity Index (PDSI) for period February- July (2009, 2012, 2013) and corresponding levels of aflatoxin B_1-lysine

		Palmer Drought Severity Index[****] Pre-harvest season								**Aflatoxin B1-lysine (pg/mg albumin)**[**]					
Year	Location	Feb	Mar	Apr	May	June	July	Mean	n	%, >LOD	G.M	25th Pct	75th Pct	90th Pct	p-value
2007	Southcentral Texas	0.63	2.26	2.22	2.44	2.88	5.67	2.68	172	27	0.62	0.4	0.61	2.96	
	Corn Belt[***]	1.82	-0.5	-0.2	-0.6	-0.9	-1	-0.23							
2012	Southcentral Texas	-3.6	-2.6	-2.9	-2.7	-3.1	-2.5	-2.89	191	79	2.48	1.17	6.2	8.18	<0.0001[*]
	Corn Belt	-0.1	-1	-1.3	-1.9	-2.8	-4	-1.85							
2013	Southcentral Texas	-3.8	-4	-3.6	-3.7	-4.2	-4.3	-3.94	177	100	2.67	2.95	3.87	5.17	
	Corn Belt	0.52	0.44	1.84	2.6	2.82	2.57	1.8							

* Comparisons of AF-alb between years were performed by the Kruskal–Wallis.

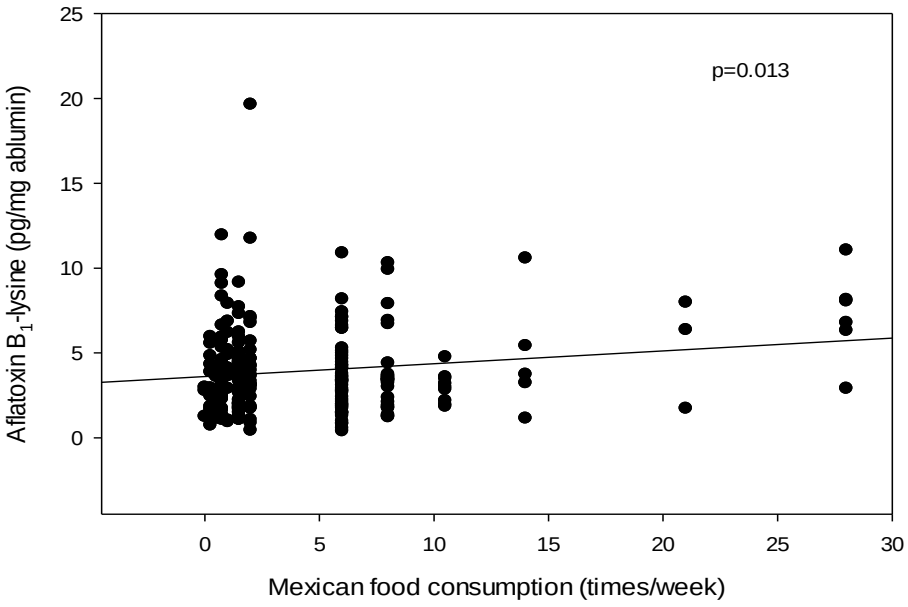
**The values in the column indicate either PDSI values or aflatoxin B_1-lysine (pg/mg albumin), otherwise stated. G.M. is a geometric mean.

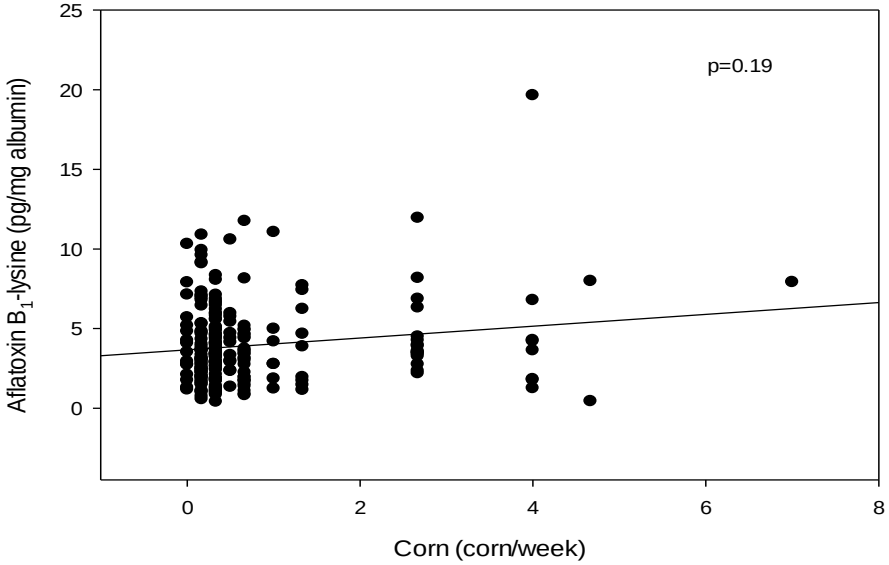
*** The boundary of the south-central Texas and primary corn/soybean belt regions were based on the NOAA U.S. Agricultural Belts definition (Accessed Oct 1, 2015). ****The PDSI accounts for a meteorological drought. It is calculated based on precipitation and temperature data, and Available Water Content (AWC) of the soil. The index values ranged from -6 (extreme drought) to 6(extreme wet).

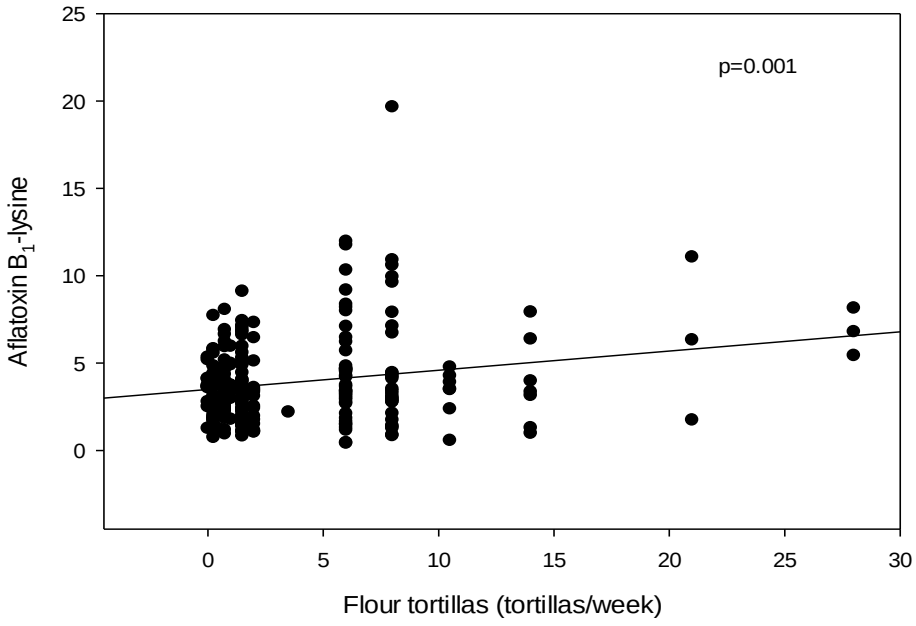
Table 6.5. Comparison of aflatoxin B1-lysine adducts among the residents of Bexar County in 2007-2008 and 2012-2014

Year	2007-2008	2012-2014
Study samples (n)	172	380
Positive samples (%)	27	90.5
Mean	1.12 ± 1.95	3.64 ± 2.82
Median	0.40	3.19
Range	0.40-16.57	0.40-19.65

***Data are aflatoxin B1-lysine (pg/mg albumin), otherwise stated**


p=0.013
Aflatoxin B1-lysine (pg/mg ablumin)
Mexican food consumption (times/week)
0
5
10
15
20
25
30

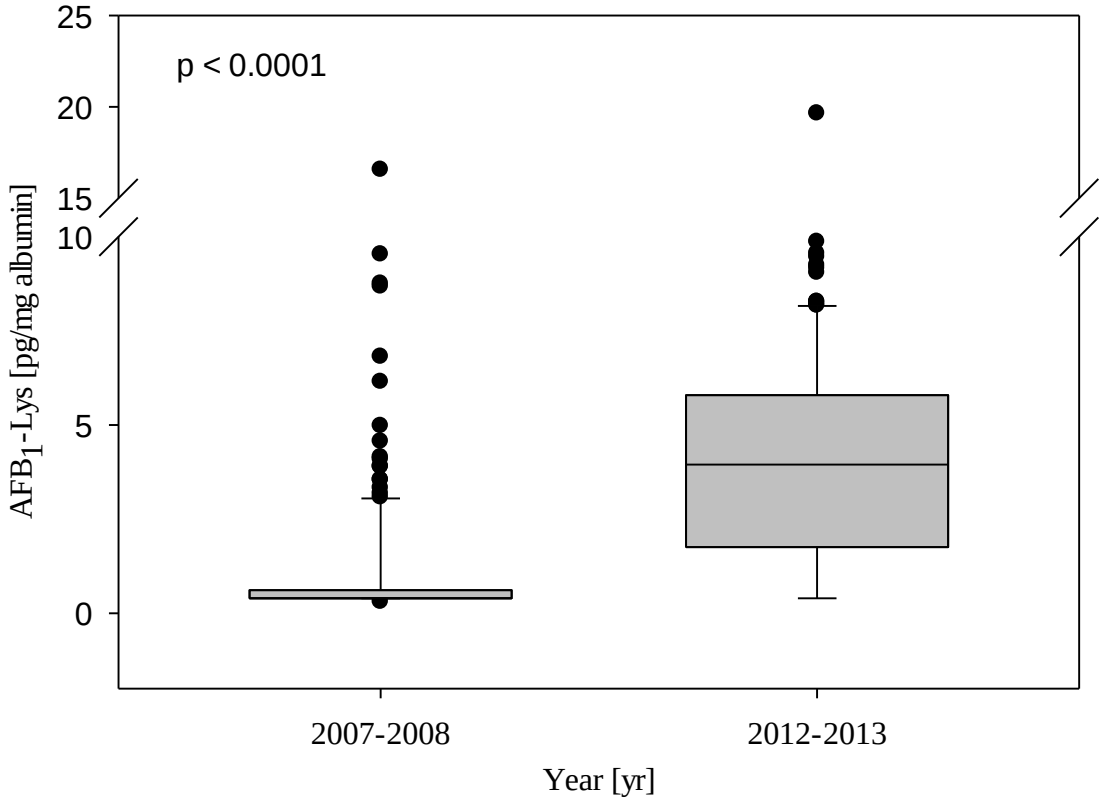

p=0.19
Aflatoxin B1-lysine (pg/mg albumin)
Corn (corn/week)
0
2
4
6
8



Graph 6.1. Linear regression of Aflatoxin B1-lysine with respect to Mexican food consumption and flour tortillas

* The amount of food consumptions per week was estimated by multiplying the frequency of food consumption and the amount of consumption per sitting.

** A linear regression is carried out for relating the level of aflatoxin B_1-lysine to the amount of Mexcian food, corn, and flour tortillas consumption per week. The p-value is obtained by linear regression ANOVA.



Graph 6.2. Comparison of aflatoxin B1-lysine levels in 2007-2008 and 2012-2014

* Comparisons of AF-alb between months were performed by the Wilcoxon Rank Sum test.

Reference

Adejumo O, Atanda O, Raiola A, Somorin Y, Bandyopadhyay R, Ritieni A. 2013. Correlation between aflatoxin m1 content of breast milk, dietary exposure to aflatoxin b1 and socioeconomic status of lactating mothers in ogun state, nigeria. Food Chem Toxicol 56:171-177.

Altekruse SF, Henley SJ, Cucinelli JE, McGlynn KA. 2014. Changing hepatocellular carcinoma incidence and liver cancer mortality rates in the united states. Am J Gastroenterol 109:542-553.

Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR, Daszak P. 2004. Emerging infectious diseases of plants: Pathogen pollution, climate change and agrotechnology drivers. Trends Ecol Evol 19:535-544.

Bardgett RD, De Deyn GB, Ostle NJ. 2009. Plant-soil interactions and the carbon cycle. Journal of Ecology 97:838-839.

Batis C, Hernandez-Barrera L, Barquera S, Rivera JA, Popkin BM. 2011. Food acculturation drives dietary differences among Mexicans, Mexican Americans, and Non-Hispanic Whites. J Nutr 141:1898-1906.

cancer-rates.info. 2014. Cancer registry, 2008-2012. Part January 2014:University of Kentucky.

Cardwell KF, Cotty PJ. 2002. Distribution of aspergillus section flavi among field soils from the four agroecological zones of the republic of benin, west africa. Plant Disease 86:434-439.

Cole RJ, Sanders TH, Hill RA, Blankenship PD. 1985. Mean geocarposphere temperatures that induce preharvest aflatoxin contamination of peanuts under drought stress. Mycopathologia 91:41-46.

Commission E. 2007. Adapting to climate change in europe– options for eu action.

Cotty PJ. 1997. Aflatoxin-producing potential of communities of aspergillus section flavi from cotton producing areas in the united states. Mycological Research 101:698-704.

Cotty PJ, Jaime-Garcia R. 2007. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. Int J Food Microbiol 119:109-115.

Cotty PJ, Jaime-Garcia R. 2007. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. Int J Food Microbiol 119:109-115.

Dobolyi C, Sebők F, Varga J, Kocsubé S, Szigeti G, Baranyi N, et al. 2013. Occurrence of aflatoxin producing aspergillus flavus isolates in maize kernel in hungary. Acta Alimentaria 42:451-459.

Dorner JW, Cole RJ, Sanders TH, Blankenship PD. 1989. Interrelationship of kernel water activity, soil temperature, maturity, and phytoalexin production in preharvest aflatoxin contamination of drought-stressed peanuts. Mycopathologia 105:117-128.

El-Serag HB. 2002. Hepatocellular carcinoma and hepatitis C in the United States. Hepatology 36: S74-S83.

Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, et al. 2012. Emerging fungal threats to animal, plant and ecosystem health. Nature 484:186-194.

Gillam C, Ingwersen J. 2013. Dog food recall underscores toxic danger in drought-hit u.S. Corn. Reuters Feb 25, 2013.

Harvell CD, Mitchell CE, Ward JR, Altizer S, Dobson AP, Ostfeld RS, et al. 2002. Climate warming and disease risks for terrestrial and marine biota. Science 296:2158-2162.

Hell K, Cardwell KF, Setamou M, Poehling H. 2000. The influence of storage practices on aflatoxin contamination in maize in four agroecological zones of benin, west africa. Journal of stored products research 36:365-382.

Hurburgh CR, Robertson AE, Hardy CL. 2012. Crop quality issues from the drought of 2012.Iowa State University.

Iha MH, Barbosa CB, Okada IA, Trucksess MW. 2013. Aflatoxin m 1 in milk and distribution and stability of aflatoxin m 1 during production and storage of yoghurt and cheese. Food Control 29:1-6.

IPCC. 2012. Managing the risks of extreme events and disasters to advance climate change adaptation. (A Special Report of Working Groups I and II of the Intergovernmental Panel on Climate Change). Cambridge,

UK and New York, NY, USA.

IPCC. 2013. 2013 fifth assessment report. (Climate Change 2013).

Johnson NM, Afriyie-Gyawu E, Huebner H, Marroquin-Cardona A, Robinson A, Tang L, et al. 2009. Pah exposure in a ghanaian population at high risk for aflatoxicosis. The Science of the total environment 407:1886-1891.

Jolly P, Jiang Y, Ellis W, Awuah R, Nnedu O, Phillips T, et al. 2006a. Determinants of aflatoxin levels in ghanaians: Sociodemographic factors, knowledge of aflatoxin

and food handling and consumption practices. Int J Hyg Environ Health 209:345-358.

Jolly P, Jiang Y, Ellis W, Awuah R, Nnedu O, Phillips T, et al. 2006b. Determinants of aflatoxin levels in ghanaians: Sociodemographic factors, knowledge of aflatoxin and food handling and consumption practices. Int J Hyg Envir Heal 209:345-358.

Kellner O, Niyogi D. 2014. Forages and pastures symposium: Assessing drought vulnerability of agricultural production systems in context of the 2012 drought. Journal of animal science 92:2811-2822.

Kočube Š, Varga J, Sigeti Đ, Baranji N, Šuri K, Tot B, et al. 2013. Aspergillus species as mycotoxin producers in agricultural products in central europe. Zbornik Matice srpske za prirodne nauke:13-25.

Kos J, Mastilović J, Hajnal EJ, Šarić B. 2013. Natural occurrence of aflatoxins in maize harvested in serbia during 2009–2012. Food Control 34:31-34.

Larsson SC, Wolk A. 2007. Overweight, obesity and risk of liver cancer: a meta-analysis of cohort studies. Br J Cancer 97:1005-1008.

Li W. 2013. Aspergillus flavus in corn ears from indiana under the drought conditions in 2012. Phytopathology 103:80-80.

Mc Millian WW, Wilson DM, Widstrom NW. 1985. Aflatoxin contamination of preharvest corn in georgia: A six-year study of insect damage and visible aspergillus flavus. Journal of Environmental Quality 14:200-202.

Medina A, Rodriguez A, Magan N. 2014. Effect of climate change on aspergillus flavus and aflatoxin b(1) production. Frontiers in microbiology 5:348.

Miraglia M, Marvin H, Kleter G, Battilani P, Brera C, Coni E, et al. 2009. Climate change and food safety: An emerging issue with special focus on europe. Food Chem Toxicol 47:1009-1021.

Mohd-Redzwan S, Jamaluddin R, Abd-Mutalib MS, Ahmad Z. 2013. A mini review on aflatoxin exposure in malaysia: Past, present and future. Frontiers in microbiology 4:334.

NOAA. Historical palmer drought indices. Available: http://www.ncdc.noaa.gov/temp-and-precip/drought/historical-palmers/ [accessed Oct 14 2015].

Odvody G, Spencer N, Remmers J. 1997. A description of silk cut, a stress-related loss of kernel integrity in preharvest maize. Plant disease 81:439-444.

Park DL. 2002. Effect of processing on aflatoxin. In: Mycotoxins and food safety:Springer, 173-179.

Paschos P, Paletas K. 2009. Non alcoholic fatty liver disease and metabolic syndrome. Hippokratia 13: 9-19.

Paterson RRM, Lima N. 2011. Further mycotoxin effects from climate change. Food Research International 44:2555-2566.

Piva G, Battilani P, Pietri A. 2006. Emerging issues in southern europe: Aflatoxins in italy.Wageningen Academic Publishers: Wageningen, The Netherlands, 139-153.

Plasencia J. 2004. Aflatoxins in maize: A mexican perspective. J Toxicol-Toxin Rev 23:155-177.

Qian G, Tang L, Liu W, Wang J-S. 2010a. Development of a non-antibody method for rapid detection of serum aflatoxin b1-lysine adduct. Toxicologist 114:1163.

Qian G, Tang L, Liu W, Wang J-S. 2010b. Development of a non-antibody method for rapid detection of serum aflatoxin b1-lysine adduct. In: Toxicologist, Vol. 114, 1163.

Qian G. 2012. Integrative toxicological evaluation of aflatoxin b_1 exposure in fischer 344 rats and biomarker based exposure assessment of aflatoxin b_1 in human populations. [electronic resource]:2012. https://getd.libs.uga.edu/pdfs/qian%5Fguoqing%5F201212%5Fphd.pdf

Robertson AE. 2013. Impacts of the midwest 2012 drought on aflatoxin contamination of maize. Phytopathology 103:175-175.

Romagnoli B, Menna V, Gruppioni N, Bergamini C. 2007. Aflatoxins in spices, aromatic herbs, herb-teas and medicinal plants marketed in italy. Food Control 18:697-701.

Sanchis V, Magan N. 2004. Environmental conditions affecting mycotoxins. Mycotoxins in food:174.

Shirima CP, Kimanya ME, Routledge MN, Srey C, Kinabo JL, Humpf HU, et al. 2015. A prospective study of growth and biomarkers of exposure to aflatoxin and fumonisin during early childhood in tanzania. Environmental Health Perspectives 123:173-178.

Shuaib FMB, Jolly PE, Ehiri JE, Ellis WO, Yatich NJ, Funkhouser E, et al. 2012. Socio-demographic determinants of aflatoxin b1-lysine adduct levels among pregnant women in kumasi, ghana. GHANA MEDICAL JOURNAL 46:179-188.

Siegel R, Ma J, Zou Z, Jemal A. 2014. Cancer statistics, 2014. CA: A Cancer Journal for Clinicians 64:9-29.

Singh BK, Bardgett RD, Smith P, Reay DS. 2010. Microorganisms and climate change: Terrestrial feedbacks and mitigation options. Nature Reviews Microbiology 8:779-790.

Solomon S, Qin D, Manning M, Alley RB, Berntsen T, Bindoff NL, et al. 2007. Climate change 2007-the physical science basis: Working group i contribution to the fourth assessment report of the ipcc. Cambridge and New York:Cambridge University Press.

Strosnider H, Azziz-Baumgartner E, Banziger M, Bhat RV, Breiman R, Brune MN, et al. 2006. Workgroup report: Public health strategies for reducing aflatoxin exposure in developing countries. Environ Health Perspect 114:1898-1903.

Tajkarimi M, Shojaee Aliabadi F, Salah Nejad M, Pursoltani H, Motallebi AA, Mahdavi H. 2007. Seasonal study of aflatoxin m1 contamination in milk in five regions in iran. Int J Food Microbiol 116:346-349.

Tóth B, Kótai É, Varga M, Pálfi X, Baranyi N, Szigeti G, et al. 2013. Climate change and mycotoxin contamination in central europe: An overview of recent findings. Review on Agriculture and Rural Development 2:461-466.

Viegas S, Veiga L, Malta-Vacas J, Sabino R, Figueredo P, Almeida A, et al. 2012. Occupational exposure to aflatoxin (AFB(1)) in poultry production. J Toxicol Environ Health A 75:1330-1340.

Wagacha JM, Muthomi JW. 2008. Mycotoxin problem in africa: Current status, implications to food safety and health and possible management strategies. Int J Food Microbiol 124:1-12.

Wood GE. 1992. Mycotoxins in foods and feeds in the United States. J Anim Sci 70:3941-3949.

Wotton HR, Strange RN. 1987. Increased susceptibility and reduced phytoalexin accumulation in drought-stressed peanut kernels challenged with aspergillus flavus. Applied and environmental microbiology 53:270-273.

CHAPTER 7

REDUCTION OF AFLATOXIN BIOAVAILABITY WITH ACCS100 IN AFLATOXIN EXPOSED SOUTH TEXAS POPULATIONS

Introduction

Aflatoxins(AFs) are the secondaryfungal metabolites of *Aspergillus* species. Major aflatoxin producers in the US, *Aspergillus flavus* and *A. parasiticus*, produce AFB_1, AFB_2, AFG_1, and AFG_2 in grains and cereals. Humans and animals are exposed to aflatoxins mainly through the consumption of AFs-contaminated grains and cereals or drinking of milk contaminated with AFM_1, a toxic metabolite of AFB_1. Inhalation exposure to AFs in occupational setting while loading or unloading the aflatoxin contaminated grains was also reported(Autrup et al. 1991; Desai and Ghosh 2003; Viegas et al. 2012; Viegas et al. 2015). Individual dietary pattern, ethnicity, weather during the harvest seasons. Acute exposure to AFs is associated to aflatoxicosis in humans and animals; the chronic exposure was linked to the risk of hepatocellular carcinoma (HCC), immune modulation, growth retardation.

The causal relation between aflatoxin B_1 (AFB_1) and HCC is well established (animal studies, human studies) (IARC 2002; 1993). Forty Percents of HCC in the Qidong, China where AFs exposure is common, is attributable to AFs exposure. Deep

understanding of the AFs metabolism enables to develop a reliable marker of AF exposure (Kensler et al. 2011), and those AF markers were used as the intermediate end points to assess the efficacy of chemoprevention(Egner et al. 2001; Kensler et al. 1998; Luo et al. 2006; Tang et al. 2008; Wang et al. 1999) . The age-adjusted incidence rate of HCC in United States is 6.60 (95% CI=6.56-6.64), whereas the age-adjusted incidence rate of HCC in Bexar County Texas was 13.8 (95% CI=13.0-14.6)(cancer-rates.info 2014). This difference may be attributable to the fact that prevalence of hepatitis C virus (HCV), 7.1% of the residents of the area, is 3.9 times higher than 1.8 %(Alter et al. 1999; N.M. Johnson et al. 2010), the prevalence in overall US population, and a large proportion of residents in the Bexar County consumes corn and corn-based diets(Batis et al. 2011). The previous study in the area reveal that the percent positive samples and average of aflatoxin B_1-lysine (AFB_1-Lys), was significantly higher than in the US general populations(Natalie M. Johnson et al. 2010; Schleicher et al. 2013), which may contribute the high incidence rate of HCC in the area

To reduce the exposure to AFs at a population level, chemoprevention and optimizing farming practice were currently applied in the high-risk population (Brown et al. 1991; Egner et al. 2001; PC Turner et al. 2005; Wang et al. 2008). ACCS100, previously known as Novasil clay is absorbing aflatoxins and reducing the bioavailability of AFs. Here, the randomized, double-blinded, placebo-controlled trial of ACCS100 was carried out in the risk population of AF exposure.

.

Materials and Methods

Study design and participants

Adult in good general health without history major chronic illness and with AFB_1-Lys ≥ 1 pg/mg albumin at baseline were randomized to three intervention arms: two tablets of placebo, 250 mg ACCS100, and 500 mg ACCS were administered three times daily. The trial included men and women and did not exclude hepatitis B surface antigen-positive and hepatitis C surface antigen-positive individuals with normal liver function. Study participants were recruited from a variety of public and residential sites in Bexar County, Texas which contains most of the population of the metropolitan area of the City of San Antonio, Texas from September 2012 to May 2014. The study was approved by the institutional review boards at the University of Texas Health Science Center at San Antonio (UTHSCSA) and Texas A&M University, and by the Protocol Review Committee of the Cancer Therapy and Research Center at UTHSCSA (*Clinicaltrials.gov* Identifier: NCT01677195) and by the Cancer Therapy and Research Center Protocol Review Committee. A medical history, physical examination, liver, hepatic, and renal function tests were used to screen the individuals, aged 18-77 years, at the first baseline visit. Individuals with a history of the uncontrolled chronic disease and women who were pregnant or breastfeeding were not allowed to participate. Two hundred and thirty-three eligible individuals (54 men and 179 women) from the screen group had AFB_1-Lys≥1.0 pg/mg albumin and were randomized. Sociodemographic and general health information was collected via a questionnaire administered at baseline including medical history and diet. Anthropometric data including age, race/ethnicity, height, weight, and vital signs were collected during the study.

Study Protocol

ACCS100 and placebo tablets containing 250 mg and 500 mg of ACCS100, and carbon carbonate were provided by Texas Enterosorbents, Inc. (Bastrop, TX). The trace elements in the material were examined to ensure compliance with federal and international standards. Dioxin and trace metals in ACCS100 were under the tolerable daily intake (TDI) or provisional tolerable daily intake (PTDI) (Marroquín-Cardona et al. 2011). Masking for the clinical trial was achieved by coating all tablets the same size and color capsules. The clinical intervention was a randomized, double-blind, placebo-controlled trial in which participants were randomly allocated to three groups (stratified on gender): High Dose (HD), Low Dose (LD) and Placebo (PL). The HD group received two 500 mg ACCS100 capsules before each meal, and the LD group received two capsules containing 250 mg ACCS100 and 250 mg calcium carbonate placebo each, whereas the PL group received the same size and color capsules of 500 mg calcium carbonate. Dosed were derived based on previous titrations with parent clay (NovaSil) *in vivo*, whereby 0.5% w/w NovaSil significantly diminished the toxicity of very high levels of AFs in the feed of experimental animals (ranging from 2.0–7.5 ppm). The protection was also significant at half the concentration; i.e., 0.25% (Phillips et al. 2002; Pimpukdee et al. 2004). The medication compliance using pill count was recorded during each visit. Adverse effects were carefully monitored throughout the trial. Daily worksheets and symptom checklists were provided to study participants as assessment tools for adverse events monitoring and were completed two times daily after ingestion of each treatment dose.

Blood was collected from participants during screening (Visit 1) at the community recruitment sites. Subsequent blood and urine specimens were obtained at

baseline (Visit 2), at weeks 4 (Visit 3), 12 (Visit 4), and 16 (Visit 5) conducted at UTHSCSA (Figure 7.2). A portion of the blood was centrifuged within 1h of the collection to separate serum, and stored at –80°C, then shipped to the University of Georgia for AFB_1-lysine adduct analysis. Aliquots were also sent to LabCorp (San Antonio, Texas) for complete blood count with differential and comprehensive metabolic profile. Premenopausal women provided urine for hCG pregnancy tests at Visits 1–3. Additionally, these participants have dispensed a pregnancy test at Visit 3 with instructions to test at 8 weeks (between Visits 3 and 4).

Materials for Laboratory Analysis

AFB1 (>98% purity), albumin determination reagent, bromocreosol purple, and normal human serum were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Protein assay dye reagent concentrate and protein standards were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). Pronase (25kU, Nuclease-free) was purchased from Calbiochem (La Jolla, CA). Mixed mode solid phase extraction (SPE) cartridges were purchased from the Waters Corp. (Milford, MA). Authentic AFB-Lys was synthesized as previously described (Sabbioni et al. 1987). The authentic AFB-Lys standard was purified and characterized by UV absorption and mass spectrometry (Sabbioni et al. 1987; Wang et al. 2001a). All other chemicals and solvents used were of the highest grade commercially available.

Measurement of Serum AFB-Lys Adduct

Serum samples were coded separately and analyzed with a newly developed HPLC-fluorescence method (Qian 2012). Briefly, thawed human serum samples were heated to 56°C for 30 min to inactivate infectious microbial agents. Total protein and albumin concentrations were determined using a modification of a previously described method (Wang et al. 1996). A portion of each serum sample (150 μL) were digested by pronase (pronase:total protein, 1:4, w:w) at 37 °C for 3 h to release AFB-Lys adduct. The serum sample digests were subjected to solid phase extraction (SPE) clean-up using Waters MAX SPE cartridges, which had been conditioned with methanol and equilibrated with water before loading with the AFB-Lys containing digests. The loaded cartridges were sequentially washed by water, 70% methanol, and 1% ammonium hydroxide in methanol at a flow rate of 1 ml/min. Purified AFB-Lys was eluted with 2% formic acid in methanol. The eluent was vacuum-dried with a Labconco Centrivap Concentrator (Kansas City, MO) and reconstituted for HPLC-fluorescence detection. The analysis of AFB-Lys adduct was conducted in an Agilent 1200 HPLC-fluorescence system (Santa Clara, CA). The mobile phases consist of buffer A (20 mM $NH_4H_2PO_4$, pH 7.2) and buffer B (100% methanol). The Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 x 250 mm) equipped with a guard column was used. Column temperature was maintained at 25°C during analysis, and a volume of 100 μL was injected into HPLC. The flow rate was kept at 1 mL/min. A gradient was generated to separate the AFB-Lys adduct within 25 min of injection. AFB-Lys adduct was detected by fluorescence at maximum excitation and emission wavelengths of 405 nm and 470 nm, respectively. Calibration curves of authentic standard were generated weekly, and the

standard AFB-Lys was eluted at approximately 13.1 min. Quality assurance and quality control procedures were conducted during analyzes, which include analysis of one authentic standard and a quality control sample daily in same sequence. The limit of detection was 0.4 pg mg^{-1} albumin. The average recovery rate with various spiked concentrations of AFB-Lys adduct standard was 90%. The serum AFB-Lys concentration was normalized to albumin content for statistical analysis and report. The technicians who perform the analyzes had no information about sample sources, which guarantees the confidentiality.

Statistical analysis

All efficacy analyzes were conducted using as per-protocol analysis for randomized subjects who received and fulfilled the protocol. The sample size was determined based on a comparison of quantitative reduction from baseline to 3-month adduct levels of at least 20% in the high dose arm compared to 0% in the placebo arm with 80% power and a two-sided α=0.05.

At each time point, a trend test (nonparametric test for trend across ordered groups) was performed to test the hypothesis that the reduction of AFB_1-Lys in serum increased with ACCS100 dose. To evaluate the overall treatment effects, log mixed-effect models for serum AFB_1-lysine adducts were constructed. The model included the intercept, indicators for treatment group, time, and a treatment by time interaction term as fixed effect terms. Then, individual-level intercept and time variables were included as random effects. The final mixed model was fitted using PROC MIXED in SAS software (Brown and Prescott 2009). Parameters of the mixed model were estimated using the Maximum Likelihood Estimation (MLE) method. The Akaike Information Criteria (AIC)

and the Bayesian Information Criteria (BIC), where smaller values for both are considered more preferable, were used as measures of the relative qualities of particular models. Both AIC and BIC dealt with the trade-off between the goodness of fit of the model and the complexity of the model, and thus provided valid means for model selection (Egner et al. 2014). The separate analyzes at different time points were conducted using the Kruskal-Wallis test. Hypothesis tests were two-tailed and assumed an α=0.05. All analyzes were conducted in SAS 9.4 (SAS Institute, Cary, NC, USA). Statistical analysis of all other data was conducted using JMP 10 software (SAS Institute, NC). Analysis of variance (ANOVA) and Tukey's tests were conducted on all demographic information for comparisons among treatment groups. Adverse events data was analyzed using a χ^2 test. A two-sided p-value $\leq$ 0.05 was considered statistically significant.

Results

Two hundred and thirty-four participants were randomized into three groups: high dose group (n=71), low dose group (n=83), and placebo group (n=80). Among them, 62.8% (147/234) of participants completed 3-months intervention. As shown in Figure 7.1., 27, 33, and 27 participants in placebo, low dose, and high dose groups, respectively, were failed to complete the trials. The incompletion was mainly due to lose on follow-up, which is irrelevant to adverse health effect of regimens. The protocol adherence was followed by counting the number of pills taken, and the overall adherence was 85.1%. At

the baseline, the three groups were not significantly different with respect to the level of AFB1-Lys, sex, education, ethnicity, and income (Table 7.1).

A total of 588 serum samples collected over a 4-month study period were analyzed for AFB_1-Lys adducts. Average levels and 95% CI of serum AFB_1-Lys adducts in three study groups, placebo, 1.5 g ACCS100/day, and 3 g ACCS100/day at different time points are shown in Table 7. 2. The distributions of overall AFB_1-Lys with respects to dose and time were shown in Figure 7.3 and 7.4, respectively. There were no significant differences in serum AFB_1-Lys adduct level by the dose of ACCS100 (Figure 7.3).The time effects of ACCS100 intervention on serum AFB1-Lys was significant at low dose group ($p \leq 0.001$); however, the time effect was not significant in placebo and high dose group. The results of the mixed model regression analysis showed a significant association between AFB_1-lysine adduct level and time ($p<0.001$) but not for the treatment group × time interaction term ($p=.722$). The model-adjusted means are shown in Figure 7.5 for the three treatment groups over time.

Discussion

The incidence of HCC among the South Texas population is much higher than US general population, and the incidence has risen (Altekruse et al. 2009; Altekruse et al. 2014; El-Serag et al. 2003; Ramirez et al. 2012; Ryerson et al. 2016). Several risk factors may attribute the higher incidence of HCC such as hepatitis C virus (HCV), obesity, lifestyle, and aflatoxin exposure (N. M. Johnson et al. 2010; Ramirez et al. 2012). The participants in this study were recruited a metropolitan area of Bexar County, TX, and incidence of HCC in the Bexar County has risen in several zipcode. The previous study reveals the exposure to poly aromatic hydrocarbons(PAH), and aflatoxins may attribute the burden of HCC. The current study revealed that the 84.0 % (316/376) of screened participants had detectable level of AFB_1-Lys in serum and the means of 3.59 (95% CI=3.31-3.86). Along with other risk factors, aflatoxin exposure possibly plays a significant role in the burden of HCC among South Texas populations.

To reduce the aflatoxin exposure at a population level, various intervention strategies have applied to the populations at risk of aflatoxin exposure: optimization of storage and harvest practice, biological control using atoxigenic strains, primary prevention through entero- sorbent, and chemoprevention (Egner et al. 2001; Kaaya et al. 2005; Thomas W Kensler et al. 1998; Luo et al. 2006; Perrone et al. 2014; Tang et al. 2008; P Turner et al. 2005; Wang et al. 1999; Wang et al. 2008). ACCS100, previously known as novasil clay, reduces bioavailability of aflatoxins by adsorbing aflatoxins. The cost and application allow this intervention to populations at risk of aflatoxin exposure in developing counties. Our previous studies showed the clay model significantly reduced the bioavailability of aflatoxins in the populations at developing county and animals (Pimpukdee et al. 2004;

Wang et al. 2008). The level of AFB1-Lys was significantly decreased after 3 months interventions with 1.5g of ACCS100. However, the mixed model analysis exhibited that the reduction of AFB1-Lys was not in a dose-dependent manner. This discrepancy may attribute to a different level of aflatoxin exposure. The study populations of our previous study in Ghana had the mean level of AFB1-Lys, 685 pg/mg albumin, and range from 438.7 pg/mg albumin to 1339.0 pg/mg albumin at the baseline. However, our current study population had the mean level of AFB1-Lys, 3.59 pg/mg albumin, and a range from 0.4 pg/mg albumin to 19.65 pg/mg albumin. Besides, our current study population in U.S. may have more diverse food intakes than the Ghanaian populations. Consumption of leafy green is linked to chlorophyll intake, which is also entero- sorbent and linked to the decrease of bioavailability of aflatoxins.

The level of AFB1-Lys in the residents of Bexar County, Texas was significant, and the aflatoxin exposure may play a role in the increasing incidence of HCC in the area. This study showed a minor evidence that 1.5 g of ACCS100 daily intake reduced the level of AFB1-Lys in Bexar County residents. However, the reduction was independent of dose of ACCS100, whereas the novasil clay, the parent model of ACCS100, reduced the level of AFB1-Lys in Ghanaians population. The discrepancy may attribute to the different level of aflatoxin exposure in two populations and dietary diversity in the U.S, developed country. Careful follow-up of dietary pattern in the study population may evaluate the efficacy of ACCS100 more successfully. Also, it is possible that ACCS100 can efficiently reduce the bioavailability of aflatoxins in a population with high level of aflatoxin exposure.

Table 7.1. Demographic characteristics of trial participants (completed)

	Treatment Group		
	Placebo	Low Dose (250 mg ACCS/day)	High Dose (500 mg ACCS/day)
N	53	50	44
Sex			
Male	10 (18.9)	10 (20.0)	14 (31.8)
Female	43 (81.1)	40 (80.0)	30 (68.2)
Age			
Ethnicity			
Hispanic	36 (67.9)	33 (66.0)	29 (65.9)
Non-Hispanic White	15 (28.3)	14 (28.0)	14 (31.8)
Non-Hispanic Black/Others	2 (3.8)	3 (6.0)	1 (2.3)
Education			
High school or less	10 (18.9)	11 (22.0)	12 (27.3)
Associate degree or certificates	27 (50.9)	24 (48.0)	17 (38.6)
Bachelor or above	16 (30.2)	15 (30.0)	15 (34.1)
Income			
<$20,000	21 (42.9)	23 (47.9)	18 (41.9)
$20,000-$50,000	14 (28.6)	11 (22.9)	16 (37.2)
>$50,000	14 (28.6)	14 (29.2)	9 (20.9)
Body weight, lbs	189.2 ± 58.1	188.2 ± 46.9	173.9 ± 32.9
Height, inches	65.0 ± 4.4	64.9 ± 3.7	64.6 ± 4.0

*Data in the column are 'n(%)'.

Table 7.2 . Levels of serum AFB_1-lysine at different time points in every group with various ACCS100 treatments

Treatment	AFB1-Lys adducts (pg/mg albumin)			
	Baseline	1-Month	3-Months	4-Months
Placebo	4.26 ± 3.21	3.05 ± 1.69	3.24 ± 1.87	3.02 ± 1.74
	(3.37, 5.14)	(2.58, 3.51)	(2.72, 3.75)	(2.54, 3.50)
Low Dose (1.5g/day)	3.77 ± 2.55	3.00 ± 1.32	2.87 ± 1.54	2.78 ± 1.45
	(3.00, 4.55)	(2.60, 3.40)	(2.40, 3.34)	(2.34, 3.22)
High Dose (3.0 g/day)	3.96 ± 2.05	2.64 ± 1.09	2.70 ± 1.37	2.99 ± 1.27
	(3.37, 4.54)	(2.33, 2.95)	(2.31, 3.09)	(2.63, 3.35)

* data in the column are 'mean ± S.D. (95% CI)

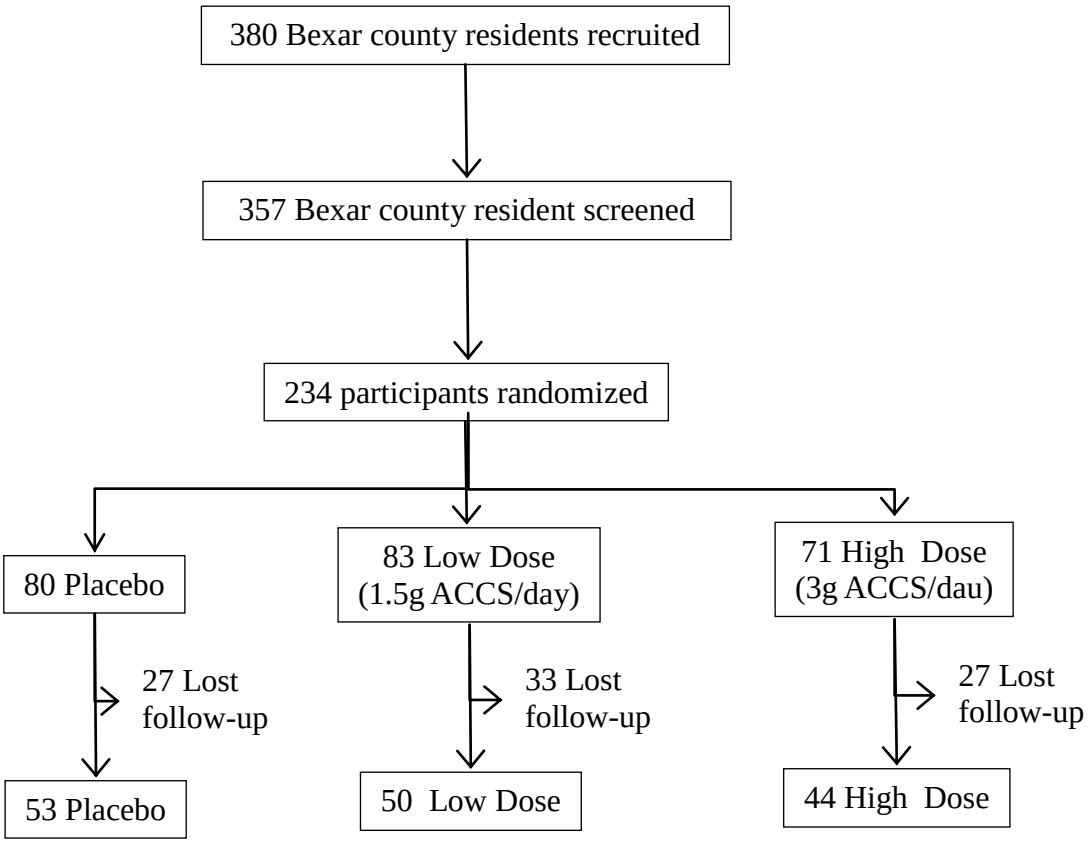


Figure 7.1. Overall study design of the ACCS intervention trial.

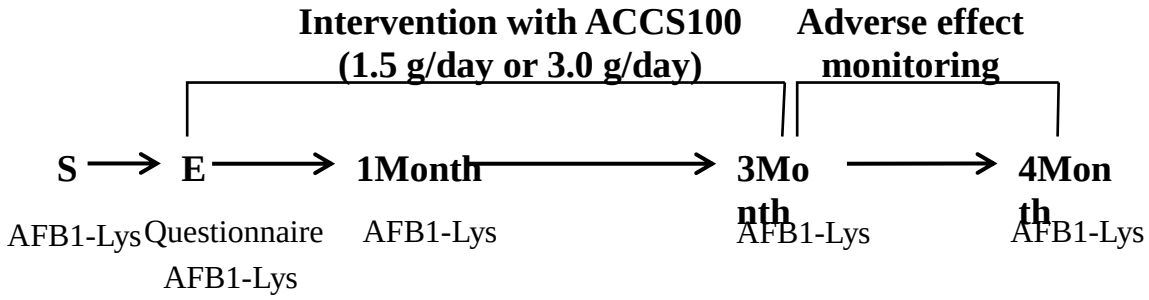


Figure 7.2. Scheme for the Phase II ACCS100 chemoprevention trials

*S, screening blood collection for biomarker and informed consent; E, enrollment (questionnaire and blood collection for biomarker); 1-4 months, blood collection for biomarker and adverse event monitoring.

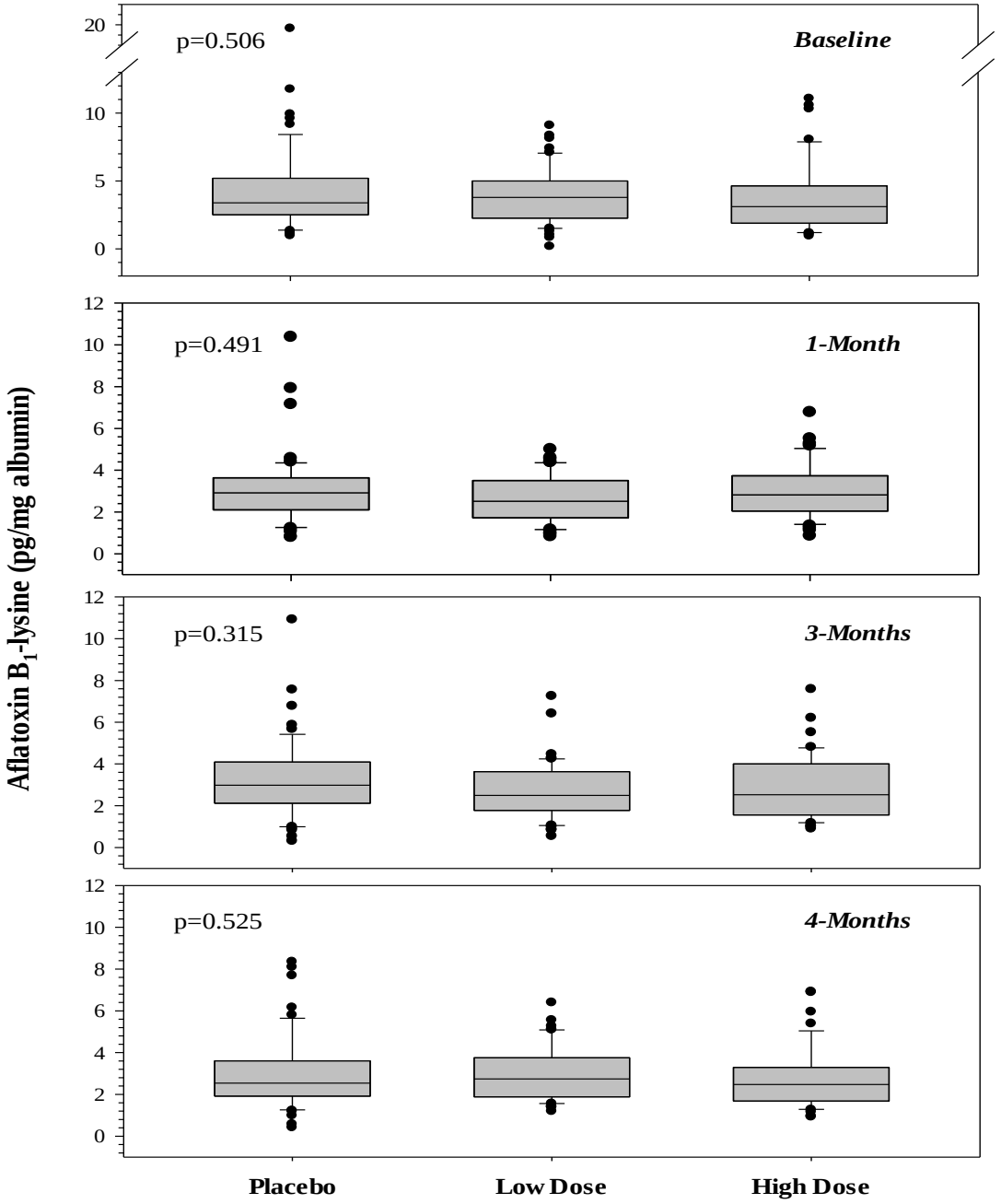


Figure 7.3. The dose effects of ACCS100 intervention on serum AFB_1-Lys adduct levels over the study duration. The box plots show distributions of AFB1-albumin adduct levels in each group at each time point. The box values ranged from 25 to 75 percentile of the total samples, the line within it indicating the median value. The bars on both sides of a box represent values ranging from 5 to 25 percentile and from 75 to 95 percentile, respectively.

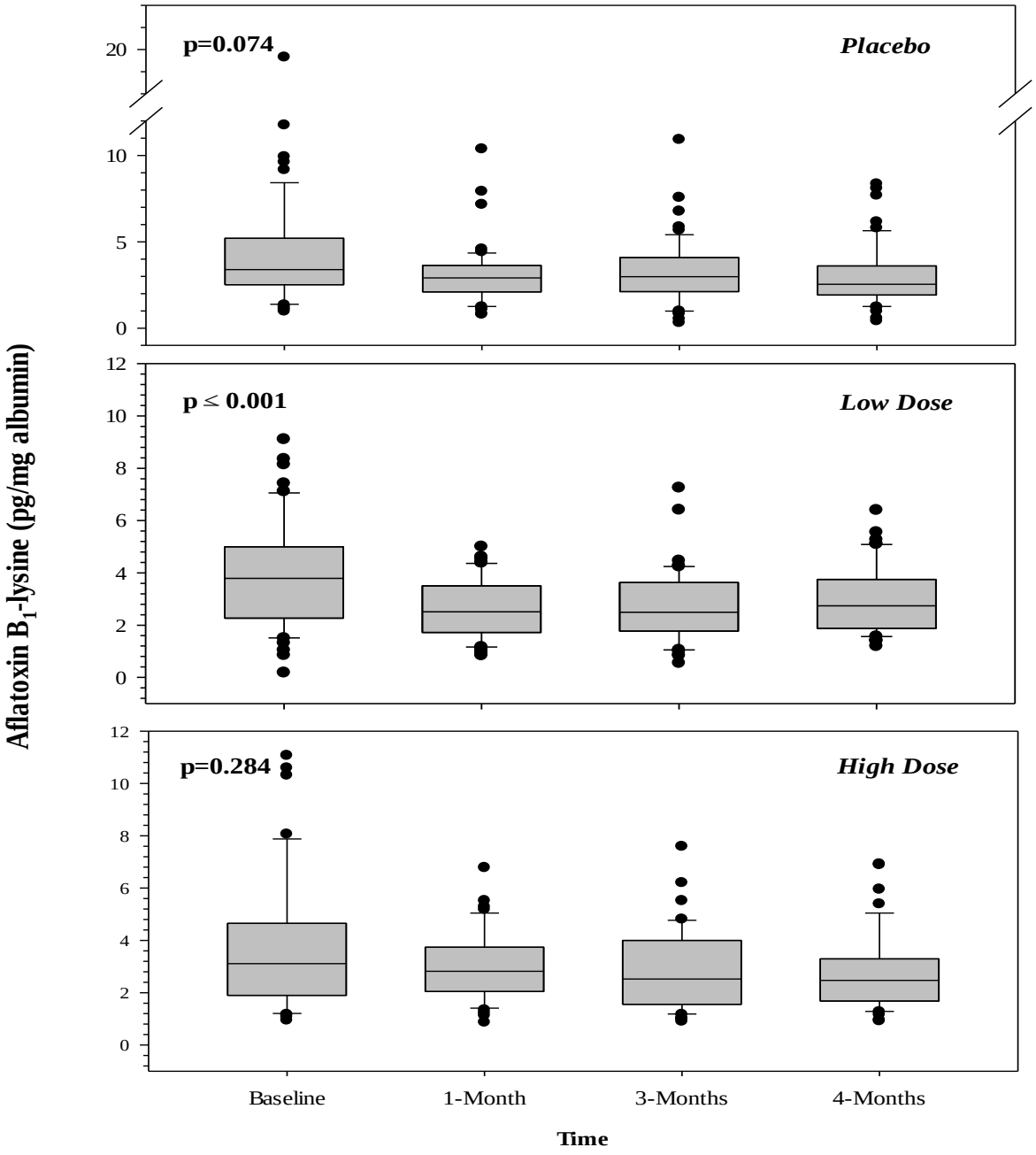


Figure 7.4. The time effects of ACCS100 intervention on serum AFB_1-albumin adduct levels over the study duration. The box plots show distributions of AFB1-albumin adduct levels in each group at each time point. The box values ranged from 25 to 75 percentile of the total samples, the line within it indicating the median value. The bars on both sides of a box represent values ranging from 5 to 25 percentile and from 75 to 95 percentile, respectively.

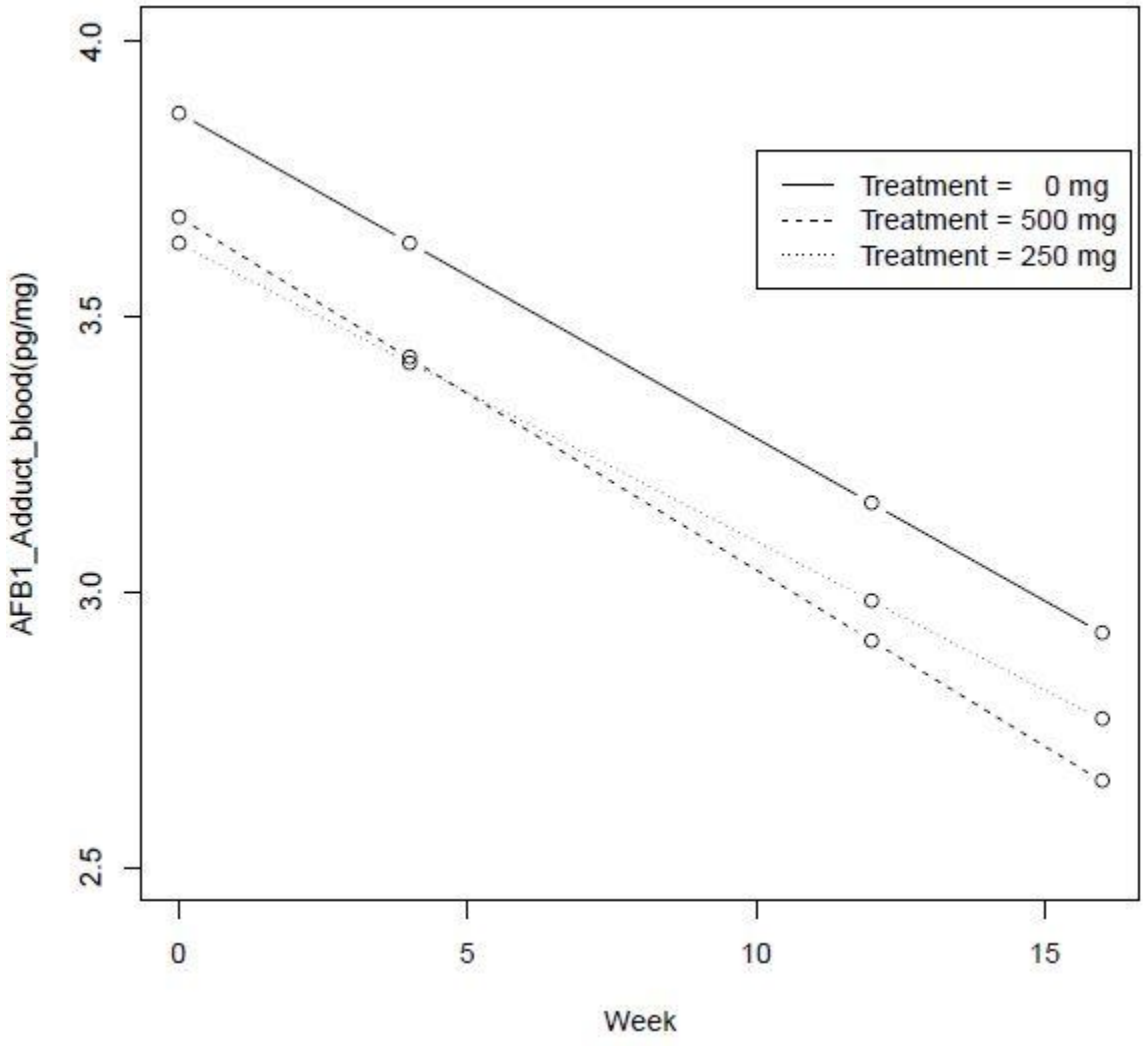


Figure 7.5. Log mixed-effect regression model estimated AFB_1-lysine adduct (pg/mg albumin) levels for each treatment group over time

References

Altekruse SF, McGlynn KA, Reichman ME. 2009. Hepatocellular carcinoma incidence, mortality, and survival trends in the united states from 1975 to 2005. J Clin Oncol 27:1485-1491.

Altekruse SF, Henley SJ, Cucinelli JE, McGlynn KA. 2014. Changing hepatocellular carcinoma incidence and liver cancer mortality rates in the united states. Am J Gastroenterol 109:542-553.

Alter MJ, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, et al. 1999. The prevalence of hepatitis c virus infection in the united states, 1988 through 1994. New England journal of medicine 341:556-562.

Autrup JL, Schmidt J, Seremet T, Autrup H. 1991. Determination of exposure to aflatoxins among danish workers in animal-feed production through the analysis of aflatoxin b_1 adducts to serum albumin. Scandinavian journal of work, environment & health:436-440.

Batis C, Hernandez-Barrera L, Barquera S, Rivera JA, Popkin BM. 2011. Food acculturation drives dietary differences among mexicans, mexican americans, and non-hispanic whites. The Journal of Nutrition 141:1898-1906.

Brown H, Prescott R. 2009. Applied mixed models in medicine (statistics in practice), 2nd edition. New York:John Wiley & Sons.

Brown RL, Cotty PJ, Cleveland TE. 1991. Reduction in aflatoxin content of maize by atoxigenic strains of aspergillus-flavus. Journal of food protection 54:623-626.

cancer-rates.info. 2014. Cancer registry, 2008-2012. Part January 2014:University of Kentucky.

Desai MR, Ghosh SK. 2003. Occupational exposure to airborne fungi among rice mill workers with special reference to aflatoxin producing a. Flavus strains. Annals of Agricultural and Environmental Medicine 10:159-162.

Egner PA, Wang JB, Zhu YR, Zhang BC, Wu Y, Zhang QN, et al. 2001. Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer. Proc Natl Acad Sci U S A 98:14601-14606.

Egner PA, Chen JG, Zarth AT, Ng D, Wang J, Kensler KH, et al. 2014. Rapid and sustainable detoxication of airborne pollutants by broccoli sprout beverage: Results of a randomized clinical trial in china. Cancer Prev Res 7:813-823.

El-Serag HB, Davila JA, Petersen NJ, McGlynn KA. 2003. The continuing increase in the incidence of hepatocellular carcinoma in the united states: An update. Ann Intern Med 139:817-823.

Johnson NM, Qian G, Xu L, Tietze D, Marroquin-Cardona A, Robinson A, et al. 2010. Aflatoxin and pah exposure biomarkers in a u.S. Population with a high incidence of hepatocellular carcinoma. Sci Total Environ 408:6027-6031.

Johnson NM, Qian G, Xu L, Tietze D, Marroquin-Cardona A, Robinson A, et al. 2010. Aflatoxin and pah exposure biomarkers in a u.S. Population with a high incidence of hepatocellular carcinoma. Science of the Total Environment 408:6027-6031.

Johnson NM, Qian G, Xu L, Tietze D, Marroquin-Cardona A, Robinson A, et al. 2010. Aflatoxin and pah exposure biomarkers in a u.S. Population with a high incidence of hepatocellular carcinoma. The Science of the total environment 408:6027-6031.

Kaaya AN, Warren HL, Kyamanywa S, Kyamuhangire W. 2005. The effect of delayed harvest on moisture content, insect damage, moulds and aflatoxin contamination of maize in mayuge district of Uganda. Journal of the science of food and agriculture 85:2595-2599.

Kensler TW, Roebuck BD, Wogan GN, Groopman JD. 2011. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. Toxicol Sci 120:S28-S48.

Kensler TW, Groopman JD, Roebuck BD. 1998. Use of aflatoxin adducts as intermediate endpoints to assess the efficacy of chemopreventive interventions in animals and man. Mutat Res-Fund Mol M 402:165-172.

Kensler TW, He X, Otieno M, Egner PA, Jacobson LP, Chen B, et al. 1998. Oltipraz chemoprevention trial in qidong, people's republic of china: Modulation of serum aflatoxin albumin adduct biomarkers. Cancer Epidem Biomar 7:127-134.

Luo HT, Tang LL, Tang M, Billam M, Huang TR, Yu JH, et al. 2006. Phase iia chemoprevention trial of green tea polyphenols in high-risk individuals of liver cancer: Modulation of urinary excretion of green tea polyphenols and 8-hydroxydeoxyguanosine. Carcinogenesis 27:262-268.

Marroquín-Cardona A, Deng Y, Garcia-Mazcorro J, Johnson N, Mitchell N, Tang L, et al. 2011. Characterization and safety of uniform particle size novasil clay as a potential aflatoxin enterosorbent. Applied clay science 54:248-257.

Perrone G, Gallo A, Logrieco AF. 2014. Biodiversity of aspergillus section flavi in europe in relation to the management of aflatoxin risk. Frontiers in microbiology 5:377.

Phillips TD, Lemke SL, Grant PG. 2002. Characterization of clay-based enterosorbents for the prevention of aflatoxicosis. Adv Exp Med Biol 504:157-171.

Pimpukdee K, Kubena LF, Bailey CA, Huebner HJ, Afriyie-Gyawu E, Phillips TD. 2004. Aflatoxin-induced toxicity and depletion of hepatic vitamin a in young broiler chicks: Protection of chicks in the presence of low levels of novasil plus in the diet. Poultry science 83:737-744.

Qian G. 2012. Integrative toxicological evaluation of aflatoxin b_1 exposure in fischer 344 rats and biomarker based exposure assessment of aflatoxin b_1 in human populations. [electronic resource]:2012. https://getd.libs.uga.edu/pdfs/qian_guoqing_201212_phd.pdf

Ramirez AG, Weiss NS, Holden AE, Suarez L, Cooper SP, Munoz E, et al. 2012. Incidence and risk factors for hepatocellular carcinoma in texas latinos: Implications for prevention research. PloS one 7:e35573.

Ryerson AB, Eheman CR, Altekruse SF, Ward JW, Jemal A, Sherman RL, et al. 2016. Annual report to the nation on the status of cancer, 1975-2012, featuring the increasing incidence of liver cancer. Cancer.

Schleicher RL, McCoy LF, Powers CD, Sternberg MR, Pfeiffer CM. 2013. Serum concentrations of an aflatoxin-albumin adduct in the national health and nutrition examination survey (nhanes) 1999-2000. Clinica chimica acta; international journal of clinical chemistry 423:46-50.

Tang LL, Tang M, Xu L, Luo HT, Huang TR, Yu JH, et al. 2008. Modulation of aflatoxin biomarkers in human blood and urine by green tea polyphenols intervention. Carcinogenesis 29:411-417.

Turner P, Sylla A, Gong Y, Diallo M, Sutcliffe A, Hall A, et al. 2005. Reduction in exposure to carcinogenic aflatoxins by postharvest intervention measures in west africa: A community-based intervention study. The Lancet 365:1950-1956.

Turner PC, Sylla A, Gong YY, Diallo MS, Sutcliffe AE, Hall AJ, et al. 2005. Reduction in exposure to carcinogenic aflatoxins by postharvest intervention measures in west africa: A community-based intervention study. Lancet 365:1950-1956.

Viegas S, Veiga L, Malta-Vacas J, Sabino R, Figueredo P, Almeida A, et al. 2012. Occupational exposure to aflatoxin (afb1) in poultry production. Journal of Toxicology and Environmental Health, Part A 75:1330-1340.

Viegas S, Veiga L, Figueiredo P, Almeida A, Carolino E, Viegas C. 2015. Assessment of workers' exposure to aflatoxin b1 in a portuguese waste industry. Annals of Occupational Hygiene 59:173-181.

Wang J-S, Shen X, He X, Zhu Y-R, Zhang B-C, Wang J-B, et al. 1999. Protective alterations in phase 1 and 2 metabolism of aflatoxin b1 by oltipraz in residents of qidong, people's republic of china. Journal of the National Cancer Institute 91:347-354.

Wang JS, Qian GS, Zarba A, He X, Zhu YR, Zhang BC, et al. 1996. Temporal patterns of aflatoxin-albumin adducts in hepatitis b surface antigen-positive and antigen-negative residents of daxin, qidong county, people's republic of china. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 5:253-261.

Wang P, Afriyie-Gyawu E, Tang Y, Johnson NM, Xu L, Tang L, et al. 2008. Novasil clay intervention in ghanaians at high risk for aflatoxicosis: Ii. Reduction in biomarkers of aflatoxin exposure in blood and urine. Food Addit Contam 25:622-634.

CHAPTER 8.

CONCLUSIONS

AFB_1 is a group 1 carcinogen by IARC. An extensive number of studies have carried out to solve the collection between AFB1 exposure and liver cancer. More than a hundred countries set a regulatory level for AFB1. Regardless of the efforts by curbing the AF contamination, cases of human aflatoxicosis in rural Kenya and Malaysia were reported. Because AFB1 occurs naturally, complete eradication of AFB1 in the food system is difficult. Besides, global warming changes the maps of the aflatoxins affected areas. Southern Europe, and the United States, where aflatoxin exposure was not frequent, experienced the high level of aflatoxin exposure in ecosystem and humans (Chap 6). The population of southern Uganda and south-central Texas chronically exposed to aflatoxins although the level of exposure is not significantly high (Chap3 and Chap6). To prevent the adverse effects of the chronic AF exposure, an intervention with ACCS100 (enterosorbent; adsorbing aflatoxins) was carried out in Bexar County, Texas (south-central Texas) (Chap7).

Hepatic toxicity of aflatoxins is well-known through extensive research. However, the effects of aflatoxins in other organs need more research: how aflatoxins affect immunity, how it interacts with malnutrition, and how it affects the developing immune system or development. Thirty years ago, Dr. Hendricks observed children in sub-Saharan Africa suffer more severe HIV infection and the rate of infection compared to the children living other parts of the world, and he related it to chronic exposure to AFs.

Based on the idea, this dissertation is further studied whether the odds of HIV infection increases on AF exposure and whether the HIV progression is more severe with AF exposure (Chap 4). The progression of HIV to AIDS was faster on the level of AFB1-Lys in serum. The odds of HIV was higher with respect to AFB1-Lys. However, the increased odds with an increment of ln(AFB1-Lys) may be affected by unmeasured confoundings due to the nature of the case-control study. For this reason, this study may need to carry out again with the better study design to test the effect of aflatoxin B1 on the infectivity of the agent. Furthermore, the cause of faster progression of HIV with respect to aflatoxins nees to be investigated.

Our unpublished data indicated that the level of AFB_1 was significantly higher in Ugandan infected with both Tuberculosis and HIV thang HIV infected population. In disregard of TB drug use, the level of AFB1-Lys indicated the predominant exposure to AFB_1.Our previous study using F344 rats indicated TNF-α and IFN-γ expression in spleen were altered (Qian et al. 2014). TNF-α and IFN-γ are known to have a critical role in resisting to the infection with *M. Tuberculosis*. Furthermore, it is known that about 90% of *M. tuberculosis* infection is latent and called latent tuberculosis infection. Only 5~10% of tuberculosis infected people will develop active tuberculosis. Risk factors associated with the reactivation are alcohol use, tobacco smokers, underweight, silica exposure, steroid treatment, rheumatologic conditions, indigenous populations, and cancer patients. Other study indicated that aflatoxin exposure can elicit the inflammatory response. It may be possible that aflatoxin exposure play a role in the activation of latent tuberculosis to active. So far, there is no study illucidated the roles of aflatoxins in acqusition of Tuberculosis and activation of latent tuberculosis.

A few studies linked the risk of being exposed AFs with socio-economic status. Because of low purchasing power, people in the low economic status are likely to be exposed to AFs to a greater extent than individuals with the higher economic power. Dietary diversity is one of preventive measure to avoid AFs exposure. However, major staples in Africa are susceptible to AF contamination, and the exposure is predominant. In Chapter 5 and 6, the level of AFB1-Lys in serums was compared on socio-economic status. Among the residents of Bexar County, participants with near poverty level had the highest level of AFB1-Lys, and the participants with an annual household income between $50,000 and $74,999 had the lowest. Educational status showed an inverse relationship with tortillas and corn consumptions. The avoidance of those foods seems to be educated behavior. In the resident of Kampala, Uganda, economic status was estimated based on household possession and housing, and there was no statistically significant difference of AFB1-Lys due to economic status. The frequent consumption of Mexican food, tortillas, and corn/rice drink were positively associated with the level of AFB1-Lys among the residents of Bexar County, Texas. The consumption of such food was higher among Hispanic populations and people with high school diploma or lower. In south-central Texas, Hispanic ethnicity, lower educational level, and lower annual income are positively associated with the serum AFB1-Lys level.

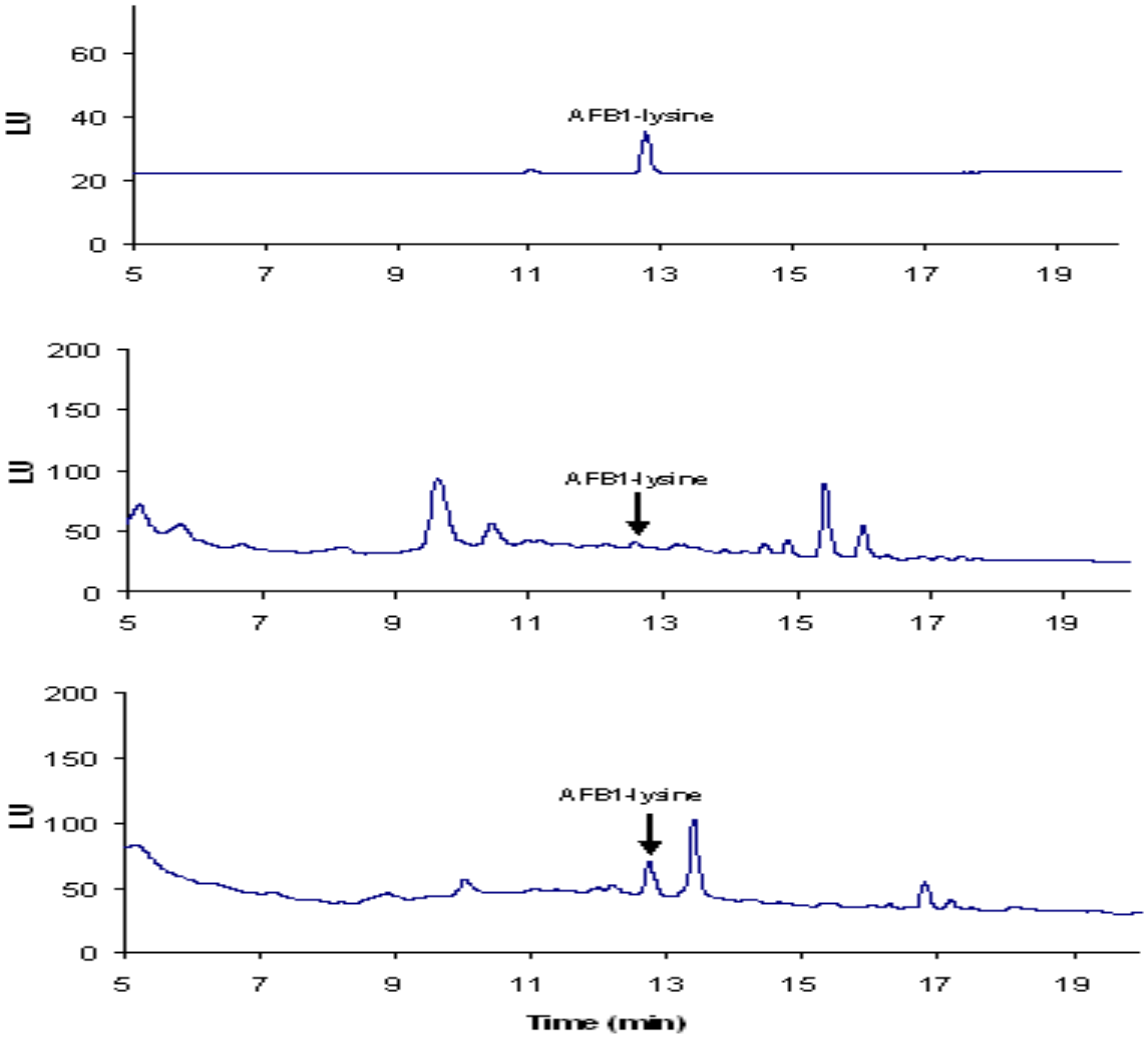
To reduce the risk of adverse effects by AF exposure, we conducted a 3-months trial with ACCS100 among the Bexar County residents with ≥1 pg AFB1-Lys/ mg albumin(Chapter7). Although we found the reduction of AFB1-Ly at low dose group (1.5g ACCS/day) after three months, there was not evidence of dose-response relation. In other words, the level of AFB1-Lys did not change in the group of participants with

high dose (3.0 g ACCS/day). The lack of efficacy may attribute to the low level of AFB1-Lys in south-central Texas populations. Ghanaian population has the level of serum AFB1-Lys adduct about 170-times higher than residents of Bexar County. Besides, leafy vegetables, which is common in the United States, contains chlorophyll that works as an enterosorbent as well; therefore, the efficacy of ACCS100 can be confoundable with the efficiency of the individual's diet.

In this dissertation, we found the increasing exposure of AFB_1 among the south-central Texas populations and linked to drought stress due to the global warming. It is necessary to study how the weather change affects prevalence and level of AFs in the foods and how it affects humans and environments as well as prevention strategy that is applicable and efficient in the United States. Accumulated knowledge shows that aflatoxins are immunotoxicants. Current studies on the immunotoxicity of aflatoxins mainly show the functional deficit or cell deaths. A mechanistic approach using omics technologies may help us to understand aflatoxin immunotoxicity holistically and even predict the future outcome. Also, many epidemiological studies proposed that chronic aflatoxin exposure increases the infectivity of the pathogens in humans. However, to my knowledge, there is no experimental study proven the theory yet. On top of that, the contamination of AF in foods frequently occurs in developing countries where the malnutrition and concatenation with other mycotoxins are common. The understanding of the interaction between those elements is critical to grabs the dynamic effects in human .

Appendix A.

Chromatograms of AFB_1-Lys A), 1ppb standard of AFB_1-Lys; B) , high level of AFB_1-Lys in human serum ; C), low level of AFB1-Lys in human serum.



Appendix B. SAS Code for survival analysis

For the clarity of epidemiological analysis, data merging and data analysis were conducted by SAS program. Data formatting, analysis, and a method to compare SAS merged data and manually merged data were included (Because of limited pages, routine data merging process was excluded).

```
/*************************************************************************/
/*                                                                       */
/*                                                                       */
/*                          Data formatting                              */
/*                                                                       */
/*                                                                       */
/*************************************************************************/
*formatting a graph;
goptions reset = all gunit=pct border cback=white
        colors=( blue green red black) ftext=swiss
        ftitle=swissb htitle=3 htext=3.5 ctitle=black ctext=black;

create variables (geometric mean of aflatoxin B1-lysine across three rounds and a natural log transformation of aflatoxin B1-lysine)
data af_demog_vst_hivcare_cd4_s;
          retain RHSP_ID afb_geo ln_afb_geo;
          set af_demog_vst_hivcare_cd4_s;
          afb_geo=geomean(aflatoxin_r1,aflatoxin_r2,aflatoxin_r3);
          *a geometric mean of aflatoxin B1-Lysine across three rounds;
          ln_afb_geo=log(afb_geo);
          *a log transformation of geometric mean of aflatoxin B1-Lysine;
          if afb_geo=. then delete;
          if hiv=. then delete;
          *the measurements without participant's demographic information and follow-up;
run;

data af_demog_vst_hivcare_cd4_m;
          retain RHSP_ID afb_geo ln_afb_geo Time aflatoxinB1 age date;
          array afb[3] aflatoxin_r1 aflatoxin_r2 aflatoxin_r3;
          array ageyrs[3] age1 age2 age3;
          array date_samp[3] date1 date2 date3;
          afb_geo=.;
          ln_afb_geo=.;
```

```
        set af_demog_vst_hivcare_cd4_s;

        do i=1 to 3;
        Time=i;
        aflatoxinB1=afb[i];
        age=ageyrs[i];
        date=date_samp[i];
        output;
        end;
        drop afb_geo ln_afb_geo aflatoxin_r1 aflatoxin_r2 aflatoxin_r3 age1 age2 age3 date1
date2 date3;
run;
data af_demog_vst_hivcare_cd4_m;
        retain RHSP_ID ln_aflatoxinB1;
        set af_demog_vst_hivcare_cd4_m;
        ln_aflatoxinB1=log(aflatoxinB1);*log transformation of aflatoxin B1-Lys;
run;

/*subsetting data*/
*HIV positive data;
data positive_m;
        set af_demog_vst_hivcare_cd4_m;
        if hiv=2 then delete;*HIV=2 (HIV negative);
run;
*HIV negative data;
data negative_m;
        set af_demog_vst_hivcare_cd4_m;
        if hiv=1 then delete; *HIV=1 (HIV positive);
run;

*HIV positive data;
data positive_s;
        retain RHSP_ID afb_geo ln_afb_geo hiv ;
        set af_demog_vst_hivcare_cd4_s;
        if hiv=2 then delete;
run;

/*classify HIV positive participants based on ln(geomean of aflatoxin-lysine), lower than median
and higher than median*/

proc means data=positive_s median;
var ln_afb_geo;
output out=median_dataset median(ln_afb_geo)=med;
```

```
run;

proc sql noprint;
select med into :median_value
from median_dataset;
quit;

%put &median_value;

data positive_s;
set positive_s;
if ln_afb_geo > &median_value then af_class=2;
else af_class=1;
run;


/*creat variables for survival analysis*/
*creat variable for survival analysis (incubation time (incubation), earliest date of HIV confirm
(hiv_confirm_d1), date of AIDS confirm(event_date), AIDS progression (event);

data positive_s;
        retain RHSP_ID afb_geo ln_afb_geo aflatoxin_r1 date1 age1 aflatoxin_r2 date2 age2
aflatoxin_r3 date3 age3 incubation event hiv_confirm ;
        format hiv_confirm date1-date3 mmddyy10.;
        set positive_s;

        *to define event (AIDS);
        if cd4_conver_date^=. then event=1;*AIDS (event=1);
        else event=0;*NO-AIDS(event=0);


        hiv_confirm=min(first_hiv_confirm, enroldate);*earliest date of hiv confirmation;

        *last visit before 01AUG2011 and the date of event (AIDS);
        if cd4_conver_date^=. then conver_date=cd4_conver_date;
        if cd4_conver_date=. then conver_date=last_visit;

        *calculating incubation time;
        incubation=(conver_date-hiv_confirm)/365;

        *calculating the age at seroconversion;
        if date3^=. then
        do;
```

```
                diff3=(date3-hiv_confirm)/365;
                age_at_seroconversion=age3-diff3;
        end;
        else if date2^=. then
        do;
                diff2=(date2-hiv_confirm)/365;
                age_at_seroconversion=age2-diff2;
        end;
        else if date1^=. then
        do;
                diff1=(date1-hiv_confirm)/365;
                age_at_seroconversion=age1-diff1;
        end;
run;

/*************************************************************************/
/*                                                                       */
/*                                                                       */
/*                    Logistic regression (HIV(+) vs. HIV(-))            */
/*                                                                       */
/*                                                                       */
/*************************************************************************/
/******Logistic regression (Odds ratio)******/
proc logistic data=af_demog_vst_hivcare_cd4_s ;*multivariate logistic regression;
        class hiv education(ref='0') occupation(ref='1') religion(ref='1') tribe(ref='1') area(ref='1')
sex(ref='1') marital_dis(ref='0')/param=ref;
        model hiv= ln_afb_geo age1 education occupation religion tribe area sex
marital_dis/selection=backward slstay=0.1;
run;
proc logistic data=af_demog_vst_hivcare_cd4_s ;*univariate logistic regression;
        class hiv;
        model hiv= ln_afb_geo;
run;

/******Case-Control (Table)****** ***/
proc freq data=af_demog_vst_hivcare_cd4_s;
        table hiv*sex/chisq;
        table hiv*education/chisq;
        table hiv*occupation/chisq;
        table hiv*marital_dis/chisq;
        table hiv*tribe/chisq;
        table hiv*area/chisq;
run;
```

```
proc npar1way data=af_demog_vst_hivcare_cd4_s;
        class hiv;
        var ln_afb_geo age1;
run;

proc print data=af_demog_vst_hivcare_cd4_m;
where hiv=2;
run;

proc freq data=af_demog_vst_hivcare_cd4_m;
table time;
run;

/*******Box Plot across rounds********/

*the effect of time on the level of aflatoxin B1-lysine using GEE in the HIV positives;
proc genmod data=af_demog_vst_hivcare_cd4_m;
   class RHSP_ID time (ref='1')/param=ref;
   model  ln_aflatoxinB1 = time;
   repeated  subject=RHSP_ID / type=ind corrw;
        contrast "time effect" time 1 -1 0, time 0 1 -1;
        where hiv=1;
run;
proc genmod data=af_demog_vst_hivcare_cd4_m;
   class RHSP_ID time/param=ref;
   model  aflatoxinB1 = time;
   repeated  subject=RHSP_ID / type=ind corrw;
        contrast "time effect" time 1 -1 0, time 0 1 -1;
        where hiv=1;
run;

*the effect of time on the level of aflatoxin B1-lysine using GEE in the HIV negatives;
proc genmod data=af_demog_vst_hivcare_cd4_m;
   class RHSP_ID time/param=ref;
   model  ln_aflatoxinB1 = time;
   repeated  subject=RHSP_ID / type=ind corrw;
        contrast "time effect" time 1 -1 0 time 0 1 -1;
        where hiv=2;
run;
proc genmod data=af_demog_vst_hivcare_cd4_m;
   class RHSP_ID time/param=ref;
   model  aflatoxinB1 = time;
   repeated  subject=RHSP_ID / type=ind corrw;
```

```
        contrast "time effect" time 1 -1 0, time 0 1 -1;
        where hiv=2;
run;
*Drawing the box plots of aflatoxinB1-lysine across times;
*in HIV positive;
proc sort data=positive_m;
        by time;
run;
proc boxplot data =positive_m;
  plot ln_aflatoxinB1*time;
run;

*in HIV negative;
proc sort data=negative_m;
        by time;
run;
proc boxplot data =negative_m;
  plot ln_aflatoxinB1*time;
run;

/*************************************************************************/
/*                                                                       */
/*                                                                       */
/*              HIV positive dataset (Survival Analysis)                 */
/*                                                                       */
/*                                                                       */
/*************************************************************************/
*Multiple Cox Proportional Hazard model;
proc phreg data=positive_s;
        class education(ref='0') occupation(ref='1') religion(ref='1') tribe(ref='1')  sex (ref='1')
        area(ref='1')       marital_dis(ref='0')/param=ref;
        model incubation*event(0)=ln_afb_geo ln_cd41 age_at_seroconversion education
occupation religion tribe area sex marital_dis/selection=backward slstay=0.1;
run;

proc freq data=positive_s;
        tables af_class*sex/chisq;
        tables af_class*education/chisq;
        tables af_class*marital_dis/chisq;
        tables af_class*tribe/chisq;
        tables af_class*area/chisq;
run;
```

```
proc npar1way data=positive_s;
        class af_class;
        var afb_geo ln_afb_geo ln_cd41 age_at_seroconversion;
run;


proc lifetest data=positive_s atrisk plots=survival(atrisk cb) outs=outrural;
        strata af_class;
        time incubation*event(0);
run;
/*************************************************************************/
/*                                                                       */
/*                                                                       */
/*                    Testting proportional hazard assumption            */
/*                    (cumulative sums of martingale residuals)          */
/*                                                                       */
/*                                                                       */
/*************************************************************************/
proc phreg data=positive_s;
    class education(ref='0') occupation(ref='1') religion(ref='1') tribe(ref='1') sex (ref='1')
area(ref='1')     marital_dis(ref='0')/param=ref;
model incubation*event(0)=ln_afb_geo ln_cd41 age_at_seroconversion education occupation
religion tribe area sex marital_dis/selection=backward slstay=0.1;
    assess ph/resample seed=385;
run;

/*************************************************************************/
/*                                                                       */
/*                                                                       */
/*              Compare merged datasets(excel_merged vs. sas_merged)     */
/*                                                                       */
/*                                                                       */
/*************************************************************************/
*import the file merged with excel;
proc import
datafile="E:\Research\Manuscript\HIVprogression\DATA_FOR_JOURNAL\02_Merged\HIV_p
articipants_survival_analysis.xls" dbms=xls out=hiv_survival replace;
run;
/*compare the aflatoxin B1-Lysine*/
data hiv_survival_afb (KEEP=RHSP_ID aflatoxin_r1 aflatoxin_r2 aflatoxin_r3) ;
        retain RHSP_ID;
        format aflatoxin_r1 7.3  aflatoxin_r2 7.3 aflatoxin_r3 7.3;
        *for matching the format of number (aflatoxin B1-lysine);
```

```
        set hiv_survival;
run;
proc sort data=hiv_survival_afb;
        by RHSP_ID;
run;

data positive_s_afb(KEEP=RHSP_ID aflatoxin_r1 aflatoxin_r2 aflatoxin_r3);
        retain RHSP_ID;
        format aflatoxin_r1 7.3  aflatoxin_r2 7.3 aflatoxin_r3 7.3;
        *for matching the format of number (aflatoxin B1-lysine);
        set positive_s;
run;

proc sort data=positive_s_afb;
        by RHSP_ID;
run;

proc compare base=hiv_survival_afb compare=positive_s_afb listall;
run;

/*compare the classification of participants based on the geometric mean of aflatoxin B1-lysine*/
data hiv_survival_af_class (KEEP=RHSP_ID level_lngeomean) ;
        retain RHSP_ID;
        format level_lngeomean 2.;*for matching the format of number (aflatoxin B1-lysine);
        set hiv_survival;
run;
proc sort data=hiv_survival_af_class;
        by RHSP_ID;
run;
data positive_s_af_class(KEEP=RHSP_ID af_class);
        retain RHSP_ID;
        format level_lngeomean 2.; *for matching the format of number (aflatoxin B1-lysine);
        set positive_s;
run;
proc sort data=positive_s_af_class;
        by RHSP_ID;
run;
proc compare base=hiv_survival_af_class compare=positive_s_af_class listall;
run;


/*compare the event variable, incubation time, and the level of aflatoxinB1-lysine(class)*/
data hiv_survival_event (KEEP=RHSP_ID event) ;
```

```
        retain RHSP_ID;
        format event;*for matching the format of number (aflatoxin B1-lysine);
        set hiv_survival;
run;
proc sort data=hiv_survival_event;
        by RHSP_ID;
run;

data positive_s_event(KEEP=RHSP_ID event);
        retain RHSP_ID;
        format event; *for matching the format of number (aflatoxin B1-lysine);
        set positive_s;
run;
proc sort data=positive_s_event;
        by RHSP_ID;
run;
proc compare base=hiv_survival_event compare=positive_s_event listall;
run;* the difference is from an error on the HIV_survival data(excel merged);

/*compare the event variable, incubation time, and the level of aflatoxinB1-lysine(class)*/
data hiv_survival_incubation (KEEP=RHSP_ID incubation2) ;
        retain RHSP_ID;
        set hiv_survival;
run;
proc sort data=hiv_survival_incubation;
        by RHSP_ID;
run;

data positive_s_incubation(KEEP=RHSP_ID incubation);
        retain RHSP_ID;
        set positive_s;
run;
proc sort data=positive_s_incubation;
        by RHSP_ID;
run;
proc compare base=hiv_survival_incubation compare=positive_s_incubation listall;
run;

/*compare the demographic variables*/
data hiv_survival_ed (KEEP=RHSP_ID educationgrp) ;
        retain RHSP_ID;
        set hiv_survival;
run;
```

```
proc sort data=hiv_survival_ed;
        by RHSP_ID;
run;
data positive_s_ed (KEEP=RHSP_ID education);
        set positive_s;
run;
proc sort data=positive_s_ed;
by RHSP_ID;
run;
proc compare base=hiv_survival_ed compare=positive_s_ed listall;run;


data hiv_survival_occ (KEEP=RHSP_ID occupation) ;
        retain RHSP_ID;
        set hiv_survival;
run;
proc sort data=hiv_survival_occ;
        by RHSP_ID;
run;
data positive_s_occ (KEEP=RHSP_ID occupation);
        set positive_s;
run;
proc sort data=positive_s_occ;
by RHSP_ID;
run;
proc compare base=hiv_survival_occ compare=positive_s_occ listall;run;


data hiv_survival_religion (KEEP=RHSP_ID religion) ;
        retain RHSP_ID;
        set hiv_survival;
run;
proc sort data=hiv_survival_religion;
        by RHSP_ID;
run;
data positive_s_religion (KEEP=RHSP_ID religion);
        set positive_s;
run;
proc sort data=positive_s_religion;
by RHSP_ID;
run;
proc compare base=hiv_survival_religion compare=positive_s_religion listall;run;
```

```
data hiv_survival_tribe01 (KEEP=RHSP_ID tribe01) ;
        retain RHSP_ID;
        set hiv_survival;
run;
proc sort data=hiv_survival_tribe01;
        by RHSP_ID;
run;
data positive_s_tribe (KEEP=RHSP_ID tribe);
        set positive_s;
run;
proc sort data=positive_s_tribe;
by RHSP_ID;
run;
proc compare base=hiv_survival_tribe01 compare=positive_s_tribe listall;run;


data hiv_survival_area (KEEP=RHSP_ID area) ;
        retain RHSP_ID;
        set hiv_survival;
run;
proc sort data=hiv_survival_area;
        by RHSP_ID;
run;
data positive_s_area (KEEP=RHSP_ID area);
        set positive_s;
run;
proc sort data=positive_s_area;
by RHSP_ID;
run;
proc compare base=hiv_survival_area compare=positive_s_area listall;run;

data hiv_survival_marital (KEEP=RHSP_ID marital_stat) ;
        retain RHSP_ID;
        set hiv_survival;
run;
proc sort data=hiv_survival_marital;
        by RHSP_ID;
run;
data positive_s_marital (KEEP=RHSP_ID marital_dis);
        set positive_s;
run;
proc sort data=positive_s_marital;
```

```
by RHSP_ID;
run;
proc compare base=hiv_survival_marital compare=positive_s_marital listall;run;
```