AFLATOXIN AND FUMONISIN EXPOSURE: INTERNATIONAL SURVEY AND ENTEROSORPTION MITIGATION STRATEGY IN HUMANS

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2016

Major Subject: Toxicology

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ABSTRACT

Aflatoxins (AFs) are toxic metabolites produced by *Aspergillus flavus* and *A. parasiticus.* Fumonisins (FBs) are also toxic products of fungi, specifically *Fusarium verticilloides* and *F. proliferatum*. Both toxins commonly contaminate staple grains and cereals such as maize and groundnuts. Aflatoxin B_1 (AFB_1) is the most toxic and prevalent of the AFs. Chronic dietary exposure to AFs is a known risk factor for hepatocellular carcinoma and may also affect protein metabolism and the immune system. Fumonisin B_1 (FB_1) is the most abundant and toxicologically significant of the congeners. In populations where AFs and FBs are inextricable contaminants, a multi-faceted approach must be implemented to reduce exposure to these toxins, especially in the young who are more susceptible. Alternative methods such as calcium montmorillonite clay (UPSN or ACCS100) as an enterosorbent therapy that focus on reducing biological exposure to AFs and FBs in foods already contaminated are desirable as a secondary defense to the harmful effects of these toxins. Therefore, I propose to test the efficacy of UPSN in food matrices, identify populations at high risk for AFs and FBs with urinary biomarkers, and finally, combine clay technology and biomarker analysis to intervene with UPSN or ACCS100 in frequently exposed human populations.

In these studies UPSN was able to significantly reduce AFB_1 under common cooking conditions in a corn meal matrix suggesting a potential delivery of the clay directly in the contaminated food. A high prevalence of exposure to variable AFB_1 and FB_1 levels in participants from Monterrey, Mexico was observed. After a two week crossover trial in a high risk area of Kenya with 3.0g ACCS100/day mixed in water, urinary aflatoxin M_1 (an

AFB_1 metabolite) was significantly reduced compared to the placebo group. ACCS100 was found to be safe and well tolerated suggesting potential for reducing exposure to AF in this particular population during outbreak situations. In a 3-month intervention with 3.0g or 1.5g ACCS100/day (encapsulated) in San Antonio, Texas, AFB_1-lysine (an AFB_1 protein adduct) was significantly reduced in the Low Dose group (1.5g) compared to Placebo. ACCS100 was well tolerated in the majority of participants and no significant changes in serum biochemistry or hematology were detected in any treatment group. Thus, use of calcium montmorillonite clay at doses as low as 1.5g/day and delivered in capsules, food, drink, or water may provide a viable strategy to reduce dietary AFB_1 bioavailability in populations exposed to this toxin for up to 3 months. Moreover, AF and FB exposure is a global and unavoidable public health concern and biomarkers are important tools for monitoring exposure.

DEDICATION

This work is dedicated to my family. To my parents, Howard and Cindy, who fostered my love of science at a young age, and gave me constant love, support, and strength throughout my education. Without them, this work would not have been possible. To my sister, Kate, who is my biggest cheerleader as well as my inspiration for the meaning of dedication and hard work. Finally, to my grandparents, Tom Gargiulo and John and Annabeth Elmore for their continued support of my education, especially Grandpa Tom who has been with me since my first science fair project and has never stopped encouraging me to pursue my dreams.

ACKNOWLEDGEMENTS

My sincerest gratitude goes to my advisor, Dr. Tim Phillips, who allowed for me to grow as a researcher and scientist. Without his mentorship, encouragement, and passion for toxicology I would not be where I am today. I would like to thank my committee members: Dr. Roger Harvey, Dr. Stephen Safe, and Dr. Alice Villalobos, for their guidance, encouragement, and academic contributions. I appreciate all of my colleagues in the Phillips' lab (past and present): Nicole Mitchell, Katie Zychowski, Alicia Marroquin-Cardona, Amelia Romoser, Natalie Johnson, and Cody Maki, who contributed to my graduate education. I am grateful for all of the friends I've made in the toxicology program especially Alex Reeder-Lacey, Xi Li, Kristal Rychlik, and Kelly Scribner. I would also like to acknowledge Emily Schmitt who alleviated much of my dissertation-stress through her friendship and guidance. Additionally, I am grateful for all of my collaborators who made much of this work possible: Dr. Wang, Dr. Pollock, Dr. Marroquin-Cardona, Dr. Yard, Johnni Daniel, Abigael Obura, and all of the team members from Mexico, San Antonio, and Kenya. Lastly, I would like to acknowledge the College of Veterinary Medicine and Biomedical Sciences whose support through GSA travel awards allowed for me to present my work at national meetings.

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF FIGURES ix

LIST OF TABLES xi

1. INTRODUCTION 1

1.1 Aflatoxin 3
1.1.1 Problem defined 3
1.1.2 Discovery 4
1.1.3 Source of contamination 6
1.1.4 Biotransformation 8
1.1.5 Biomarkers of exposure 18
1.1.6 Carcinogenicity 24
1.1.7 Toxicity 33
1.2 Fumonisin 43
1.2.1 Problem defined 43
1.2.2 Discovery 44
1.2.3 Source of contamination 45
1.2.4 Adsorption, biodistribution, and pharmacokinetics 47
1.2.5 Biomarkers of exposure 49
1.2.6 Mechanism of action 51
1.2.7 Carcinogenicity 55
1.2.8 Toxicity 58
1.3 Aflatoxin B_1/fumonisin B_1 co-exposure 61
1.4 Reducing exposure in human populations 67
1.5 Research objectives 85

2. COMMON AFRICAN COOKING PROCESSES DO NOT AFFECT THE AFLATOXIN BINDING OF REFINED CALCIUM MONTMORILLONITE CLAY 88

2.1 Introduction 88

Page

2.2 Materials and methods 90
2.2.1 Materials 90
2.2.2 Cornmeal preparation 91
2.2.3 Base product 91
2.2.4 Fermented product 92
2.2.5 Sterilized product 92
2.2.6 Extraction and quantification of aflatoxin B_1 92
2.2.7 Calculations and statistical analysis 96
2.3 Results 96
2.3.1 UPSN reduction of aflatoxin B_1 in unfermented products 96
2.3.2 UPSN reduction of aflatoxin B_1 in fermented products 96
2.3.3 HPLC verification 97
2.4 Discussion 102

3. EPIDEMIOLOGICAL SURVEY OF AFLATOXIN AND FUMONISIN IN MONTERREY, MEXICO 107

3.1 Introduction 107
3.2 Materials and methods 109
3.2.1 Chemicals 109
3.2.2 Participant recruitment and sample collection 109
3.2.3 Determination of aflatoxin M_1 in urine 111
3.2.4 Determination of fumonisin B_1 in urine 112
3.2.5 Statistical analysis 113
3.3 Results 113
3.4 Discussion 117

4. EFFICACY OF DIETARY INTERVENTION DELIVERED IN WATER TO REDUCE EXPOSURE TO AFLATOXIN IN EASTERN KENYA 127

4.1 Introduction 127
4.2 Materials and methods 129
4.2.1 Materials 129
4.2.2 Aflatoxin B_1 sorption analysis with ACCS100 130
4.2.3 Study design and procedures 130
4.2.4 Study population and enrollment 133
4.2.5 Palatability 134
4.2.6 Adherence and acceptability 134
4.2.7 Determination of urinary aflatoxin M_1 and serum aflatoxin B_1-lysine adduct level 134
4.2.8 Statistical analysis 137

4.3 Results ... 138
4.3.1 Aflatoxin B_1 sorption analysis with ACCS100 ... 138
4.3.2 Study population and demographics ... 138
4.3.3 Compliance ... 139
4.3.4 Analysis of urinary aflatoxin M_1 and serum aflatoxin B_1-lysine levels ... 140
4.3.5 Palatability ... 144
4.3.6 Acceptability ... 145
4.3.7 Adverse events ... 147
4.4 Discussion ... 147

5. ACCS100 CLAY INTERVENTION IN A U.S. POPULATION WITH A HIGH INCIDENCE OF HEPATOCELLULAR CARCINOMA ... 153

5.1 Introduction ... 153
5.2 Materials and methods ... 154
5.2.1 Recruitment and eligibility ... 154
5.2.2 ACCS100 dosing and treatment schedule ... 155
5.2.3 Adverse effects monitoring ... 157
5.2.4 Blood and urine collection and processing ... 158
5.2.5 Analysis of serum aflatoxin B_1-lysine adduct ... 158
5.2.6 Analysis of urinary aflatoxin M_1 ... 159
5.2.7 Statistical analysis ... 160
5.3 Results ... 162
5.3.1 Sample collection and demographics ... 162
5.3.2 Trial efficacy—aflatoxin B_1-lysine adduct ... 166
5.3.3 Trial efficacy—aflatoxin M_1 ... 171
5.3.4 Adverse events and serum biochemistry ... 171
5.3.5 Dietary survey ... 174
5.4 Discussion ... 175

6. SUMMARY ... 181

REFERENCES ... 188

LIST OF FIGURES

FIGURE | Page

1 Chemical structures of naturally occurring aflatoxins B_1, B_2, G_1, and G_2 . 4

2 Metabolism of aflatoxin B_1 by phase I and phase II enzymes 15

3 Molecular structure of fumonisin B_1. Molecular structures of sphinganine, sphingosine, dihydroceramide, and ceramide 46

4 A simplified scheme on the pathway of de novo sphingolipid synthesis and turnover in mammalian cells and the sites of action of FB_1-induced inhibition of the enzyme ceramide synthase 52

5 Biochemical and cellular consequences of fumonisin inhibition of ceramide synthase(s) and global disruption of lipid metabolism.............. 60

6 Calcium montmorillonite clay structure 70

7 Reduction of AFB_1 in unfermented cornmeal with UPSN....................... 95

8 Reduction of AFB_1 in fermented cornmeal with UPSN........................... 98

9 Reduction of AFB_1 in cornmeal in the presence of UPSN after heat exposure or sterilization... 99

10 HPLC detection of AFB_1 levels in unfermented, fermented, and sterilized samples 100

11 Recruitment area in Monterrey, Nuevo Leon, Mexico............................. 110

12 Overall study design and participant flow for ACCS100 crossover trial ... 132

13 AFB_1 isotherm onto ACCS100 at pH 6.5 139

14 AFM_1 excretion by tea consumption.. 141

15 AFM_1 distribution within groups and treatment arms.............................. 143

16 Daily median AFM_1 levels for 2 weeks of crossover study...................... 144

FIGURE Page

17 A summary of study procedure for 3 month intervention in San Antonio . 156

18 A flow diagram from recruitment to completion 164

19 Distribution of AFB_1-lys adduct over time by group 168

20 Log mixed-effect regression model showing estimated AFB_1-lys adduct . 169

21 Distribution of AFB_1-lys adduct by recruitment year 170

22 AFM_1 distribution within groups over the study period............................ 172

23 Percent area of Texas in drought from January 2011 to April 2014 178

LIST OF TABLES

TABLE Page

1 Comparative toxicity of AFB_1 in various species of vertebrates 32

2 AF and growth faltering 40

3 Reported co-occurrence of AFB_1 and FB_1 in maize worldwide 63

4 Safety and efficacy studies with calcium montmorillonite clay in animals and humans 76

5 Percent AFB_1 reduction trends in cornmeal verified by HPLC 101

6 Demographic characteristics of study participants from Monterrey, Mexico 114

7 Level of urinary AFM_1 and FB_1 in study participants in Mexico independent of co-exposure status 115

8 Level of urinary AFM_1 and FB_1 in study participants in Mexico by co-exposure status 116

9 Percent food consumption in Mexico study population by co-exposure status 118

10 Kenya study population demographics, by group 140

11 Kenya study palatability ratings reported by treatment 145

12 Kenya study adverse events reported by treatment 146

13 San Antonio study demographic distribution of enrolled participants by treatment group 163

14 San Antonio study participant adherence and completion of treatment regimen 165

15 Change in AFB_1-lys adduct over time in serum from San Antonio study participants 166

TABLE Page

16 Change in AFM_1 over time in urine from San Antonio study participants 170

17 Hematological analysis and serum biochemistry analysis in San Antonio study participants 173

1. INTRODUCTION

Aflatoxins (AFs) are known human carcinogens and common contaminants of globally important commodities, such as corn and groundnuts. Historically, AFs have been of significant interest in the areas of food safety and public health in developing countries. However, due to ongoing global climate change, AFs are of increasing concern in parts of the developed world, including North America and Europe. Aflatoxin B_1 (AFB_1) is one of four secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus* and is the most prevalent and toxic of the AF congeners (Wild *et al.*, 2002). Chronic AF exposure is greatest in communities that produce and consume their own food (Wild and Gong, 2010) but is of universal concern due to its association with an increased risk of hepatocellular carcinoma (HCC) (Eaton and Gallagher, 1994; IARC, 1993; IARC, 2002; Wild and Turner, 2002). HCC is the 5th and 3rd leading cause of cancer-related mortality in females and males, respectively. Based on the results from a 2012 meta-analysis of AF-related HCC studies, the population at risk for AF-related HCC was estimated to be 17% worldwide (Liu *et al.*, 2012). Although the hepatotoxicity and genotoxicity of AFs have been studied extensively in relation to HCC, AFB_1 is also known to be immunosuppressive and anti-nutritional (IARC, 2002, 2015), with these health effects becoming an increasing focus in high risk and vulnerable populations.

Fumonisins (FBs) are also common contaminants of maize and have been found in high levels in Sub-Saharan Africa, Central America, and Southeast Asia. FBs, which are structurally different than AFs, are primarily produced by *Fusarium verticilloides* and *F. proliferaturm*. Of the naturally occurring homologues and derivatives, fumonisin B_1

(FB_1) is the most abundant and most toxicologically significant (JECFA, 2001). Although epidemiological studies have demonstrated correlations between FB-contaminated food with increased incidence of esophageal cancer in regions of China and South Africa, neural tube defects along the Texas-Mexico border, and primary liver cancer in patients from China (Chu and Li, 1994; Marasas *et al.*, 2004; Shephard *et al.*, 2007b; Ueno *et al.*, 1997), the actual impact on human health has not been well delineated.

Geography and climate change are of increasing importance with regard to AF and FB contamination in foods and subsequent human exposure. AF contamination occurs mainly in tropical and semi-tropical climates, often called the "hot zone" between 40° north and south of the equator. *Aspergillus* fungi typically grow at temperatures >25° C (Cotty and Jaime-Garcia, 2007) in moist conditions, which leads to the production of AFs in mature crops (Cotty, 1991). However, higher temperatures during droughts have also been associated with increased AF contamination (Sanders *et al.*, 1984). As climate warms and weather patterns become less predictable, countries such as the United States may become more vulnerable to these toxicants. When patterns change, good agricultural practices and proper planning may not be sufficient to prevent AF contamination in the food supply. It has been postulated that contamination may become widespread in areas such as the US Midwest that were previously unaffected (Cotty and Jaime-Garcia, 2007). Furthermore, poor grain storage practices that lead to higher moisture levels can cause increased AF levels in harvested grains previously infected with fungi in the field (CAST, 2003).

Since ubiquitous AF and FB contamination of the food supply exists in developing countries due to lack of proper food quality control measures and food insecurity, methods to decrease exposure in foods already contaminated are needed. One strategy to ameliorate mycotoxin exposure is high affinity sorption of AFs and FBs with specific clays in the diet to decrease toxin bioavailability (Phillips, 2002). Such a strategy could positively affect outcomes in cancer development, growth, and immune function associated with mycotoxin exposure.

1.1 Aflatoxin

1.1.1 Problem defined

AFs are fungal toxins that are members of a larger family of mycotoxins. Mycotoxins are structurally diverse chemical compounds produced by fungi and their carcinogenicity in humans and animals has been suspected for centuries. The word mycotoxin was first used by in 1955 (Forgacs *et al.*, 1955) and derived from mycotoxicosis, or diseases of animals caused by fungal toxins. Subsequently, mycotoxin was defined as a toxin produced by a fungus. The primary route of exposure is ingestion; however dermal or inhalational exposure may occur. Due to their ubiquitous nature, mycotoxin contamination of food and feed supplies have the potential to increase the economic risks and affect the health humans and animals. Of the 300 naturally occurring mycotoxins, AF is the most toxic and therefore most widely studied. AFs are largely produced by the common fungi *Aspergillus flavus* and the closely related species *A. parasiticus*. To understand the risks associated with AF, knowledge of the following is required: (1) the toxicology of the compounds (2) recorded effects on exposed human and

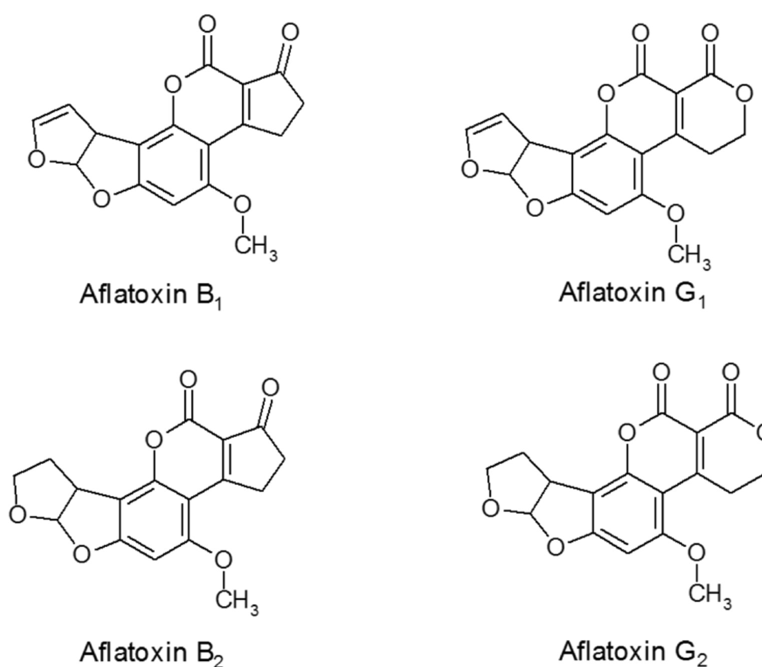

Figure 1. Chemical structures of naturally occurring aflatoxins B_1, B_2, G_1, and G_2. AFs are produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus* fungi, and their series nomenclature denotes a characteristic fluorescence emission under UV light, that is, (B) blue and (G) green fluorescence.

animal populations, (3) effects of acute and chronic exposure, (4) human and animal exposure to the toxins (molecular epidemiology), and (5) method to mitigate exposures.

1.1.2 Discovery

Aflatoxins (AFs) are known human and animal carcinogens and common contaminants of globally important commodities, such as corn and groundnuts. Historically, AFs have been of significant interest in the areas of food safety and public health in developing countries but weren't discovered until 1960 when turkey poults in England died of acute hepatotoxicity attributed to contaminated groundnut meal from

Brazil (Blount, 1961). This event, reported as turkey "X" disease, was followed by similar poisonings in chickens and ducklings (Blount, 1961). The characteristics of the acute hepatotoxicity included necrosis, marked bile duct hyperplasia, acute loss of appetite, wing weakness, and lethargy and resulted in the attention of many scientific laboratories worldwide. A year later, Asplin and Carnaghan (Asplin and Carnaghan, 1961) identified the toxins as metabolites of *Aspergillus flavus* strains. This identification was followed by chemical characterization and the designation of the name AFs by Asao et al (1963). It was discovered that the AF metabolites consist of four major congeners designated as B_1, B_2, G_1, and G_2 (Figure 1) based on their fluorescence and R_f values from thin-layer chromatography. *A. flavus* produces only B aflatoxins, while *A. parasiticus* produces both B and G AFs (Diener *et al.*, 1987; Klick and Pitt, 1988). In light of the findings resulting from the turkey "X" disease incident, it was suggested that several events preceding 1960 could be attributed to AF poisoning including the death of hunting dogs fed a peanut-based diet (Newberne *et al.*, 1955), high incidence of liver tumors in a colony of rats fed a diet containing peanut meal (Le Breton *et al.*, 1964), and toxicosis in swine and cattle fed moldy corn (Burnside *et al.*, 1957). Susceptibility to the toxic effects of AF varies widely between species with the duckling and rainbow trout being the most sensitive animals. Halver et al. (1967) was the first to demonstrate the carcinogenicity of AF in rainbow trout and subsequently recognizing AF as one of the most potent liver carcinogens. A variety of similar fungal species have also been identified as AF producers, however *A. flavus* and *A. parasiticus* are responsible for the overwhelming proportion of AFs found in foodstuffs throughout the word and have been studied in great detail.

1.1.3 Source of contamination

Following the Turkey X incident in 1960, accumulation of AF was primarily considered a storage issue and thus research was focused on preventing post-harvest contamination (Asao *et al.*, 1965; Trenk and Hartman, 1970). However, a severe outbreak in the early 1970s in U.S. maize led to the discovery that *A. flavus* could both colonize and produce its toxic metabolites pre-harvest in developing maize kernels. Studies have shown that Aspergillus fungi colonize and inoculate crops through three main routes: 1) airborne spores, 2) soil contact, and 3) insect transfer. This initial colonization and inoculation is crop specific. *A. parasiticus* appears to be adapted to a soil environment and therefore prominent in peanuts, whereas *A. flavus* is adapted to the aerial and foliar environment based on its dominance in corn, cottonseed, and tree nuts (Diener *et al.*, 1987). Furthermore, it has been suggested that insects facilitate infection pre-harvest by transporting Aspergillus spores; *A. flavus* was found more often on insects inhabiting corn and *A. parasiticus* was found more often on insects associated with soil (Lillehoj *et al.*, 1980). The vectors do not need to be pests that cause damage but a relationship between insect injury and AF contamination in not surprising. Lee et al. (1980) demonstrated that this relationship between insect damage and AF contamination is related to enhanced AF production in damaged areas; however, the two are not mutually exclusive (Widstrom *et al.*, 1976).

Since the discovery of pre-harvest contamination, AF contamination is frequently broken down into two phases: the first phase occurring on the developing crop (pre-harvest) and the second phase affecting the crop after maturation (harvest and post-

harvest) (Cotty and Jaime-Garcia, 2007). It is important to note however, that crops infected with Aspergillus fungi do not always contain AF. Although contamination is usually associated with one phase or the other, contamination during both phases isn't uncommon (Cotty and Jaime-Garcia, 2007). Generally, pre-harvest (or immediately following harvest) contamination by *A. flavus* only occurs in maize, cottonseed and by both *A. flavus* and *A. parasiticus* in peanuts. *A. flavus* lacks this affinity for other crops, so it is not normally a concern pre or during harvest (IARC *et al.*, 2002). Rain and temperature largely influence the phases differently with dry, hot conditions favoring the first and warm, wet conditions favoring the second. However, in warm humid, subtropical, and tropical climates the storage fungi (second phase) often become the field (first phase) as well as the storage fungi (Wilson and Abramson, 1992). Generally, *A. flavus* and *A. parasiticus* fungi produce AFs when the temperatures are between 24 and 35° C, and will contaminate many commodities if the moisture content exceeds 7% (10% with ventilation) (Williams *et al.*, 2004). The amount of contamination varies with climate both temporally and spatially. This becomes increasingly important as droughts become more frequent and persistent and global temperatures rise. What used to be considered the hot zone for AF contamination, 20° north and south of the equator, may now extend into areas the southern half of the US and Europe (at 40°).

In addition to the effects of climate and weather conditions, factors that make crops vulnerable to Aspergillus growth and AF production include genetics, soil type, and insect activity. Timely harvest and rapid and adequate drying before storage are also important. However, even commodities dried to a satisfactory degree are vulnerable to isolated

pockets of fungal growth and AF production due to moisture generated by insect respiration and local condensation. Many of these facts are managed in AF-prone crops in developed countries, but this comes as a cost due to investments in production, drying and storage facilities. Despite these investments, sizable losses still occur regularly in the United States when farmers are unable to meet the more relaxed standards allowed for animal feed.

1.1.4 Biotransformation

1.1.4.1 Introduction

Biotransformation is a necessary step regarding the toxicity and carcinogenicity of AFB_1; it is important for the disposition and biological activity of the compound. Toxicity or carcinogenicity of the parent compound is directly related to the proportion of the mycotoxin converted to metabolites that bind to critical cellular macromolecules. In this regard, exposure to dietary compounds that affect the rates of AFB_1 activation or elimination can ultimately affect AFB_1 carcinogenicity. It is important to note that far less attention has been given to the role of biotransformation in acute AF toxicity. Biotransformation of AF can be broken down into several detoxification pathways: oxidation, reduction, and conjugation. Not all metabolites have been identified in all species and therefore significant quantitative differences in the formation of the various products may exist.

1.1.4.2 Absorption

After rapid absorption in the gastrointestinal tract, AFB_1 is distributed among various tissues including the liver (Dalezios *et al.*, 1973; Dalezios and Wogan, 1972;

Steyn *et al.*, 1971; Wogan *et al.*, 1967). Adsorption appears to occur in the small intestine (Wogan *et al.*, 1967) with the specific site undecided. Earlier research demonstrated absorption from the jejunum after direct injection in anaesthetized rats (Shantha *et al.*, 1970). In separate rat studies, results indicate that absorption is most efficient in the duodenum (Hsieh and Wong, 1994; Kumagai, 1989). The rate of absorption may be influenced by endocrine activity due to observed changes with age and reproductive stage, with the greatest absorption of AFB_1 from the small intestine occurring in suckling rats (Kumagai, 1989). This could explain the higher susceptibility of the young rat to acute toxicity of AFB_1 (Newberne and Butler, 1969). This is due to changes in lipid composition in the epithelial cell membrane during growth (Schwarz *et al.*, 1985) since AF absorption is due to passive diffusion due to its lipid solubility (Shantha *et al.*, 1970). Following absorption, AFB_1 is almost exclusively transferred to the mesenteric blood (Shantha *et al.*, 1970; Wilson *et al.*, 1985a) indicating that although a hydrophobic compound, AFB_1 does not partition into lipoproteins and circulate via intestinal lymphatic drainage like compounds with similar properties such as Benzo(a)pyrene. Transfer to the vascular system ultimately results in its immediate transport to the liver which contributes to AF's hepatotoxicity; however, total hepatic exposure to AFB_1 is limited by both incomplete absorption and gastrointestinal metabolism (Hsieh and Wong, 1994) (to be discussed later).

1.1.4.3 Distribution

Following absorption from the intestine, AFB_1 enters the liver through the hepatic portal blood supply (Wilson *et al.*, 1985a), and to a much lesser extent, the kidneys.

Radiolabeled AFB_1 studies in rats (Wogan *et al.*, 1967) and male rhesus monkeys (Dalezios and Wogan, 1972) found that 17 and 19% of the radioactivity was located in the liver, and 5 and 0.9% was contained in the kidneys, respectively, within the first 30-45 min following intraperitoneal (ip) injection. This rapid hepatic uptake of AFB_1 is consistent with studies in perfused rat liver and isolated rat hepatocytes (Chih and Devlin, 1984; Unger *et al.*, 1977). AFB_1 appears to be retained in the liver very effectively with most of it bound irreversibly to tissue macromolecules (Holeski *et al.*, 1987). Based on the studies of Wong and Hsieh (1980), the volume of distribution, using the one-compartment pharmacokinetic model, was consistent with the relative susceptibility for the monkey, rat, and mouse (ranked from most to least susceptible), i.e., greater volume, greater susceptibility Furthermore, the same order of susceptibility holds true for the first-order rate constant for AFB_1 elimination from plasma (K_E), which were 1.1, 1.4, and 3.2 hr^{-1} and plasma biological half-lives ($t_{1/2}$), 36.5, 28.9, and 12.9 min. for the monkey, rat, and mouse, respectively. Using a two-compartment open model in the monkey and rat, the results further confirm that the rat has a slower rate of plasma to tissue transfer, meaning the tissue is less concentrated and therefore less susceptible to acute toxicity than the monkey (Wong and Hsieh, 1980).

1.1.4.4 Metabolism

Metabolism of the parent compound, AFB_1, undergoes two phases as demonstrated in Figure 2. This process begins in the intestine where lower levels of the same biotransformation abilities in the liver can be found (Hartiala, 1977). The gastrointestinal mucosal cells possess the enzyme capability necessary for many

biotransformation reactions (Hartiala, 1977). AFB_1 can be metabolized to several active forms (discussed further in the following paragraphs) which interact with protein at the gastrointestinal mucosa (Hsieh and Wong, 1994). These metabolites include the highly reactive AFB_1-epoxide (AFBO), the AFB_1-dihydrodiol, and $AFB_2\alpha$. One of the products of these reactive metabolites formed at or during absorption is the serum albumin adduct of AFB_1. AFB_1 is able to form a Schiff base between the lysine residues of serum albumin to produce a protein adduct in the blood. A serum albumin adduct indicates that AFB_1 is metabolized to the epoxide either in the gut lumen, the gut wall, or in some blood components. This is not unlikely due to the presence of important metabolizing enzymes within the gut mucosa. Furthermore, the absence of phase I enzymes in the blood suggests that metabolites are rapidly conjugated by phase II enzymes or are already bound to proteins as previously mentioned (Hsieh and Wong, 1994). Although little attention has been paid to this step, it warrants attention, especially considering that the gut is the first line of immune defense. It is an intriguing hypothesis to go a step further and suggest AFB_1 not only affects liver and kidney, but also can harm the integrity of the gut, and directly affect nutrient uptake and immune health. Importantly, the strong detoxification pathway in gastrointestinal metabolism of AFB_1 limits the concentration of the parent compound in the portal inflow to the liver leading to the first line of defense from classic symptoms of AFB_1 toxicity and carcinogenicity.

As previously mentioned, AFB_1 enters the liver through the hepatic portal blood supply (Wilson *et al.*, 1985a) where a majority of the metabolism of the circulating AFB_1 occurs. In the liver, phase I microsomal cytochrome P450-dependent oxidation of the

double bond on the terminal furan of both AFB_1 and AFG_1 generates a very potent electrophilic species, AFB_1-8,9-epoxide (AFO) (Eaton and Gallagher, 1994; Essigmann *et al.*, 1977; Swenson *et al.*, 1977). This process yields *exo*- and *endo*-AFB_1 epoxide stereoisomers (Raney *et al.*, 1992a). The initial critical lesion, 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB_1, is formed in the reaction of DNA with AFBO (Iyer, 1994). Of the reactive stereoisomers, the *exo*-epoxide is the dominant form and is the most reactive with DNA (Raney *et al.*, 1992a). AFBO has not been isolated from biological systems due to its high reactivity, but was indirectly isolated from the products of its reactions with DNA and the detoxification product, GSH (Degen and Neumann, 1978; Essigmann *et al.*, 1977). Ultimately, the proportion of AF activated by cytochrome P450 to the epoxide determines the amount of AFB_1 that will bind to DNA.

Much of the species difference in carcinogenicity is related to the biotransformation of AFB_1 and the subsequent ratio of metabolites and detoxification processes. Cytochrome (CYP) activity contributes a significant portion of the observed species differences. Human liver microsomes are approximately one-fourth as efficient at activating AFB_1 as are rat microsomes (Ramsdell and Eaton, 1990) and mice have an even higher activity for AFB_1-8,9-epoxide production but are resistant to hepatocarcinogenic effects due to additional enzyme differences (Monroe and Eaton, 1987). Importantly, the microsomes active in AFB_1 metabolism differ with substrate concentration. The proportion of AFB_1 converted to AFBO was increased at the lower substrate concentrations representing dietary exposure in the rat and human microsome, but not with

the mouse or monkey microsome resulting in striking differences in the metabolites observed across species (Ramsdell and Eaton, 1990).

Multiple studies have demonstrated that the biotransformation of AFB_1 in human liver involves multiple CYP enzymes, each exhibiting different characteristics (Aoyama *et al.*, 1990; Forrester *et al.*, 1990; Gallagher *et al.*, 1994; Ramsdell *et al.*, 1991). At least five CYP enzymes have been implicated in activating AFB_1 to its mutagenic metabolites including CYP 1A2, 2A6, 2B7, 3A3, and 3A4 (Aoyama *et al.*, 1990) with CYP 1A2 and 3A4 being the predominant isoforms active in the conversion to AFBO (Gallagher *et al.*, 1994; Raney *et al.*, 1992a). *In vitro* data suggests that the dominant route for *in vivo* AFB_1 activation at dietary concentrations is primarily through CYP 1A2, although urinary metabolite data evidence suggests that both forms are involved (Gallagher *et al.*, 1994). However, contrasting results from an early study in Thailand indicated a significant correlation between the formation of AFBO (as measured by AFB_1 tris-diol formation) and AFQ_1 (the major metabolite measured) with CYP3A4 expression at intermediate AFB_1 concentrations (Kirby *et al.*, 1993) suggesting that concentration influenced CYP activity. The same study also measured individual variation in expression of the various CYPs resulting in a >10-fold variation, including CYP3A4 (57-fold). This variation in expression is possibly be due to genetic polymorphisms or environmental factors and may be an important risk factor of liver cancer development in AFB_1-exposed populations. Importantly, this also has implications for the analysis of the various metabolites and measurements of toxicant exposure.

In addition to the formation of AFBO, oxidation of AFs by microsomal cytochromes P450 1A2 and 3A4 at the 3 and 9a positions produces the metabolites AFQ_1 and AFM_1 (Figure 2). O-demethylation at the 9a position by the same enzymes produces the metabolite, aflatoxin P_1 (AFP_1). In some species, reduction to aflatoxicol occurs, although it is not a major metabolite and is readily oxidized back to AFB_1 (Salhab and Edwards, 1977). These metabolites are referred to as detoxification products due to their relatively low mutagenicity and carcinogenicity compared to the reactive AFBO and resulting DNA adduct. In human liver microsome studies, AFQ_1, in addition to AFBO, are the major oxidative products formed from AFB_1 at all substrate concentrations (Raney *et al.*, 1992c). AFM_1 and AFBO formation correlates (r^2=0.976) under low substrate concentrations. Conversely, at high AFB_1 concentrations, the correlation between AFBO and AFQ_1 becomes much higher (r^2=0.550). These results suggest that at low substrate concentrations, the "high affinity" (CYP1A2) form of P450 is responsible for the epoxidation of AFB_1 and the oxidation of AFM_1, whereas the "low affinity" form (CYP3A4) produces AFQ_1 as well as AFBO (Ramsdell *et al.*, 1991; Raney *et al.*, 1992c). The CYP P450 and metabolite patterns are similar to earlier work where phenobarbital induction of CYP 3A4, increased levels of AFQ_1 and decreased levels of AFM_1; however, the decreased AFM_1 may have been due to increased conjugation enzymes (Monroe and Eaton, 1987).

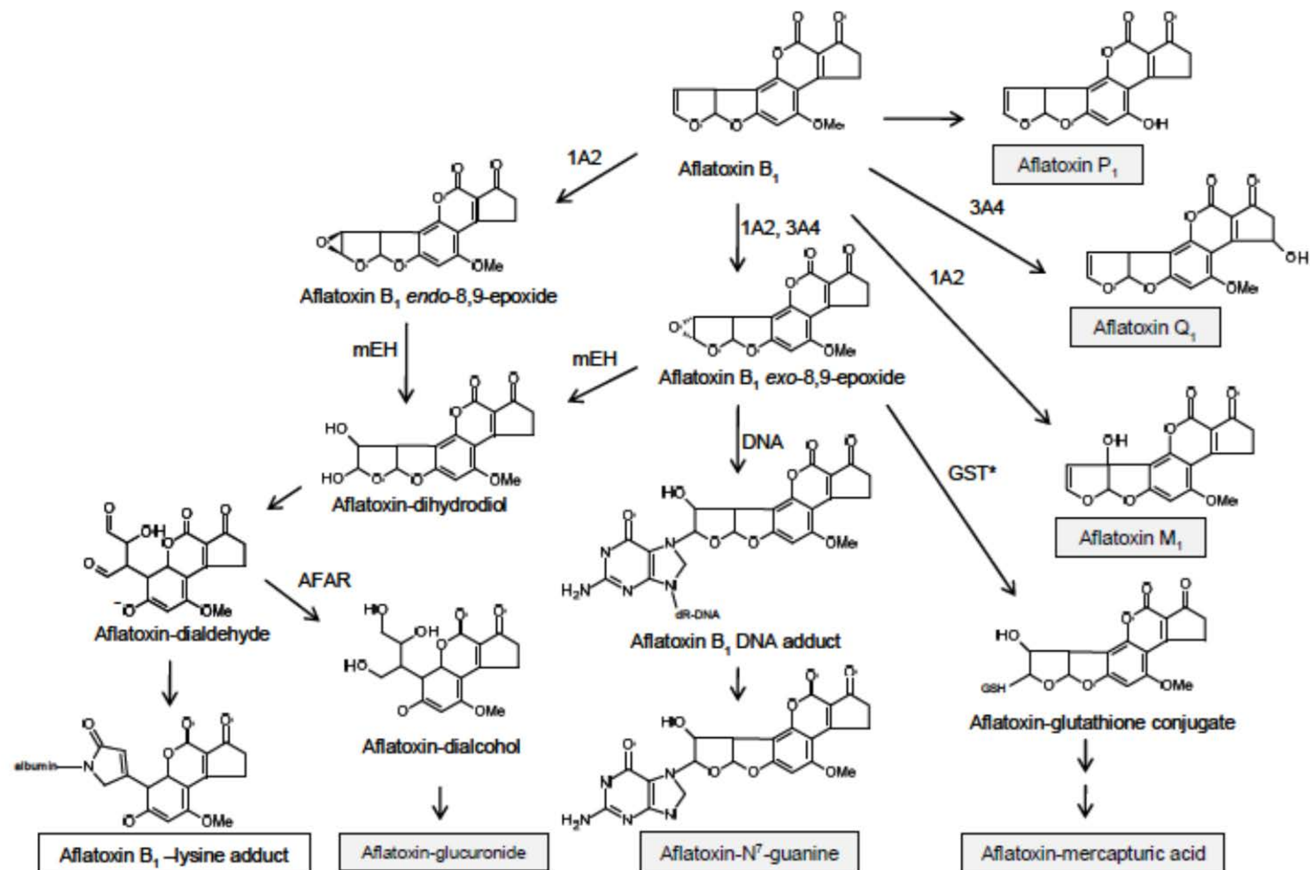

Figure 2. Metabolism of aflatoxin B_1 by phase I and phase II enzymes. Phase I enzymes include CYP 3A4 and 1A2. Biomarkers are highlighted in blood (white box) and urine (gray box). Adapted from Wild and Turner 2002**.**

AFB_1 is further metabolized by phase II conjugation enzymes to form more water soluble that are excreted. The main phase II pathway for AFBO is through conjugation with glutathione by way of the cytosolic enzyme glutathione-S-transferase (GST). GST enzymes efficiently conjugate the tripeptide GSH with lipophilic electrophiles including the carcinogen benzo(a)pyrene diol epoxide (Robertson *et al.*, 1986) and AFBO (Raney *et al.*, 1992b). GST are a multigene family containing many enzymes with variable patterns of gene regulation and catalytic specificity that can act as an alternative nucleophilic site to the nucleophilic portions of DNA (Coles and Ketterer, 1990). Conjugation of AFBO with GSH gives the primary metabolite in the bile following AFB_1 treatment in rats (Degen and Neumann, 1978; Holeski *et al.*, 1987; Raney *et al.*, 1992b); about 10% of the administered dose was identified as the glutathione conjugate (Degen and Neumann, 1978). Conjugation of AFBO with GSH is typically followed by conversion to an AF-mercapturic acid residue by acetylases and peptidases which is then excreted in the urine (Wild and Turner, 2002).

GST activity is an important factor in determining the susceptibility of different species to the carcinogenic effects of AFB_1. This is evident in the species difference between rats and mice. Although AFB_1 is readily bioactivated to the reactive AFBO in mice, they are resistant to AFB_1 induction of tumors due to high levels of cytosolic GST which form an AFB-conjugate that is excreted. Rats on the other hand, poorly convert AFB_1 to AFBO, which in turn is a poor substrate for GST enzymes. For example, oral doses as high as 10,000 ppb AFB_1 do not cause liver cancer in mice while levels as low as 15 ppb cause increased tumors in rats (Wogan and Newberne, 1967). It was later

discovered that mice constitutively express murine GST A3-3, which allows conjugation of AFBO to occur up to 50 times faster than in sensitive species such as the rat (Eaton and Gallagher, 1994). Human cytosolic fractions and liver slices demonstrate relatively low AFBO conjugation (Heinonen *et al.*, 1996; Kirby *et al.*, 1993; Moss and Neal, 1985). In one particular study comparing the conjugation activities in mouse, rat and human liver cytosols, the human liver cytosol appears to conjugate AFBO, however, with lower efficiency than the rat (Raney *et al.*, 1992b). GST mediated conjugation of AFBO in humans was supported by identification of the urinary mercapturic acid metabolite in the course of chemoprevention studies in the People's Republic of China (Wang *et al.*, 1999).

The same hydroxylated AF products can undergo phase II conjugation with glurcuronide via UDP-glucuronyltransferase. Holeski et al. reported that AFP_1-glucuronide made up 4-15% of total biliary AFB_1 metabolites, which is second only in rat bile to AFB-GSH (1987). Additional studies have confirmed that AFP_1-glucuronide is the only significant glucuronide or sulfate conjugate of hydroxylated AFB_1 metabolites (Eaton *et al.*, 1994).

1.1.4.5 Excretion

Absorbed AFB_1 and its metabolites are excreted in urine, while elimination in feces is a route for both unabsorbed AFB_1 and the biliary excretion of metabolites. Early work identified biliary exertion as the primary route in rats with 60% of the administered radio-labeled AFB_1 excreted within the first 24 hr, followed by 20% excreted in the urine (Wogan *et al.*, 1967). Rhesus monkeys administered a low or high dose of radio-labeled AFB_1 excreted 40% of the dose in urine and 42% in the feces within 7 days, independent

of the dose (Dalezios *et al.*, 1973). The urine and blood still contained detectable radioactivity 5 weeks after administration. In this study, AFM_1 was the major urinary metabolite during days 1-4 (18-20%), and unmetabolized AFB_1 was excreted in small amounts on day 1 (0.05-0.2%). Eighty-three Chinese males with detectable levels of AFM_1 were recruited to asess the fecal and urinary excretion of AFM_1, AFQ_1, and AFB-N7-guanine. The concentration of fecal AFQ_1 was 60 times higher than that of AFM_1. Similarly, excretion of AFQ_1 in urine was greater than that of AFM_1 and AFB-N7-guanine (Mykkanen *et al.*, 2005). Additionally, mammals excrete AFM_1 in milk while nursing. In fact, the designation "M" comes from its discovery in milk, initially identified as a compound related to AFB_1 (De Iongh *et al.*, 1964). Figure 2 depicts the fate of the various AFB_1 metabolites. Many of these metabolites serve as important biomarkers of AFB_1 exposure.

1.1.5 Biomarkers of exposure

Several AFB_1 metabolites have been studied as potential biomarkers of exposure and biological effect. Typically, biomarkers are chemical compounds that can be measured and correlated with specific endpoints or molecular and cellular events that may be predictive of health risks. These compounds or events fall into the categories of exposure (exposure to a parent compound), effect (biological responses to an exposure), and susceptibility (individual response to an environmental agent) (Groopman, 1994). Importantly, AF biomarkers can serve as intermediate endpoints for assessing the efficacy of cancer prevention interventions. In 1989, Wogan defined the important attributes necessary for measuring exposure to environmental carcinogens such as AF. Viable

biomarkers must: have adequate analytical methods to detect and quantify exposure to carcinogens/mutagens at ambient levels in the environment; be applicable to cells or body fluids that are readily accessible; have measured values quantitatively related to exposure levels over a wide range; and integrate consequences of intermittent or continuous exposures to multiple agents (Wogan, 1989). The development of molecular biomarkers for AFs is based on the extensive data available about their metabolism, macromolecular adduct formation, and general mechanisms of actions which have been previously discussed. Several AFB_1 serum and urinary markers fall into these categories and have been thoroughly researched and utilized in epidemiological studies and clinical intervention trials. These biomarkers include AFB_1-N7-guanine, AFB_1-albumin adduct, AF-mercapturic acid and AFM_1.

1.1.5.1 Aflatoxin M_1

Of the urinary metabolites assessed as biomarkers, only AFB-N7-Gua and AFM_1 have shown a dose-dependent relationship between AF intake and urinary levels. This is an important correlation for determining the risk associated with dietary AF exposure and therefore serves as an important measure of internal dose. Zhu et al. (Zhu *et al.*, 1987) analyzed AFM_1 concentration in urine samples by enzyme-linked immunoabsorbent assay and noted correlations between levels of AFM_1 excretion and levels of AFB_1 in corn and peanut oil samples collected from different households in Fusui County, Guangxi Autonomous Region, People's Republic of China. A good correlation (r= 0.65) between total dietary AFB_1 intake and total AFM_1 excretion in human urine was observed during a 3-day period. However, one study did not report AFM_1 as a major urinary metabolite

after using immunoabsorbent assays (Groopman *et al.*, 1992a). Alternately, the same group was able to use immunoaffinity and HPLC methods (Groopman, 1994) and confirmed a strong and highly statistically significant correlation between AF intake with measurements of urinary exertion of AFM_1 and DNA adduct AFB_1-N7-guanine in samples from Shanghai, China. Several studies report similar correlations, as well as estimate that 1.2-2.2% of the total AFB_1 intake is excreted as AFM_1 in urine (Groopman *et al.*, 1992a; Nyathi *et al.*, 1987). Rat dosimetry studies confirmed this work demonstrating excellent correlation between amount quantified in the urine and the dose administered resulting with a correlation coefficient of 0.93 (Groopman *et al.*, 1992b). Because AFM_1 is formed by the same cytochrome P450 that yields the 8,9-epoxide, AFM_1 serves as a reasonable surrogate for the genotoxic potential of AF exposures in individuals in addition to measuring internal dose.

Furthermore, urinary levels of AFM_1 may provide some index of altered risk for use in intervention studies aimed to reduce exposure and prevent cancer. Sun *et al*. (1999) utilized AFM_1 to calculate risk of developing HCC due to AF exposure, concomitant exposure to hepatitis C virus or a family history of HCC and was able to show that exposure to AFM_1 can account for a substantial part of the risk of developing HCC. When oltipraz, a chemopreventative agent that affects phase I and phase II metabolism of AF, was administered to rats exposed to AFB_1, AFM_1 levels were significantly reduced during the intervention followed by a rapid rebound following completion of treatment (Scholl *et al.*, 1996). Continued work with oltipraz demonstrated similar decreases in AFM_1 in addition to AF-mercapturic acid conjugate following 1 month of treatment in humans

(Wang *et al.*, 1999). Intervention trials using an AF binder in a humans showed significant decreases in both AFB_1-albumin adduct and AFM_1 following 3-months of treatment (Wang *et al.*, 2008). Mitchell et al. (2013) reported 55% reduction in urinary AFM_1 levels by a clay treatment compared to the placebo. Furthermore, this was the first to show that daily urinary AFM_1 levels can be used as a biomarker of internal AFB_1 exposure in a short-term intervention trial. Due to the ease of sample collection, this biomarker proved to be especially useful in measuring the efficacy of the same clay binder in children (Mitchell *et al.*, 2014a). It is important to note however, that AFM_1 is indicative of AF exposure within the past two days due to its rapid excretion, which typically occurs in the first 12 hr and decreases to undetectable levels within 48 hr.

As previously indicated, AFM_1 is also measured in milk. Numerous studies have detected AFM_1 in human breast milk with varying frequency, presumably due to differences in AF intake. These incidences range from 22% in Iran (Mahdavi et al. 2010) to 92% in the United Arab Emirates (Abdulrazzaq et al 2003). Recently, Columbian women were surveyed about their dietary habits and provided breast milk for HPLC analysis. AFM_1 was detected in 90% of the milk samples with a mean of 5.2 pg/ml. In an earlier study conducted in Zimbabwe, 54 samples were obtained and 11% were found to contain up to 50 pg/ml in breast milk (Wild *et al.*, 1987). In further studies, carryover from AFB_1 intake to AFM_1 in milk has ranged from 0.09 to 0.43% (Zarba *et al.*, 1992) to 0.3-6.2% (Creppy, 2002). AFM_1 in milk provides an additional source of exposure. This source is of special concern due to the fact that milk is frequently consumed by children and as a result, the FDA action limit is 0.5 ppb AFM_1 in milk.

1.1.5.2 Aflatoxin B_1-N7-guanine

As previously mentioned, multiple studies report a linear correlation with AFM_1 and AFB1-N7-Gua and AF intake and therefore serve as important biomarkers of exposure. However, if the measurement of carcinogen-DNA adducts is of primary interest and the subsequent representation of macromolecular damage and cancer initiation, AFB-N7-Gua may serve as a more relevant biomarker of effect since it represents a surrogate measure of covalent binding to DNA. Furthermore, AFB_1-N7-Gua was shown to be exclusively excreted in the urine of rats, which simplifies pharmacokinetics (Bennett *et al.*, 1981). AFB_1-N7-Gua was also determined to be a short-term biomarker due to its half-life of 8-10 hr in rats (Groopman *et al.*, 1980). Groopman and colleagues reported a correlation coefficient of 0.82 between AFB_1-N7-Gua levels and dietary intake from daily samples collected for four consecutive days from Gambian participants. This same study raises the issue of rapid urinary excretion of AFs and the rapid fluctuation in urinary AF levels. Samples analyzed from 20 of the Gambian participants demonstrated marked fluctuation in urinary AF levels, exceeding two orders of magnitude in some cases. This was overcome by the integration of urinary levels over a number of days by collecting consecutive 24-hr urine samples. The mean daily urinary AF levels over the four days were then compared with the mean daily AF intake for each individual and resulted in the correlation previously reported. Importantly, formation and excretion of AFB_1-N7-Gua in urine are similar in F344 rats and humans, thereby adding an important confirmation of the rat to human extrapolation (Groopman, 1994). A comparison of the dose-dependent levels of AF binding to liver DNA with the amount of urinary AFB_1-N7-Gua in rats

demonstrated a correlation coefficient of 0.98 highlighting the use of the biomarker as a surrogate of AFB_1-DNA binding (biomarker of effect) as well as a surrogate for AF intake (biomarker of exposure).

1.1.5.3 Aflatoxin B_1-albumin/Aflatoxin B_1-lysine adduct

The AF-adduct with serum albumin has been examined as a biomarker of exposure. Because of the longer in vivo half-life of albumin compared to the urinary DNA adduct, the serum albumin adduct can integrate AF exposures over longer time periods and act as a surrogate measure of covalent binding to DNA. Albumin is the only protein in serum that binds AFB_1 to any significant extent in both monkeys and rats. Wild *et al.* (1986) found that 25 hr after a single dose of AFB_1 (3.5-200u/kg AFB_1), a total 0.98-2.15% of the amount administered was bound to plasma protein in rats. Importantly, in a chronic study where animals were sacrificed on days 2, 3, 7, 14, 21, and 24, binding of AF to albumin accumulated to a 3-fold higher level than observed after a single dose. Furthermore, AFB_1-albumin (AFB_1-alb) binding reached a plateau between days 7 and 14 of treatment indicting that this biomarker may represent stable chronic or long-term exposure.

Gan *et al.* (1988) analyzed AFB_1-alb adduct in samples collected from individuals living in Guangxi Province, P.R.C. and a high significant association between adduct and dietary intake was observed. It was determined in this study that 1.4-2.3% of the ingested AFB_1 was covalently bound to serum albumin which was very comparable to the data observed in rats. Furthermore, the data for the DNA-adduct in urine and serum albumin adduct correlated significantly with a coefficient of 0.73. Similarly, in samples collected

from The Gambia, another region of high incidence of HCC, the correlation between dietary AF intake and AF-alb biomarkers was equal to 0.83 (Groopman *et al.*, 1992a). However, a separate study reported a weaker correlation (.0.55) between AFB_1-alb and intake of AF-contaminated food in the Gambia (Wild *et al.*, 1992). This conflicting data could be due to inaccurately measured dietary intake of AF or due to the fact that AF-alb biomarker is a measure of exposure over the past 2 to 3 months, and therefore dietary intake of AF prior to the 7-day study period would contribute to the adduct level observed at the end of the study period. AFB_1 can decrease albumin levels as observed in swine fed AF-contaminated diets (Annau *et al.*, 1964). This is not surprising considering the fact that the liver is the site of albumin synthesis as well as the site of AF-albumin adduct formation (Wild *et al.*,1992). However, the effects of decreased albumin levels following AF exposure on the stability of the AF-alb adduct have not been explored.

Despite the animal model validation and epidemiological support, AF biomarker measurements have only been used as categorical data with simple dichotomization into positive or negative or high or low levels. Therefore, no quantitative data on dose-response from dietary exposure data can be generated.

1.1.6 Carcinogenicity

Following the identification of the hepatotoxic effects of AF in the 1960s, it was discovered that AF contamination was responsible for an outbreak of HCC in rainbow trout, a species not usually plagued with such malignancies (Halver, 1967). This finding led to the extensive study of chronic AF exposure. These studies utilized a variety of protocols including different dosing methods, rat strains, different periods of

administration and observation, etc. All reported the potency of AF in inducing HCC in rats when fed for periods of 20 weeks or longer. By 1977, three independent laboratories identified covalent modification of DNA by AF (Swenson *et al.*, 1977). Additionally, some of the first epidemiological work in this field reported AF exposure as a risk factor in the development of HCC in Mozambique, Swaziland, and Philippines (Bulatao-Jayme *et al.*, 1982; Peers and Linsell, 1977; Van Rensburg *et al.*, 1985). Results from all three studies positively correlated mean AF exposure with HCC. Moreover, daily AF exposure in HCC cases was estimated to be 4.5 times higher than in controls. Due to strong epidemiological evidence the International Agency for Research on Cancer (IARC) classified AFB_1 as a Group 1 carcinogen: human carcinogen, following multiple epidemiological studies in populations with high HCC incidence (IARC, 1993; IARC, 2002).

Covalent modification of DNA through alkylation of nucleic acids is a critical reaction in tumor induction with AFB_1. As previously mentioned, the reactive epoxide (AFBO) that is responsible for the carcinogenicity of the parent compound is formed following CYP oxidation. The alkylation of nucleic acid by AFBO occurs with high regiospecificity at the N7 position of guanine residues in DNA. AFBO can also form derivatives with RNA and proteins. Early work demonstrated that the *exo*-epoxide reacts with DNA by attack of the nitrogen atom at the 7 position of guanine on C8 of the epoxide. This reaction yields a *trans* DNA-adduct via an S_N2 reaction to produce 8,9 dihydro-8-(N7 guanyl)-9-hydroxdy AFB_1 adduct (AFB_1-N7-gua). The *endo*-epoxide fails to form an adduct at N7 or any site in DNA (Iyer *et al.*, 1994). The positive imidazole ring on AFB_1-

N7-gua promotes depurination and results in the formation of an apurinic site. Additionally, under slightly alkaline conditions the imidazole ring opens and forms the more stable and persistent AFB_1 formaminopyrimidine adduct (AFB_1-FAPY adduct) (Wild and Turner, 2002). Both AFB_1-N7-gua and AFB_1-FAPY adducts cause guanine (G) to thymine (T) transversion mutations (Foster *et al.*, 1983; Levy *et al.*, 1992; Lin *et al.*, 2014; Trottier *et al.*, 1992). It is likely that these adducts and the AP site individually or collectively represent the chemical precursors to the genetic effects of AFB_1; the dominant precursor to mutations induced by AFB_1 is still unconfirmed (Wild and Turner, 2002).

This AF-induced mutational hotspot (G→T transversion) occurs at the third position of codon 249 resulting in the Arg→Ser alteration in of the p53 gene. The p53 gene, associated with tumor suppression, is mutated in >50% of tumors, including HCC (Bressac *et al.*, 1991; Hsu *et al.*, 1991) and it can behave as a dominant oncogene. This gene fragment extends from the third position of codon 247 to the middle positon of codon 250 (Aguilar et al 1993). The GC→TA transversion is the most frequently observed mutation induced by AFB_1 in vitro followed by the transversion of C→A in the adjacent first position of codon 250 (Aguilar *et al.*, 1993). These findings correlate with *in vivo* reports of G→T hotspots in high AFB_1 regions in east Asia and Africa (Bressac *et al.*, 1991; Hsu *et al.*, Harris, 1991; Li *et al.*, 1993; Murakami *et al.*, 1991). It is important to note that many of the early studies identifying this hotspot in human populations did not take hepatitis status into account; this confounder will be discussed in a later section. Recently, early life exposure, specifically during the embryonic period, was demonstrated

to be strikingly susceptible to the mutagenic effects of AFB_1. AFB_1-DNA adducts in the embryos of C57BL/6J mice dosed on gestation day 14 were shown to be 20-fold more potent inducers of mutagenesis than adducts in parallel-dosed adults (Chawanthayatham *et al.*, 2015). The data revealed mutation spectra dominated by GC→TA mutations in both the mother and offspring.

The ras oncogene has also been identified at a molecular target in AF carcinogenesis. Studies using DNA from both AFB_1-induced transformed cell lines and primary liver tumors, demonstrated that AFB_1 produces mutations in codon 12 of all three types of c-ras oncogene (ha-ras, Ki-ras, and N-ras). However, few studies have identified activated c-ras oncogenes in human HCC obtained from AF-endemic areas (Eaton and Gallagher, 1994). One recent toxicogenomic study has also looked at microRNA expression responses following genotoxic doses of AFB_1 in HepaRG cells (Marrone *et al.*, 2015). In this study, miR-410 was over-expressed as compared to exposure to the non-carcinogenic analogue (AFB_2). Previous studies have identified overexpression of miR-410 in both rodent and human hepatocellular carcinogenesis (Luk *et al.*, 2011). Furthermore, miR-122 was inhibited in a dose- and time-dependent manner providing a novel insight to a genotoxic mode of action (Marrone *et al.*, 2015). miR-122 is a liver-specific miRNA and is the most abundant miRNA accounting for 52% of the total miRNAs in the livers of adult humans and plays a central role in normal liver function (Lewis and Jopling, 2010). Furthermore, associations between reduced expression of miR-122 and liver diseases have been reported, including (but not limited to) HBV, HCV, and hepatocellular carcinogenesis (Marrone *et al.*, 2015). However, the exact mechanism of

miR-122 down-regulation during hepatocellular carcinogenesis is still poorly understood. Studies focusing on the abnormal expression of miRNAs in human cancers have suggested that the presence of miRNA could have some consequential effect on tumorigenesis, such as hepatocellular carcinogenesis (Fang *et al.*, 2013).

Despite the highly mutagenic potency of AFBO and the associated adducts, there is variability among species susceptibility to AF-induced HCC. As previously described, biotransformation of AFB_1 appears to be a major determinant of the potency of its effects. Furthermore, glutathione activity and/or metabolism to a less mutagenic metabolite (i.e., AFQ_1) also serve as crucial roles in the dose that ultimately binds to DNA. For example, GST activity is inversely related to the susceptibility of rodent species to AFB_1-induced HCC (Degen and Neumann, 1981; Monroe and Eaton, 1987). Alternately, primates appear to have low GST activity but metabolism favors the production of AFQ_1 therefore limiting the amount converted to the reactive epoxide (Moss and Neal, 1985; Roebuck and Wogan, 1977).

In general, a species that is sensitive to acute toxic effects of AFB_1 is more susceptible to hepatic cancers by some degree of exposure. For example, the LD_{50} of ducks is 0.34 mg/kg and ducks exposed to AF develop HCC. In comparison, chickens have a LD_{50} twice that of ducks and (15-18 mg/kg), however hepatic cancer from AF exposure has never been reported in chickens (Roebuck and Maxuitenki, 1994). Additionally, the level of liver DNA adduction per unit AFB_1 dosage generally correlates with species susceptibility (Cole *et al.*, 1988; Lutz *et al.*, 1980). After a single exposure, concentration of the initial AFB_1-DNA adduct decreases at varying rates in different organisms or cell

types, based on conversion to persistent FAPY ring-opened derivatives, enzymatic DNA repair, spontaneous depurination, and growth dilution (Bailey, 1994).

The AFB_1 dose-response relationship has been explored, particularly regarding the possibility of a no-adduct threshold at low doses. Single-dose exposures of 10-1000 ng/kg in male F344 rats produced AFB_1-DNA adducts in the liver in a dose-dependent manner. Macromolecular adduct formation was observed at 10 ng/kg, which is within the human exposure range. However, the curves produced were linear at low doses and less than linear at higher doses (Appleton *et al.*, 1982). Male F344 rats chronically exposed to a series of AFB_1 doses maintained levels of DNA-adduct that did not increase significantly after 4 weeks. These results indicate that a steady-state adduct formation and removal can be reached. Furthermore, the adduct levels were proportional to the dose given (Buss *et al.*, 1990). These studies demonstrate DNA adduction by AFB_1 at doses that extend as low as human exposure levels and provide no indication of a threshold dose below which AFB_1 exposure might impose no genotoxic risk. Similar effects were also observed in the extremely sensitive rainbow trout. Trout treated with carcinogenic doses of AFB_1 for 2-4 weeks showed dose-linear accumulation of liver AFB_1-DNA adducts (Dashwood *et al.*, 1988). Importantly, this study also demonstrated a linear inhibitory response with low doses of indole-3-carbinol indicating the possible absence of any significant threshold of I3C protection against AFB_1-DNA binding; even at low levels, I3C may offer some protection against chemically induced neoplasia. Similarly, Johnson et al. (2014) recently demonstrated complete protection from AF-induced liver tumors with a 66% reduction in urinary AFB_1-N^7-guanine through the use of a triterpenoid (CDDO-Im), compared to 96%

HCC incidence in the AFB_1 treatment group. This study suggested that a threshold for AF-induced cancer might exist.

HBV infection is a risk factor for HCC and is often prevalent in areas where AF exposure is endemic. Based on the results from a 2012 meta-analysis of AF-related HCC studies, the population at risk for AF-related HCC was estimated to be 17% worldwide (Liu *et al.*, 2012). However, when factoring for individuals chronically exposed to HBV infection and AF, the risk of developing HCC nearly doubles (Groopman *et al.*, 2008) suggesting an additive or synergistic response. AF also appears to have a synergistic effect on hepatitis C virus-induced liver cancer (Wild and Montesano, 2009), although the quantitative relationship is not as well established as that for AF and HBV in inducing HCC. It should be noted that while HBV infection occurs most commonly in developing countries early in life, infection with HCV normally manifests much later, thus affecting the time period over which AF and HCV may interact (Wild and Montesano, 2009).

Studies quantifying the relationship between AF and HBV are well documented. Several have strongly demonstrated that concomitant exposure to AFs and HBV strengthens the risk of developing HCC. Groopman *et al.* (2008) reports the risk of liver cancer in individuals exposed to chronic HBV infection and AF to be up to 30 times greater than those exposed to AF alone. Another study in Shanghai revealed that hepatitis B virus surface antigen positive (HBsAg+) individuals exhibited a much greater risk for developing HCC when AF was detectable in the urine [59 risk ratio (RR)] compared to those with no detectable urinary AF (7 RR) (Qian *et al.*, 1994; Ross *et al.*, 1992). Similar trends were observed in the Guangzi Shuang Autonomous Region of China (Yeh *et al.*,

1985; Yeh *et al.*, 1989) and Taiwan (Sun *et al.* 2001). In a follow-up of the Taiwan cohort, it was discovered that HBsAg carriers who had detectable AF-alb adduct were more likely to develop HCC. In a recent prospective study in the Guangxi Province of China, tumor tissue and adjacent liver tissue from 397 HCC patients, and normal hepatic tissues from 68 cases of hepatic hemangioma, liver resection and liver transplant donors were collected and subdivided by AF and HBV status (Qi *et al.*, 2015). Interestingly, when the p53 mutation spectrum was analyzed, the patients positive for AF and independent of HBV status, had a significantly higher p53 mutation rates compared to AF negative groups thus suggesting an AF-specific mutation. Furthermore, 93.3% of the 223 HCC specimens exhibited positive staining for p53 mutations. The authors suggest that the positive staining seen in the HCC specimens indicate the presence of mutant p53 protein, which has a longer half-life and is easier to detect compared to the wild type p53 protein with a short half-life. One of the potential mechanisms of AF and HBV interactions could possibly explain this observation with p53. Chronic liver injury and regenerative hyperplasia resulting from HBV infection are critical to the development of HCC (Hussain *et al.*, 2007) and the HBV x gene (HBx) is frequently included in sequence of the virus that is integrated into cellular DNA (Kew, 2003). Cells transfected with the HBx gene are more prone to apoptosis and to induction of mutations at codon 249 of the p53 gene possibly by inhibiting excision repair thus leading to increased AFB_1 DNA adduct persistence and mutation induction (Hussain *et al.*, 2007; Kew, 2003), i.e., greater expression of the mutant p53 protein. Inflammation and oxidative stress associated with chronic active hepatitis may also result in DNA damage and mutations (Wild and

Table 1. Comparative toxicity of AFB_1 in various species of vertebrates[a]

Species	Strain	Sex	Age or weight	Route of administration[b]	LD_{50} (mg/kg)
Duck	Khaki-Campbell	M, F	1 day	P.O.	0.36
	Pekin	M, F	1 day	P.O.	0.34
Chicken		M	21 days	P.O.	18.00
Turkey	Beltsville	M	15 days	P.O.	3.20
Trout	Mt. Shasta	M, F	9 months	I.P.	0.81
Catfish	Channel	M, F	9.3-0.5 kg	P.O.	11.50
Mouse	Swiss	M, F	newborn	P.O.	1.50
	CD_1	M	weanling	P.O.	7.30
Rat	Porton	M	42 days	P.O.	6.25
		F	42 days	P.O.	18.00
	Fischer	M	42 days	I.P.	4.20
Hamster	Syrian	F	42 days	I.P.	5.85
		M	30 days	P.O.	12.80
Guinea Pig		M	56 days	P.O.	1.00
		F	56 days	P.O.	1.80
Rabbit	Dutch breed	M, F	90 days	I.P.	0.30
Cat	Mixed breed	M, F	adult	P.O.	0.55
Dog	Mixed breed	M, F	weanling	P.O.	0.80
Pig	Poland China	M	weanling	P.O.	0.62
Sheep	Cross breed	M	2 years	P.O.	2.00
Baboon	Wild	M	adult	P.O.	2.20
Monkey					
Cynomolgus		M	adult	P.O.	2.20
Macque		F	adult	P.O.	8.00

[a]Data shown in this table is adapted from Eaton and Groopman (1994). [b]P.O., Per os; I.P., intraperitoneal.

Montesano, 2009). Finally, AFB_1 is known to be immunospressive in animals and may affect susceptibility to chronic viral infection in exposed individuals (Turner *et al.*, 2003).

AFM_1 is approximately only 10% of the mutagenicity of AFB_1 (Wogan *et al.*, 1974), however the acute toxicity of AFM_1 is both quantitatively and qualitatively similar to AFB_1 in ducklings and rats. A test for covalent binding to rat liver DNA revealed a covalent binding index of 2100 demonstrating that AFM_1 must also be regarded as a strong

hepatocarcinogen (Lutz *et al.*, 1980). For this reason, AFM_1 is strongly regulated in diary which is frequently consumed by the young.

1.1.7 Toxicity

AF toxicity principally leads to structural and functional damage to the liver. The effects of toxic doses are well known in most laboratory animals and in several domestic animal species and are summarized in Table 1. Susceptibility to AF is greatest in the young, and there are significant differences between species, within species, and sexes (according to the concentrations of testosterone). The toxicity of AF also varies according to many nutritional factors (Pier, 1985). For instance, recovery from protein malnutrition is severely delayed by AF exposure (Rogers, 1993). In laboratory and domestic animals, chronic exposure to AFs compromises immunity and interferes with protein metabolism and multiple micronutrients that are critical to health. However, clinical data in humans is still limited. Acute AF poisoning, or aflatoxicosis in humans has been observed in multiple outbreaks and is characterized by vomiting, abdominal pain, pulmonary edema, and fatty infiltration and necrosis of the liver. Aflatoxicosis is the poisoning that results from AF exposure. Two forms of aflatoxicosis have been identified: the first is acute severe intoxication, which results in direct liver damage and subsequent illness or death, and the second is chronic subsympotmaic exposure (Williams *et al.*, 2004). Depending on the dose and duration of the exposure, AF toxicity may lead to acute illness and death, usually through liver cirrhosis, nutritional and immunologic consequences, and cumulative effect on the risk of cancer. One of the most devastating outbreaks occurred in the winter through early summer of 2004 in eastern Kenya (Azziz-Baumgartner *et al.*, 2005). This outbreak

resulted in 317 cases and 125 deaths. Health officials sampled maize from the affected area and measured AFB_1 concentrations 220 times greater than the 20 ppb limit for food suggested by Kenyan authorities. Similar to studies conducted in animals, early symptoms of aflatoxicosis include anorexia, malaise, low-grade fever and progress to acute hepatitis with vomiting, abdominal pain, and death (Etzel, 2002). The first sign of exposure in all animal species is decreased growth and loss of appetite. This initial observation led to the early studies demonstrating the role of AF in nutritional modulation, growth suppression, and immune system impairment.

Micronutrient deficiencies commonly occur in populations of developing countries and exposure to dietary AF can further aggravates the deficiencies. Animal studies indicate that exposure to AF may reduce plasma and tissue vitamin A and E concentrations. Broiler chicks and barrows dosed with AFB_1 showed a depression of hepatic vitamin A (Harvey *et al.*, 1994; Pimpukdee *et al.*, 2004). Vitamin A is vital for vision, programming of epithelial cell differentiation in the digestive tract and respiratory system, skin, bone, nervous system, and immune system, and for hematopoiesis (Sahin *et al.*, 2002). Similarly, vitamin A and vitamin E serum levels decreased by half in young pigs exposed to AF-contaminated corn (500 ng/g AFB_1) for 21 days (Harper *et al.*, 2010). Vitamin E also plays an important role in maintenance of the immune system, possibly through enhancing T-cell proliferation and lowering measure of oxidative stress due to its antioxidant properties (Lee and Man-Fan Wan, 2000). Like vitamins A and E, vitamin D is strongly involved in the maintenance of immune system competence and has been shown to be affected by AFs in the diet in broiler chickens. AF (1ppm in the diet) reduced

plasma vitamin D concentrations after 5 days of treatment (Glahn *et al.*, 1991). Results from a population in Ghana found a significant inverse relationship between AF-alb levels and both vitamin A and E concentrations (Obuseh *et al.*, 2010; Tang *et al.*, 2009). Furthermore, participants who had high detectable levels of AF-alb ($\geq$0.8 pmol/mg albumin) had increased odds for vitamin A deficiency compared to those with lower levels. In contrast, a study measuring the modification of immune function through exposure to AF in Gambian children only observed a negative correlation with AF-alb and vitamin C (Turner *et al.*, 2003). The authors of this study point out that seasonal variations exist for a number of micronutrients, which should be considered in future studies. Although AFB_1 appears to modulate vitamin A and E levels in animal studies, there is an important need to clarify this role in relation to humans.

AFs have been shown to have an effect on zinc and selenium concentrations. Importantly, these minerals are essential for healthy immune systems. Specifically, zinc is required to activate a thymic hormone, thymulin (ZnFTS), which is responsible for cell-mediated immunity (Mocchegiani *et al.*, 1998). Intestinal malabsorption occurs in piglets from AF-exposed sows with defects related to a reduced zinc intestinal absorption (Miller *et al.*, 1981). Thymic involution, associated with depletion of thymocytes and reduction of both thymus and body weights, is an event that has been reported in animals exposed to AF (Harvey *et al.*, 1988; Panangala *et al.*, 1986). This is possibly due to AF-associated zinc malabsorption. Piglets born to mothers exposed to AFB_1 and G_1 were deficient in zinc and had decreased thymic endocrine activity despite normal zinc milk concentrations from the lactating sows (Mocchegiani *et al.*, 1998; Silvotti *et al.*, 1995). Active ZnFTS

were decreased and inactive thumulin (FTS) concentrations were high. The low peripheral zinc bioavailability induced by maternal AF exposure appears to be associated with the depletion of thymulin-secreting cells and cortical thymocytes. Interestingly, zinc levels in the AF-exposed sows were not affected (Mocchegiani *et al.*, 1998). The authors suggest that the observed reduced thymus and body weights of the piglets may be largely due to the presence of AF in sow milk. Reduced zinc concentrations following AF exposure has also been observed in rats (Doyle *et al.*, 1977; Ikegwuonu, 1984) and lambs (Ramos *et al.*, 1996). Importantly, the rat studies utilized intraperitoneal injections of AF rather than fed AF-contaminated diets like the lamb and pig studies suggesting that the observed depression in minerals is a biochemical effect rather than a consequence of AF-associated malabsorption. Similarly, selenium levels were found to be inversely related to AF exposure (Chen *et al.*, 1982; Chen *et al.*, 2000; Hegazy and Adachi, 2000). Early work in chickens demonstrated the depressing effect on Se by AF in a salmonella challenge model (Chen *et al.*, 1982). Authors from this study also report increased liver and plasma GSH levels and decreased AF-DNA adducts with Se supplementation. Interestingly, an inverse association between plasma selenium levels and AFB_1-alb adducts in men from Matzu, Taiwan was statistically significant but only among those with null genotypes of GSTM1 and GSTT1 (Chen *et al.*, 2000). These results suggest that Se may be involved in AF metabolism through modulation of GSH and GST levels.

AF exposure can occur in infancy, through weaning, and into adulthood, providing a strikingly dynamic pattern. The interference of AFB_1 with prenatal development has been reported in mice (Arora *et al.*, 1981), hamsters (Schmidt and Panciera, 1980), and

rats (Butler and Wigglesw, 1966; Geissler and Faustman, 1988; Mayura *et al.*, 1998). Results from animal studies with *in utero* exposure demonstrate a delay of early response development, impaired locomotor coordination, impaired learning ability, and growth retardation (Kihara *et al.*, 2000). Moreover, reduced feed intake and subsequent weight loss in animals exposed to AF have been reported in ducklings (Cheng *et al.*, 2001), mice (Kocabas *et al.*, 2003), Japanese quail (Sadana *et al.*, 1992), chickens (Bryden *et al.*, 1979; Doerr *et al.*, 1983; Pimpukdee *et al.*, 2004), turkeys (Giambrone *et al.*, 1985), pigs (Harvey *et al.*, 1995; Harvey *et al.*, 1994; Lindemann *et al.*, 1993), red drum (Zychowski *et al.*, 2013a), and Nile tilapia (Zychowski *et al.*, 2013b).

AF biomarkers have played an important role in supporting the growth inhibitory effects in humans. AFB_1, AFG_1, and AFQ_1 were detected in 17 out of 35 Thai cord blood samples as compared to 2 out of 35 maternal blood samples indicating transfer of AFs from mothers to fetuses (Denning *et al.*, 1990). Following analysis of results demonstrating much greater levels (up to 10 times) of AF-alb in the venous blood of Gambian mothers compared with those in matched cord blood samples, it has been suggested that fetal metabolism of AF to AF-alb may occur with low efficiency or that transplacental transfer may be low (Wild *et al.*, 1991).

Extensive research in West Africa has demonstrated a significant role for AF in early life growth faltering where dietary insufficiency and infectious disease only explain about half of the restricted growth (Turner, 2013). Importantly, children who are stunted often develop long-term developmental and cognitive problems and are more vulnerable to infectious diseases (Ricci *et al.*, 2006). In separate surveys conducted in The Gambia,

Benin and Togo, a dose dependent association between AF-alb and growth/growth velocity was observed in children 16 weeks (Gambia) (Turner *et al.*, 2007), 9-60 months (Benin and Togo) (Gong *et al.*, 2002; Gong *et al.*, 2003), 16-37 months (Benin) (Gong *et al.*, 2004), and 6-9 years (Gambia) (Turner *et al.*, Wild, 2003) of age. Furthermore, mothers with high AF-alb adduct levels in Ghana were more likely to have low birth weight babies demonstrating a significant inverse relationship between birth weight and maternal AF-alb adduct (Shuaib *et al.*, 2010). Findings from studies associating growth and AF have been summarized in Table 2.

Even though *in utero* exposures can have a significant effect on faltering in infant growth (Shuaib *et al.*, 2010; Turner *et al.*, 2007), AF exposure increases most dramatically after children are weaned from breastfeeding (Gong *et al.*, 2003). In many parts of Africa, weaning food typically consist of varying ratios of maize meal and groundnuts. Weaned children in Benin and Togo had approximately two-fold higher mean AF-alb adduct levels than those receiving a mixture of breast milk and solid foods (Gong *et al.*, 2003). It was recently determined that a highly exposed population in Ghana was consuming homemade "Weanimix" with average contamination levels exceeding 200 ppb AFB_1 and some containing up to 500 ppb (Kumi *et al.*, 2013). For comparison, after dosing for 4 or 10 weeks, decreases in weight gain were observed in young pigs exposed to similar levels (Panangala *et al.*, 1986; Schell *et al.*, 1993).

Although AF's mode of action for growth stunting has not been definitively established, the role for DNA, RNA, and protein has been implicated. Recently, the contribution of hepatotoxic effects on growth hormone (GH) signaling and growth

stunting has been studied. A rat model studying liver injury demonstrated dose-dependent wasting and stunting, liver pathology, and suppression of hepatic targets of GH signaling in AFB_1-exposed rats. The authors suggest a toxin-dependent liver injury and hepatic GH-resistance mechanism for growth impairment (Knipstein *et al.*, 2015). Another hypothesis that deserves attention is the combined damaging effect of AFs and other infectious agents on the intestinal epithelium leading to chronic inflammation, lowered resistance to systemic infection, and abnormalities in nutrient absorption. For the first time, exposure to AF was associated with childhood chronic hepatomegaly in school-age children from the Makueni district in Kenya (Gong *et al.*, 2012). The long-term effects of this condition have yet to be defined but recent analysis suggests an association with slowed child growth (Wilson *et al.*, 2010).

AFB_1 acts as a "force multiplier" synergizing the adverse effects of microbial pathogens and other agents or factors detrimental to health (Monson *et al.*, 2015) including immune suppression. The effects of AF on the immune system have been well established in a number of animal species as reviewed by Bondy and Pestka (2000). The main target of such studies have been cell-mediated immune responses (Ali *et al.*, 1994; Bondy and Pestka, 2000; Neiger *et al.*, 1994). As previously implied, growth faltering and micronutrient deficiencies are associated with decreased immune and non-immune host defenses that increase susceptibility to infectious diseases (Wild, 2007) which contributes to the term immunotoxin.

A cohort of Gambian children 6-9 years was studied to determine the effect of dietary AF exposure on immune parameters reflecting T-cell, B-cell, and mucosal

Table 2. AF and growth faltering

Country	Sample size, n	Method	Results	Reference
Benin and Togo	480	Children (9-60 months) from 16 villages in 4 geographic zones were included. Anthropometric measures wre taken, z-scores calculated and AF-alb adducts analyzed.	Association: AF-alb adduct and HAZ**; AF-alb adduct and WAZ**; AF-alb adduct and WHZ*	Gong et al. 2002
Benin	200	Fifty children from 4 villages (2 high-contamination risk areas and 2 low-contamination risk areas). Measurements were conducted in February and October.	Mean AF-alb and height increase after 8 months. Negative association between height and AF-alb quartiles.	Gong et al. 2004
Benin and Togo	479	A cross-sectional study that included children of weaning age. Anthropometric measures were taken, z-scores calculated and AF-alb adducts tested.	Association:AF-alb adduct and HAZ**; AF-alb adduct and WAZ**; AF-alb adduct and WHZ	Gong et al. 2003
The Gambia	138	Infants were included at birth and followed up monthly for 12 months. Serum AF-alb levels were measured in maternal blood during pregnancy, in cord blood and infants at 16	Maternal AF-alb levels and infant weight, multiple regression coefficient: Maternal AF-alb and infant WAZ: -0.249*; Maternal AF-alb and infant WAZ	Turner et al. 2007
Kenya	242	Maize was collected from households with children ages 3-36 months and analyzed. Anthropometric measures of the children were taken.	Wasted children: 53.8% AF consumers vs. 27.7% non-AF consumers*; Stunted children: 32.4% AF consumers vs. 28.9% non-AF consumers; Underweight children: 41.4% AF consumers vs. 27.3% non-AF consumers	Okoth et al. 2004

HAZ= Height-for-age z-score; LAZ = Length-for-age z-score; WLZ = weight-for-lenth z-score; * p,0.05; ** p<0.01.

[a] According the Wellcome classification. Adapted from Lombard et al. 2014

secretion as measured by secretory IgA (sIgA) levels in saliva, cell-mediated immunity (CMI) multi-test, and antibody responses to both rabies and pneumococcal polysaccharide vaccines. The study reported that sIgA was markedly lower in children with detectable AF-alb compared to those with non-detectable levels. There was a weak association with one of four pneumococcal serotypes (no response to rabies) and no association between CMI responses to test antigens of AF (Turner et al., 2003). The IgA suppression observed in this study suggests that AF exposure may influence susceptibility to infectious disease. In addition, the authors question the relevancy of using a long-term biomarker, specifically concerning the lack of an association between AF exposure and CMI, which is evidenced in animal studies. It is unknown whether recent or past exposure is important in determining immune modulation. In a separate study, the role of AFs in modifying the distribution and function of leukocyte subsets in Ghanaians was assessed (Jiang et al., 2005). Correlation analyses indicate that high levels of AFB1 are significantly associated with low levels of CD3+ (T cells) and CD19+ (B cells) cells showing the CD69 activation marker. The CD69 activation marker is a co-stimulatory molecule for T-lymphocyte proliferation and ultimately results in amplified immune responses. CD8+ T cells that express perforin and granzyme A were also inversely associated with AF in this study. Overall, alteration of immunological parameters could result in impairments in cellular immunity that decrease host resistance. Importantly, Jiang and colleagues attempted at calculating general dietary exposure levels for this Ghanaian population estimating that 10 µg of AFB1 a day would result in the effects observed. High AF-alb levels also appeared to accentuate some HIV associated changes in T-cell phenotypes and in B-cells

in HIV positive participants from a more recent study in the same Ghanaian population (Jiang *et al.*, 2008). Additionally, vitamin A induces lymphoproliferation resulting in a stimulated immune system; a deficiency can decrease specific antibody production, the number of circulating lymphocytes, and lymphocyte proliferation (West *et al.*, 1991). As previously mentioned, AF has been associated decreased vitamin A levels therefore imparting an indirect effect on immune function.

Mechanistically, the reactive AFBO has been associated with acute toxicity through multiple pathways via adduct formation with DNA, RNA, and proteins. Dramatic decreases in nuclear and nucleoar RNA synthesis has been observed in AFB_1-treated animals (Yu, 1983; Yu *et al.*, 1988a; Yu *et al.*, 1988b) resulting in inhibition of the chromatin template and inhibition of RNA polymerase. Guanine nucelotides of RNA adducts are believed to interfere with cellular protein synthesis and to inhibit protein translation at ribosomes in acute aflatoxicosis (Sarasin and Moule, 1975). This RNA injury is considered the major factor in diminished cellular protein synthesis (Yu *et al.*, 1988a). Recently, Zhuang et al. (2016) utilized immobilized affinity chromatography and liquid chromatography-tandem mass spectrometry to identify AFB_1-binding proteins and observed AFB_1 binding with RPSA (Zhuang *et al.*, 2016). RPSA is a ribosomal subunit that can be found in cytoplasmic membrane, cytoplasm, and cell nuclei. This subunit is involved in ribosomal RNA processing and precursor assembly and is critical for 40S ribosome subunit maturation. Furthermore, overexpression of RPSA is common in a number of cancers and is hypothesized to play a role in chromatic regulation through interactions with histone proteins. Although these results represent one study in a limited

field, they would suggest a strong role for RNA and histone binding in the mechanism of toxicity through inhibition of RPSA. In addition to DNA and RNA binding, AFBO can bind to various proteins affecting structural and enzymatic protein functions (Viviers and Schabort, 1985), as well as inhibit cellular respiration through mitochondrial damage (Doherty and Campbell, 1972).

1.2 Fumonisin

1.2.1 Problem defined

Similar to AF, Fumonisins (FB) are fungal toxins, or mycotoxins, produced by *Fusarium verticilloides* and *F. proliferatum*. Of the naturally occurring homologues and derivatives, fumonisin B_1 (FB_1) is the most abundant and most toxicologically significant (JECFA, 2001) analog. FB_1 was involved in the fatal toxicosis of horses caused by equine leukoencephalomalacia (ELEM) (Kriek *et al.*, 1981a; Wilson *et al.*, 1990) and exposure to higher levels can also cause porcine pulmonary edema in swine (Kriek *et al.*, 1981a). A few studies have linked FB dietary exposure with oesophageal cancer in human populations in South Africa and China, as well as neural-tube defects along the Texas-Mexico border (Missmer *et al.*, 2006); however, the overall impact on human health has not been well delineated. To understand the risks associated with FB, knowledge of the following is required: (1) pre-harvest contamination and natural occurrence of FBs in food and feeds, (2) the toxicology of FB and the recorded effects on exposed humans and animals, (3) the effects of acute and chronic exposure, (4) the extent of human and animal exposure to the toxin (molecular epidemiology), and additionally, the methods to mitigate the exposure.

1.2.2 Discovery

FBs were first discovered as toxic metabolites produced by *Fusarium* species by Gelderblom and colleagues (1988). Prior to this discovery, *Fusarium* fungal species had been known, to cause leukoencephalomalacia (ELEM) in horses (Marasas *et al.*, 1976), and to be highly toxic in vervet monkeys, horses, pigs, sheep, rats, ducklings, (Jaskiewicz *et al.*, 1987a; Kriek *et al.*, 1981a; Kriek *et al.*, 1981b) and hepatocarcinogenic in rats (Jaskiewicz *et al.*, 1987b; Marasas *et al.*, 1984). In early studies, the toxic compound, was identified in *F. moniliforme*-infected corn after it was implicated in field outbreaks of ELEM. Samples from the outbreak were later reported to be hepatocarcinogenic in rats (Wilson *et al.*, 1985b) suggesting that the toxicant and carcinogen were the same. Gelderblom and colleagues were able to isolate the cancer-promoting compounds in a short-term cancer initiation-promotion assay from *F. moniliforme* culture material and diethylnitrosamine. Two pure compounds were isolated, chemically characterized, and given the names fumonisin B_1 and fumonisin B_2 (FB_1 and FB_2, respectively) (Gelderblom *et al.*, 1988). Fumonisin B_1 was found to be the most dominant analog, although others in addition to FB_2 have been identified (Voss *et al.*, 2001). The toxic effects associated with the consumption of *F. moniliforme*-contaminated corn, including ELEM and porcine pulmonary edema, have been experimentally reproduced using purified FB_1 (Alberts *et al.*, 1993; Harrison *et al.*, 1990; Haschek *et al.*, 1992; Kellerman *et al.*, 1990; Marasas *et al.*, 1988b). The initial studies have resulted in a great interest in the toxicity and carcinogenicity of FB_1 in humans in the past decades; however, the overall impact on human health is still unclear.

1.2.3 Source of contamination

FBs are long-chain polyhydroxyl alkylamines fungal metabolites with two propane tricarboxylic acid moieties esterified to hydroxyls on adjacent carbons (Figure 3) (Bezuidenhout *et al.*, 1988). Although *Fusarium proliferatum* is also capable of producing FBs, *F. moniliforme* is responsible for most of the contamination and is one of the most prevalent fungi found with maize and related crops (Marasas, 1995; Shephard *et al.*, 1996a). *Fusarium moniliforme* has been shown to produce FB_1, FB_2, and Fumonisins B_3 (FB_3) in substantial quantities (Cawood *et al.*, 1991). As previously mentioned, FB_1 and FB_2 were identified and characterized in 1988 (Gelderblom *et al.*, 1988). Following the identifications of these novel mycotoxins, other members of the FB family have been isolated and characterized including: FA_1 and FA_2 (Gelderblom *et al.*, 1988); FB_3, FB_4, and FB's hydrolysis products (Cawood *et al.*, 1991); FC_1 and FC_2 (Branham and Plattner, 1993); and FP_1, FP_2, and FP_3 (Musser *et al.*, 1996).

Many of the *F. moniliforme* strains cover five continents including Africa and North American and have been isolated from not only corn and corn feeds, but also sorghum and millet (Scott, 1993). *Fusarium* can contribute to both nutritional losses and toxic contamination of staple substrates (Marin *et al.*, 1999). A strain of *F. moniliforme*, MRC 826, was demonstrated to produce maximum yields of FB_1 when incubated at 20° C for 13 days (Alberts *et al.*, 1990). This temperature was later confirmed to be optimal for the production of FB_1 on corn (La Bars *et al.*, 1994). Further work demonstrated that biosynthesis of FBs by *F. moniliforme* appeared to primarily be a function of water activity (a_w) as it grows on maize (Cahagnier *et al.*, 1995): a 5% decrease in a_w didn't affect fungal

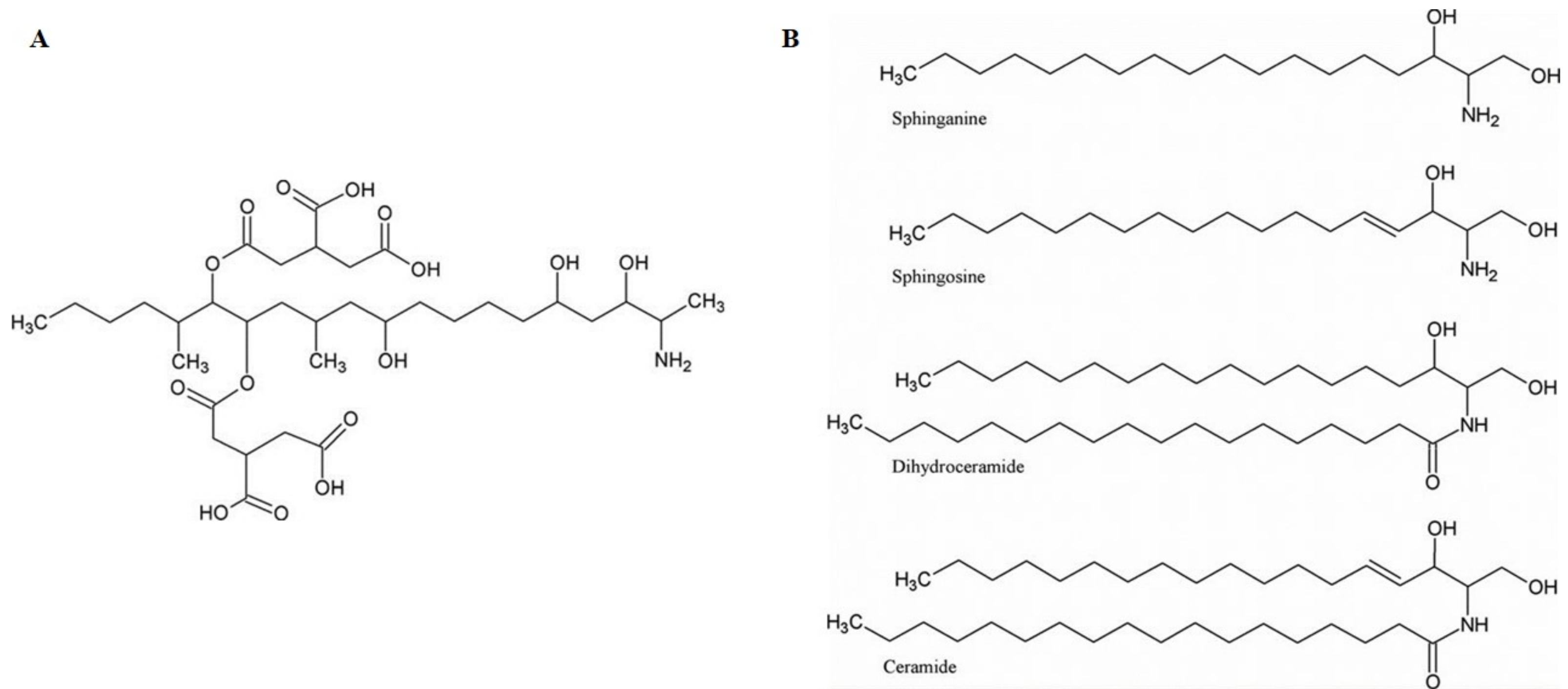

Figure 3. A) Molecular structure of fumonisin B_1 B) Molecular structures of sphinganine, sphingosine, dihydroceramide, and ceramide.

growth but FB biosynthesis was reduced by threefold and a 10% reduction in a_w from 1.0 to 0.9 resulted in a 300-fold fumonisin reduction and 20-fold drop in fungal growth. The authors determined the threshold a_w for growth of *F. moniliforme* to be around 0.85-0.86; at this activity, basic metabolism and growth was inhibited. Another study reported optimal conditions for growth of the Spanish strain of *F. moniliforme* and *F. proliferatrum* on maize to be 30° C with better growth with increasing a_w; FB_1 and FB_2 were synthesized regardless of a_w or temperature (Marin *et al.*, 1995). In this study, the maximum mycotoxin production occurred at $a_w > 0.95$ at both 25° C and 30° C. In general, fumonisin production decreases with temperature and high a_w conditions (Doohan *et al.*, 2003) but significant production of FBs by *F. moniliforme* have been observed at temperatures as high as 37° C (Marin *et al.*, 1999). Furthermore, conditions such as drought, high temperatures, and insect damage exert stress to the plant and increase the likelihood of *Fusarium* growth (Miller, 2001). The influence of climatic conditions on the incidence of *Fusarium* species is probably both direct (e.g. an effect on mode of reproduction) and indirect (e.g. an effect of soil and vegetation type) (Doohan *et al.*, 2003).

1.2.4 Adsorption, biodistribution, and pharmacokinetics

FBs are poorly absorbed and rapidly excreted and the accumulation is found in small amounts mainly in liver and kidney. Biliary secretion appears to be an important route of excretion as a majority of FB_1 is found in the feces (Norred *et al.*, 1993). Early work demonstrated a mono-exponential elimination phase that fitted a one-compartment model in rats dosed intraperitoneally (Shephard *et al.*, 1992a). However, no data for metabolism by liver, kidney, or other tissues exists for the rat (Voss *et al.*, 2001); no

research has been reported in non-human primates and the kinetics and metabolism of FB_1 in humans has not been reported.

FB_1 is poorly absorbed in the gastrointestinal tract as demonstrated by comparisons of the different routes of exposure. In rats dosed intraperitoneally, 0.5% of the radiolabeled FB1 dose was primarily detected in the liver followed by the blood and kidney; FB_1 was not detected in other tissue. Conversely, 45% of the radiolabeled FB_1 dose injected intravenously appeared in the liver within 1 hr; measurable levels were also detected in muscle, fat, skin, and other tissues (Norred *et al.*, 1993). Absorption of FB1 in rats is rapid, with maximum plasma level reached within 20 min (Shephard *et al.*, 1992a). Norred *et al.* (1993) suggests the efficiency of absorption could be attributed to gut content. In rats fasted overnight prior to dosing, 70-80% of the dose was recovered in feces (Norred *et al.*, 1993) and in another study 101% of the dose was recovered in feces with no major retention in tissues (Shephard *et al.*, 1992b). Overall, absorption has been estimated to be 1% to 6% in non-ruminants (Martinez-Larranaga *et al.*, 1999).

A majority of the FB_1 absorbed is rapidly eliminated through biliary secretion (Shephard *et al.*, 1994a) and the remaining FB_1 is circulated to the liver. After 4 hr of an oral dose of radiolabeled FB_1 in rats, 0.5% of the dose was detected in the liver where it remained for at least 96 hr (Norred *et al.*, 1993). In a continuation of the study, consecutive dosing revealed peak liver- and kidney-activities occur 24 hr after the final dose which persisted for another 48 hr. Secondary to the liver, FB_1 was found in blood and kidney. Importantly, the amount of FB_1 that is persistent is not metabolized in animals (Martinez-Larranaga *et al.*, 1999; Shephard *et al.*, 1992a; Shephard *et al.*, 1994a) with the exception

of vervet monkeys and they have been shown to partially hydrolyze FB_1 in the gut (Shephard *et al.*, 1994b). The apparent lack of metabolism (except for vervet monkeys) was demonstrated in a rat study by Norred *et al.* in which 80% of a radiolabeled dose was recovered from feces within 48 hr and less than 3% from urine within 96 hr (1993).

1.2.5 Biomarkers of exposure

In comparison to AF, very few FB biomarkers of exposure exist. This is due in part to the lack of metabolism of the parent compound providing no measurable metabolites. As a result, the limited data regarding the human health effects from FB_1 exposure can likely be attributed to the lack of sensitive and validated methods for determining exposure (Shephard *et al.*, 2007a; Shephard *et al.*, 1996b). Despite their limitations, biomarkers have proven to be useful in assessing exposure at the population level. The most extensively studied FB biomarkers are the sphingoid bases, sphinganine (Sa) and sphingosine (So), followed by unmetabolized urinary FB_1.

Use of the sphingoid bases as biomarkers requires understanding of FB's mechanism of action and this will be explained in greater detail in the following section (Fumonisin Toxicity). In short, FB disrupts sphingolipid metabolism thereby increasing the ratio of the two sphingoid precursors, sphinganine and sphingosine (Sa:So). This increased ratio has been detected in tissue, blood, and urine of vervet monkeys, ponies, chickens, rabbits, and rats and it was also utilized as a biomarker of exposure (Riley *et al.*, 1994b). However, other inhibitors of ceramide synthase exist (i.e., alternaria toxin and australifungins) which can decrease the specificity for Sa:So as a biomarker for FB_1 exposure (Merrill *et al.*, 1997). Furthermore, the sensitivity of Sa:So method appears to

be an issue because this biomarker was developed based on limited non-human primate data. The levels fed (300 and 800 ug FB_1 kg^{-1} b.wt. day^{-1}) to vervet monkeys gave significantly altered Sa:So ratios in serum in contrast a non-significant increase of Sa:So in urine was detected (Shephard *et al.*, 1996b). Although the administered doses from the vervet monkey feeding study are comparable to estimates for human populations consuming moldy corn (Thiel *et al.*, 1992), most human exposures are 100-1000 times lower (de Nijs *et al.*, 1998). Further evidence of limited sensitivity of this method was evident in mice and rat studies fed with smaller concentrations where only marginal changes in serum Sa:So ratios were observed (Castegnaro *et al.*, 1998). Moreover, investigations into the possible elevation of Sa:So ratio in human blood and urine have generally failed to correlate with estimates of dietary fumonisin exposure (Shephard *et al.*, 2007b). The Sa:So ratios in the plasma and urine of male and female volunteers consuming a staple diet of maize from the Transkei region of the Eastern Cape and KwaZulu-Natal province, South Africa and in the Bomet district of western Kenya failed to correlate with mean total FB levels in food collected from the respective regions (van der Westhuizen *et al.*, 1999). Overall, Sa:So ratio may indicate dietary FB_1 exposure, but this method is currently not sufficiently sensitive or specific for determining associations between individual exposure and health outcomes.

The detection of free fumonisin has been developed in recent years as an alternative to the Sa:So ratio method but the effective use of this biomarker is matrix specific. Free fumonisin in human blood is unlikely to provide a useful biomarker due to rapid clearance from the bloodstream (i.e., a few hr in vervet monkeys) (Shephard *et al.*,

1995). Biliary FB_1 excreted from feces has been successfully detected (Chelule *et al.*, 2000; Chelule *et al.*, 2001; Shephard *et al.*, 2007b), however this method is not very sensitive (detection limit: 50 ng g^{-1}) nor practical due to the difficult nature of the matrix in collection, extraction, and scale necessary for analysis. Urinary FB however appears to show promise as a valid biomarker of FB exposure. Shetty and colleagues (1998) were the first to publish a method for measuring FB_1 in human urine with a detection limit of 10 ng mL^{-1}. This detection limit could be used to monitor populations consuming > 28 ug FB_1 kg $^{-1}$ b.wt. day^{-1} assuming a ~0.5% transfer to urine in a 70-kg adult producing 1 L of urine a day (Turner *et al.*, 1999). Improvements have been made over the years with increased sensitivity using HPLC with fluorescence detection following derivatization or LC/MS-MS. Using the latter detection method, Gong and colleagues reported a 75% incidence of urinary FB_1 (UFB_1) in samples collected from a cohort of women recruited in Morelos County, Mexico (Gong *et al.*, 2008). This method permits the detection of 20 pg FB_1/mL urine in a 10 mL urine sample. Furthermore, this study was able to correlate biomarker levels with dietary intake demonstrating increased concentrations of FB_1 in the urine with increased consumption of corn tortillas. Similarly, urine samples provided by participants from communities of high- and low-exposure in Guatemala (previously determined by FB-maize survey results) were measured for UFB_1 and the results mirrored FB intake (Torres *et al.*, 2014). Overall, UFB_1 appears to be a good proxy for dietary FB_1 exposure.

1.2.6 Mechanism of action

In contrast to AFB_1, metabolic activation is not required for FB_1 toxicity. Overwhelming weight of evidence indicate that the biochemical mode of action of FB_1 is

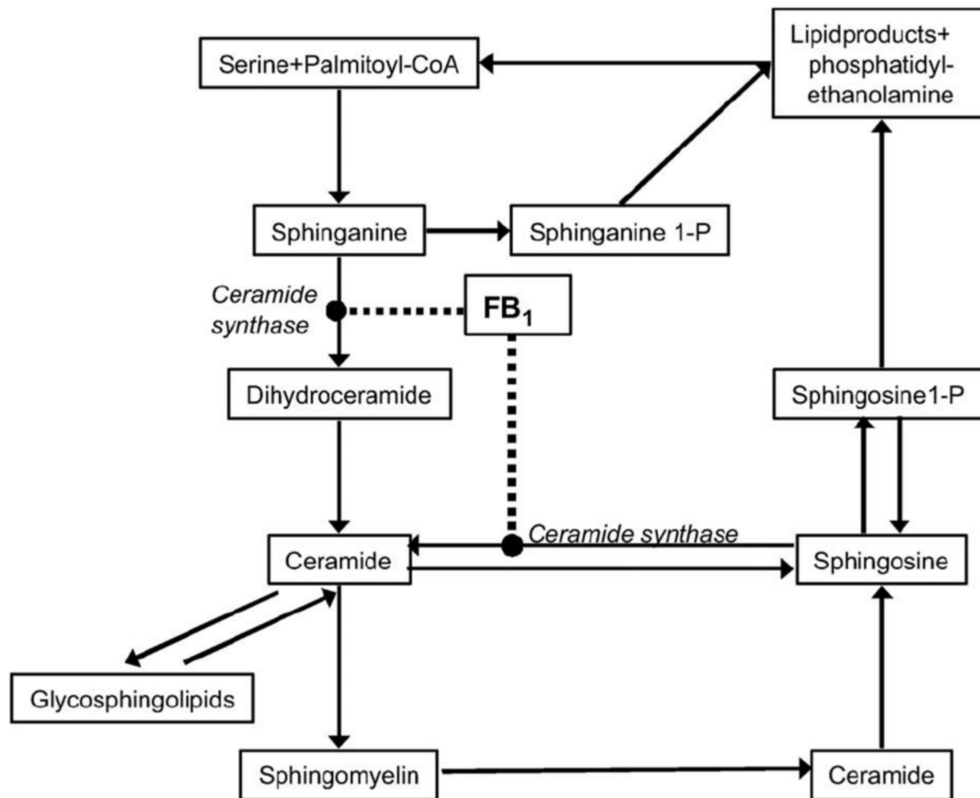

Figure 4. A simplified scheme of *de novo* sphingolipid synthesis and turnover in mammalian cells and the sites of action of FB_1-induced inhibition of the enzyme ceramide synthase. (Modified from WHO, 2000; Merrill et al., 2001).

through the inhibition of the enzyme sphingosine (sphinganine) N-acyltransferase (ceramide synthase). In the sphingolipid biosynthetic pathway (Figure 4), ceramide synthase acylates the amino group of sphinganine with a fatty acid moiety. This process yields dihydroceramide, which is then converted to ceramide by the addition of the 4,5-trans-double bond and finally to the more complex sphingolipids, such as sphingomyelin

and glycosphingolipids, by the addition of the appropriate headgroup (Riley *et al.*, 1996). Sphingolipids are critical components of the water barrier of skin by affecting the properties of cell membrane and lipoproteins. They serve both as ligands for extracellular matrix proteins and receptors for neighboring cells, enteric bacteria, and viruses. Additionally, sphingolipids can act as modulators of growth factor receptors and secondary messengers for agonists including but not limited to tumor necrosis factor-α, interleukin-1β, nerve growth factor, and 1α,25-dyhydroxyvitamin D3 (Merrill *et al.*, 1997). FB_1 bears a structural similarity to free sphingoid bases, most notably 1-deoxysphinganine (Zitomer *et al.*, 2009), and as a result, it inhibits ceramide synthase. When ceramide synthase is inhibited, the *de novo* formation of ceramide from free sphingoid bases and fatty acyl-CoAs is blocked and a similar sphingolipid salvage pathway is also inhibited. This allows for accumulation of the sphingoid bases and their metabolites in liver, kidney, blood, and other tissues (Torres *et al.*, 2015). This inhibition response that is the rationale for the Sa:So ratio biomarker method.

Ceramide synthase is a major cellular target of FB (Wang *et al.*, 1991), and FB can block the biosynthesis of complex sphingolipids resulting in accumulation of sphinganine which is toxic at high concentrations. Importantly, increases in sphinganine and its 1-phosphate in tissues and blood are tightly correlated with the onset and severity of toxic, responses in target organs in multiple species studied (Voss and Riley, 2013). Cellular deregulation can also result in the increase in degradation products from catabolism of free sphingoid bases, lipid products derived from sphingoid base degradation, and free sphingosine from the inhibition of reacylation of sphingosine derived from either the diet

or catabolism of complex sphingolipids (Riley *et al.*, 1994b). In sum, it is difficult to clearly demonstrate a single biochemical pathway that could account for this specific effect on cell behavior.

This disruption of *de novo* sphingolipid biosynthesis by FB has been demonstrated in rat liver microsomes (Wang *et al.*, 1991), cultured renal cells (Yoo *et al.*, 1992), cultured cerebellar neurons (Merrill *et al.*, 1993), and in rat primary hepatocyte cultures (Gelderblom *et al.*, 1995). FB-induced inhibition of sphingolipid biosynthesis and the consequent disruption of sphingolipid metabolism has been observed in all animal species studied thus far including horses, ponies, pigs, rats, chickens, catfish, and rabbits (Riley *et al.*, 1994b). In a study using pigs, it was concluded that 1.) disruption of sphingolipid biosynthesis in liver, lung, and kidney occurs at lower concentrations of FB than concentrations that cause tissue lesions observed by light microscopy, 2.) elevation of free sphinganine and free Sa:So ratio and depletion of complex sphingolipids occur before serum indicators for tissue injury are elevated, and 3.) pure FB_1 and diets containing contaminated corn both disrupt sphingolipid biosynthesis in liver, lung, and kidney to a much greater extent than other tissues (Riley *et al.*, 1994b). In this study, the kidney was more sensitive to FB-induced toxicity than other tissue based on the relatively high level in free sphinganine and the free Sa:So ratio reported in the pigs. Similarly, in Sprague Dawley rats fed FB_1 for 4 weeks, a close correlation between the severity of the ultrastructural changes in liver and kidney was observed; the greater sensitivity that was observed in the kidney likely is due to the increased concentration of free sphinganine and Sa:So ratio in the urine compared to serum (Riley *et al.*, 1994a).

1.2.7 Carcinogenicity

Based on the sufficient evidence in experimental animals and insufficient evidence in humans for the carcinogenicity of FB, FB_1 is classified as possibly carcinogenic to humans (Group 2B) (IARC, 2002). FB_1 is a carcinogen in rodents, exhibiting both cancer-initiating and -promoting effects. Cancer promoting effects were observed in a short-term cancer initiation-promotion assay using a dietary level of 0.1% FB_1 in diethylnitrosamine (DEN)-initiated rats (Gelderblom *et al.*, 1988). In this study, FB_1 significantly induced the formation of GGT^+ foci in the DEN-initiated group. The induction of GGT^+ is widely used as a marker of preneoplastic hepatic lesions during chemical carcinogenesis (Hanigan and Pitot, 1985). In another study, when rainbow trout were fed a diet containing 0, 3, 23, or 104 mg/kg FB_1 for 34 weeks, no liver tumors were seen at termination (60 weeks) (Carlson *et al.*, 2001). However, when the fish were pre-treated with 100 mg/kg AFB_1 or 35 mg/kg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, liver tumors were observed at varying incidences and doses.

In a long-term study in male BD IX rats, liver tumors developed in rats that died or were euthanized between 18 and 26 months (10/15 animals) after exposure to 50 mg/kg FB_1 in the diet (estimated 1.6 mg/kg/day) (Gelderblom *et al.*, 1991). The livers of all of the rats euthanized at 6 months and onwards presented hepatocyte nodules; the hepatocellular carcinoma that developed in the rats after 18-26 months were found to originate within one of these large regenerative nodules. In a subsequent 21-day feeding study, the initiation potential of FB_1 was studied in rats fed 250, 500, or 750 mg FB_1/kg diet. Results from this study indicate that FB_1-induced hepatocellular carcinogenesis is a

function of time since a dosage of 29.7 mg/100 mg body wt. over 7 days did not initiate cancer while a similar dose over 21 days induced liver tumors (Gelderblom *et al.*, 1994). The authors also suggest that a balance exits between compensatory cell proliferation and toxicity-induced inhbitory effect. In contrast, no liver tumors were observed in a 2-year National Toxicology Program feeding study using 50 male and female Fischer 344/N/Nctr BR rats (Howard *et al.*, 2001). However, male rats did develop kidney adenomas and carcinomas. Results suggest a no-observed-effect-level (NOEL) for the induction of renal tumors in male rats that is between 15 and 50 ppm FB_1. Interestingly, female B6C3F1/N/Nctr BR mice from the same study showed an increased incidence of hepatocellular adenomas and carcinomas with dietary levels >50 mg FB_1/kg. The differences in organ and dose response observed in the contrasting studies have been postulated to occur as a result of dietary differences or deficiencies (Gelderblom *et al.*, 2004).

In a single IV dose study, Sprague-Dawley rats were injected with FB_1 at 0 or 1.25 mg/kg and were euthanized at 12 hr or, 1, 2, 3, or 5 days (Lim *et al.*, 1996). Results demonstrated that the kidney was the primary target organ of FB_1 in rats. Neoplasia also occurred in the liver and esophagus, suggesting that the liver and esophagus are also targets for fumonisin toxicity. An early response in these target organs is apoptosis with some evidence of oncogenic necrosis following FB_1 administration, especially in the liver (Dragan *et al.*, 2001). This effect was thought to be modulated through tumor necrosis factor α (TNFα) production. In short-term studies from the NTP study, increases in tissue

Sa:So ratio correlated with apoptosis and hepatotoxicity was ameliorated in mice lacking either the TNFR1 or the TNFR2 TNFα receptor.

Epidemiological data initially correlated esophageal cancer with *Fusarium* contamination in food samples from the Transkei region of South Africa and the Henan Province of China (Luo *et al.*, 1990; Marasas *et al.*, 1988a; Zhen *et al.*, 1984). Following the identification of FBs in the 1980s, correlation studies were improved to focus on the specific toxin. Sydenham *et al.*(1990) measured FB_1 and FB_2 levels in uncontaminated corn samples collected from high and low prevalence areas for esophageal cancer in Transkei. Significantly higher toxin levels were present in samples from the high esophageal cancer area confirming previous studies correlating *Fursarium* species with cancer in this region. Similar trends were observed in Cixian and Linxian counties (Chu and Li, 1994) and Linxian and Shangxui counties (Yoshizawa *et al.*, 1994), People's Republic of China. Interestingly, Chu and Li (1994) noted that in addition to fusaria mold, nitrosamines in the diet were a major contributing factor in the etiology of esophageal cancer in this part of China (1994). A number of fungi, including *F. moniliforme*, are capable of forming secondary amines and nitrosamines presenting an interesting possibility of synergism or additively in diet related carcinogenesis which has not been further investigated.

A critical component of FB_1 carcinogenicity is apoptosis and continuous regeneration to compensate for apoptosis (Dragan *et al.*, 2001). This is likely a consequence of ceramide synthase inhibition, TNFα production, and induction of apoptosis (Voss *et al.*, 2002). The sphingoid bases, sphingoid base metabolites such as So-

1-phosphate, ceramide, and other sphingolipids stimulate cellular events leading to apoptosis or mitosis. As a result, disruption of metabolism and cellular functions by these toxins is likely to be critical for cytotoxicity, apoptosis and carcinogenicity resulting from FB exposure (Voss *et al.*, 2002). Additional evidence also supports the possibility that TNFα is involved in regulation of apoptosis and cell replication (Bazzoni and Beutler, 1996). According to current data, FB_1 may be the first example of an apparently nongenotoxic (non-DNA reactive) agent that induce tumors through a mode of action involving apoptosis, necrosis, atrophy, and consequent cell regeneration.

1.2.8 Toxicity

FB_1 is neurotoxic, hepatotoxic, and nephrotoxic in animals and many of the effects are species and gender dependent (Dragan *et al.*, 2001; Stockmann-Juvala and Savolainen, 2008). Furthermore, FB_1 caused hepatic injury in all species regardless of the route of administration (Wild and Gong, 2010). The primary role of FB_1 in equine leukoencephalomalcia (Kellerman *et al.*, 1990; Marasas *et al.*, 1988b) and pulmonary edema syndrome in swine has been well documented (Harrison *et al.*, 1990). In these diseases, as well as in FB_1-induced carcinogenesis, the main mechanism is the inhibition of the enzyme ceramide synthase. However, there are several additional proposed mechanisms for different types of FB_1-mediated toxicity and it seems that no one common pathway exists. Potential mechanisms include oxidative stress, cytotoxic effects, apoptosis, and immunotoxic effects, which have been demonstrated in various animal models and cell lines (Figure 5).

Recently, it was demonstrated that ceramide synthase inhibition by FB_1 is dependent on the six mammalian ceramide synthase isoforms in a tissue-specific pattern (Loiseau *et al.*, 2015). The results demonstrated that in a piglet model gavaged with 1.5 mg FB_1/kg body weight daily for 9 days, FB_1 preferentially binds two isoforms of CS (CerS4 and CerS2) to deplete specific ceramides in lung while enriching them in liver. The authors postulate that this enzyme expression might explain the organ-specific adverse effects observed in various species.

Due to the lack of data regarding the kinetics and metabolism of FB_1 in humans, little is known about the toxic effects in humans. However, FB has been linked to neural tube defects (NTD; embryonic defects of the brain and spinal cord resulting from failure of the neural tube to close) in populations along the Texas-Mexico border. In 1990-1991, six anencephalic births occurred in 6 weeks at one hospital in Cameron County, Texas (Missmer *et al.*, 2006). Already having linked an extreme cluster of ELEM to FB_1 present in contaminated corn-fed horses, this outbreak of NTDs was hypothesized to be the consequence of exposure to high levels of FB_1 (Missmer *et al.*, 2006). Other regions with high corn-based food consumption and documented FB contamination also have high prevalence of NTD including regions of China and South Africa (Cornell *et al.*, 1983; Moore *et al.*, 1997; Ncayiyana, 1986).

Folate deficiency has been identified as a major risk factor for NTD. FB_1 has been shown to disrupt folate transport and utilization in Caco-2 cells *in vitro* and in LM/Bc mice *in vivo* and this effect is associated with reduced complex sphingolipids (Gelineau-van Waes *et al.*, 2012; Gelineau-van Waes *et al.*, 2005). Therefore, it has been proposed that

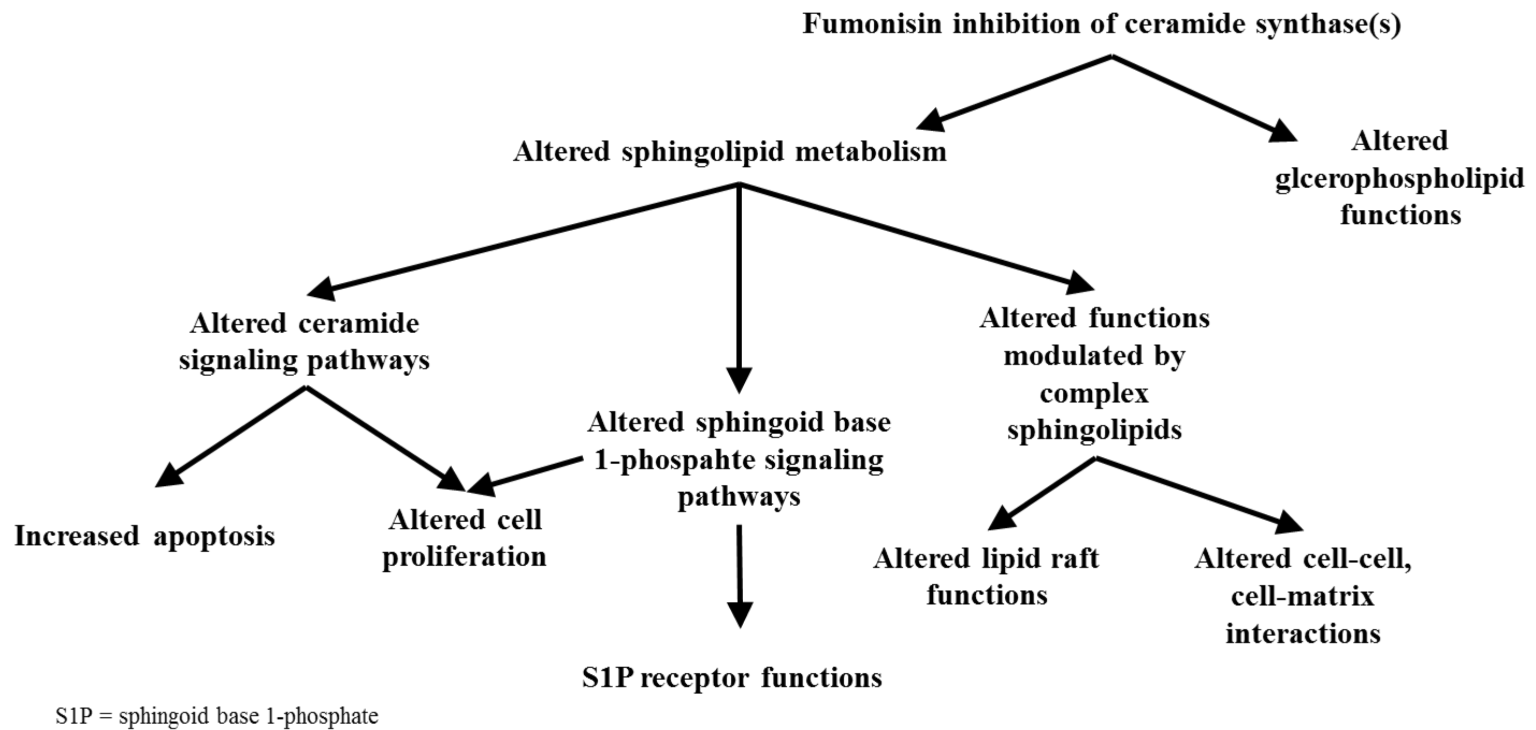

Figure 5. Biochemical and cellular consequences of fumonisin inhibition of ceramide synthase(s) and global disruption of lipid metabolism. Adapted from Bulder *et al.* 2012.

the disruption of sphingolipid metabolism caused by FB_1 may affect folate uptake and result in an increased risk for NTD.

Importantly, exposure to FB alone or co-exposure with AFs may contribute to child growth impairment in Tanzania (Shirima *et al.*, 2015). A prospective study of 166 children 6-14 months of age demonstrated negative associations with length-for-age z-scores (LAZ) and UFB_1 at recruitment and 6 and 12 months following recruitment. AF-alb and child growth did not reach statistical significance. However, causal mechanisms need to be investigated.

1.3 Aflatoxin B_1/fumonisn B_1 co-exposure

AFs and FBs are increasingly identified as a dual public health concern. Analysis of AF and FBs mixtures and investigating their possible antagonistic, additive, or synergistic toxic effects is ongoing. This is primarily due to their overlap in target organs (liver), common growth niches of the fungi, and shared nutrient source, i.e., maize. The combination of AF and FB exposure was of particular concern to the 74th meeting of the Joint FAO/WHO Expert Committee on Food Additives because of the well-known genotoxicity of AFB_1 and the ability of FB_1 to induce regenerative cell proliferation (Bulder *et al.*, 2012).

AFs and FBs have been simultaneously detected in maize in areas where maize consumption, HCC, chronic liver disease, and growth retardation in children are high. For example, in Guatemala, a country with a high incidence of children born with NTD and the fifth highest prevalence of stunting worldwide, a survey of 640 maize samples revealed a high risk of co-exposure (Torres *et al.*, 2015). Their highest co-occurrence rate was

observed in stored maize (Hove *et al.*, 2016). Another recent survey in sub-Saharan Africa found that of 388 samples analyzed, 81% and 65% were positive for FB and AF, respectively (Probst *et al.*, 2014). Additional reports of co-exposure are presented in Table 3. Based on this data, co-exposure in contaminated maize occurs on average of about 50%. Exact ratios of AFB_1 and FB_1 in stored maize have not been documented, however co-exposure data suggests the occurrence of FBs in maize is at least 10 times greater than that of AFs (Hove *et al.*, 2016). Additionally, biomarkers for both toxins were found in high frequency of an adult population residing in a high-risk area of rural Ghana (Robinson *et al.*, 2012; Wang, Afriyie-Gyawu *et al.*, 2008).

Additive and synergistic toxic effects from co-exposure to AF and FB have been reported in multiple animal models. Hydra maintained at pH 6.9-7.0 were exposed to AFB_1, FB_1, and AFB_1/FB_1 for 92 hr and measured for toxic responses based on morphology and mortality (Brown *et al.*, 2014). Results demonstrated that combined AFB_1 and FB_1 exposure was more toxic than either individual mycotoxin with the hydra disintegrated by hr 92. McKean *et al.* (2006) demonstrated that AFB_1 and FB_1 interacted to produce alterations in the toxic responses with a strong additive interaction in F344 rats and mosquitofish. In combinative toxicity studies in rats, 25 mg/kg bw of FB_1 additively increased the mortality of rats caused by the acute toxicity of AFB_1 (dosed in various fractions of the AFB_1 LD_{50} value, 2.71 mg/kg) evidenced by the interaction index value (K; [theoretical] LD_{50}/Experimental [measured] LD_{50}) of 1.98, indicating an additive interaction. Acute toxic effects such as depression and diarrhea occurred after a two lowest

Table 3. Reported co-occurrence of AFB_1 and FB_1 in maize worldwide

Region	Country	Year	Source	No. of samples	Co-occurrence (%)	Mean AFB_1 (µg/kg)	Mean FB_1 (µg/kg)	Max AFB_1 (µg/kg)	Max FB_1 (µg/kg)	Reference
Asia	India	1997	Stored	35	63	3	620	4,030	4,740	Shetty & Bhat, 1997
	China	2009	Field	25	0	n.d.	360	n.d.	3,480	Feng *et al*. 2011
	China	2011	Stored	31	90	1[a]	300[a]	2	12,500	Sun *et al*. 2011
	China	2011	Stored	43	100	14[a]	2,600[a]	137	5,900	Sun *et al*. 2011
	China	2011	Stored	29	85	2[a]	400[a]	50	37,000	Sun *et al*. 2011
	Vietnam	2004	Stored	25	28	NS	1,100[a]	47	3,300	Trung *et al*. 2008
Europe	Croatia	2007	Stored	12	25	3	7,630	5	20,700	Šegvić Klarić *et al*. 2009
	Italy	2007	Harvest	36	94	26[b]	5,727[b]	820[b]	76,232[b]	Covarelli *et al*. 2011
	Turkey	2002-2003	Field	19	100	11[b]	88,240[b]	32	356,800	Oruc *et al*. 2006
	Serbia	2011-2012	Stored	12	100	1	1,610.80	2	2,950	Krnjaja *et al*. 2013
South America	Argentina	1999-2010	Harvest	1655	6	NS	NS	711	498,212	Garrido *et al*. 2012
	Argentina	1999-2010	Stored	1591	10	NS	NS	464	31,701	Garrido *et al*. 2012
	Argentina	2003	Harvest	14	50[b]	3[b]	1,200[b]	11[b]	34,700[b]	Broggi *et al*. 2007

Table 3. Continued

Region	Country	Year	Source	No. of samples	Co-occurrence (%)	Mean AFB_1 (µg/kg)	Mean FB_1 (µg/kg)	Max AFB_1 (µg/kg)	Max FB_1 (µg/kg)	Reference
	Argentina	2004	Harvest	17	41[b]	2[b]	4,700[b]	22[b]	16,100[b]	Broggi *et al.* 2007
	Brazil	2003-2004	Harvest	300	9-17	23-40	1,310-3,120	56	18,780	Moreno *et al.* 2009
	Brazil	2005-2006	Harvest	74	16	<LOD	1,300[a]	3	6,000	Souza *et al.* 2013
	Guatemala	2012	Stored	640	NS	63	1,800	2,655	17,100	Torres *et al.* 2015
	Venezuela	1993	Stored	37	17	NS	NS	50	15,050	Medina-Martinez *et al.* 2000
Africa	Burkina Faso	2010	Stored	26	50	24[a]	269[a]	636	1,343	Warth *et al.* 2012
	Cameroon	2004-2005	Stored	40	55	2	3,684	15	24,225	Njobeth *et al.* 2010
	Cameroon	2009	Stored	75	NS	35	1,329	345	5,412	Njumbe Ediage *et al.* 2014
	Cameroon	2010-2011	Stored	90	NS	81	2,072	645	4,030	Njumbe Ediage *et al.* 2014
	Cote D'Ivoire	2006	Stored	10	100	NS	900	20	1,500	Sangre-Tigori *et al.* 2006
	Egypt	2012	Stored	20	30[b]	11[b]	33[b]	19[b]	947[b]	Madbouly *et al.* 2012

Table 3. Continued

Region	Country	Year	Source	No. of samples	Co-occurrence (%)	Mean AFB_1 (µg/kg)	Mean FB_1 (µg/kg)	Max AFB_1 (µg/kg)	Max FB_1 (µg/kg)	Reference
	Ghana	200	Stored	15	53	NS	NS	338	1,655	Kpodo *et al*. 2000
	Mozambique	2010	Stored	13	46	70[a]	869[a]	363	7,615	Warth *et al*. 2012
	South Africa	2010	Stored	40	70	94	331	741	892	Chilaka *et al*. 2012
	South Africa	2011	Stored	54	0	n.d.	2083	n.d.	14,990	Shepard *et al*. 2013
	Tanzania	2006	Stored	120	12[b]	44[b]	2,157[b]	151[b]	11,048[b]	Kimnya *et al*. 2008
USA	Georgia	1991	Field	28	86	73	870	321	1,820	Chamberlain *et al*. 1993

NS--not specified; n.d.--not detected; [a] Median. [b] Total aflatoxins or total fumonisins. Adapted from Hove et al., 2016.

doses; dose dependent mortality also occurred. In the combinative toxicity study in mosquito fish there was no dose-dependent mortality, however a dose response in acute toxic symptoms such as reduction of activity and loss of righting reflex was observed at high concentrations. Similarly, the combinative toxicity index, K, was 1.98. In a 90-day feeding study, male Wistar rats were fed 40 μg/kg AFB_1or a mixture containing 40 μg/kg AFB_1 and 100 mg/kg FB_1 (Theumer *et al.*, 2010; Theumer *et al.*, 2008). The co-exposed group had higher numbers of tubular apoptotic cells in the kidney, which are signs of cellular mitosis and apoptosis in the liver compared to single mycotoxin treatment. The Joint FAO/WHO committee considers the data from Theumer et al. (2010) to be additive of the effects of AFB_1 and FB_1. Furthermore, a co-exposure study in growing barrows resulted in additive to synergistic responses in the barrows (Harvey *et al.*, 1995). Less than additive to additive responses were observed in turkey poults fed AFB_1/FB_1 contaminated diets (Kubena *et al.*, 1995). Another study showed no additive effects of AFB_1/FB_1 co-exposure in barrows except for a decrease in feed consumption and feed conversion (Dilkin *et al.*, 2003). However, this data was sufficient for the Joint FAO/WHO committee to consider the data additive (Bulder *et al.*, 2012).

The cancer initiation potential of AFB_1 and FB_1 was studied in F344 rats (Gelderblom *et al.*, 2002). The effect of sequential exposure was investigated by treatment with AFB_1 via gavage over a period of 14 days followed by FB_1 treatment 3 weeks later over a period of 21 days. Treatment groups with individual mycotoxin exposure were also included. Individually, both toxins exhibit low cancer initiating potency as measured by the induction of foci and nodules stained positively for placental form of glutathione-*S*-

transferase ($GSTP^+$). However, when rats were co-treated sequentially, the number and size of $GSTP^+$ lesions significantly increased. Furthermore, the sequential treatment induced cellular apoptosis and proliferation at the same time, leading cirrhotic livers with numerous dysplastic nodules. Although the authors suggested that that FB_1 acted as a promoter, this cannot be concluded only based on the increase in foci and nodules; however, the data clearly suggest a synergistic effect on cancer initiation. Interestingly, in a one-week single-dose co-exposure study measuring urinary metabolites, F344 rats co-exposed to AFB_1 and FB_1 excreted significantly less AFM_1 than the AFM_1 only group (Mitchell *et al.*, 2014b). This study suggest that FB_1 modulated the metabolism of AFB_1 to favor the formation of DNA-adduct-forming AFB_1-expoxide. This modulation could possibly explain some of the additive or synergistic effects observed in AFB_1 and FB_1 carcinogenesis.

1.4 Reducing exposure in human populations

In high-risk populations, where AFs and FBs are inextricable contaminants of food, a multi-faceted approach must be implemented to reduce exposure to these toxins, especially in the young population who are more susceptible. The same can be true for at risk populations in developed countries that may be highly exposed and vulnerable due to dietary and cultural practices. However, many of the proposed methods are difficult to implement and maintain in underserved communities.

Innovative strategies that significantly diminish AF and, to a lesser extent, FB bioavailability and mitigate human and animal exposures from contaminated food and feed, have been reported. Based on the extant scientific literature, some of these

approaches are already in the stage of clinical intervention and translation. Studies describing materials that tightly adsorb AFs onto internal and/or external surfaces interfering with toxin uptake and bioavailability have recently been reviewed (Kensler *et al.*, 2013; Miller *et al.*, 2014). Extensive studies with calcium montmorillonite clay and dietary chlorophyllin in humans and animals indicate that they are approaching implementation, but still require further clinical evaluation in the field to delineate the effects of dose and time on efficacy and safety as well as acceptability (Phillips *et al.*, 2002; Wild and Turner, 2002). Other AF sequestering materials that have limited evidence of efficacy require preclinical trials in animals to confirm safety followed by clinical intervention trials in humans prior to implementation. Before full-scale implementation, all of these products should be rigorously evaluated *in vitro* and *in vivo*, and should meet the following criteria: (1) favorable thermodynamic characteristics for AF sorption, (2) tolerable levels of potential hazardous contaminants, (3) safety and efficacy in multiple animal species, (4) safety and efficacy in long-term studies, (5) negligible interactions with vitamins, iron and zinc and other micronutrients. Based on these criteria, calcium montmorillonite clay is one of the most thoroughly characterized and has produced the most consistent clinical results across studies. Moreover, the use of calcium montmorillionte clay has demonstrated potential application for the remediation of FB and is the focus of this section.

The concept of eating dirt (clay) falls under the scientific term, geophagy, which is practiced by humans and observe in animals. For centuries, people have used clays in food preparation for toxin removal, condiments or spices, or food during famine

(Callahan, 2003). Other common clay consumption practices include that for medication or during pregnancy with the latter being most common in cultures of sub-Saharan Africa (Callahan, 2003). This use for toxin removal was expanded further for the reduction of dietary AFs by pioneering work from the Phillips' laboratory in the early 1980s. The observation that populations at high risk for exposure commonly engaged in geophagy, as well as the apparent success of zeolite, bentonite and spent bleaching clay from canola oil refining in reducing the effects of the T-2 and zearalenone mycotoxins in swine, led to the investigation of the sorbent properties of montmorillonite clays. Phillips and coworkers reported that a calcium montmorillonite is efficacious to decrease the negative health effects from AF exposure in multiple animal species in the 1980s. Furthermore, isothermal analyses and molecular modelling techniques have been employed to validate and characterize clay-based materials for the enterosorption of AFs and FBs.

Clay minerals are structurally and chemically diverse. Many are ineffective and/or nonselective. However, research supports the hypothesis that calcium montmorillonite clay (CM) has a notable preference (and capacity) for AFB_1. This is due to the structural and chemical composition of CM clay as well as the AFB_1 molecule. The solid particles of soil are classified into three categories based on size: sand (0.05 – 2 mm), silt (0.002 – 0.05 mm), and clay (less than 2 µm). The relative contribution of each type of particle to a particular soil determines its texture and other physical attributes and is used to name soil classes (Phillips *et al.*, 2002). The soil mineral classes are divided based on the density of the dominant anionic group with silicates making up the largest class. The basic structural unit for the silicates is a SiO_4 tetrahedron in which the Si^{4+} is located at the

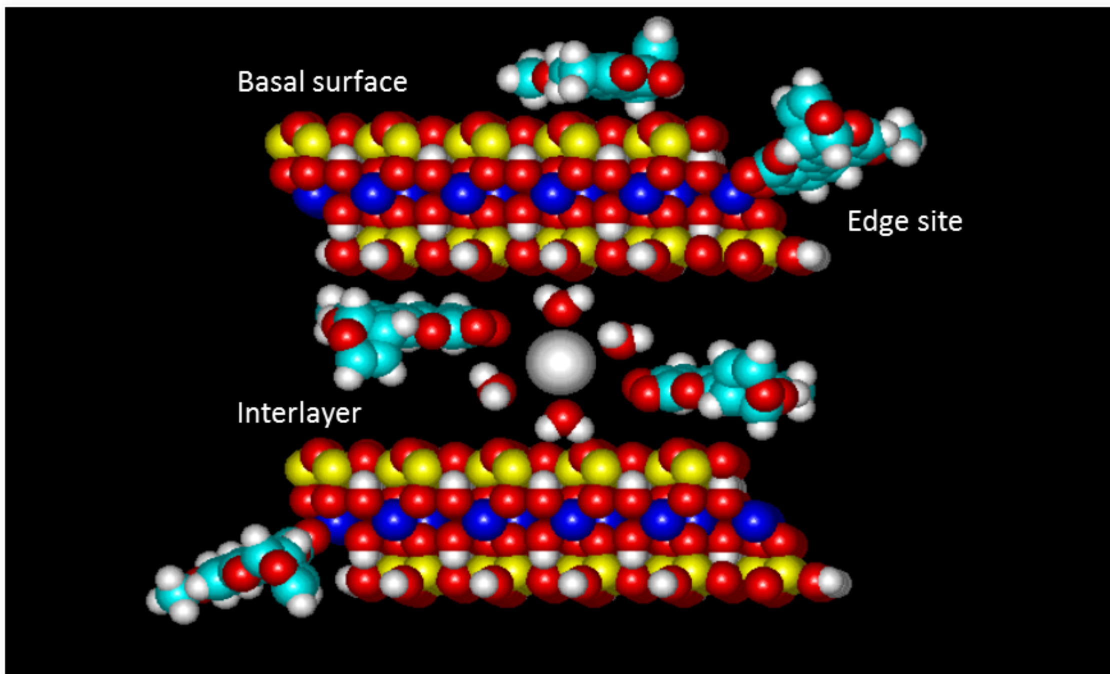

Figure 6. Calcium montmorillonite clay structure: $Al_2Si_4O_{10}(OH)_2$; Si^{4+} yellow (Silica tetrahedra); Al^{3+} blue (Aluminum octahedra); Hydrated Ca^{2+} green (Interlayer) Common substitutions: Mg^{2+} for Al^{3+}, Al^{3+} for Si^{4+} (Phillips and Grant, 1998).

center and together can form a variety of more complex structures including rings (cyclosilicates), chains (inosiliates), sheets (phyllosilicates) and three-dimensional arrangements (tectosilicates) (Phillips *et al.*, 2002).

CM falls under the phyllosilicate class. The functionality of this class of minerals is a result of the distinctive structural and chemical properties of the silicate layers containing both tetrahedral and octahedral sheets (Figure 6). The tetrahedral sheets are composed of the SiO_4 tetrahedra linked together and each sheet shares three O^{2-} ions with adjacent tetrahedral. Together, this forms a plane of basal oxygens. The fourth O^{2-} of each tetrahedron is referred to as the apical oxygen and is free to bind to other elements. The

octahedral sheet is comprised of two planes of OH^- groups that form a hexagonal close packing arrangement. In the case of CM, to counter the negative charge of this structure, Al^{3+} fills two out of every three spaces to produce a dioctahedral arrangement. With this structure, the apical oxygens from the tetrahedral layer coordinate with Al^{3+} to link the octahedral and tetrahedral layers in a 2:1 layer structure in which an octahedral layer is bound on either side by a tetrahedral layer. Frequently, cations in either the tetrahedral or octahedral layers are missing or have been replaced through an isomorphic substitution with another cation of lesser charge which results in a permanent negative charge. To counteract the negative charge, CM clays attract Ca^{2+} into the region between the layers (i.e., the interlayer). This Ca^{2+} attraction into the interlayer creates the space responsible for the high binding capacity of CM for AF. The amount of charge necessary to balance the negative charge of the clay is referred to as the cation exchange capacity (CEC). As a phyllocilicate that is grouped as a smectite, CM has a layer charge per formula unit of 0.25 to 0.6 (Phillips *et al.*, 2002; Schulze *et al.*, 1989).

Due to the overall negative charges on CM clay platelets, compounds with areas of electron deficiencies can also be attracted to these areas. The partial positive dicarbonyl system along with the general planarity of AFB_1 (with the exception of the terminal furan) has been shown to be essential to the adsorption process. Therefore, AFB_1/CM binding is likely the result of a chemisorptive mechanism, through direct ion-dipole interactions and electron sharing (Deng *et al.*, 2010; Grant and Phillips, 1998; Phillips, 1999; Phillips *et al.*, 2002). Early work demonstrated the importance of the spatial orientation of AFB_1 in the CM/AFB_1 binding phenomenon. In adsorption studies, data was fitted to multiple

isotherm equations, as described in detail by Kinniburgh (1986) and Grant (1988). Briefly, an isotherm is a plot of the concentration of a ligand left in solution versus the concentration bound to the surface of a solid which can then be utilized in the Langmuir equation to determine the capacity (Q_{Max}) and affinity of adsorption. The shapes of the plots have been given classifications that describe the types of binding occurring (Giles *et al.*, 1960; Giles *et al.*, 1974a; Giles *et al.*, 1974b). More specifically, the isotherm of AFB_1 adsorption onto CM can be categorized as an L2 plot that is reaching a plateau. The maximum amount of AFB_1 that was adsorbed onto CM was 0.336 mol/kg which equates to 72.9% of the binding capacity (Q_{max}) as derived from fitting the Langmuir model to the data. The LM was also used to estimate the Q_{max} at different isotherm temperatures and to calculate individual K_ds for the calculation of enthalpy of adsorption. These results confirmed multiple sites (with dissimilar thermodynamic properties), and that all sites were probably involved in a chemisorption mechanism, since the enthalpy was near, or above, -40 jK/mol.

The isotherm evidence combined with molecular modeling suggests that AF may react at multiple sites on CM clay particles, however, the interlayer region is the major site of chemisorption of AFB_1 (Grant and Phillips, 1998). The importance of the interlayer in the sorption of AF was indirectly demonstrated by the decreased binding after heat-collapsing the clay (removing the interlayer) and performing isothermal analyses. Results indicated that steriochemical differences of some AF analogues significantly affected the tightness of binding. Therefore, it was concluded that the molecular mechanism for the adsorption of AFB_1 onto CM may favor the furan alignment away from the surface.

However, based on the good correlation between the magnitude of partial positive charges on carbons C11 and C1 of the β-dicarbonyl system and the strength of adsorption of planar analogs and derivatives of AFB_1, an electron donor acceptor mechanism appears to be responsible for the AFB_1 sorption. Recent characterizations have indicated a similar binding capacity and affinity for AFB_1 with a CM marketed as NovaSil (NS) clay and refined to the final product called Uniform Particle Size NovaSil (UPSN) (Marroquin-Cardona *et al.*, 2011).

The binding of FB_1 by CM *in vitro* and *in vivo* was reported by Lemke (2000) who observed interlayer binding of FB_1 by CM at acidic pH *in vitro* suggesting a possible cationic exchange reaction at negatively charged surfaces of the clay. These findings provide a possible mechanism for NS efficacy. This conclusion was a result of comparisons between various clays, including four different montmorillonites, and their binding capacity for FB_1. Isothermal analysis data suggest that an exchange mechanism in the interlayer could be important to biding since this feature is characteristic of montmorillonite clays. The difference between the binding capacities of the montmorillonite clays tested is likely a product of the hydronium ion, which could aid protonation of FB_1 and subsequent cation exchange. As previously described, CM contains hydronium ions within its interlayer. Importantly, sorption at pH 7 was not significant, supporting the hypothesis that only positively charged FB_1 molecules participate in exchange.

Although *in vitro* studies have confirmed that NS successfully binds AFB_1 and FB_1, *in vivo* short-term studies show that NS only protects animals against toxic levels of

AF. Importantly, it does not interfere with the utilization of essential vitamins and micronutrients in the diets (Phillips, 2002). Initially, CM was sold as an anticaking additive for animal feed and was identified as an attractive mitigating agent due to its GRAS (Generally Recognized as Safe) classification. Radiolabeled AFB_1 ($[^{14}C]AFB_1$) studies in chicks demonstrated markedly diminished radioactivity in the blood and hepatic tissues in animals dosed with either 0.1 or 0.5% CM suggesting that CM decreased AF bioavailability *in vivo* (Davidson *et al.*, 1987). Furthermore, the addition of 0.5% CM in the diet rescued broiler and leghorn chicks from the toxic effects of 7.5 ppm AFB_1 (Phillips *et al.*, 2006; Phillips *et al.*, 1988). Although the levels in this study were exceedingly high, they are still within the realm of possibility for acute outbreak situations (Phillips *et al.*, 1988). Following these initial studies, the efficacy of CM clays for AF protection has been confirmed in multiple animal species including pregnant rodents (Mayura *et al.*, 1998), chickens (Kubena *et al.*, 1990b; Phillips *et al.*, 1988; Pimpukdee *et al.*, 2004), turkeys (Kubena *et al.*, 1991), swine (Lindemann *et al.*, 1993), and lambs (Harvey *et al.*, 1991a). These studies have shown that CM is a preferential enterosorbent for AFs when included in the diet from 0.25 to 2% (w/w) in animal models (Phillips *et al.*, 2002). A study in which Sprague-Dawley rats ingested CM clay at dietary concentrations as high as 2% throughout pregnancy showed neither maternal nor fetal toxicity, and did not show significant trace metal bioavailability in a variety of tissues (Wiles *et al.*, 2004). A large volume of scientific literature has indicated that dietary inclusion of CM clay is effective for reducing AF exposure in multiple animal models (Table 4). Moreover, CM rescued diminished levels of vitamin A after AFB_1 exposure in chickens (Pimpukdee *et al.*, 2004)

and reduced the effects of AFB_1 on serum concentrations of cholesterol, albumin, triglycerides, calcium, glucose, and total protein with 0.5% clay (Abo-Norag *et al.*, 1995; Kubena *et al.*, 1990a; Kubena *et al.*, 1993). In sum, no observable adverse effects were reported following ingestion of the dietary CM clay in these short-term animal studies (Phillips *et al.*, 2002). Importantly, it was determined that the minimal effective dose to significantly reduce aflatoxicosis was 0.5% w/w CM clay (Phillips *et al.*, 1990; Phillips *et al.*, 1995). Although CM at 2% in the diet of broiler chicks was not able to rescue the birds from FB_1-induced toxicity, it is important to note that no LD_{50} exists for FB and that levels needed to induce toxicity in this study were very high (Lemke, 2000). The clay appears to have partially ameliorated the lowered weight gain and increased activity of alkaline phosphatase.

In the early 1990s, urinary and milk AFM_1 biomarkers were employed in CM safety and efficacy studies. These studies were able to demonstrate reduced bioavailability of AFB_1 due to clay binding in the GI tract based on decreased metabolite measurements. In other words, the addition of clay decreased levels of parent compound available to metabolize. Inclusion of 1% CM clay was able to reduce excretion of AFM_1 in the milk of dairy cows and goats by 44% and 51.9%, respectively (Harvey *et al.*, 1991b; Smith *et al.*, 1994). Urinary AFM_1 measurements revealed reductions of 48.4% in dogs (Bingham 2004) and >90% in rats (Sarr *et al.*, 1995). Ultimately, these studies demonstrated the use of biomarkers in measuring the efficacy of CM but also the differences in efficacy because of species differences in digestion, metabolism, dietary habits, and excretion.

Table 4. Safety and efficacy studies with calcium montmorillonite clay in animals and humans

Animals fed clay	Mycotoxin in feed	Clay in feed (duration)	Major effects of the clay reported in the study	References
Chickens	Aflatoxins	0.5% (28d)	Growth inhibition diminished; gross hepatic changes prevented.	Phillips *et al*. 1988
Chickens	Aflatoxins	0.5% (28d)	Growth inhibition diminished; decreased mortality.	Kubena *et al*. 1990
Chickens	Aflatoxins	0.1%; 0.5% (24h)	Reduced bioavailability of Aflatoxin to the liver and blood in a dose-dependent manner.	Davidson *et al*. 1987
Chickens	Aflatoxins	0.5%; 1.0% (21d)	Growth inhibitory effects reduced.	Araba & Wyatt 1991
Chickens	Aflatoxins	0%-1.0% (21d)	Feed conversions improved; growth inhibition diminished.	Doerr 1989
Chickens	Aflatoxins	1.0% (21d)	Growth inhibition completely prevented.	Ledoux *et al*. 1999
Chickens	Aflatoxins Ochratoxin A	0.5% (21d)	Decreased growth inhibitory effects; no effect against ochratoxin.	Huff *et al*. 1992
Chickens	Aflatoxins Trichothecenes	0.5% (21d)	Diminished growth inhibition; no effect against trichothecenes.	Kubena *et al*. 1990
Chickens	None	0.5%; 1.0% (14d)	NS did not impair phytate or inorganic phosphorous utilization.	Chung & Baker 1990
Chickens	None	0.5%; 1.0% (14d)	NS did not impair utilization of riboflavin, vitamin A, or Mn; slight reduction of Zn.	Chung *et al*. 1990
Chickens	Aflatoxins	0.1%; 0.2%	0.2% significantly reduced toxicity in the liver, 0.1% was not able to prevent toxicity.	Jayaprakash *et al*. 1992
Chickens	Aflatoxins	0.125%; 0.25%; 0.5% (21d)	Protected against vitamin A depletion in the livers of chicks exposed to aflatoxins.	Pimpukdee *et al*. 2004

Table 4. Continued

Animals fed clay	Mycotoxin in feed	Clay in feed (duration)	Major effects of the clay reported in the study	References
Chickens	Aflatoxins	0.5 HCSAS; 0.5 HCSAS + 16.5 mg VM/Kg (28d)	HSCAS and HSCAS+VM (virginiamycin) counteracted some of the toxic effects of AF in growing broiler chicks.	Abo-Norag *et al.* 1995
Chickens	Cyclopiazonic acid	1.0% (21d)	Clay did not significantly prevent the adverse effects of clyclopiazonic acid.	Dwyer *et al.* 1997
Chickens	Aflatoxins	0.5%; 0.5% + 0.5 TMP (3wks)	Improved feed intake and weight gain. Alleviated the adverse effects of AFB_1 on some serum chemistry.	Gowda *et al.* 2008
Chickens	Aflatoxins	0.1%; 0.2% (21d)	Clay effectively alleviated the negative effect of AFB1 on growth performance and liver damage.	Zhao *et al.* 2010
Chickens	Aflatoxin, Ochratoxin, T-2 toxin	0.2% (42d)	Increased feed intake and apparent retention of phosphorus. Prevented adverse effects to mycotoxins.	Liu *et al.* 2011
Turkeys	Aflatoxins	0.5% (21d)	Decreased mortality.	Kubena *et al.* 1991
Turkeys	Aflatoxins	0.5% (21d)	Decreased urinary excretion of aflatoxin M_1.	Edrington *et al.* 1996
Pigs	Aflatoxins	0.50%	Decreased DNA adducts in the liver and reduced tissue residues of total aflatoxins.	Beaver *et al.* 1990
Pigs	Aflatoxins	0.5% (42d)	Diminished growth inhibition.	Lindemann *et al.* 1993
Pigs	Aflatoxins	0.5%; 2.0% (28d)	Decreased growth inhibition; prevention of serum effects and hepatic lesions.	Harvey *et al.* 1994
Pigs	Aflatoxins	0.5%; 2.0% (28d)	Diminished growth inhibition, hepatic lesions and immunosuppression.	Harvey *et al.* 1998
Pigs	Aflatoxins	0.5% (35d)	Growth inhibitory effects reduced.	Schell *et al.* 1993

Table 4. Continued

Animals fed clay	Mycotoxin in feed	Clay in feed (duration)	Major effects of the clay reported in the study	References
Pigs	Ochratoxins	1.00%	No significant effect.	Bauer 1994
Pigs	Trichothecenes	0.5%; 1.0% (7-13d)	No significant effect.	Patterson & Young 1993
Dogs	Aflatoxins	0.5% (48h)	Significantly reduced the bioavailability of aflatoxins and excretion of M_1 in urine.	Bingham *et al*. 2004
Lambs	Aflatoxins	2.0% (42d)	Diminished growth inhibition and immunosuppression.	Harvey *et al*. 1991
Mink	Aflatoxins	0.5% (77d)	Mortality was prevented.	Bonna *et al*. 1991
Dairy Cows	Aflatoxins	0.5%; 1.0% (28d)	Reduction of aflatoxin M_1 in milk.	Harvey *et al*. 1991
Dairy Goats	Aflatoxins	1.0%; 2.0%; 4.0% (12d)	Reduction of aflatoxin M_1 in milk.	Smith *et al*. 1994
Mice	Zearalenone	400 mg/kg bw; 5 g/kg bw (48h)	Prevented the general toxicity of ZEN.	Abbès *et al*. 2006
Rats (& Sheep)	Ergotamine	Rats: 2.0% (28d) Sheep: 20% (17d)	HSCAS did not significantly protect rats or sheep from fescue toxicosis.	Chestnut *et al*. 1992
Rats	Aflatoxins	0.1%; 1.0% (8 wks)	Partial protection against lesions in the liver.	Voss *et al*. 1993
Rats	Aflatoxins	0.5% (21d)	Significant prevention of maternal and developmental toxicity.	Mayura *et al*. 1998
Rats	Aflatoxins	0.5% (21d)	Decreased growth inhibition in pregnant rats.	Abdel-Wahhab *et al*. 1998
Rats	Aflatoxins	0.5% (48h)	Decreased urinary excretion of Aflatoxin metabolites (M_1 & P_1).	Sarr *et al*. 1995

Table 4. Continued

Animals fed clay	Mycotoxin in feed	Clay in feed (duration)	Major effects of the clay reported in the study	References
Rats	None	2.0% (16d)	In pregnant rats, Rb was reduced in groups with clay. Neither NSP nor SWY-2 influenced mineral intake.	Wiles *et al*. 2004
Rats	Aflatoxins, Fumonisins	0.25%; 2.0% (1wk)	Reduced bioavailability of AFB_1 and FB_1 individually and in combination.	Mitchell *et al.* 2013
Humans	None	1.5 g; 3 g (2wks)	Mild GI effects, not significantly different. No difference in hematology, electrolytes, liver and kidney function.	Wang *et al*. 2005
Humans	None	1.5 g/day; 3 g/day (3mo)	Moderate effects, though not significant. No significant difference in hematology, electrolytes, liver and kidney function.	Afriyie-Gyawu *et al*. 2008
Humans	None	In capsules: 1.5 g/d; 3 g/d (3mo)	Significantly reduced AFM_1 biomarker in urine and AFB_1-albumin biomarker in serum.	Wang *et al*. 2008
Humans	None	1.5 g/d; 3 g/d (3mo)	No significant effects in vitamins A & E and micronutrients, except for Strontium.	Afriyie-Gyawu *et al*. 2008
Humans (children)	N/A	6 g/d; 12 g/day (3d)	Significantly reduced stool output in children with acute watery diarrhea	Dupont *et al*. 2009
Hydra	None	0.1%; 0.3%; 0.5% (92hr)	No toxicity from NS	Marroquin-Cardona *et al.* 2009
Hydra	Aflatoxins, Fumonisins	0.01%; 0.7%; 1.4%; 2.0% (92hr)	Protection from AFB_1, FB_1, and co-exposure to AFb_1/FB_1	Brown *et al.* 2014

From this point on, the clay discussed in the various studies is still a CM but due to product source and branding, different nomenclature is used. Three of the clays that will be referenced are refined versions of CM. This was done to concentrate the smaller particle size to increase the overall uniformity of the product and make the product more palatable.

A refined CM clay under the name Uniform Particle Size NovaSil (UPSN) has been investigated for its potential to mitigate co-exposures in animal models. NS was used to predict the detoxification efficacy of the clay and evaluate the toxicity of AFB_1, FB_1, and AFB_1/FB_1 using a hydra bioassay (Brown *et al.*, 2014). The results confirmed earlier work with individual AFB_1 and FB_1 isotherms. The inclusion of an isothermal analysis using the toxin mixture revealed site-specific competition between the toxins and UPSN. However, when hydra were exposed to the AFB_1/FB_1 mixture in the presence of UPSN at 1.4%, no toxic responses were observed based on morphology and mortality within 92-hr. The 0.01% UPSN + 400 µg/mL FB_1 + 10 ug/mL AFB_1 group was significantly different from controls at 4, 28, 68, and 92 hr, while the 0.7% UPSN group was significantly different at the 68-92 hr time points only. Based on urinary AFM_1 and FB_1 and AF-albumin biomarkers, USPN (0.25% or 2%) significantly reduced the bioavailability of both AFB_1 and FB_1 in rats when dosed in combination (Mitchell *et al.*, 2014b). With 2% inclusion, AFM_1 was reduced by 97% and 99% when dosed with AFB_1 and 95% and 76% when co-exposed with FB_1 at 12 and 24 hr post-gavage, respectively. Interestingly, FB_1 reductions were not as marked in the co-exposed group. At 2% UPSN, urinary FB_1 was reduced by 85% and 98% in the FB_1-treatment only and by 51% and 59% in the co-

exposed group by 12 and 24 hr after gavage, respectively. This is consistent with isothermal analysis indicating a site-specific competition.

To determine the potential toxicity of long-term dietary exposure to NS, 5-6-week-old male and female Sprague Dawley rats were fed rations containing 0, 0.25, 0.5, 1.0 or 2.0% (w/w) of NS for 28 weeks (Afriyie-Gyawu *et al.*, 2005). The parameters measured during this study included: body weight gain, feed conversion efficiency, relative organ weight, gross and histological appearance of major organs, hematological and serum biochemistry parameters, and essential nutrient levels including vitamins A and E, and Zn. There were very few statistically significant differences between rats consuming treated versus untreated diets with most differences not NS-related or dose dependent. Overall, the authors of this study concluded that ingestion of up to 2% NS was safe in a sub-chronic protocol. Notably, serum and hepatic vitamin A and E levels were slightly increased in the 1% NS-females compared to untreated female rats. Dioxin and furan levels in NS were measured and showed negligible levels. In a more recent study, UPSN was utilized due to its more palatable texture and when Sprague-Dawley rats were dosed for 3 months study, no overall toxicity was observed for UPSN (Marroquin-Cardona *et al.*, 2011). No changes were observed for most of the blood and serum biochemical parameters; increased serum Na, Ca, vitamin E and Na/K ratio and the reduction of serum K and Zn were reported in males with all parameter within the normal ranges reported for rats and no dose trends. The authors of this study conclude that the ingestion of low levels of UPSN does not present a health risk.

As a result of the extensive safety data in animal models, it was hypothesized that NS may be safe and beneficial for humans. A randomized and double-blinded phase I clinical trial was conducted to evaluate the safety and tolerance of NS, and to establish dosimetry protocols for long-term efficacy studies. The doses used for this study were extrapolated from dosimetry data in animal models (Phillips, 1999; Phillips *et al.*, 2002). The high dose (3.0 grams per day) was selected based on the fact that no toxic effects were demonstrated in experimental animals dosed at levels approximately ten times higher (Afriyie-Gyawu *et al.*, 2005). The low dose (1.5 grams per day) was equivalent to the minimal effective concentration (minimal effective dose; MED) that reduced the effects of AF in animals. NS was measured for various environmental contaminants, including dioxins and heavy metals, with everything falling under JECFA or USEPA standards. NS capsules were then manufactured in the same color and size under sterile conditions using US Good Manufacturing Practices. Following the treatment of fifty healthy adult volunteers for two weeks, no significant differences for adverse effects, hematology, liver and kidney function, electrolytes, vitamins A and E, and minerals were observed between to the two randomized dosage groups. The only symptoms reported were gastrointestinal in nature and included abdominal pain (6%, 3/50), bloating, (4%, 2/50), constipation (2%, 1/50), diarrhea (2%, 1/50), and flatulence (8%, 4/50). The results demonstrated the relative safety of NS clay in human subjects and served as a basis for long-term human trials in populations at high risk for aflatoxicosis.

NS was then investigated for safety, tolerance, and AF-sorption efficacy in a 3-month double-blind and placebo-controlled, phase IIa clinical trial in the Ejura-

Sekyedumase district of the Ashanti region of Ghana (Afriyie-Gyawu *et al.*, 2008a; Wang *et al.*, 2008). This region was chosen as the intervention study site based on a report that AFB_1-alb adducts and AFM_1 metabolites were detected in 100% of the 140 sera samples and 91.2% of 91 urine samples collected from study participants in the area (Jolly *et al.*, 2006), which is consistent with reports of 75-100% incidence of exposure in people of East and West Africa (Wild *et al.*, 1992; Wild and Turner, 2002). The NS dosimetry protocol was the same as reported by Wang *et al.* in 2005. Individuals who qualified as study subjects met the following criteria: healthy status based on physical examination results, age 18-58 years, intake of corn and/or groundnut-based foods at least four times a week, blood AFB_1-alb adduct levels >0.5 pmol AFB_1 per mg alb adducts, no history of chronic disease(s), no use of prescribed medications for chronic or acute illness, non-pregnant and/or –breastfeeding females, normal ranges of hematological parameters, liver and renal function indicators (blood and urine parameters), and a signed consent form. The subjects who met the recruitment criteria were randomly divided into three study groups (60/group) based on serum AFB_1-alb adduct levels to avoid selection bias. Importantly, this study employed the use of well-trained study monitors who delivered the capsules daily, witnessed ingestions, and recorded any symptoms that subjects might have experienced; NS was delivered before meals via capsules. Urine and blood samples from each participant were collected at the baseline, at months one, two, and three of treatment, and at month four to represent one month off treatment. Overall, there was a 92% completion for the study and compliance was over 97%. Similar to the safety study, adverse events were minimal and no significant differences were shown in hematology,

liver and kidney function, or electrolytes in the three treatment groups, nor did it interfere with levels of serum vitamins A and E, iron, or zinc (Afriyie-Gyawu *et al.*, 2008b). Importantly, levels of AFB_1-alb adduct were significantly decreased (>40% reduction) in HD and LD groups by month 3. Similarly, levels of AFM_1 in urine samples were decreased by up to 58% in the median level of AFM_1 in samples collected at 3 months in the HD group as comparted to the PL group. This study effectively demonstrated that NS clay capsules can be used to effectively reduce the bioavailability of dietary AF thus confirming earlier work in animal models.

Samples from this study were later analyzed to evaluate the ability of NS clay to reduce urinary FB_1. Fifty-six percent of the samples had detectable levels of FB_1 and >90% of the median urinary FB_1 was significantly decreased in the high dose NS group (2% w/w) (Robinson *et al.*, 2012). Robinson *et al.* (2012) also demonstrated a significant decrease in UFB_1 in rats after treatment with 2% clay by 20% at 24 hr post gavage and 50% at 48 hr post-gavage.

Implementation of CM as a food additive was investigated in a 2010 human crossover designed trial in the same region of Ghana. In this study, UPSN or placebo were included in prepared foods at 0.25% (w/w) for two weeks (Mitchell *et al.*, 2013). Participants exhibited significantly decreased levels of urinary AFM_1 compared to placebo groups (55% reduction) and reported no adverse reactions. Results indicated that UPSN can safely and effectively reduce AF exposure when included in food. Utilization of the clay as a food additive could allow for lowered cost of production, decreased impact of daily life (i.e., eliminate the daily routine of taking pills), and improved sustainability.

Results from the phase I and II clinical trials, in addition to the years of safety testing in animals, demonstrate that ingestion of up to 3 g/day in adults is safe for up to 3 months. Based on these detailed studies, it was determined that ingestion of UPSN at levels efficacious for reducing AFB_1 biomarkers would be reasonably safe in children. A phase I clinical intervention in children ages 3-9 was completed in the Ejura-Sykedumase district of Ghana. The study followed a double-blind, placebo-controlled trial design for 2-weeks (Mitchell *et al.*, 2013). The three treatment arms consisted of a placebo group, which received 0.75 g calcium carbonate two times daily, a low-dose group, which received 0.375 g UPSN two times daily, and a high-dose group, which received 0.75 g UPSN two times daily. Results indicated a significant reduction of AFM_1 biomarkers, with serum biochemical and hematological parameters within the normal range for all groups. This study demonstrated, for the first time, that UPSN is a safe and effective product in children.

1.5 Research objectives

It has been well established that a number of detrimental effects result from the consumption of grains and peanuts contaminated with mycotoxins. Much of this research has focused on the more potent AFB_1. However, recent work by the scientific community has allowed for greater insight into the toxicity and carcinogenicity of FB_1. This is especially true for human public health implications of AFB_1 and FB_1 co-exposure.

Despite advances in agricultural technology and education in methods to reduce mycotoxins, AF and FB contamination is an inextricable issue regarding the consumption of these important commodities. What was once considered a mainly a public health

concern in developing countries that subsist off these foods, is increasingly identified as a global health issue due to climate change and the resulting expansion of agroecological zones of mycotoxin-producing fungi.

Due to government regulations and heterogeneous diets, developed nations face the possibility of chronic low-exposure. Conversely, in developing nations, such as those in sub-Saharan Africa, exposure can be both acute (aflatoxicosis) and chronic. Interestingly, there is few data regarding AF and FB exposure in Latin American. However, exposure is assumed to be high based on the frequent consumption of maize and maize-based products.

Calcium montmorillonite has shown promising results in reducing bioavailability of both AFB_1 and FB_1. Until recently, human efficacy has only been invested in the high-risk Ejura-Sykedumase district region of Ghana. Although determined to be safe in both children and adults, cultural and dietary habits are important determining factors of efficacy for reducing a dietary toxin. Therefore, the principle goals of this research were to:

1) Determine the AFB_1 binding capacity of UPSN in a food matrix under various cooking conditions typical of sub-Sahara African. The binding capacity of UPSN was determined by measuring AFB_1 concentrations with and without the application of clay after exposure to various cooking challenges using a fluorometer and confirmed by HPLC with fluorescence detection.

2) Evaluate AF and FB exposure in participants from Monterrey, Mexico in relation to various sociodemographic and dietary factors through use of a questionnaire and one-time analysis of AFM_1 and FB_1 in urine.

3) Determine efficacy, palatability, and acceptability of ACCS100 as a strategy to reduce AF exposure in a vulnerable Kenyan population when delivered in drinking water. A double-blind cross-over clinical trial design was followed for two weeks. AFM_1 and AFB_1-lys biomarkers were analyzed in serum and urine and used to determine efficacy. Dietary survey, palatability, and acceptability data were collected and analyzed.

4) Determine the efficacy of ACCS100 in reducing biomarkers of AF exposure after three months of treatment in Bexar County, Texas. AFM_1 and AFB_1-lys biomarkers were analyzed in serum and urine and used to determine efficacy. Dietary survey data were collected and analyzed.

2. COMMON AFRICAN COOKING PROCESSES DO NOT AFFECT THE AFLATOXIN BINDING EFFICACY OF REFINED CALCUIM MONTMORILLONITE CLAY

2.1 Introduction

In many parts of West Africa, populations are chronically exposed to AFs beginning *in utero* (Partanen *et al.*, 2010; Turner *et al.*, 2007; Turner *et al.*, 2012). Exposure typically continues through the first years of life, with the presence of the toxic secondary metabolite AFM_1 in breast milk (Gong *et al.*, 2003; Shephard, 2008; Zarba *et al.*, 1992), and well into childhood and adulthood where exposure to AFB_1 can be present in fermented corn meals and porridges (Andah, 1972; Lartey *et al.*, 1999). Recently, a sampling of corn-based weaning foods intended for children between the ages of 6 months and 2 years in the Ashanti region of Ghana were found to contain high levels of AFs (Kumi, 2011). All of the 36 samples tested were contaminated with AFs, with 83% containing concentrations above the U.S. FDA action level of 20 ppb AFB_1 and some samples ranging as high as 500 ppb. Despite the fact that fermenting and heating these weaning foods and breakfast gruels may prevent spoilage and enhance food safety, AFs are resistant to degradation by thermal inactivation and fermentation (Christensen *et al.*, 1977) and therefore remain a constant source of concern.

Methods that focus on reducing dietary exposure to AFs in contaminated foods are

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*Reprinted from *Food Control*, **37**, by SE Elmore, N Mitchell, T Mays, K Brown, A Marroquin-Cardona, et al., "Common African cooking processes do not affect the aflatoxin binding efficacy of refined calcium montmorillonite clay", 27-32, Copyright 2014, with permission from Elsevier Ltd., doi.org/10.1016/j.foodcont.2013.08.037.

highly desirable as a practical strategy to mitigate the harmful effects of this toxin (Williams *et al.*, 2004). Preferential sorption of AFs in the gastrointestinal tract with the inclusion of certain clays in the diet is one example of this type of approach. NS is a calcium montmorillonite clay with high binding affinity and capacity for AFB_1. NS has been shown to be safe and effective in preventing aflatoxicosis in animals and reducing biomarkers of AF exposure in humans and animals (Harvey *et al.*, 1991a; Harvey *et al.*, 1991b; Kubena *et al.*, 1991; Lindemann *et al.*, 1993; Phillips, 1999; Phillips *et al.*, 2006; Phillips *et al.*, 1988; Phillips *et al.*, 2002; Pimpukdee *et al.*, 2004). These studies have shown that NS is effective as an enterosorbent for AFs when included in the diet at levels ranging from 0.25 to 2% (w/w) in animals (Phillips *et al.*, 2002). Additionally, a minimal effective dose of NS 0.25% w/w delivered in capsules for three months in a high risk Ghanaian population was successful in decreasing biomarkers of AF exposure and did not interfere with the levels of serum vitamins A and E, iron, or zinc (Afriyie-Gyawu *et al.*, 2008b). Parent NS clay was refined to form UPSN (Uniform Particle Size NovaSil) through a process that served to improve the palatability and consistency of the clay for food delivery. The refining process resulted in a higher percentage of NS particle sizes between 45-100 µm and lower levels of quartz; however, NS and UPSN were compared and shown to have similar AF sorption properties (Marroquin-Cardona *et al.*, 2011). Rats fed UPSN at levels as high as 2% (w/w) for 13 wk displayed no detectable toxicity (Marroquin-Cardona *et al.*, 2011). Recently, UPSN inclusion in foods has been investigated in populations at high-risk of AF exposure. In a crossover study in Ghana, UPSN was shown to be palatable and well-tolerated when added to fermented foods.

Moreover, those participants consuming 0.25% UPSN exhibited significantly decreased levels of urinary AFM_1 compared to the placebo group. Also, no adverse reactions from the treatment or placebo were reported. This study indicated that UPSN (when delivered in common fermented foods) was acceptable and could safely and effectively reduce AF exposure when included in contaminated diets (Mitchell *et al.*, 2013).

Fermentation of corn-based foods in West Africa is common and the effects of acidity and ethanol production during this process are important parameters that could interfere with toxin sorption by clay (UPSN) and thus need to be investigated. Also, knowledge regarding the effects of different cooking conditions (temperature and fermentation time) on the AF-clay complex is needed to determine AF adsorption ability of the clay in a cornmeal matrix. Hence, the objective of the present study was to determine UPSN stability and AFB_1 sorption during fermentation and heating protocols that typify the production of common corn-based foods intended for consumption in this region.

2.2 Materials and methods

2.2.1 Materials

Acetonitrile (ACN) and methanol (MeOH) utilized were HPLC analytical grade (Fisher Scientific, Fair Lawn, NJ). Ultrapure deionized water (18.2 MΩ) was generated using an Ultrapure automated filtration system (Elga™ Woodridge, IL). Aflatoxin B_1 was purchased from Sigma-Aldrich Corporation (St. Louis, Mo). Cornmeal was purchased from a local grocery store in College Station, TX. Extraction equipment, including AflaTest® immunoaffinity columns, was purchased from Vicam® (Watertown, Ma) and utilized according to the manufacturer's instructions. Uniform Particle Size NovaSil

(UPSN) was obtained from Texas Enterosorbents (Bastrop, TX). Quantitative analysis of AFB_1 was performed using a Vicam Series 4 Fluorometer and verified on a Waters HPLC with fluorescence detection (Watertown, Ma).

2.2.2 Cornmeal preparation

Purchased cornmeal contained an average of 1 ppb AFB_1 as measured by Vicam analysis (see section 2.2.6). AFB_1 standard was diluted in water to obtain a 25 ppm stock solution for spiking cornmeal samples. The concentration was verified daily using UV spectrophotometry (Shimadzu UV-1800). Cornmeal samples (50 g) were prepared in triplicate, containing 5, 50, 100, 300, 500, and 1000 ppb AFB_1, for both the control and UPSN-treated groups. UPSN (1.5 g) was added to AFB_1-spiked cornmeal samples to comprise the clay test group. The amount of UPSN was calculated based on the high dose of UPSN that was delivered per person in clinical intervention trials in Ghana (1.5 g in each meal) (Mitchell *et al.*, 2013). AFB_1-spiked samples and clay were mixed together for 15 seconds to disburse the clay throughout the cornmeal. This procedure was repeated for all samples and served as a base mixture before additional processing steps were applied as described in the following Sections 2.2.3, 2.2.4, and 2.2.5.

2.2.3 Base product

AFB_1 was extracted from samples immediately following the base (non-processed) cornmeal product to assess binding capacity of UPSN for AFB_1 within the cornmeal matrix without any fermentation or cooking. The same procedure was also repeated at pH 3.5 to simulate the average pH observed during the fermentation of corn dough (Plahar

and Leung, 1983). This was achieved by adjusting the mixture with HCl until stabilized at pH 3.5.

2.2.4 Fermented product

Fermentation of the base product was allowed to occur naturally in covered flasks simulating the process used in Africa. Water (50 mL) was added and mixed by agitating the flask until uniform in appearance and thoroughly moist. Mixtures were allowed to ferment for 24 or 72 hr in a NUAIRE™ TS Autoflow Incubator (Plymouth, Mn) maintained at 30° C. This procedure represents both the average temperature and typical fermentation environment that would occur in West Africa. Additionally, samples with the same AFB_1 concentrations and controls were subjected to heat treatment by adding 50 mL boiling water following fermentation. Then, the dough and water were mixed together without further heating, resulting in a matrix with the consistency of a thick soup or gruel. Mixtures were allowed to sit at room temperature for 10 min prior to processing.

2.2.5 Sterilized product

Sterilized samples were produced by autoclaving the base products and water at 220°C for 30 min prior to the 72 hr incubation period. Throughout this publication, the term "fermentation" will refer to samples that were not sterilized and allowed to ferment by naturally present species of bacteria and other fermenting microorganisms, whereas "sterilized" will refer to samples that were autoclaved and incubated but did not ferment.

2.2.6 Extraction and quantification of aflatoxin B_1

The USDA-FGIS (Federal Grain Inspection Service) single filtration procedure for corn (0-1000 ppb) was used with modifications for the extraction of AFB_1. In brief, 250

mL of water was added to each 50 g cornmeal sample and allowed to mix (887 xg) for 30 min on a plate shaker. This step allowed for thorough UPSN interaction with AFB_1 and maintained the clay's interlayer structure responsible for sequestering AFB_1 prior to extraction with a solvent. AFB_1 extraction procedures require use of alcohols and solvents which may disrupt the interlayer structure and decrease AFB_1 binding potential. To circumvent this problem, all samples were first mixed in water to allow for UPSN to bind AFB_1 prior to toxin extraction procedures (Phillips TD, 1990). Following mixing with water, 100 mL of 85:15 acetonitrile:water was added and samples were shaken again for 30 min to extract AFB_1 from the corn matrix. The slurry was then filtered through 8-12 µm X 24 cm fluted filter paper (Vicam). The extract was collected in a clean vessel and filtered a second time through a 1.5 µm X 11 cm glass microfiber filter (Vicam). The final filtered extract was stirred for 10 sec, and 2 mL were passed through an AflaTest immunoaffinity column (Vicam) at a rate of 1-2 drops/sec. Columns were washed twice with 3 mL water and eluted with 1 mL MeOH into a glass cuvette. AflaTest developer was added and mixed with the eluate prior to fluorescence detection on a Series 4 Vicam fluorometer with excitation and emission wavelengths of 365 nm and 440 nm, respectively.

To verify overall trends observed with the Vicam analysis, AFB_1 concentrations from representative samples were also measured by HPLC with fluorescence detection. Unlike HPLC, the Vicam method accounts for a loss of AF during immunoaffinity extraction through internal calibrations. AOAC AF extraction protocol (991.31) was utilized for HPLC-fluorescence analysis. For HPLC sample extraction, fermented and

sterilized samples spiked with 5, 500, and 1000 ppb AFB_1 were prepared as previously mentioned. The samples were then transferred to a blender jar with 250 mL extraction solvent (70:30 MeOH: water) and blended at high speed for 2 min. The slurry was filtered through 8-12 µm X 24 cm fluted filter paper (Vicam®) into a clean vessel. The extract was then divided into 10 mL aliquots and diluted with 20 mL water and vortexed. The diluted extract was filtered again through a 1.5 µm X 11 cm glass microfiber filter (Vicam®). For samples spiked with 5 ppb AFB_1, 6 mL extract was passed through an AflaTest immunoaffinity column at a rate of 1-2 drops/sec. For 500 and 1000 ppb samples, 2 mL extract was passed through the columns. Columns were washed twice with 10 mL water and eluted with 1 mL MeOH into a glass cuvette. Water (1 mL) was added to each cuvette and mixed well prior to syringe filtering. A mobile phase of 3:1:1 water:ACN:MeOH with a flow rate of 1.0 mL/min was utilized with a Waters Spherisorb C18 column for separation. Corresponding external standards were prepared for each level of AFB_1 and injected (100 µL) prior to sample injection. Concentrations of AFB_1 from 0.1 to 10 µg/mL were linear (r^2= 0.99) using this method. Excitation and emission wavelengths were set at 360 and 420 nm, respectively. Quantification was achieved by peak-area measurement using Breeze™ HPLC analysis software (Waters).

Although different extraction methods were utilized for Vicam and HPLC analysis, comparison of the overall trends between sample groups was the primary focus of this manuscript, rather than comparison of absolute extractable values between AF analytical methods.

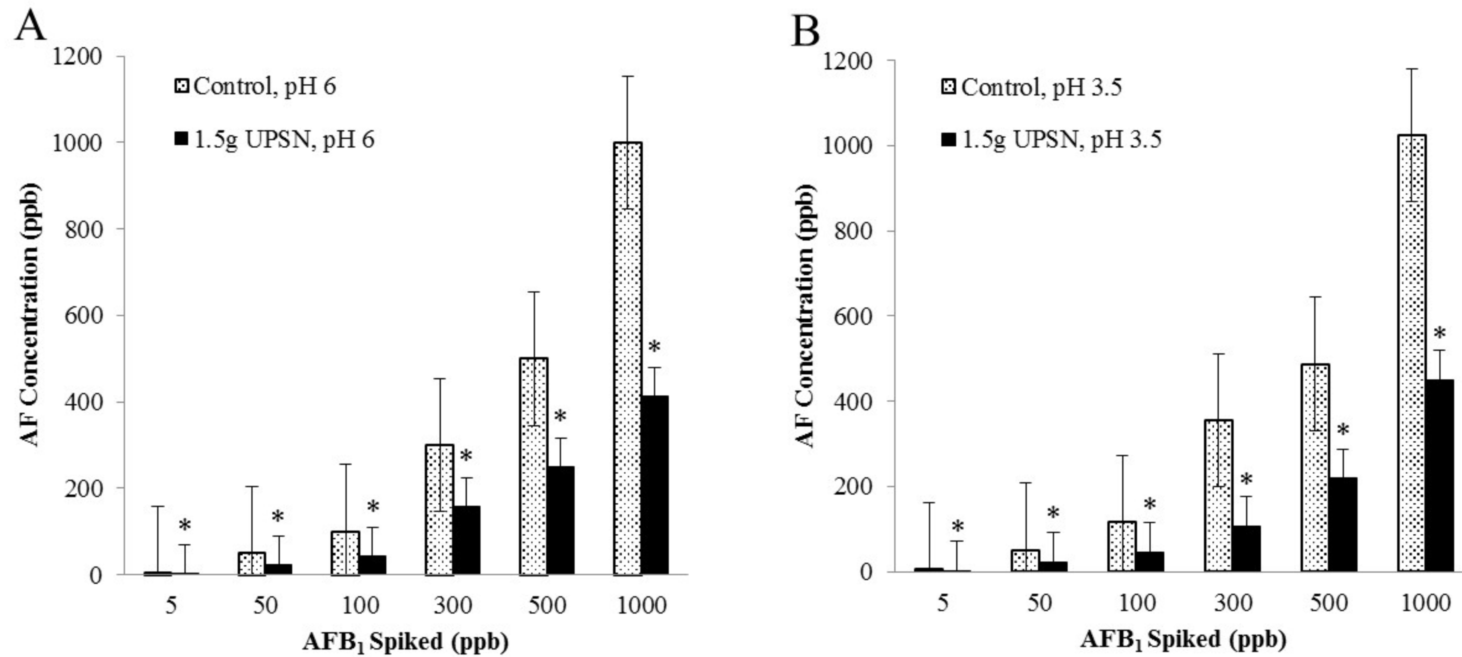

Figure 7. Reduction of AFB_1 in unfermented cornmeal with UPSN. Cornmeal was spiked (5-1000 ppb) in the presence of UPSN at pH 6 (A) and pH 3.5 (B). Differences between control and UPSN-treated samples were considered significant at $*p < 0.05$. Error bars represent standard error of the mean.

2.2.7 Calculations and statistical analysis

All data obtained with the Series 4 Vicam® fluorometer was standardized with the values obtained for unfermented cornmeal control samples. For HPLC data, a dilution factor of 5 was applied to samples spiked with 5 ppb AFB_1 and a factor of 15 to the 500 and 1000 ppb AFB_1 samples. JMP 9 software (SAS, Carry, NC) was used to perform factorial analysis of treatments and included Summary of Fit, Analysis of Variance and Student t-test analyses. Percent reduction of AFB_1 was calculated by dividing the treatment average by the control average for each sample concentration. Results were considered significant at $p \leq 0.05$.

2.3 Results

2.3.1 UPSN reduction of aflatoxin B_1 in unfermented products

Figure 7 demonstrates the binding capacity of UPSN in cornmeal samples at unadjusted (pH 6, Fig. 7a) and acidified pH (pH 3.5, figure 7b). A significant decrease in the amount of extractable AFB_1 was observed in the presence of UPSN at both pH values ($p < 0.002$) and percent reductions for unadjusted and pH 3.5 samples ranged from 68-77% and 49-70%, respectively. UPSN-treated sample values were compared between unadjusted and pH 3.5 conditions to assess the effect of acidic pH on the efficacy of UPSN binding. There was no significant difference between 5, 50, 100, 500, and 1000 ppb AFB_1 samples.

2.3.2 UPSN reduction of aflatoxin B_1 in fermented products

In samples that were allowed to ferment for 24 or 72 hr, UPSN significantly reduced the amount of AFB_1 compared to controls (figure 8). Percent reduction ranged

from 79-88% for 24 hr and from 98-100% for 72 hr fermented products. UPSN binding capacity at 72 hr was enhanced; these samples contained significantly lower AFB_1 levels in the presence of UPSN compared to the 24 hr data at AFB_1 concentrations between 50 to 1000 ppb. Since there was no dose-dependent difference between fermented controls at 24 and 72 hr, these results suggest that AFs were not significantly degraded by fermentation (consistent with the literature), but instead were reduced due to clay sorption processes that were time dependent.

Similarly, samples containing UPSN that were fermented for 72 hr. followed by the addition of boiling water (simulating the production of corn-based porridge or gruel) significantly reduced AFB_1 levels compared to controls with percent reduction ranging from 91-100% (figure 9a). Samples that were treated with UPSN and incubated for 72 hr. following sterilization demonstrated significant reduction of AFB_1 in the presence of UPSN ($p < 0.0001$) and total sorption ranged from 85-100% (figure 9b).

2.3.3 HPLC verification

HPLC fluorometric analysis of sample extracts confirmed UPSN sorption efficacy observed using the Vicam method. The overall trend remained the same between HPLC and Vicam samples. UPSN significantly reduced the amount of AFB_1 present in samples that were unfermented, fermented for 72 hr., or incubated for 72 hr. following sterilization (figure 10). Percent reductions ranged from 66-92%, 99-100%, and 99-100% for unfermented, fermented, and sterilized samples with UPSN, respectively (Table 5).

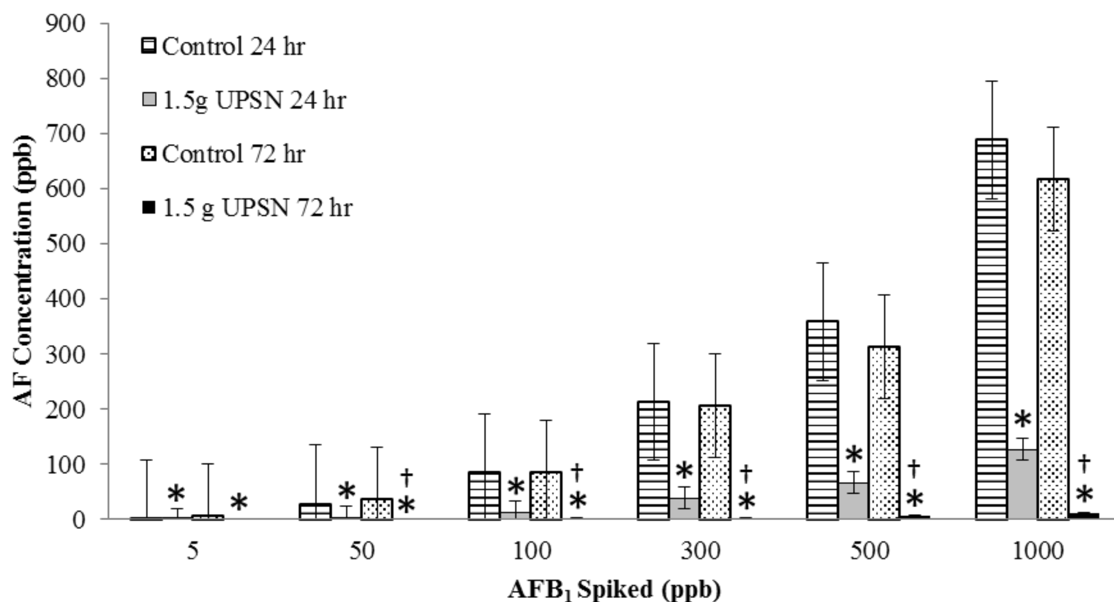

Figure 8. Reduction of AFB_1 in fermented cornmeal with UPSN. AFB_1 (5-1000) was added to cornmeal and allowed to ferment for 24 or 72 hr. AF reduction between control and UPSN-treated samples at either time point was considered significant at * $p < 0.05$. Significant differences ($p < 0.05$) in AF reduction between 24 and 72 hr are indicated by †. Bars represent standard error of the mean.

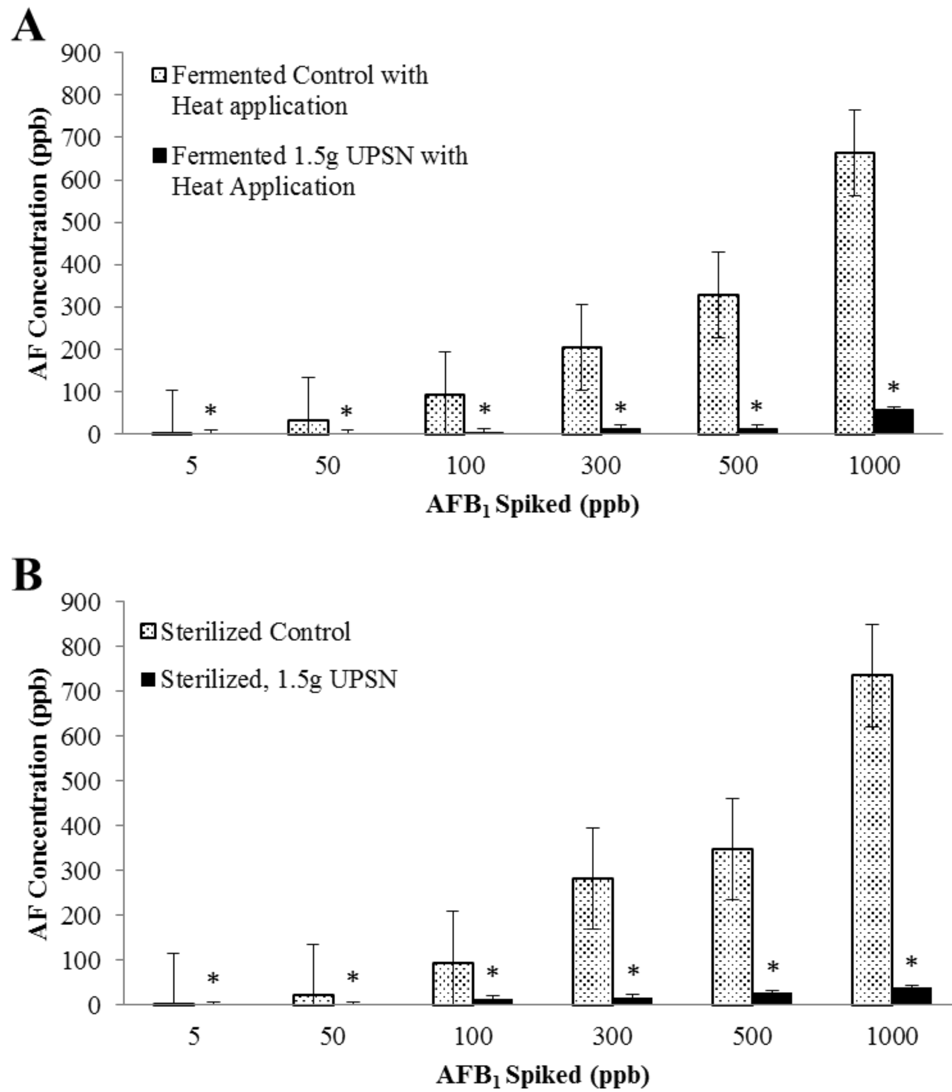

Figure 9. Reduction of AFB_1 in cornmeal in the presence of UPSN after heat exposure or sterilization. Cornmeal samples spiked with 5-1000ppb were allowed to ferment for 72 hr, then exposed to boiling water (A) or sterilized and allowed to incubate without fermentation for 72 hr (B). UPSN reduction of AFB_1 compared to controls (no UPSN) was considered significant at $*p < 0.05$. Bars represent standard error of the mean.

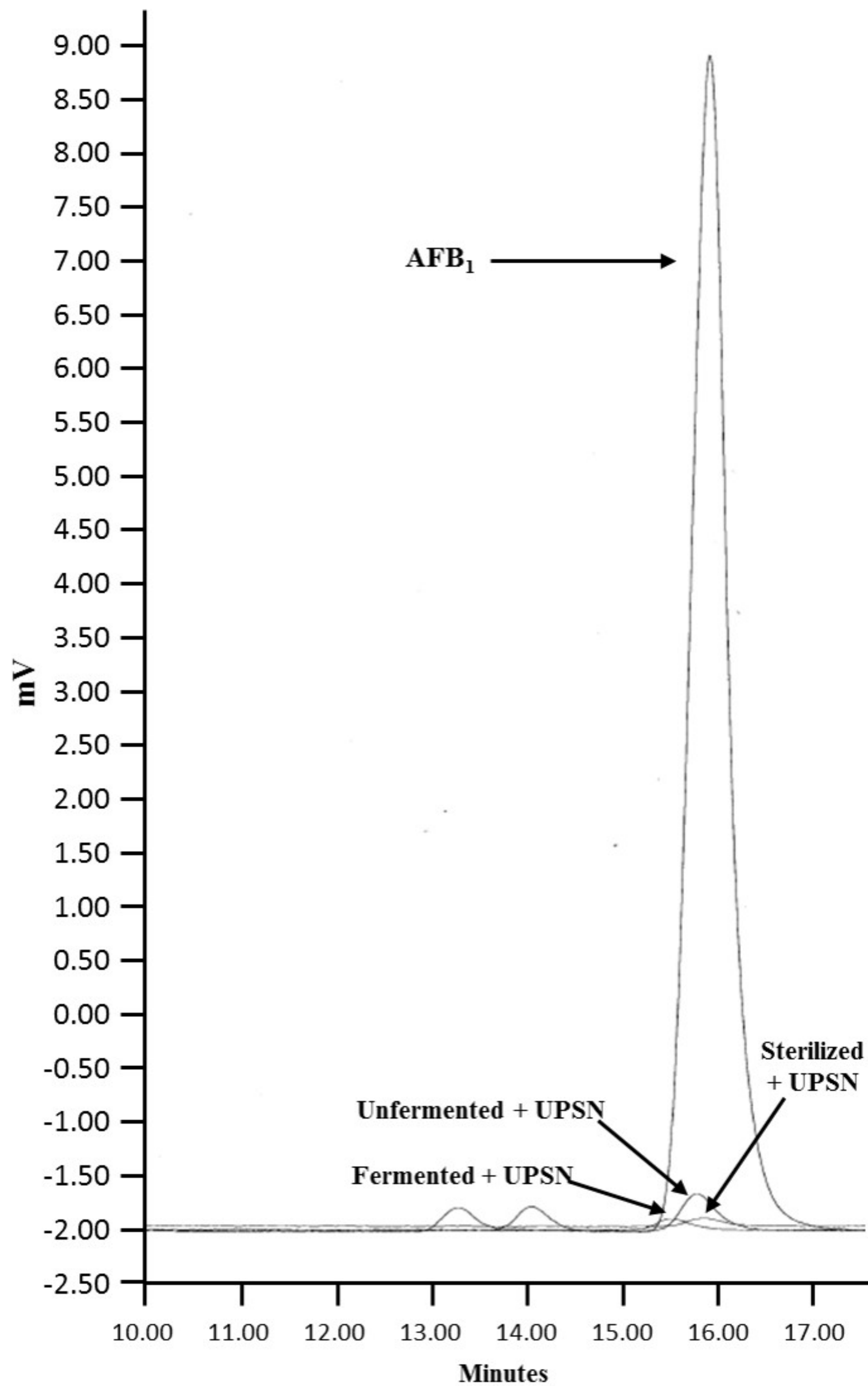

Figure 10. HPLC detection of AFB_1 levels in unfermented, fermented, and sterilized samples. All samples were spiked with 1000 ppb AFB_1.

Table 5. Percent AFB_1 reduction trends in cornmeal verified by HPLC.
Comparison of samples unfermented, fermented for 72 hr, or incubated for 72 hr with and without UPSN.

AFB_1 Spiked (ppb)	UPSN (g)[1]	Unfermented 0 hr (ppb)[2]	% Reduction[3]	Fermented 72 hr (ppb)[2]	% Reduction[3]	Sterilized & Incubated 72 hr (ppb)[2]	% Reduction[3]
5	0	18.38		2.78		2.35	
	1.5	6.25	66.0	ND	100.0	ND	100.0
500	0	372.98		254.32		324.11	
	1.5	49.57	86.7	0.77	99.7	4.54	98.6
1000	0	692.41		522.82		617.04	
	1.5	57.11	91.8	57.14	99.2	3.65	99.4

[1]Three samples per treatment (n=3).
[2]AFB_1 was recovered at 0 hr (prior to fermentation), at 72 hr with fermentation, and at 72 hr in sterilized samples.
[3]In all samples containing UPSN, significantly less AFB_1 was detected compared to control. Values were considered significant at $p < 0.05$.

2.4 Discussion

AF contamination in African cornmeal products is a major public health issue that has yet to be resolved; importantly, the vulnerable in susceptible regions are at considerable risk for life-long exposure (IARC, 2012). One suggested solution that has shown promise for wide-scale application is the use of UPSN enterosorption therapy. Since the fermentation of corn-based foods in West Africa is common, the objective of the present study was to determine UPSN stability and AFB_1 sorption during fermentation and heating protocols that typify the production of common corn-based foods intended for consumption in this region. This is a concern because heat treatment via cooking and acid and ethanol created during the fermentation process could potentially interfere with the sorption of AFB_1 on the surfaces of UPSN. Fermentation results in the production of ethanol, which can affect the interlayer stability of calcium montmorillonites such as UPSN. The effects of acidity, fermentation, time of fermentation, and heat application on the ability of UPSN to bind AFB_1 were investigated using a Vicam fluorometer with HPLC validation. By testing cornmeal under these conditions, we were able to determine the difference in UPSN's sorption of AFB_1. In all corn samples, UPSN was able to significantly reduce the amount of extractable AFB_1. This suggests that the clay is stable during fermentation (72 hr) and in the presence of heat while in a food matrix. Thus, the addition of UPSN to foods prior to processing could help to reduce hazardous exposures to AFB_1 from contaminated food sources.

The effect of acidic pH, similar to that produced by the process of fermentation, was assessed and compared to unfermented cornmeal at unadjusted pH. In both conditions,

AFB_1 was significantly reduced by UPSN. The lack of significant difference in binding due to alteration of pH is consistent with *in vitro* binding models conducted in our laboratory, where UPSN has previously been shown to be an effective AFB_1 sorbent at a pH as low as 2 from a series of isothermal analyses (Marroquin-Cardona *et al.*, 2011). This preliminary data supported further work to assess the effects of relevant African food preparation techniques on AF binding by UPSN.

Results from this study indicate that length of fermentation influenced sorption ability, as UPSN was significantly more effective in reducing AF at 72 hr compared to the earlier time points. Our findings suggest that small amounts of UPSN (1.5 g) included in foods, prior to fermentation, could significantly reduce physiologically relevant levels of AFs in corn-based foods. It is possible that this dose delivery platform could result in increased efficacy *in vivo*. When the majority of binding occurs within foods that are hydrated prior to ingestion, the likelihood of potential physiological interactions is reduced, therefore decreasing the risk of exposure and associated toxicities.

Overall, samples that were exposed to heat following fermentation (72 hr) were statistically similar to samples that were fermented without heat. These findings are supported by previous research showing that calcium montmorillonite interlayer structures are stable at temperatures up to 400° C, where the clay can delaminate (Deng Y., 2010) and become inactivated. In order to decrease variability due to time of cooking, the primary preparation step for gruel was simulated in this study. Traditionally, boiling water is added to reconstitute the matrix and allowed to cook for 2-20 min. This boiling time (and the recipe) can vary according to the region and community. Thus, our design was

focused on the addition of boiling water to bring the matrix (and AFB_1) to a uniform slurry prior to extraction and analysis. Importantly, we demonstrated that boiling water and/or sterilization temperatures had no effects on the ability of UPSN to bind AFB_1.

Although fermented samples with UPSN exhibited decreased levels of AFB_1, we wanted to determine if treatment time contributed to the effect of UPSN. Sterilized samples were incubated for the same length of time (72 hr) without fermentation. Although comparison between the fermented and sterilized cornmeal did show significantly decreased binding in the sterilized samples with 100 and 500 ppb AFB_1, this trend was not dose-dependent. These findings suggest that the fermentation process itself did not enhance binding, but that time appeared to be the primary factor in the increased sorption of AFB_1 following fermentation. Importantly, inclusion of UPSN in foods before fermentation or cooking does not negatively affect the binding capacity, but allows more opportunity for interaction between UPSN and AFB_1, enhancing its efficacy.

Dietary exposure in Africa is often associated with consumption of both groundnuts and corn; however, it is likely that the majority of AF ingestion occurs through corn-based foods. Exposure assessment and risk characterization for AF-induced health disparities in several African countries indicated that corn consumption, particularly in Ghana, could be as high as 1,000 g per day (Shepard, 2008). Furthermore, dietary intake questionnaires administered by our laboratory in various regions in Ghana revealed that 62% of adults reported consuming corn or corn-based products every day, while only 12% reported daily consumption of groundnuts or groundnut products (Mitchell *et al.*, 2013). However, it is important to note that clays similar to UPSN can effectively bind AF in a

peanut matrix (Seifert *et al.*, 2010), therefore it is likely that UPSN would be effective in a diet consisting of both corn and groundnuts.

Another objective of this study was to develop practical strategies and analytical protocols that would be applicable in parts of the world where AF contamination is often coupled with food scarcity and lack of access to sophisticated analytical technology. Since most field laboratories in areas affected by frequent and high level exposures to AF have the capacity to perform the Vicam assay, we designed this study to use this method. The Vicam assay is relatively inexpensive and field practical which is ideal for situations where rapid screening is desired to monitor and mitigate AF outbreaks. HPLC (with fluorescence detection) was used as a secondary method to validate the ability of UPSN to sorb AF from a food matrix. Although different extraction methods were utilized for Vicam and HPLC analysis, comparison of the overall trends between sample groups was the primary focus of this manuscript, rather than comparison of absolute extractable values between methods. Preliminary work indicated that both methods were similar in their ability to detect the binding of AFs by clay (data not shown).

Furthermore, we hope that this technology can be translated at the village level by including UPSN or similar clays at local mills during corn processing. In the case of AF outbreaks, this would empower the village millers to help prevent aflatoxicosis by adding AF binders to the cornmeal prior to distribution, not unlike iodine inclusion in salt. This novel application could afford villages increased self-sufficiency and entrepreneurial capability. Additionally, advantages of adding the UPSN at the mill include assurance of uniform UPSN distribution within the cornmeal and added consumer convenience.

In this study we were able to demonstrate that a refined calcium montmorillonite clay, UPSN, was able to significantly reduce AFB_1 under common cooking conditions in a corn meal matrix using a field-practical technique. Therefore, the addition of UPSN to foods at any stage of preparation could be a sustainable approach to alleviate AF-associated public health issues in high-risk populations, enhance the benefits of potentially contaminated foods and nutritional supplements, and empower high risk populations during times of food scarcity and high AF exposures.

3. EPIDEMIOLOGICAL SURVEY OF AFLATOXIN AND FUMONISN IN MONTERREY, MEXICO

3.1 Introduction

The assurance of food safety and quality represents a major challenge worldwide. Contamination of foods with fungal metabolites known as mycotoxins is an unavoidable problem, particularly in regions with limited resources. AF and FB are mycotoxins commonly found as co-contaminants of staple foods (e.g., corn and nuts) and produced by certain strains of *Aspergillus* and *Fusarium* species, respectively. Of the toxic congeners, AFB_1 and FB_1 are the most potent and prevalent analogs of the AFs and FBs. AFB_1 is listed as a group 1 human carcinogen (IARC, 2012), and has been associated with child growth impairment (Gong *et al.*, 2002; Gong *et al.*, 2004; Shouman *et al.*, 2012), suppressed immune function (Turner *et al.*, 2003), hepatomegaly (Gong *et al.*, 2012), and death due to acute poisoning (Probst *et al.*, 2007). FB_1 is a group 2 compound, i.e., "possibly carcinogenic in humans" (IARC, 2002), which is found to be associated with esophageal cancer (Rheeder *et al.*, 1992), neural tube defects in humans (Missmer *et al.*, 2006) and possibly primary liver cancer (Ueno *et al.*, 1997). FB-AF co-exposure data is limited, with much of it lacking causal mechanisms. However, co-exposure has been shown to increase toxicity in a hydra model (Brown, *et al.*, 2014), alter toxic responses in rats and mosquito fish (McKean *et al.*, 2006), lower feed conversion and mean body weight in pigs (Dilkin *et al.*, 2003), and increase the development of hepatocyte nodules in rodents (Gelderblom *et al.*, 2002). Several studies indicate the need for further

investigation into the prevalence and source of AF and FB co-exposure, especially for liver cancer risk.

AF and FB have been detected in maize samples worldwide. According to Food and Agriculture Organization data for the year 2010, per capita maize consumptions were 13 and 117 kg in the United States and Mexico, respectively (United Nations, 2015). Mexico has a long history of maize consumption with daily consumption ranging from 400 to 600 g (Plasencia, 2004). Due to high consumption levels of maize, the co-exposure to AF and FB is expected to be one of the highest in the world for Mexican populations. Consumption of maize (as corn ear or powdered products) by Mexican populations or Hispanic populations in other countries has resulted in measurable levels of AFs and FBs. Previous data from our laboratory conducted in a Hispanic population with high incidence of HCC in the U.S. suggested that AF exposure may play a role in the carcinogenesis. Questionnaires which investigated the dietary habits of this population reported that consumption of corn tortillas, rice, and nuts were related to AF exposure (based on measurement of urine biomarkers) (Johnson *et al.*, 2010). In a cohort from Morelos County, Mexico, women with high intake of maize-based tortillas had a 3-fold increase in average FB_1 levels comparing to that of the low intake control group (Gong *et al.*, 2008).

Although the individual exposure to both AF and FB in Mexican populations has been previously shown using blood (Soini *et al.*, 1996) and urine biomarkers (Gong *et al.*, 2008), respectively, AF and FB co-exposure in Mexicans has not been investigated. Furthermore, the potential source of exposure due to the high consumption of corn products is unknown. We report here co-exposure to AFs and FBs in Mexican volunteers

using urine biomarkers of exposure and investigate the role of corn products from street markets as a potential source of exposure.

3.2 Materials and methods

3.2.1 Chemicals

AFM_1 was obtained from Sigma Aldrich (St. Louis, MO, USA). FB_1 was purchased from PROMEC Unit of South Africa Medical Research Council (Tygerberg, South Africa). AflaTestWB and FumoniTestWB Immunoaffinity columns were purchased from VICAM (Watertown, MA, USA). All solvents were purchased from Fischer and were LC/MS grade. Ultrapure deionized water (18.2 MΩ cm) was used in all extractions.

3.2.2 Participant recruitment and sample collection

Institutional Review boards from Texas A&M University (TAMU, College Station, TX) and Universidad Automonoma de Nuevo Leon (UANL, Monterrey, Mexico) reviewed and approved protocols for analysis of human samples. Enrollment of participants was achieved in 9 cities from the metropolitan area of the city of Monterrey, Nuevo Leon state in Mexico. These cities are the most populated urban areas in the state and include Apodaca, Escobedo, Garcia, Guadalupe, Juarez, Monterrey, San Nicolas, San Pedro, and Santa Catalina (Figure 11). Recruitment criteria for participants included: age (18 and 75), no history of chronic kidney disease or liver disease, consumption of maize or maize products from street markets (at least one a week), and written informed consent. Recruitment teams visited homes from February 2015 to April 2015. Homes were located near street markets (same zip code). Upon arrival to participants' home, teams explained the study and obtained informed consent and at the same time a dietary questionnaire was

administered. The questionnaire was designed to investigate the frequency of maize and maize products consumption. Questions focused on exploring the role of dietary toxins on mycotoxin excretion in urine. Urine collection flasks were given to each participant along with instructions for collecting the first morning urine sample. Teams collected the urine in the morning (7:00 am-9:00 am). Upon collection, participants were given a $100 pesos gift card. Urines collected were kept in a cooler with refrigerant during transfer to the laboratory where samples were placed in a -80 °C freezer. A urine subsample (2 mL) was sent for creatinine analysis within 5 hr of urine collection. Urine samples were maintained at -80°C until shipment for analysis at TAMU. An aliquot of urine remained at UANL for creatinine analysis.

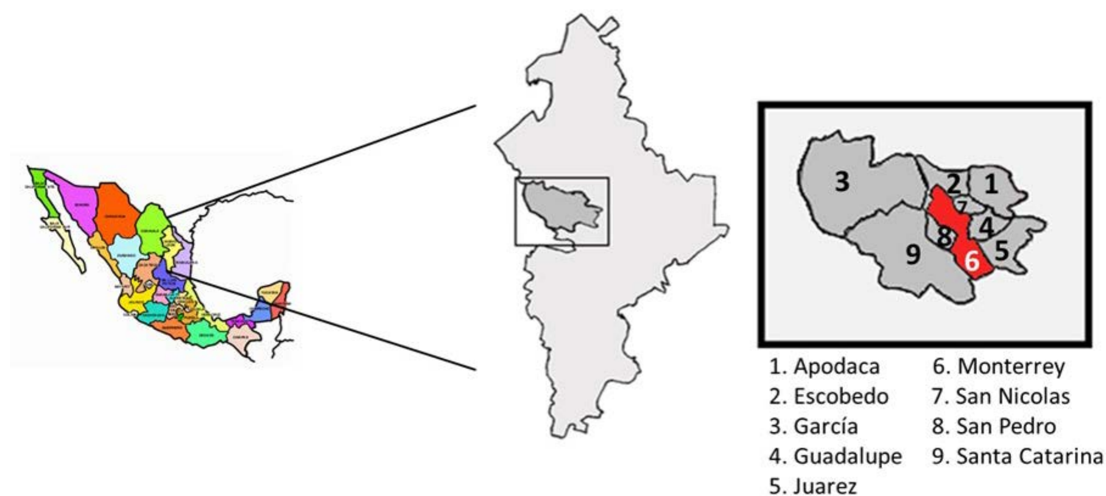

Figure 11. Recruitment area in Monterrey, Nuevo Leon, Mexico

3.2.3 Determination of aflatoxin M_1 in urine

Extraction of urinary AFM_1 followed the method of Groopman (1992) with modifications of Sarr (1995) and Wang (1999). Urine samples were centrifuged at 887 xg, and 5.0 mL of supernatant was collected and diluted with water to a total volume of 10.0 ml. Samples were then loaded onto a 3 mL preparative Aflatest® WB immunoaffinity column (VICAM) at a flow rate of 1 ml/min. Following washing of the column, the AF fraction was eluted from the column with 2 ml of 80% methanol, dried under N^2 and re-suspended in 200 µL of a 1:1 solution of methanol:water.

Samples were analyzed using a Waters Acquity H-Class UPLC-MS/MS (Waters Corporation) and separation using a 2.1 x 50 mm Acquity UPLC BEH C18 column with a particle size of 1.7 µm. Isocratic separation was achieved with 70% water buffered with 1% formic acid and 30% ACN buffered with 1% formic acid. Samples (10 µL) were injected onto the column and the elution rate was 0.325 ml/min. The column effluent was directly coupled to the MS, which was operated in the positive electrospray ionization mode. MS/MS conditions were optimized for AFM_1 and based on Warth *et al.* (2012). The precursor ion was set to 329.00 Da and the two product ions were 273.00 Da (quantifying ion) and 259.1 Da (qualifying ion). Urinary AFM_1 concentrations were expressed as pg/mg creatinine (pg/mg crt) to correct for variations in urine dilution among samples. External AFM_1 standards were prepared weekly and injected following every five injections of samples. LOD for this method is 3 ppt. Recovery from extractions was greater than 85% with a relative standard deviation of less than 5%.

3.2.4 Determination of fumonisin B_1 in urine

Extraction of urinary FB_1 levels followed methods by Robinson (Robinson *et al.* 2012). Urine samples were centrifuged at 887 xg, and 5.0 mL of supernatant was collected and loaded onto a preparative Fumonitest® WB immunoaffinity column (Vicam) at a flow rate of 1mL/min. The column was then washed with 6 mL of PBS, followed by 6 mL H_2O. After the washes were complete, the FB_1 fraction was eluted from the column with 2 mL methanol, dried under a gentle stream of Nitrogen gas, and re-suspected in 1 mL of a 1:1 solution of acetonitrile and water. Detection and quantification was performed using a Waters Acquity H-Class UPLC-MS/MS (Waters Corporation). Separation was achieved using a 2.1 x 50 mm Acquity UPLC BEH C18 column with a particle size of 1.7 µm. Both eluents contained 1% formic acid and were composed of water (eluent A) and ACN (eluent B). After an initial time of 2.69 min at 90% A and 10% B, the proportion of B was increased linearly to 90% within 1.71 min, followed by a hold-time of 1.4 min, then a steep return and column re-equilibration for 1.10 min, and 5 min wash before the next injection. The flow rate was 0.4 ml/min. The column effluent was directly coupled to the MS, which was operated in the positive electrospray ionization mode. MS/MS conditions were optimized for FB_1 as described by Warth *et al.* (2012). The precursor ion was set to 722.5 Da and the two product ions were 334.4 Da (quantifying ion) and 352.2 Da (qualifying ion). Urinary FB_1 concentrations were expressed as pg/mg creatinine (pg/mg crt) to correct for variations in urine dilution among samples. External FB_1 standards were prepared weekly and injected daily. LOD for the method was determined to be 40 ppt.

Recovery from extractions was greater than 85% with a relative standard deviation of <5%.

3.2.5 Statistical analysis

The GLIMMIX procedure was used in SAS University Edition software (SAS Institute, Cary, NS, USA) because the original dependent variables (adjusted toxin levels in urine) did not follow a normal distribution. The lognormal distribution was used to fit the data. The following variables were tested in order to explain the toxin urine levels: Age, sex, the other toxin levels, the frequency of and amounts of traditional Mexican food consumption, corn, and corn products in the food survey. Possible interactions and inclusion of continuous covariates were also analyzed in order to explain the variability in the dependent variables.

Secondary analyses with AFM_1 were done using JMP version 9 software (SAS Institute, Cary, NC, USA). Toxin levels were analyzed as categorical variables and used in chi-square analyses with demographic and dietary survey information. For all comparisons, $p < 0.05$ was considered to be statistically significant.

3.3 Results

Table 6 represents the study population statistics. Males and females were evenly represented in this study. All were of Mexican nationality at an average age of 44 years old (median: 44; range: 18-81 years). The majority of participants were originally from Nuevo Leon (91%), the state in which the study was conducted. Most participants claimed an average yearly income of less than 10,000 Pesos (82.4%). Majority of study participants were on no special diet at the time of recruitment. Of those with children, there was only

Table 6. Demographic characteristics of study participants from Monterrey, Mexico

Characteristic		n	(%)
Sex			
	Male	53	(49.1)
	Female	55	(50.9)
Age			
	18-29	29	(26.9)
	30-39	15	(13.9)
	40-49	17	(15.7)
	50-59	24	(22.2)
	60-69	15	(13.9)
	>70	8	(7.4)
Nationality			
	Mexican	108	(100)
State			
	Coachuila	5	(4.6)
	Nayarit	2	(1.9)
	Nuevo Leon	91	(84.3)
	San Luis Potosi	2	(1.9)
	Tamaulipas	6	(5.6)
	Veracruz	1	(0.9)
	No Answer	1	(0.9)
Income			
	< 10,000	89	(82.4)
	10,000-20,000	14	(13.0)
	20,000-30,000	3	(2.8)
	No Answer	2	(1.9)
Special diet			
	Reduced Sugar and Salt	1	(0.9)
	Reduced Sugar and Salt	4	(3.7)
	Reduced Salt	1	(0.9)
	Reduced Fat	1	(0.9)
	Reduced Carbohydrates	2	(1.9)
	Arthritic Diet	1	(0.9)
	Gluten-Free	1	(0.9)
	No special Diet	97	(89.8)
Children with birth defects			
	Yes	1	(0.9)
	No	104	(96.3)
	No Answer	3	(2.8)

Table 7. Level of urinary AFM_1 and FB_1 in study participants in Mexico independent of co-exposure status

AFM_1 levels (pg/mg creatinine)		**FB_1 levels (pg/mg creatinine)**	
Number Positive	73	Number Positive	76
Mean ± SD	4.29 ± 3.76	Mean ± SD	49.40 ± 51.50
Median	2.82	Median	29.1
Range	0.29-25.98	Range	1.11-248.47
Percentiles		Percentiles	
25	2.19	25	10.4
50	2.82	50	29.1
75	6.72	75	68.13

one report of a child born with a defect and it was not related to the neural tube.

Of the 108 participants recruited, 106 urine samples were available for analysis. Table 7 shows the descriptive statistics for each mycotoxin. Urinary AFM_1 was detectable in 68.9% (73/106) of samples analyzed with the average level of 4.29 pg/mg creatinine (median: 2.82; detectable range: 0.29-25.98 pg/mg creatinine). There is no significant difference in demographics among the participants with detectable levels of AFM_1. Urinary FB_1 was detectable in 71.7% (76/106) of samples analyzed with the average level of 43.40 pg/mg creatinine (median: 29.10; detectable range: 1.11-248.57 pg/mg creatinine). Similarly, there was not a significant difference in demographics among the participants with detectable FB_1. Of those participants with detectable levels of AFM_1 or FB_1, 55.7% were co-exposed to both toxins. When grouped by co-exposure, there were no

differences in the mean and median levels as compared to independent mycotoxin analysis (Table 8). Geographic distribution of co-exposure was significant by χ^2 analysis ($p = 0.03$) with a majority of those co-exposed concentrated in the cities of Guadalupe, Juarez, and San Nicolas (data not shown).

Table 8. Level of urinary AFM_1 and FB_1 in study participants in Mexico by co-exposure status

AFM_1 levels (pg/mg creatinine)			**FB_1 levels (pg/mg creatinine)**		
Number Positive		56	Number Positive		56
Mean ± SD		3.80 ± 2.66	Mean ± SD		45.76 ± 48.95
Median		2.69	Median		24.6
Range		0.29-10.38	Range		1.11-193.98
Percentiles			Percentiles		
	25	1.9		25	9.4
	50	2.69		50	24.6
	75	5.07		75	67.86

A dietary survey was administered to determine dietary factors that may contribute to AF and FB exposure (Table 9). Results from questions on food consumption show that participants consume traditional Mexican food between one and five times a week. Of the corn products questioned in the survey, corn tortillas were the most frequently consumed with 40% of the study population consuming them more than twice a day. No significant

associations were observed between frequency of consumption of the various foods and detection of AFB_1, FB_1, or co-exposure. However, the amount of corn tortilla and rice was significantly associated with detectable level of AFM_1 ($p = 0.044$ and $p = 0.005$, respectively). Detection of FB_1 was positively associated with corn tortilla consumption ($p = 0.06$); 92.11% of participants with detectable levels reported consuming more than two tortillas a day. Consumption quantity of corn ear or canned corn and corn tortillas was statistically significant when categorized by co-exposure status ($p = 0.03$ and $p = 0.01$, respectively). Twice as many participants in the co-exposure group reported consuming more than one ear or cup of corn (canned or fresh) daily. Similarly, 96% of those in the co-exposed group reported eating more than two tortillas a day compared to 78% of participants who did not have detectable levels of AFM_1 and FB_1.

No evidence was found to support the premise that individual toxin urine levels (pg/mg crt) vary according to any dependent variable tested (i.e., sex, food consumption) ($p>0.1$ Tukey's adjusted, data not shown).

3.4 Discussion

Results from this study demonstrate a high prevalence of AFB_1 and FB_1 exposure in participants recruited from Monterrey, Mexico, a metropolitan area of northeast Mexico. Although frequently exposed, LC-MS/MS data revealed relatively low levels of AF exposure. Comparisons with dietary data did not indicate any associations with toxin concentrations. In contrast, other studies have shown associations between food consumption and toxin levels in urine (Gong *et al.*, 2008; Torres *et al.*, 2014; Zhu *et al.*, 1987). Our analyses do not support such associations and this is possibly due to the fact

Table 9. Percent food consumption in Mexico study population by co-exposure status

Variable		ALL n= 106	CO-EXPOSED YES n= 56	NO n=50	p-value
Frequency of consumption:					
Traditional Mexican food					0.3122
	Greater than twice per day	2 (1.9)	2 (3.6)	0 (0.0)	
	Once a day	6 (5.7)	4 (7.1)	2 (4.0)	
	2-5 times a week	38 (35.9)	20 (35.7)	18 (36.0)	
	Once a week	38 (35.9)	22 (39.3)	16 (32.0)	
	Less than once per week	22 (20.8)	8 (14.3)	14 (28.0)	
Ear of corn or canned Corn					0.5705
	Once a day	1 (0.9)	0 (0.0)	1 (0.9)	
	2-5 times a week	13 (12.3)	5 (8.9)	8 (16.0)	
	Once a week	24 (22.6)	15 (26.8)	9 (18.0)	
	Less than once per week	47 (44.3)	24 (42.9)	23 (46.0)	
	Never	20 (18.9)	1 (1.8)	0 (0.0)	
	No answer	1 (0.9)	1 (1.8)	0 (0.0)	
Maize products					0.2701
	Greater than twice per day	5 (4.7)	2 (3.6)	3 (6.0)	
	Once a day	2 (1.9)	2 (3.6)	0 (0.0)	
	2-5 times a week	20 (18.9)	9 (16.1)	11 (22.0)	
	Once a week	37 (34.9)	17 (30.4)	20 (40.0)	
	Less than once per week	37 (34.9)	24 (42.9)	13 (26.0)	
	Never	4 (3.8)	1 (1.8)	3 (6.0)	
	No answer	1 (0.9)	1 (1.8)	0 (0.0)	

Table 9. Continued

Variable		ALL n= 106	CO-EXPOSED YES n= 56	NO n=50	p-value
Frequency of consumption:					
Corn tortilla					0.79
	Greater than twice per day	43 (40.6)	23 (41.1)	20 (40.0)	
	Once a day	40 (37.7)	20 (35.7)	20 (40.0)	
	2-5 times a week	18 (17.0)	11 (19.6)	7 (14.0)	
	Once a week	3 (2.8)	1 (1.8)	2 (4.0)	
	Less than once per week	1 (0.9)	1 (1.8)	0 (0.0)	
Flour tortilla					0.24
	Greater than twice per day	7 (6.6)	2 (3.6)	5 (10.0)	
	Once a day	16 (15.1)	11 (19.6)	5 (10.0)	
	2-5 times a week	27 (25.5)	13 (23.2)	14 (28.0)	
	Once a week	24 (22.6)	16 (28.6)	8 (16.0)	
	Less than once per week	18 (17.0)	8 (14.3)	10 (20.0)	
	Never	13 (12.3)	5 (8.9)	8 (16.0)	
Rice					0.14
	Greater than twice per day	5 (4.7)	2 (3.6)	3 (6.0)	
	Once a day	15 (14.1)	12 (21.4)	3 (6.0)	
	2-5 times a week	50 (47.2)	25 (44.6)	25 (50.0)	
	Once a week	20 (18.9)	10 (17.9)	10 (17.9)	
	Less than once per week	13 (12.3)	7 (12.5)	6 (12.0)	
	Never	3 (2.8)	0 (0.0)	3 (6.0)	

Table 9. Continued

Variable		ALL n= 106	CO-EXPOSED YES n= 56	 NO n=50	p-value
Frequency of consumption:					
Peanut butter					0.74
	Once a day	2 (1.9)	0 (0.0)	2 (4.0)	
	2-5 times a week	8 (7.6)	3 (5.4)	5 (10.0)	
	Once a week	9 (8.5)	5 (8.9)	4 (8.0)	
	Less than once a week	24 (22.6)	13 (23.2)	11 (22.0)	
	Never	60 (56.6)	33 (58.9)	27 (54.0)	
	No answer	3 (2.8)	2 (3.6)	1 (2.0)	
Nuts					0.09
	Once a day	6 (5.7)	1 (1.8)	5 (10.0)	
	2-5 times a week	23 (21.7)	10 (17.9)	13 (26.0)	
	Once a week	31 (29.3)	21 (37.5)	10 (20.0)	
	Less than once a week	31 (29.3)	14 (25.0)	17 (34.0)	
	Never	14 (13.2)	9 (16.1)	5 (10)	
	No answer	1 (0.9)	1 (1.8)	0 (0.0)	
Corn chips					0.72
	Once a day	9 (8.5)	6 (10.7)	3 (6.0)	
	2-5 times a week	17 (16.0)	8 (14.3)	9 (18.0)	
	Once a week	25 (23.6)	15 (26.8)	10 (20.0)	
	Less than once a week	28 (26.4)	15 (26.8)	13 (26.0)	
	Never	27 (25.5)	12 (21.4)	15 (30.00)	

Table 9. Continued

Variable		ALL n= 106	CO-EXPOSED YES n= 56	NO n=50	p-value
Quantity consumed:					
Ear of corn or canned corn					0.03*
	More than 1 ear or cup	23 (21.7)	16 (28.6)	7 (14.0)	
	1 ear or 1/2-1 cup	38 (35.9)	15 (26.8)	23 (46.0)	
	Less than 1 ear or 1/2-1 cup	38 (35.9)	19 (33.9)	19 (38.0)	
	Doesn't know	5 (4.7)	5 (8.9)	0 (0.0)	
	No answer	2 (1.9)	1 (1.8)	1 (2.0)	
Maize products					0.32
	Greater than or equal to 1 cup	52 (49.1)	30 (53.6)	22 (44.0)	
	1-1/2 cup	19 (17.9)	10 (17.9)	9 (18.0)	
	Less than 1-1/2 cup	29 (27.4)	12 (21.4)	17 (34.0)	
	Doesn't know	5 (4.7)	4 (7.1)	1 (2.0)	
	No answer	1 (0.9)	0 (0.0)	1 (2.0)	
Corn tortilla					0.01*
	More than 2	93 (87.7)	54 (96.4)	39 (78.0)	
	1-2	10 (9.4)	2 (3.6)	8 (16.0)	
	Less than 1	2 (1.9)	0 (0.0)	2 (4.0)	
	Doesn't know	1 (0.9)	0 (0.0)	1 (2.0)	
Flour tortilla					0.22
	More than 2	69 (65.1)	41 (73.2)	28 (56.0)	
	1-2	9 (8.5)	5 (8.9)	4 (8.0)	
	Less than 1	23 (21.7)	9 (16.1)	14 (28.0)	
	Doesn't know	2 (1.9)	0 (0.0)	2 (4.0)	
	No answer	3 (2.8)	1 (1.8)	2 (4.0)	

* $p \leq 0.05$ in comparison of distribution in co-exposed and not co-exposed groups in Fisher exact test

that an individual's levels of toxin in urine are unique and the time of year when samples were collected could reflect different exposure levels.

A dietary survey could be an inadequate method to make conclusions on toxin exposure and dietary habits in a population with a high frequency of maize consumption and low toxin exposure levels; significance can be masked. Previous studies suggest that 24 hr recall is the best method for dietary assessment in low-income households, followed by weighted inventory, food checklist, and semi-weighted method (Holmes and Nelson, 2009). However, when exposure is treated as a categorical variable (detectable vs non-detectable; co-exposed or not co-exposed), results from the current study demonstrate an association between the quantity of corn and corn tortillas consumed and co-exposure or detectable toxin status (Table 9). In a study conducted in a primarily Hispanic population of San Antonio, TX, similar results were reported with AFM_1 exposure (detectable v. non-detectable) and dietary survey data (Johnson *et al.*, 2010). Future investigations could be improved by matching 24 hr dietary data with short-term biomarkers such as AFM_1 and FB_1. In addition, co-exposed participants appear to be concentrated in the cities of Guadalupe, Juarez, and San Nicolas and the product quality in those areas and product origin should be determined.

In the course of this study, much of Mexico was experiencing moderate to high levels of precipitation (Hydroclimate Research Lab, 2016). Drought and high ambient temperatures during certain stages of maize growth have been identified as the environmental conditions most conducive to AF contamination in maize (Lisker and Lillehoj, 1991; Vincelli *et al.*, 1995). Moreover, significantly higher biomarker levels of

AF have been observed during dry season in the Gambia than in the rainy season (Turner *et al.*, 2000; Wild *et al.*, 2000). This could be one explanation for the overall low levels of AF exposure as reflected by the urinary biomarkers results reported in the current study as compared to other high-risk areas such as Ghana (Jolly *et al.*, 2006). Therefore, the sampling from February to April may not represent the highest risk associated with mycotoxins for Northern Mexico.

The ratios of AFM_1 and FB_1 levels are comparable to previous reports supporting a higher frequency of FB exposure. This could be a reflection of competition in the field between the mycotoxigenic fungi. Marin *et al.* reported that the activity of *Fusarium moniliforme* and *F. proliferatum* in grain reduced the presence of *Aspergillus flavus*, *A. niger*, and *A. ochraveus*, particularly at 15°C and higher water availabilities (Marin *et al.*, 1998b). In general, *F. moniliforme* and *F. proliferatrum* are very competitive and dominant against *A. flavus* (Marin *et al.*, 1998a). Although an overlap of niches between the fungi exist and is influenced by both temperature and water availability, the mechanism of how this overlap affects mycotoxin production is unknown.

When discussing AF and FB exposure, the idea of mixtures and modulation of metabolism should be addressed. In a co-exposure rat study with AFB_1 and FB_1, lower levels of AFM_1 were reported when rats were dosed with both toxins as compared to the AFB_1 or FB_1 exposure groups (Mitchell et al., 2014b). The highest average AFM_1 concentration in animals given the AFB_1/FB_1 mixture was approximately two times lower than the average concentration in animals dosed with AFB_1. Considerable variation was observed in the overall excretion of AFM_1 in this treatment group. The authors suggest

that AFB_1 and FB_1 could be altering each other's absorption from the gut since FB_1 levels were unchanged. While AFM_1 levels were reduced, the blood AFB_1-alb biomarker was increased in the presence of FB_1. This could be a result of modulation in the AF metabolic pathway by FB_1 to favor the formation of the albumin adduct and as a result, reducing levels of AFM_1 excreted in the urine. More studies are warranted to investigate the consequence of this toxin combination in the diet and the implication for biomarkers of exposure. Although AFM_1 concentrations remain consistent regardless of co-exposure status in the current study, measurements of toxin concentrations in foods is necessary for any conclusion regarding metabolism modulation to be made in this scenario.

Unlike sub-Saharan Africa where deaths from aflatoxicosis have occurred, none have been reported in Mexico despite the daily consumption of maize and maize products. A cooking process that is practiced in Latin America, including Mexico, is the inclusion of an alkaline-lime treatment to corn products. Termed nixtamalization, maize is cooked and steeped in alkaline water, separated from the water, rinsed, then further processed to make masa flour, tortillas, fried chips, and other foods (Torres *et al.*, 2015). This process has been demonstrated to effectively reduce FB concentrations in maize foods (Palencia *et al.*, 2003) and reduce toxicity in animal models (Burns *et al.*, 2008; Voss *et al.*, 2013; Voss *et al.*, 2009). However, reductions are incomplete. Results from the current data support the findings of incomplete FB_1 reductions via nixtamalization since a high percentage of FB_1 was detected in participant urine. At the same time, the consumption of non-alkalized maize must be considered. Future surveys in this region should include questions on the employment of nixtamalization in food preparation.

Protection from AF-toxicity by nixtamalization in animal models is unclear. The breakdown of AFB_1 during nixtamalization is attributed to the hydrolytic opening of the lactone ring, thus increasing solubility and allowing for extraction into the cooking-liquid, which is typically poured off. However, under acidic conditions, such as the low pH in the stomach, this open structure can revert to the original configuration and undergo normal metabolism once becoming bioavailable. Some studies have indicated protection from masa (alkaline-processed maize). For example, chickens fed contaminated masa (AFB_1= 260 μg over five days) survived and showed no obvious differences from the controls other than poor plumage. The contaminated maize-fed group died after five days (Anguiano-Ruvalcaba *et al.*, 2005). Juvenile Wistar rats that were fed with tortillas prepared from nixtamalized-contaminated-maize exhibited decreased weight gain and food consumption compared to control; negative control juveniles died within two weeks (Torres *et al.*, 2015). Comparisons of HCC burden and nixtamalization have also been made; HCC burden can be much higher in parts of Africa and Asia where nixtamalization is not practiced (Liu and Wu, 2010). However, it is important to factor HBV prevalence in such associations. HBV is more prevalent in the high AF exposure regions/countries in Africa compared to Latin America (Liu and Wu, 2010). In contrast to the observations made by Liu and Wu (2010), Latin American countries with the highest incidence of HCC, cirrhosis and chronic liver disease such as Mexico are also the countries that are most likely to consume nixtamalized maize-based foods (Torres *et al.*, 2015). In conclusion, nixtamalization appears to provide some reduction to the toxic effects of AF and FB, but the extent of the protection to public health is unclear and warrants further investigation.

The current study is the first report of urinary AFM_1 levels in a Mexican population. The only previous report of AF biomarker exposure was of the AF-albumin adduct, which represents long-term exposure. FB_1 levels unadjusted for creatinine (data not shown) reported here were similar to those by Gong (2008) in Mexico and by Torres (2014a) et al. in Guatemala. Torres *et al.* (2015) measured AFB_1 levels in maize from Guatemala with 78% < 20 ppb, but also detected the toxin in levels known to be harmful to animals and humans. Using Mexico and Guatemala as proxies, evidence suggests that much of Latin America is exposed to both AFs and FBs with a high risk of co-exposure. Implications of co-exposure for human health are numerous, but one aspect of particular concern is the potential of FB_1 to modulate AFB_1 hepatotoxicity and/or hepatocarcinogenicity. Recently, Goss et al. stated that the incidence of HCC in Guatemala and Mexico is attributed to high rates of chronic viral hepatitis, alcohol use, and AF exposure (2013). It is hypothesized that HCC cancer risk is increased by AF/HBV interaction through mechanisms of chronic inflammation and cell proliferation (Groopman and Kensler, 2005). It is not unlikely for FB to synergize the cell proliferation process due to its ability to alter the balance of biochemical mediators of cell death and survival in target tissues through the inhibition of ceramide synthase (Bulder *et al.*, 2012). However, this interaction has not been definitively demonstrated. Therefore, based on the variable toxin exposures in this region of Northern Mexico and the implication for AF and FB in HCC development, more data on the risk of mycotoxins to public health is warranted.

4. EFFICACY OF DIETARY INTERVENTION DELIVERED IN WATER TO REDUCE EXPOSURE TO AFLATOXIN IN EASTERN KENYA

4.1 Introduction

Acute exposure to AFs can lead to aflatoxicosis and liver failure (Wild & Gong 2010), with documented fatality rates in Kenya as high as 40% (Centers for Disease Control and Prevention, 2004). In Kenya, the first reported aflatoxicosis outbreak occurred in 1981 (Ngindu, 1982). In the period from 2004 to 2014, subsequent outbreaks have affected nearly 600 individuals and caused 211 deaths (personal communication with Kenya Ministry of Health).

There is a need to implement and evaluate evidence-based interventions in Kenya to decrease AF exposure and subsequently avert adverse health effects, particularly in regions with recurring outbreaks. One possible approach to prevent illness associated with acute AF exposure is through the use of processed calcium montmorillonite clay (ACCS100). ACCS100 is produced from Hydrated Sodium Calcium Aluminosilicate, a clay composition that is generally recognized as safe by the U.S. Food and Drug Administration (U.S. Federal Drug Agency). ACCS100 clay can be included in the diet to tightly adsorb AFs in the gastrointestinal tract, leading to decreased bioavailability and prevention of toxin-induced disease. High affinity binding of AFs to clay (Grant and Phillips, 1998; Phillips, *et al.*, 2002), reduction of AF bioavailability, prevention of aflatoxicosis, and safety at doses as high as 2% clay in the diet have been well-documented in animal studies (Afriyie-Gyawu *et al.*, 2005; Harvey *et al.*, 1993; Harvey *et al.*, 1991a; Mayura *et al.*, 1998; Phillips, 1999; Phillips *et al.*, 1988; Phillips *et al.*, 2002; Phillips

et al., 1995). Following successful results in animal models, a two-week study was carried out in 50 adults in the U.S. (Wang *et al.*, 2005). Following demonstration of safety, trials were moved to Ghana where a three month phase IIa clinical trial in adults, a two-week crossover trial in adults, and a two-week safety study in children indicated that the clay was safe for human consumption at levels as high as 0.5% in the diet and deemed to be efficacious (40-58% reduction in serum AFB_1-albumin adduct and urine AFM_1 biomarkers of exposure) (Afriyie-Gyawu *et al.*, 2008a; Mitchell *et al.*, 2014a; Mitchell *et al.*, 2013; Wang *et al.*, 2008). No significant differences in hematology, liver and kidney function, electrolytes, or minerals were evident between placebo and active treatment groups. Furthermore, ACCS100 did not affect blood levels of micro- and macro-nutrients, such as vitamins A and E (Afriyie-Gyawu *et al.*, 2008).

Although long- and short-term biomarkers of AF exposure were both utilized in previous intervention trials with CM clay conducted in Ghana, Mitchell et al. was the first to report a clinical trial supported only by AFM_1 biomarker data (Mitchell *et al.*, 2013). AFM_1 in urine reflects recent AFB_1 exposure (Zhu *et al.*, 1987) and can be easily collected. Use of a urinary biomarker is desirable, especially for use in shorter pilot trials in children and other vulnerable groups, where appropriate dosimetry has not yet been defined. This approach was applied in a crossover study in a region of Ghana with historically high levels of AF exposure. Data showed that a refined CM clay (UPSN) reduced AFM_1 biomarkers by 55% compared to the placebo in as little as five days. Furthermore, Mitchell and colleagues demonstrated that urinary AFM_1 levels can be used as a biomarker of internal AFB_1 exposure in short-term intervention trials to determine efficacy. This finding

was followed-up by a short-term safety and efficacy trial in children from the same region (Mitchell *et al.*, 2014a). Healthy children ages 6-9 with parental consent, ingested 0.75 or 1.5 g UPSN or Placebo for 14 days. Urine samples were collected at baseline, halfway through the study (day 7), and the morning after the final dose (day 15). A significant reduction in median AFM_1 was observed in the high-dose group by 52% compared to placebo. Furthermore, these studies demonstrate that utilization of the AFM_1 biomarker to prove efficacy could significantly decrease the time participants need to be treated with an investigational therapy and also to lower overall invasiveness of such intervention trials. Based on the results by Mitchell *et al.* in 2013 and 2014, detection of urinary AFM_1 could facilitate rapid surveillance of aflatoxicosis outbreaks and rapid identification of effective strategies to mitigate aflatoxicosis.

Despite the apparent effectiveness of CM, there is a need to establish feasibility and efficacy in Kenya—a country that has a well-documented history of aflatoxicosis outbreaks. There are differences between Ghana and Kenya (e.g., different cultures, ethnicities, diets, institutional policies, infrastructure, etc.) that could affect the efficacy, palatability, and acceptability of CM. Thus, our objectives were to assess these qualities of ACCS100, a CM clay, in a population with a known history of aflatoxicosis outbreaks in the Eastern Province of Kenya.

4.2 Materials and methods

4.2.1 Materials

AFM_1 was obtained from Sigma Aldrich (St. Louis, MO, USA). AflaTestWB columns were purchased from VICAM (Watertown, MA, USA). All solvents were

purchased from Fischer and were of the highest grade. Ultrapure deionized water (18.2 MΩ cm) was used in all extractions. ACCS100 and calcium carbonate were purchased from Texas Enterosorbents, Inc. (Bastrop, TX). All materials designated for human consumption were treated with electron beam radiation prior to study initiation.

4.2.2 Aflatoxin B_1 sorption analysis with ACCS100

Isothermal analyses of AFB_1 sorption onto surfaces of ACCS100 at equilibrium were performed according to methods reported by Grant and Phillips (1998) and described in detail by (Marroquin-Cardona *et al.*, 2009). ACCS100 clay or calcium carbonate (50 ng) were mixed with 11 different concentrations of AFB_1, all done in triplicate, for 2 h at a pH 6.5. Samples were then centrifuged and the absorbance read at 362 nm using a Shimadzu scanning UV visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Computer-generated equilibrium isotherms were fit to the Langmuir model (based on r^2 values and randomness of the residuals). The parameters of Q_{max} and K_d were estimated to determine the maximum sorption to the surface and the affinity of the sorption interaction. Isothermal analyses of the placebo material, calcium carbonate, indicated that it was a very weak sorbent of AFB_1 at pH 2 and 6.5, with 13% and 5% bound, respectively (Mitchell *et al.*, 2013). As the concentration of AFB_1 increased, the percent bound to carbonate became negligible at pH 2, suggesting that calcium carbonate dissolves under acidic conditions.

4.2.3 Study design and procedures

The study protocols (ID 2603, 6535.0 and 2013-0311F) were approved by the Institutional Review Boards at Kenya Medical Research Institute (KEMRI), Centers for

Disease Control and Prevention (CDC) and Texas A&M University, respectively. The study was conducted in July 2014 through August 2014. A double-blinded, crossover clinical trial was employed in which 50 participants were randomly assigned to Group I or Group II (Figure 12). Group I participants ingested 1 g calcium carbonate placebo in water three times per day for seven days, then entered a 5-day washout period during which no test material was consumed. Following the washout period, Group I participants ingested 1 g of ACCS100 mixed in water three times per day for the last seven days. Group II participants followed the same schedule, except they began with ACCS100 first and finished with placebo.

Texas EnteroSorbents Inc. provided ACCS100 and placebo in 1 g dose foil sachets packaged for individual use. Confectioners sugar was added to each treatment to enhance the taste. We asked participants to consume 3 g of ACCS100 or placebo per day during the study arms. We also instructed them to consume one sachet with each of their three main meals by diluting the sachet in water provided by the study. The sachets were coloured as pink or green to designate the content (ACCS100 or placebo). Each day during the study, we provided each participant with a sterile urine cup, three treatment sachets, and three 500-mL bottles of clean water

Trial efficacy was assessed by comparing urinary AFM_1 while on ACCS100 or placebo treatment to urinary AFM_1 at baseline. Participants provided a first morning void urine sample at baseline, and again for each of the seven days of each treatment arm. Urine samples were collected, aliquoted and frozen each morning, then kept frozen until laboratory analysis.

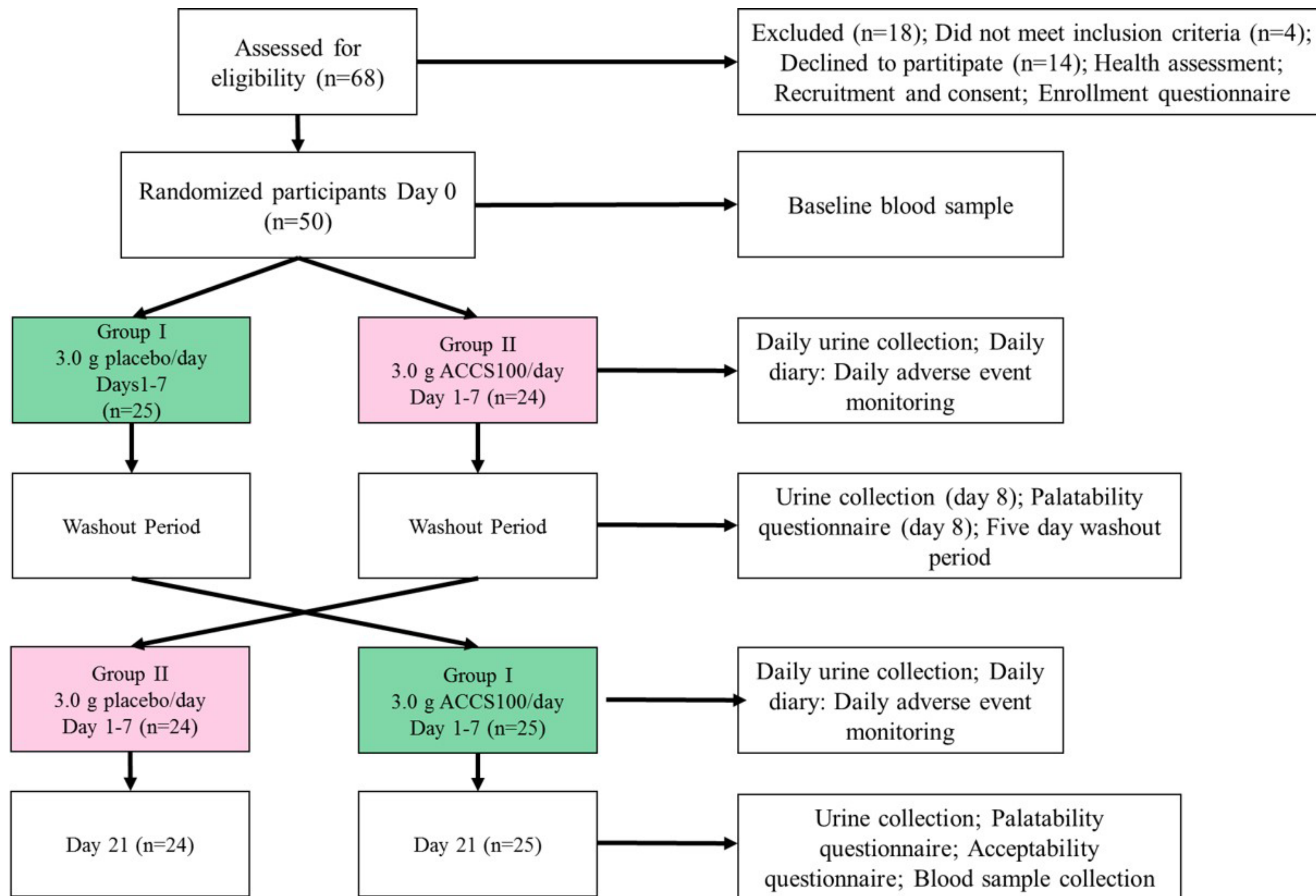

Figure 12. Overall study design and participant flow for ACCS100 crossover trial

4.2.4 Study population and enrollment

The study was implemented in the Kalimani and Kamboo villages of Kamboo sub location in Makueni County, Kenya. The region is predominately agricultural and composed of subsistence farming, where the residents maintain a maize-based diet. These villages were chosen because they had experienced multiple aflatoxicosis outbreaks within the past decade.

Field teams employed convenience sampling and went door-to-door to recruit participants, enrolling one adult per household. Inclusion criteria consisted of the following: 1) aged ≥18 years; 2) consumed maize and/or groundnuts at least four times per week; and 3) agreed to participate. Once a participant was identified, written informed consent was obtained. Consent forms were supplied in the local language (Akamba) as well as the national language (Kiswahili). Participants could decline to participate in any part of the study and were free to withdraw at any point. Upon completion, participants were reimbursed with cooking items worth approximately 400 Kenya shillings (4 U.S. dollars).

After providing consent, each person's medical history, height, and weight was recorded and their current health status assessed. Urine glucose and protein levels were tested using Chemistrip®2 GP from Roche Diagnostics (Indianapolis, IN, USA). Pregnancy was tested using Sure-Vue® Urine hCG strips (Fisher Healthcare, Pittsburgh, PA, USA). Participants who were pregnant or who had a history of thyroid disease, heart disease, lung disease, kidney disease, gastrointestinal disease, or diabetes were excluded. Participants who were not excluded were randomized into the study. An enrollment survey

was administered that included questions regarding duration of residency in the village, source of maize, and the shelf life of their maize stock.

4.2.5 Palatability

Palatability was assessed by administering a questionnaire after each arm that asked participants to rate the taste, aftertaste, smell, texture, appearance, and color of the sachet contents they had been consuming using a 5-point Likert scale (1 = really bad, 5 = really good).

4.2.6 Adherence and acceptability

Study monitors completed a worksheet each day during the study arms to record participant adherence to the study protocol (i.e., daily use of ACCS100 or placebo), the occurrence of side effects, and diet. Additional information was collected regarding the side effect severity (i.e., mild, moderate, or severe), time of day the effect occurred (i.e., AM, Noon, or PM), and whether or not the participant sought treatment. We assessed acceptance by administering a questionnaire at the end of the study to collect the participant's perceptions of ACCS100, information sources regarding AF, and whether they would find ACCS100 to be acceptable as a future intervention to reduce AF exposure.

4.2.7 Determination of urinary aflatoxin M_1 and serum aflatoxin B_1-lysine adduct level

Analysis of urinary AFM_1 levels followed methods by Groopman *et al*. (1992) and the modifications of Sarr (1995) and Wang (1999). Urine samples were centrifuged at 887 xg, and 5.0 mL of supernatant was collected, acidified with 0.5 mL of 1.0 M ammonium formate (pH 4.5) and diluted with water to a total volume of 10.0 ml. Samples were then loaded onto a 3 mL preparative Aflatest® WB immunoaffinity column

(VICAM, Watertown, MA, USA) at a flow rate of 1 mL/min. Following washing of the column, the AF fraction was eluted from the column with 2 ml of 80% methanol, dried under N^2 and re-suspended in 200 µL of a 1:1 solution of methanol:20mM ammonium formate. Samples were analyzed using a Shimadzu HPLC system (Waters, Watertown, MA, USA) with fluorescence detection capabilities. A 50 x 4.6 mm Luna C-18 column with pore size 100 Å and particle size 5 µm (Phenomenex, Torrance, CA USA) was used to resolve AF metabolites. The mobile phase consisted of 22% ethanol buffered with 20 mM ammonium formate (pH 3.0) in water. Isocratic elution of the mobile phase for 20 min at a rate of 1 ml/min allowed for proper chromatographic separation. External AFM_1 standards were prepared weekly and injected following every 5 injections of samples. The limit of detection for this method was 1.2 pg for AFM_1.

Aliquots of random samples were collected for additional verification using a Waters Acquity H-Class UPLC-MS/MS (Waters Corporation, Milford, MA, USA). Separation was achieved using a 2.1 x 50 mm Acquity UPLC BEH C18 column with a particle size of 1.7 µm. Isocratic separation occurred with 70% water buffered with 1% formic acid and 30% ACN buffered with 1% formic acid. Samples (10 µL) were injected onto the column and the elution rate was 0.325 ml/min. The column effluent was directly coupled to the MS, which was operated in the positive electrospray ionization mode. MS/MS conditions were optimized for AFM_1 and based on Warth *et al.* (2012). The precursor ion was set to 329.00 Da and the two product ions were 273.00 Da (quantifying ion) and 259.1 Da (qualifying ion).

Urinary AFM_1 concentrations were expressed as pg/mg creatinine to correct for variations in urine dilution among samples. Creatinine concentrations were measured at Baylor Scott & White Hospital (Temple, TX, USA).

Previous measurements of AF exposure in Kenya have been based on the serum AFB_1-lys, a biomarker for long-term AF exposure. For this study, two blood samples were also collected from each participant at baseline (Day 0) and completion (Day 21) of the trial for AFB_1-lys analysis. This allowed for comparisons of AF exposure to levels recorded during previous outbreaks. CDC's National Center for Environmental Health Division of Laboratory Sciences analyzed serum specimens for AFB_1-lys adduct, which consisted of two measurements: 1) analysis of AFB_1-lys by LC-MS/MS (McCoy *et al.*, 2005); and 2) albumin measurement. To allow the release of AFB_1-lys from albumin, protein in serum specimens was digested in the presence of stable-isotopically labeled internal standard ($^2H_4$-AFB_1-lys) for at least 15 hr at 37 °C by use of a commercially available mixture of proteinases (Pronase™). AFB_1-lys and $^2H_4$-AFB_1-lys were then extracted by use of mixed-mode anion exchange reversed-phase solid-phase extraction. Each solid-phase extraction eluate was evaporated, reconstituted in mobile phase, and injected onto a reversed-phase C_{18} column. AFB_1-lys was chromatographically separated from other compounds using gradient mobile phase. Both AFB_1-lys and $^2H_4$-AFB_1-lys were detected with positive electrospray ionization (ESI) in selective reaction monitoring mode using tandem quadruple mass spectrometry (McCoy *et al.*, 2005). Quantitation was based on peak area ratios interpolated against a seven-point aqueous linear calibration curve with 1/x weighting. The limit of detection (LOD) for AFB_1-lys was 0.02 ng/mL.

Serum albumin was analyzed on the Hitachi Modular P clinical analyzer using the Roche® colorimetric assay. The LOD for albumin was 0.2 g/dL.

4.2.8 Statistical analysis

Data were entered into Epi Info™ 7 (CDC, Atlanta, GA, USA) and analyzed using SAS Enterprise Guide version 4.3 (SAS Institute, Cary, NC, USA) or JMP version 9 software (SAS Institute, Cary, NC, USA). All statistical analyses were blinded. For urinary AFM_1 and serum AFB_1-lys, levels below the LOD were substituted with LOD divided by two. Urinary AFM_1 and serum AFB_1-lys levels were not normally distributed.

AFM_1 levels were analyzed under nonparametric conditions (Kruskal-Wallis) and parametric conditions (ANOVA) following a log transformation of the data. Both parametric and nonparametric analyses were used to compare groups by days and by treatment arms. A p-value <0.05 (two-tailed) was considered significant. Statistical significance was not changed between parametric and nonparametric testing. Data was analyzed with participants acting as their own controls over two different time periods and with AFM_1 levels being compared between participants during a common time period. Data was also grouped by treatment for days 1-5 and grouped separately for days 8-12 and analyzed by ANOVA.

Serum AFB_1-lys levels were compared before and after the study using a paired t-test of the log-transformed data. To compare the palatability data, a Wilcoxon rank sum test was performed to account for the matched design. For all comparisons, $p < 0.05$ was considered to be statistically significant.

Questionnaire data was analyzed categorically with a chi-square test by treatments. Tea was chosen for additional analyses due to its frequency of consumption in this region and possible modulating effects of polyphenols naturally present in tea. Baseline levels of log transformed AFM_1 in Arm 1 and Arm 2 were compared against the report of tea or not in the dietary survey for that day and analyzed by ANOVA.

4.3 Results

4.3.1 Aflatoxin B_1 sorption analysis with ACCS100

The parameters of Q_{max} and K_d were derived for sorption of AFB_1 at pH 6.5 onto ACCS100. Isotherms are run at both a pH of 6.5 to simulate conditions the clay would encounter in the intestine. The sorption of AFB_1 onto surfaces of ACCS100 fit the Langmuir model ($r^2 \geq 0.92$) with an L-shape pattern indicating saturable binding at sites similar to those shown on parent NS clay (Figure 13). As previously reported by Marroquin-Cardona *et al.* (2011) the theoretical Q_{max} values calculated for UPSN, or ACCS100, were 0.44 ± 0.05 mol AFB_1 kg^{-1} at pH 2 and 6.5, respectively (Marroquin-Cardona *et al.*, 2011). At a pH of 6.5, ACCS100 demonstrated a Q_{max} of 0.524 indicating a high binding capacity with an affinity of $13x10^5$ (K_d). This demonstrates that the sugar added to ACCS100 for taste enhancement did not interfere with the binding of AFB_1.

4.3.2 Study population and demographics

In order to enroll our study population of 50 participants, a total of 68 potential participants were assessed. Eighteen participants were not enrolled (Figure 12). Study retention for randomized participants was 98%. One male participant dropped out on day three after being randomized and completing two treatment sessions. Thus, we included

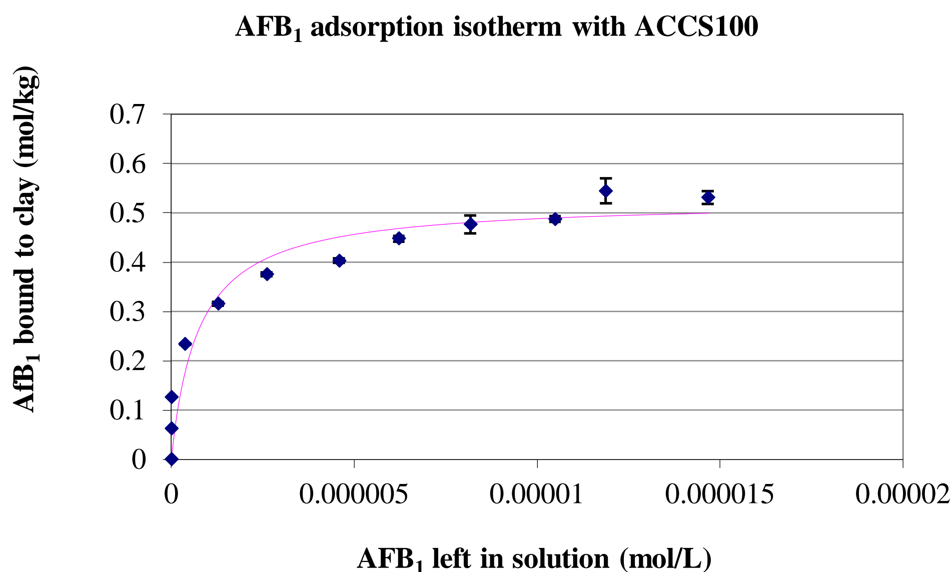

Figure 13. AFB_1 isotherm onto ACCS100 at pH 6.5. ACCS100 is depicted in a L-shape curve characteristic of a saturation of binding sites by AFB_1 in a planar configuration (Marroquin-Cardona *et al*. 2011).

49 participants in our statistical analyses. The majority of participants were female (n=36, 72%) (Table 10). Participants ranged in age from 21 to 75 years (Mean=39.3 years). Participant sex, age, weight, height, and amount of time living in the village did not differ by group.

4.3.3 Compliance

The majority of participants consumed all 21 sachets during both the ACCS100 (n=45) and the placebo (n=44) treatment. The majority (n=46) of participants always ingested the sachet with water; four individuals each reported consumption of a sachet without water once throughout the study (but with food). There were 23 participants who consumed a sachet without food; this occurred between one and three times per person.

Table 10. Kenya study population demographics, by group

Demographic	Overall	Group I (Placebo then ACCS100)	Group II (ACCS100 then Placebo)
Sex (%)			
n	50	25	25
Female	36 (72%)	17 (68%)	19 (76%)
Male	14 (28%)	8 (32%)	6 (24%)
Age (in years)			
n	48	25	23
Range	21–75	21–68	21–75
Mean	39.3	40.6	37.9
StdDev	12.3	12.8	11.9
Weight (kg)			
n	47	23	24
Range	40.0–94.5	41.5–88.0	40.0–94.5
Mean	58.4	61.8	55.1
StdDev	7.8	12.7	10.8
Height (cm)			
n	47	23	24
Range	142.2–185.4	151.0–185.4	142.2–173.5
Mean	161.6	163	160.2
StdDev	7.8	8.1	7.5
Duration of stay in the village			
n	50	25	25
Range	1–55	4.0–55.0	1.0–51.0
Mean	20	22.5	17.4
StdDev	15.2	16	14.3

4.3.4 Analysis of urinary aflatoxin M_1 and serum aflatoxin B_1-lysine levels

All 49 participants contributed data to both arms of the study and thus were included in the efficacy analyses. Participants provided 784 (98%) of the intended 800 urine samples. Overall, 48% of samples contained detectable levels of urinary AFM_1

(Range: <LOD–1986 pg/mg crt). Participants consumed an average of 1.9 maize-containing meals per day; this did not differ by treatment (Placebo, 1.8 maize-containing meals per day; ACCS100, 1.9 maize-containing meals per day). Thus, the number of maize-containing meals as a confounder was not assessed. Baseline urinary AFM_1 levels did not vary statistically by treatment. Furthermore, there was little correlation in an individual's urinary AFM_1 levels over time, either when comparing the baseline of Arm 1 to the baseline of Arm 2, or when comparing Day 1 to Day 2. Thus, we did not include baseline urinary AFM_1 levels in the efficacy analyses.

Interestingly, a significant difference was observed when baseline AFM_1 levels were categorized by consumption of tea that day (Figure 14). Pooled AFM_1 baseline levels

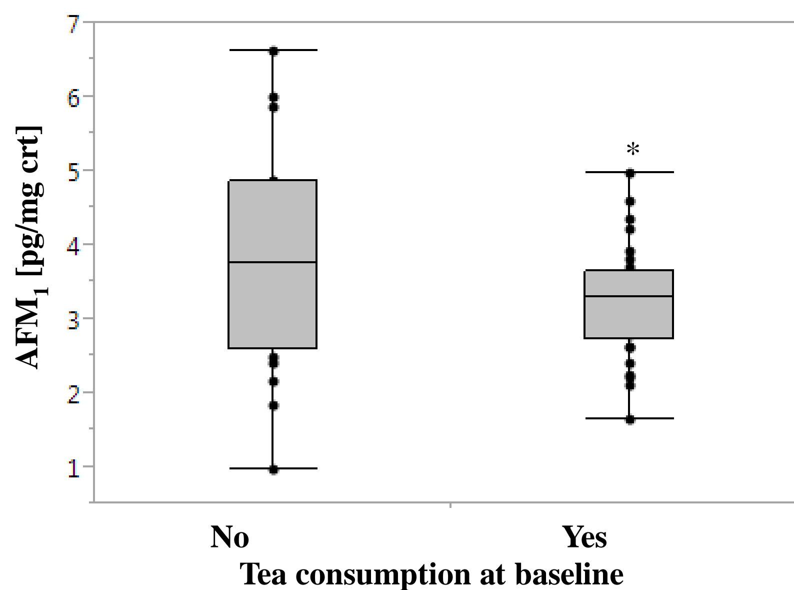

Figure 14. AFM_1 excretion by tea consumption. Baseline mean AFM1 levels based on report of tea consumption in daily diary. *p = 0.05

revealed lower concentrations of the metabolite in individuals who had consumed tea. No significant differences in tea consumption between treatment groups or study arms were observed and therefore not included as a confounder in trial efficacy.

Average AFM_1 levels from days 2-8 during both study arms were significantly lower in participants on ACCS100 when compared to placebo within groups and treatment arms (Figure 15). Figure 16 shows daily median urinary AFM_1 over the full 14 days of treatment and demonstrates the crossover in treatments for the groups with a switch in AFM_1 levels occurring by the 15th treatment day of the study. Comparisons between the ACCS100 treated groups and placebo groups were conducted for each time point by a one-way analysis. Significance was achieved in arm 1 by day 8 (day 7 on treatment) by nonparametric analysis with lower AFM_1 levels in Group 2 (ACCS100) than Group 1 (Placebo) ($p = 0.0386$). By day 2 on treatment in the second arm, Group 1 (ACCS100) had significantly lower levels of AFM_1 than Group 2 ($p = 0.0235$). This trend was maintained throughout arm 2, with significance achieved on day 18 ($p = 0.0112$) (Figure 16). Pooled AFM_1 levels by treatment group revealed a 46.6% reduction in AFM_1 excretion.

Thirty-nine participants provided serum data at both time points and thus were included in this analysis. Serum levels exhibited a statistically significant decrease from Day 0 (GM: 12 pg/mg albumin, 95% CI: 8.7–16 pg/mg albumin) to Day 20 (GM: 5.4 pg/mg albumin, 95% CI: 3.9–7.4 pg/mg albumin). The difference remained statistically significant when analyzed by treatment (data not shown) and resulted in a 55% reduction of AFB_1-levels in the ACCS100 group.

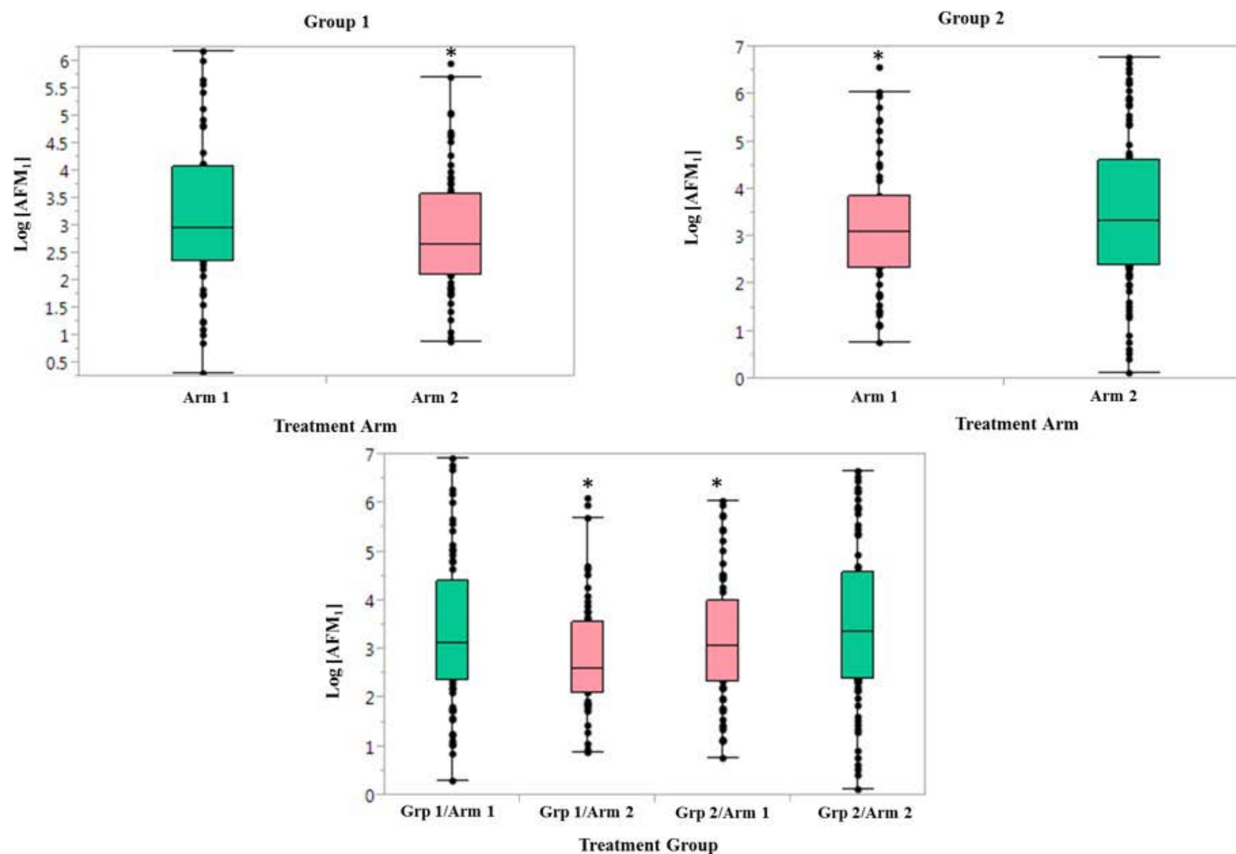

Figure 15. AFM_1 distribution within groups and treatment arms. The box values ranges from 22 to 75 percentiles of the total samples, the line within it indicated the median value. The bars on both sides of a box represent values ranging from 5 to 25 and from 75 to 95 percentiles, respectively. A) Comparison of ACCS100 and placebo treatment with the same group. Analysis of the data in this manner allowed for each person to be used as their own control and account for inter-individual differences in AFB_1 metabolism and AFM_1 excretion. B) Distribution of AFM_1 levels by arm/group. Comparison of median AFM_1 levels were compared between groups based on time points (Arm 1: days 1-7; Arm 2: days 14-21) to account for differences in daily dietary AFB_1 intake. Placebo data in Arm 1 were compared with ACCS100 data in Arm 1 and the same was calculated for Arm 2. *$p < 0.05$ as compared with placebo.

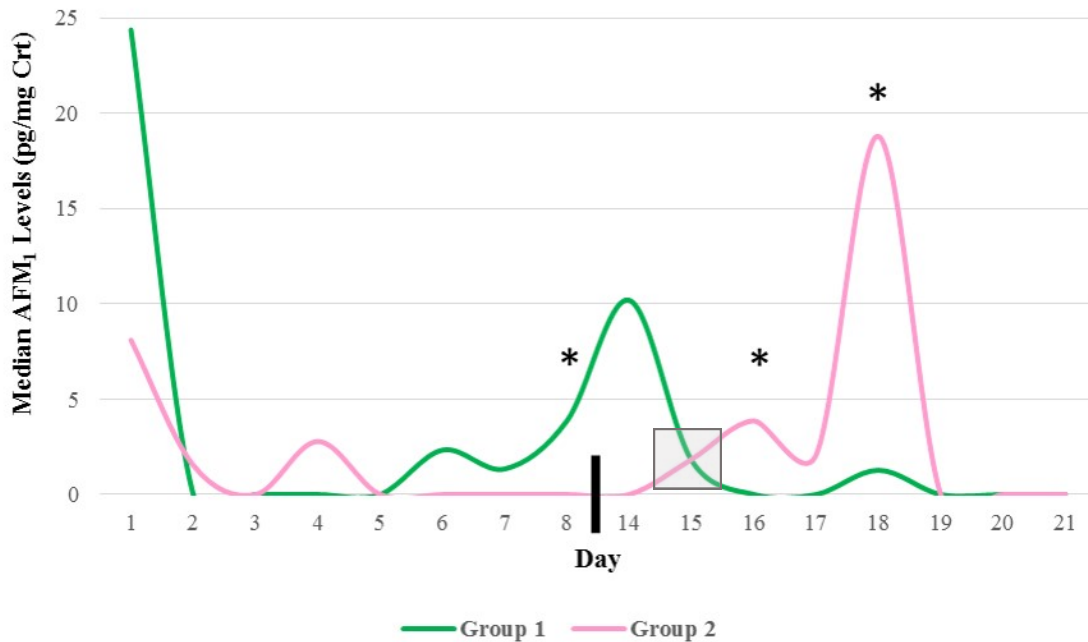

Figure 16. Daily median AFM_1 levels for 2 weeks of crossover study. Day 1 and 14 represent baseline urine samples taken for participants. Group 1 (green) was on placebo treatment days 2-8 and ACCS100 treatment days 15-21. Group 2 (pink) was on ACCS100 treatment days 2-8 and placebo days 15-21. * $p < 0.05$ as compared with placebo at the same time point. Bar between days 8 and 14 represent the washout period. Switch in treatments is highlighted by the grey box.

4.3.5 Palatability

There was a statistically significant difference in smell by treatment, with participants on average rating the placebo as 0.25 Likert points better than ACCS100. There were no statistically significant differences in ratings of taste, aftertaste, appearance, color, or texture by treatment (Table 11). The majority of participants rated aftertaste,

texture, appearance, smell, and color as either okay, good, or really good for both the placebo and ACCS100. No one reported appearance, color and taste as bad for the placebo. However, ACCS100 received a "bad" rating from one participant (2.2%) for appearance, one (2.2%) for color, one (2.2%) for taste, and one (2.2%) for texture.

Table 11. Kenya study palatability[a] ratings reported by treatment

	Placebo n=48	ACCS100 n=45
Aftertaste	3.7	3.8
Appearance	3.7	3.7
Color	3.8	3.9
Smell*	4	3.8
Taste	4	4.1
Texture	3.8	3.6

*p<0.05 comparing ACCS100 to placebo using Wilcoxon rank sum test; [a]Ratings reflect a 5-point Likert scale (1=really bad, 2= bad, 3=okday, 4=good, 5=really good)

4.3.6 Acceptability

Forty-seven participants completed the end of the study questionnaire. Most participants (96%) had heard of AF prior to the study, and most (91%) worried about becoming sick as a result of exposure to it. Over half of participants (67%) knew of someone who had become sick from AF exposure in the past, and 9% believed they

themselves had been sick from AF exposure in the past. Most participants (98%) did not have any concerns about ACCS100 and would be willing to take the ACCS100 (98%) or give it to their children (98%) if they knew it would provide them protection against aflatoxicosis. The majority of participants (72%) reported that they would prefer to take the clay in water as they had done during the study; the other commonly mentioned option was taking the clay plain (i.e., licking it; 13%). Participants would be willing to take the clay for at least two weeks (40%) or as long as it was recommended (38%). Similarly, participants would be willing to let their children take the clay for at least two weeks (36%) or as long as it was recommended (47%).

Table 12. Kenya study adverse events reported by treatment

Side Effect	Placebo (Participants: n=50) (Possible times: n=350)[a]	ACCS100 (Participants: n=49) (Possible times: n=343)
Abdominal discomfort		
# participants reporting	4 (8%)	7 (14%)
# times reported	5 (1%)	9 (3%)
Nausea		
# participants reporting	7 (14%)	2 (4%)
# times reported	8 (2%)	4 (1%)
Increased appetite		
# participants reporting	3 (6%)	4 (8%)
# times reported	6 (2%)	4 (1%)

[a]Possible tiems was calculated by multiplying the number of participatns by the numbers of days of treatment

4.3.7 Adverse events

Approximately one-quarter of participants in both the placebo and ACCS100 groups reported at least one adverse event (n=12, 26% and n=14, 28% respectively). The most commonly reported adverse events for placebo were nausea (14%) and abdominal discomfort (8%), while for ACCS100 abdominal discomfort (14%) and increased appetite (8%) occurred (Table 12). Other side effects reported at least once were diarrhea (reported once, by one individual while on ACCS100), and dizziness with headache (reported once, by one individual while on ACCS100). Frequency of an adverse event was reported as the potential number of times when it could have been reported. There were no statistically significant differences in the reporting of adverse events by treatment. No adverse events were graded as severe. Approximately one-third of side effects on ACCS100 (n=6; 35%) and placebo (n=7; 39%) were rated as moderate.

4.4 Discussion

ACCS100 was found to be safe, with no relationship between adverse events and ACCS100 use. Two of the most commonly reported side effects in our study were abdominal discomfort and nausea, and both have been reported in previous studies. One notable difference in the present study is that a small number of participants reported increased appetite. It is possible that participants may have paid more attention to their level of hunger since we performed our study during a time of relatively high food insecurity. Another difference between our study and earlier work is in the delivery mechanism insecurity. Another difference between our study and earlier work is in the delivery mechanism. Previously, ACCS100 had been delivered in food or capsule. This is

the first study to show that ACCS100 can be palatable and acceptable when mixed with water which would be a rapid and effective method of delivery and therapy during an outbreak period.

We found ACCS100 to be effective in reducing AF exposure from the diet. Participants in both groups had lower urinary AFM_1 levels while taking ACCS100 compared to placebo. During a similar cross-over study in Ghana, participants also exhibited lower urinary AFM1 levels, though the effect was statistically significant in only one group but pooled AFM_1 levels revealed a 55% reduction in the clay treatment group (Mitchell *et al.*, 2013). Similarly, the current study reports a 46% reduction in urinary AF levels.

Baseline urinary AFM_1 levels were approximately 80 times higher during the clinical trial in Ghana than observed in Kenya, suggesting that participants in our study had lower exposure to AF. This could be a result of lower daily caloric intake of AF-containing foods. Our ability to observe statistical significance in both of our study groups could also be a reflection of participants' exposure to AF remaining relatively stable throughout the study period, as evidenced by the average number of meals containing maize remaining fairly constant across each day of the study. Results from prior outbreaks have shown that this community is at high risk for AF exposure, with exposure typically originating from the consumption of homegrown maize (Daniel *et al.*, 2011). During this clinical trial, this region was in a drought. There had been no recent harvest, and thus all participants were eating maize from the market, instead of homegrown maize. This might have been one reason why AF exposure was relatively low. Another possibility is the

modulating effect of tea on AF metabolism (Tang *et al.*, 2008; Qin *et al.*, 1997). Results from a study examining the use of green tea polyphenols (GTP) as a chemopreventative agent for lowering AF biomarkers indicated that 500 mg of GTP reduced up to 43% median AFM_1 as compared with the placebo (L. Tang 2008). Furthermore, significant elevations in median AFB_1-mercapturic acid were found with GTP intervention therefore suggesting modulation of the metabolic pathway to favor the GST conjugation of AFB_1-exo 8,9-epoxide followed by urinary excretion as AFB_1-mercapturic acid over CYP 1A2 hydroxylation to AFM_1. A majority of study participants from the current study consumed tea on a daily basis. In this region, black is more readily consumed over green tea; however, studies have reported similar levels of bioavailable polyphenols in both teas (Henning *et al.*, 2006). When baseline levels were analyzed by tea consumption, mean, median, and range were significantly lower in the tea consuming group consistent with modulation of metabolism as previously reported (Tang *et al*, 2008). Although, determined not to be a confounder in trial efficacy based on even distribution between treatment groups, this modulation could explain the overall low-level exposure for such a high-risk area as compared to previous reports of AFM_1 in Ghana. Importantly, this is the first report of urinary AFM_1 levels in this region. Follow-up measurements are necessary to associate exposure levels with source (homegrown vs. imported maize) and to identify tea as a metabolic moderator for this particular population. Analysis of an additional short-term biomarker should also be employed in this scenario and the possibility of clay delivered in tea presents an interesting opportunity for increased protection. This delivery would allow for the reduction of bioavailability of the toxin by clay and chemoprevention

from the tea. In other words, the amount of toxin absorbed would be significantly reduced and what does make it into circulation and metabolism will favor rapid excretion as AFB_1-mercapturic acid therefor limiting the number of AFB_1-DNA adducts.

Median AFB_1-lys levels in our study participants (9.2 pg/mg alb at the beginning and 6.4 pg/mg alb at the end) were similar to median levels reported during a previous study that measured AFB_1-lys levels in Kenya's Eastern Province during a non-outbreak period (7.9 pg/mg alb) (Yard et al. 2013). Notably, AFB_1-lys levels were much lower during this study compared to levels reported among patients with potential liver dysfunction during aflatoxicosis outbreaks in Kenya in 2004, 2005, and 2010, when geometric mean levels ranged from 120–1,200 pg/mg alb (Azziz-Baumgartner *et al.*, 2005). Interestingly, a reduction of 55% of AFB1-lys levels was observed after seven days of clay treatment and two-weeks between sampling. Previous intervention studies have only observed a treatment effect after 1 month citing the stability of albumin and the time required to see reductions in this biomarker (Wang *et al.*, 2008).

There are multiple strategies to prevent AF contamination and exposure. A variety of long-term solutions include reducing AF contamination in maize through improved harvesting, drying, and storage (Turner *et al.*, 2005); planting resistant cultivars (Hell *et al.,* 2008), biocontrol (Cotty *et al.*, 2007; Yin *et al.*, 2008), and/or a gradual shift to a more diverse diet (Wu *et al.*, 2014). However, implementation of these strategies has been limited due to the difficulty of eliminating AFs completely. Thus, short-term interventions are needed in Kenya during times of outbreak. Currently, there are limited interventional options associated with aflatoxicosis outbreak responses in Kenya. The current outbreak

response activities include characterizing the outbreak, identifying risk factors associated with sickness, testing AF levels in food, and educating the community on AF prevention strategies. If ACCS100 clay continues to prove to be efficacious in future studies, it has the potential to be incorporated in outbreak response activities with an aim of preventing poisoning and protecting people during high risk or outbreaks.

Baseline AF exposure was relatively low during the study period. This prevented our ability to determine whether ACCS100 would be effective at the higher exposure levels typically seen during acute outbreaks. However, this allowed us to test the safety, acceptability, palatability, and delivery mechanism, during a relatively lower exposure burden. Compliance with the study protocol was measured via self-reported data, which may have biased results towards the null. However, this appeared not to have been an issue, as we still observed statistical significance. Finally, this study was conducted in a small region of Kenya that had a very high awareness of AF due to prior outbreaks. It is possible that other regions of Kenya may not have been as accepting of ACCS100 or found it as palatable. However, this is the region of Kenya where ACCS100 would most likely be used in the future.

ACCS100 shows promise as a potential method for reducing exposure to AF in this particular high-risk population in Kenya during outbreak situations; we found it to be safe, effective, acceptable, and palatable. More work is still needed to better understand whether ACCS100 could be used during a time of crisis to decrease the risk of aflatoxicosis. Further studies might characterize urinary AF levels during aflatoxicosis outbreaks and determine if ACCS100 remains effective at these extreme levels of

exposure. There is also a need to test the safety, efficacy, acceptability, and palatability among vulnerable populations, such as children, individuals in poor health, and pregnant women who are often most at-risk for aflatoxicosis.

5. ACCS100 CLAY INTERVENTION IN A U.S. POPULATION WITH A HIGH INCIDENCE OF HEPATOCELLULAR CARCINOMA

5.1 Introduction

Although chronic exposure to AFs is one of the major risk factors of HCC in many developing regions of the world, such as Southeast Asia and Sub-Saharan Africa (Kensler *et al.*, 2003; Turner *et al.*, 2002), the U.S. food supply is highly regulated and typically presents less risk for exposure (Brown *et al.*, 1999; Phillips *et al.*, 1994). However, there is a potential for increased exposure in individuals consuming diets that can be relatively high in foods prone to contamination, such as corn and corn-based products (e.g., cornmeal, corn tortillas, etc.). It has been estimated that there will be 35,660 new cases and 24,550 deaths in the U.S. due to HCC in 2015 (American Cancer Society, 2015). The State of Texas reports the highest HCC mortality in the U.S. and end-stage liver disease mortality is significantly higher in Hispanic populations (Perez *et al.*, 2004). Specifically, South Texas Hispanics have the highest HCC rates in the country, which are 3 to 4 times higher than those of non-Hispanic whites (Ramirez *et al.*, 2014). Although the causative factors for this disparity are not well delineated and may be attributed to a variety of factors including hepatitis infection, exposure to environmental and dietary carcinogens are also potential risk factors in this population.

Previously, we have shown that AF exposure in a predominantly Hispanic community in San Antonio, Texas may be a contributing factor in the significantly increased incidence of HCC in the region, where levels of AF in blood and urine correlated with consumption of corn tortillas and rice (Johnson *et al.*, 2010). Since it can be difficult

to avoid staple dietary components (and potential exposure to AF), intervention therapies to alleviate AF-induced liver disease and cancer in such populations are high priorities. To address this need, the efficacy of a refined calcium montmorillonite clay (ACCS100) intervention, as measured by AF biomarkers of exposure, was evaluated in 640 serum samples collected from 234 study participants during a 3-month randomized phase II intervention trial in Bexar and Medina Counties, Texas. Biomarker levels were evaluated prior to the beginning of the study (baseline), at 1 and 3 months, and 1-month post-intervention.

5.2 Materials and methods

5.2.1 Recruitment and eligibility

The study was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki), and approved by the institutional review boards at the University of Texas 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 (*Clinicaltrials.gov* Identifier: NCT01677195) and by the Cancer Therapy and Research Center Protocol Review Committee. After obtaining written informed consent (from September, 2012 to May, 2014), 380 participants were screened from a variety of public and residential sites in Bexar County and adjacent Medina County, Texas. These sites contain most of the population of the metropolitan area of the City of San Antonio, Texas. Many of the participants were not fluent in English. To address this, our recruitment team was bilingual and sensitive to the cultural dimensions of the study population. Individuals with a history of uncontrolled chronic disease and women who

were pregnant or breastfeeding were not allowed to participate. Eligibility for the randomized trial portion of this study was restricted to the 234 recruited participants (54 men and 180 women, ages 18-77) with AFB_1-lys adduct $\geq$ 1.0 pg/mg albumin. Sociodemographic and general health information was collected via a questionnaire administered at baseline including medical history and diet. Dietary survey included frequency and amount of consumption focused around corn- and peanut-based foods. Anthropometric data including age, race/ethnicity, height, weight, and vital signs were collected during the study. A schedule of procedures and tests performed is shown in Figure 17. An independent Data and Safety Monitoring Committee (DSMC) oversaw the study protocol and procedures, and reviewed the trial data at least biannually.

5.2.2 ACCS100 dosing and treatment schedule

This clinical study was conducted under U.S. FDA IND #114005. The test article was produced by Premier Research Laboratories (PRLabs, Austin, TX) and provided at no cost by Texas EnteroSorbents Inc. (TxESI, Bastrop, TX). This material was examined for various environmental contaminants, including dioxins and heavy metals, to ensure compliance with federal and international standards. Metal and dioxin analyses of ACCS100 were reported to be well under the tolerable daily intake or provisional tolerable daily intake set forth by the World Health Organization and the Joint Food and Agriculture Organization/WHO Expert Committee on Food Additives (Marroquin-Cardona, *et al.,* 2011). ACCS100 was sterilized by gamma radiation (Sterigenics, Fort Worth, TX) to prevent any possible bacterial or viral contamination before trial initiation. The clinical intervention was a randomized, double-blind, and placebo-controlled trial in which

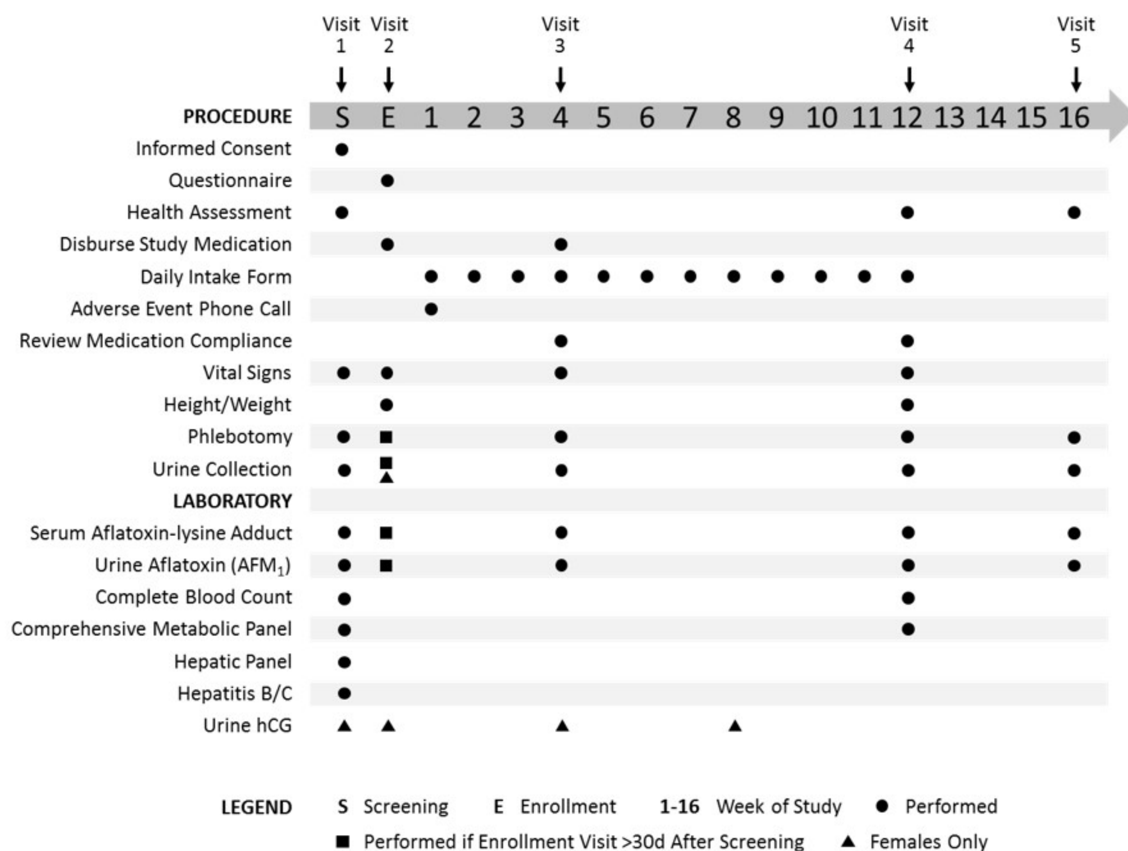

Figure 17. A summary of the study procedure for 3 month intervention in San Antonio.

participants were randomly allocated to three groups (stratified on gender): High dose, Low dose and Placebo. The High dose group received two 500 mg ACCS100 capsules before each meal and the Low dose group received two capsules containing 250 mg ACCS100 and 250 mg calcium carbonate placebo each. The Placebo group received the same size and color capsules containing 500 mg calcium carbonate. Doses were derived based on previous titrations with parent clay (NovaSil) *in vivo*. More specifically, the High dose of ACCS100 (3 g) represented an inclusion rate of only 0.25% (w/w) in the diet

which was equal to the MED required for efficacy in earlier work in animals (Phillips *et al.*, 2002; Pimpukdee *et al.*, 2004). Participant study medication compliance using pill count was recorded during each visit.

5.2.3 Adverse effects monitoring

Based on the existing literature describing consumption of dioctahedral smectite clays (including CM) in adults and children, no severe toxicity was expected as a result of ACCS100 treatment. Adverse effects were carefully monitored throughout the trial. Daily diary 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. Adverse events are described as percentages of the total numbers of adverse event reports out of the total numbers of completed daily diary worksheets per treatment group. In the event of an adverse treatment effect or unrelated condition, medical treatment was available to participants at no cost to the participant. Adverse events (AEs) and symptoms were graded according to the following criteria:

Grade 1	Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
Grade 2	Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily life.
Grade 3	Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care.

Grade 4 Life threatening consequences; urgent intervention indicated.

Grade 5 Death related AE

Any participant experiencing a severe symptom was advised to seek immediate medical attention. Any symptoms that were attributed to the ACCS100 treatment by the study physicians on the DSMC would result in immediate discontinuation of treatment for that subject.

5.2.4 Blood and urine collection and processing

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) (Figure 17) conducted at UTHSCSA or community recruitment sites. A portion of the blood was centrifuged within 1hr of collection to obtain the serum fraction, which was then stored at –80°C, and shipped to the University of Georgia for AFB_1-lys adduct analysis. Aliquots were also sent to LabCorp (San Antonio, Texas) for complete blood count to generate a differential and comprehensive metabolic profile. Premenopausal women provided urine for hCG pregnancy tests at Visits 1–3. Additionally, these participants were provided with a pregnancy test at Visit 3 with instructions to test at 8 weeks (between Visits 3 and 4).

5.2.5 Analysis of serum aflatoxin B_1-lysine adduct

Detection and quantification of serum AFB_1-lys adducts was performed as previously reported (Qian *et al.*, 2010; Wang *et al.*, 1996). In brief, serum samples were digested with Pronase to release mono-AFB_1-lysine adduct. The digests were loaded onto

an Oasis Max cartridge (Waters, Milford, MA) which was sequentially washed to concentrate and purify the adduct. The adduct was eluted with 2% formic acid in methanol. The eluents were evaporated to dryness and reconstituted with 10% methanol prior to HPLC analysis (Agilent 1200). Chromatographic separation was performed on an Agilent C18 column (5 µm particle size, 250 X 4.6 mm) and the mobile phase consisted of 20 mM ammonium phosphate monobasic (pH 7.2) and methanol in a linear gradient. The concentration of AFB_1-lys adducts were monitored at 405 nm (ex) and 470 nm (em). Authentic AFB_1-lys adduct standard was spiked into normal human serum (Sigma-Aldrich) for generating a standard curve and for quality control. AFB_1-lys adduct levels were adjusted by serum albumin concentration and expressed as the amount of AFB_1-lys adducts in pg/mg albumin. The limit of detection was 0.4 pg/mg albumin and the average recovery with various spiked AFB_1-lys adduct concentrations was 92%.

5.2.6 Analysis of urinary aflatoxin M_1

Analysis of urinary AFM_1 levels followed methods by Groopman *et al.* (1992b) and modifications by Sarr *et al.* (1995) and Wang *et al.* (1999). Urine samples were centrifuged at 887 xg, and 5.0 mL of supernatant was collected, acidified with 0.5 mL of 1.0 M ammonium formate (pH 4.5) and diluted with water to a total volume of 10.0 mL. Samples were then loaded onto a 3 mL preparative Aflatest® WB immunoaffinity column (VICAM, Watertown, MA, USA) at a flow rate of 1 mL/min. Following washing of the column, the AF fraction was eluted from the column with 2 mL of 80% methanol, dried under N_2 and re-suspended in 200 µL of a 1:1 solution of methanol:20 mM ammonium formate. Samples were analyzed using a Shimadzu HPLC system (Waters, Watertown,

MA, USA) with fluorescence detection capabilities. A 250 x 4.6 mm LiCrospher RP-18 column with pore size 100 Å and particle size 5 µm (Alltech Associates, Deerfield, IL, USA) was used to resolve AF metabolites. The mobile phase consisted of 22% ethanol buffered with 20 mM ammonium formate (pH 3.0) in water. Isocratic elution of the mobile phase for 20 min at a rate of 1 mL/min allowed for proper chromatographic separation. External AFM_1 standards were prepared weekly and injected following every five injections of samples. The limit of detection for this method was 4.8 pg for AFM_1 and the average recovery with various spiked AFM_1 adduct concentrations was great than 85%. Urinary AFM_1 concentrations were expressed as pg/mg creatinine to correct for variations in urine dilution among samples. Creatinine concentrations were measured by auto-analyzer at Baylor Scott & White Hospital (Temple, Texas).

5.2.7 Statistical analysis

All efficacy analyses were conducted using an intent-to-treat approach for randomized subjects who received and took at least one dose of the test article (placebo or ACCS100). 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.

The statistical analysis for the intervention data was comprised of four steps: a comparison of serum AFB_1-lysine adduct levels among treatment arms at the baseline prior to ACCS100 administration; an evaluation of the overall effects of ACCS100 on serum AFB_1-lysine adduct levels; an evaluation for effects underlying the baseline values adjustment, and analyses of each time point for treatment groups. Response variables that

were not normally distributed were logarithmically transformed to improve normality. To evaluate the overall treatment effects, log mixed-effect models for serum AFB_1-lysine adducts were constructed. The models included the intercept, indicators for treatment group, time, and a treatment × 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 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 analyses at different time points were conducted using the Wilcoxon rank-sum test. Hypothesis tests were two-tailed and assumed an $\alpha=0.05$. All analyses 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 and Tukey's tests were conducted on all demographic, hematological, and biochemical parameters for comparisons among treatment groups. Adverse events and dietary survey data was analyzed using a χ^2. A χ^2 ratio was generated for the relationship between various dietary factors and AF biomarkers. A two-sided p-value ≤ 0.05 was considered statistically significant.

5.3 Results

5.3.1 Sample collection and demographics

A total of 380 participants were screened in the Bexar and Medina Counties region, including urban and rural locations. Demographic characteristics of the study population and anthropometric information, including age, weight, height, blood pressure, and pulse are presented in Table 13. The three treatment groups were similar in terms of gender, age, height, and ethnicity. Although randomized evenly across treatment groups, significantly more females were recruited into the study than males ($p < 0.0001$*). Physical parameters such as body weight, systolic blood pressure, diastolic blood pressure, and pulse were not significantly affected after 3 months of ACCS100 treatment and were not different among treatment groups. Of those participants, 234 subjects who had detectable serum adduct (234/380, 61.5%) with levels>1 pg/mg alb, were randomized to a High dose group (n=71), Low dose group (n=83), or Placebo group (n=80). A flow diagram from recruitment to completion which is presented in Figure 18. Out of 355 urine samples collected at recruitment, 21.69% (77/355) had detectable levels of AFM_1. The overall study completion was similar between treatment groups (Table 14). A total of 147 subjects (62.8%) completed the 3-month trial. The study regimen had an overall adherence (number of times capsules were taken) of 85.1%. However, the High dose had significantly better adherence than the Placebo group ($p < 0.0001$ for both treatments). Detailed information about medication adherence is listed in Table 14.

Table 13. San Antonio study demographic distribution [% (n) or mean ± s.d.] of enrolled participants by treatment group.

	Treatment group		
	Placebo	**Low Dose**	**High Dose**
Participants completed trial	52	51	44
Gender			
Male (percent)	10 (19)	10 (20)	14 (32)
Female (percent)	42 (81)	41 (80)	30 (68)
Age (years)	42.2 ± 13.6	39.2 ± 13.9	41.9 ± 14.2
Ethnicity			
Hispanic (percent)	35 (67)	34(67)	32 (73)
Non-Hispanic (percent)	17 (33)	17(33)	12 (27)
Body weight (lbs)			
Baseline	185.0 ± 54.8	189.1 ± 46.9	173.9 ± 32.3
Post-treatment	184.3 ± 54.0	190.1 ± 48.0	176.2 ± 32.8
Height (ft)	5.4 ± 0.4	5.4 ± 0.3	5.4 ± 0.3
Systolic blood pressure (mm/Hg)			
Baseline	130 ± 19	129 ± 17	129 ± 17
Post-treatment	125 ± 15	126 ± 19	127 ± 18
Diastolic blood pressure (mm/Hg)			
Baseline	80 ± 11	80 ± 11	80 ± 10
Post-treatment	79 ± 10	79 ± 10	78 ± 11
Pulse (BPM)			
Baseline	71 ± 11	75 ± 11	74 ± 13
Post-treatment	74 ± 9	77 ± 11	74 ± 13

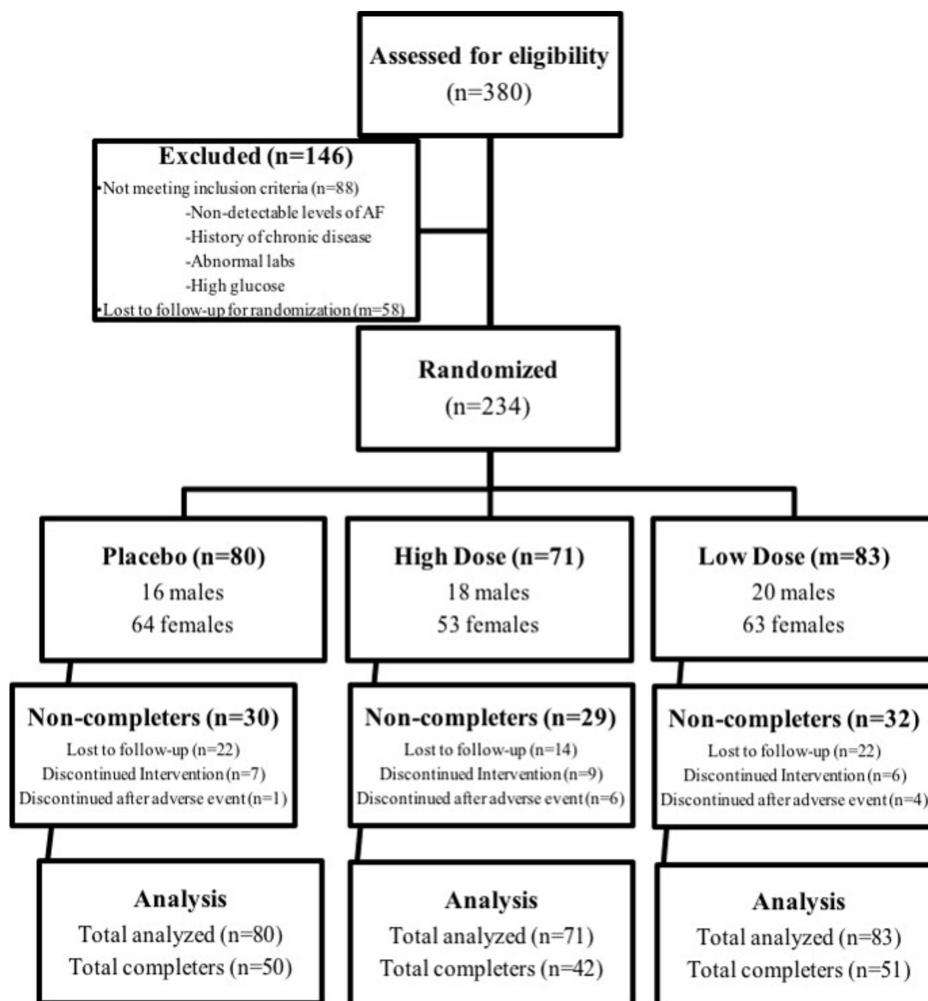

Figure 18. A flow diagram from recruitment to completion.

Table 14. San Antonio study participant adherence and completion of treatment regimen

	Treatment group			
	Placebo	**Low Dose**	**High Dose**	**Overall**
Participants				
Randomized	80	83	71	234
Completed (3 months treatment)	52	51	44	147
Completion (%)	65.0	61.4	62.0	62.8
Treatment Regimen				
Capsules to be ingested	27216	27888	23688	78792
Capsules missed[a]	4504	3967	3236	11707
Capsules taken[a]	22712	23921	20452	67085
Total reported adherence (%)	83.5	85.8[b]	86.3[b]	85.1

[a]Reported at visits 3 and 4; [b]$p < 0.05$ compared to Placebo group

Table 15. Change in AFB_1-lys adduct (pg/mg albumin) over time in serum from San Antonio study participants.

	Placebo	**Low Dose**	**High Dose**
Week	**Mean ± s.d.**	**Mean ± s.d.**	**Mean ± s.d.**
0	4.26 ± 3.15	4.09 ± 2.25	3.77 ± 2.52
4	3.06 ± 1.66	2.62 ± 1.08	3.00 ± 1.30
12	3.22 ± 1.85	2.71 ± 1.35	2.87 ± 1.53
16	3.03± 1.77	2.99 ± 1.26	2.74 ± 1.44

5.3.2 Trial efficacy—aflatoxin B_1-lysine adduct

Out of 640 samples collected from trial participants over 4 months, all had detectable levels of the serum AFB_1-lys adduct (>0.4 pg/mg albumin). In Table 15, mean values for AFB_1-lys adduct are shown for the treatment groups at baseline (0 weeks), the first follow-up (4 weeks), the second follow-up (12 weeks), and 4 weeks after

discontinuation (16 weeks). There were no significant differences in mean serum AFB_1-lys adduct level at the baseline among treatment groups (4.26, 4.09, and 3.77 pg/mg albumin for the Placebo, Low dose, and High dose group, respectively). Overall, log transformed adduct levels decreased for all groups over time ($p = 0.0448$, data not shown). Following baseline adjustment of serum AFB_1-lys adduct values across the treatment arms, it was determined by nonparametric analysis that the adduct levels Low dose group was significantly decreased at 1 month compared to Placebo ($p < 0.003$). Levels of AFB_1-lys adduct at 1, 3, and 4 months in the active treatment groups were compared to Placebo. Although AFB_1-lys adduct levels were decreased by month 3 for both treatment groups, the Low dose was the only treatment that was significant and resulted in 33% reduction from baseline levels ($p = 0.0005$) (Figure 19).

The results of the mix model regression analysis showed a significant correlation between AFB_1-lysine adduct level and time ($p < 0.001$), but not for the treatment group × time interaction term ($p = 0.99$). The model-adjusted means are shown in Figure 20 for the three treatment groups over time. Considering the rolling recruitment, a mixed effects model was run with treatment group, visit (time), and year of enrollment which were set as fixed effects. The results of the model showed a significant correlation between AFB_1-lys level and time ($p = 0.0002$) and a significant correlation between AFB_1-lys level at visit 1 (baseline) and year of enrollment (2012) ($p = 0.0049$). A oneway ANOVA analysis of baseline AFB_1-lys levels by year enrolled confirms this association ($p = 0.0011$); further post hoc analysis reveals that baseline measurements taken in 2012 were significantly higher than those measured in 2013 or 2014 (Figure 21).

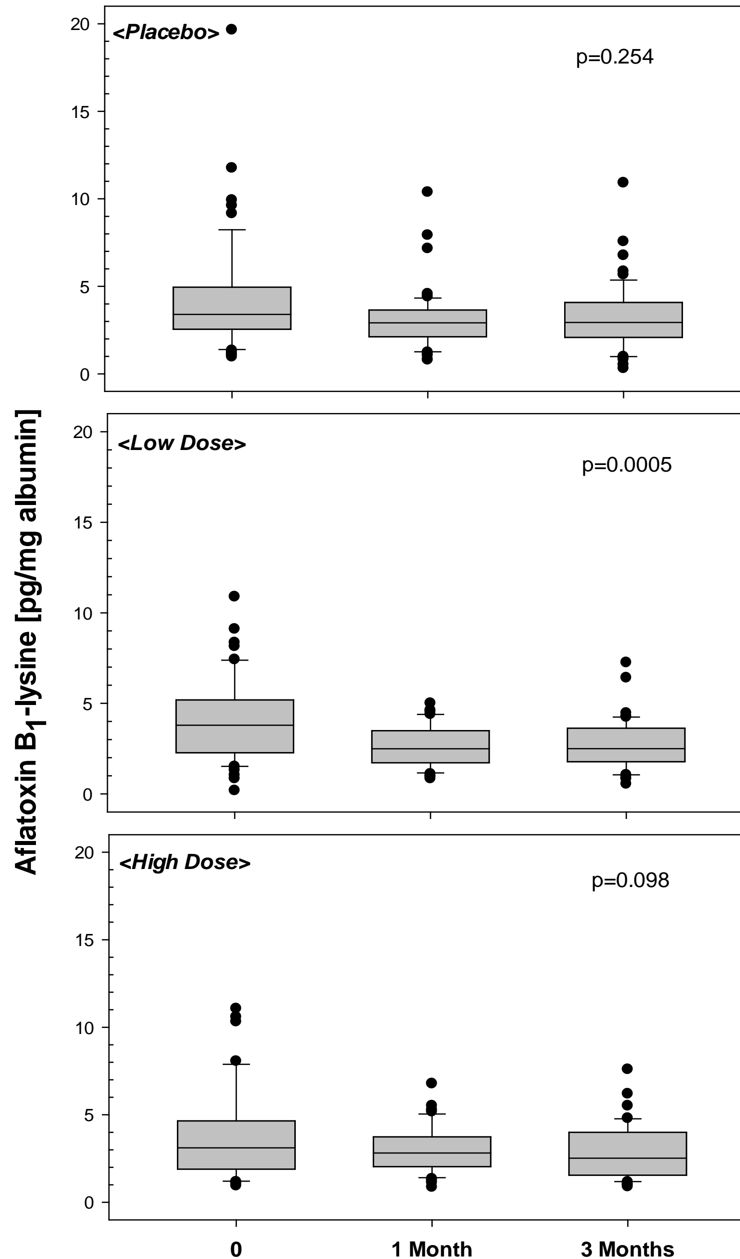

Figure 19. Distribution of AFB$_1$-lys adduct over time by group. Box and whisker plot showing the distribution of aflatoxin B$_1$-lysine adduct (pg/mg albumin) over time for each of the treatment groups. The upper, middle, and lower horizontal lines of the box represent the 75th percentile, 50% percentile, and 25th percentile, respectively. The whiskers represent the 95% confidence interval and the extreme values are shown as dots.

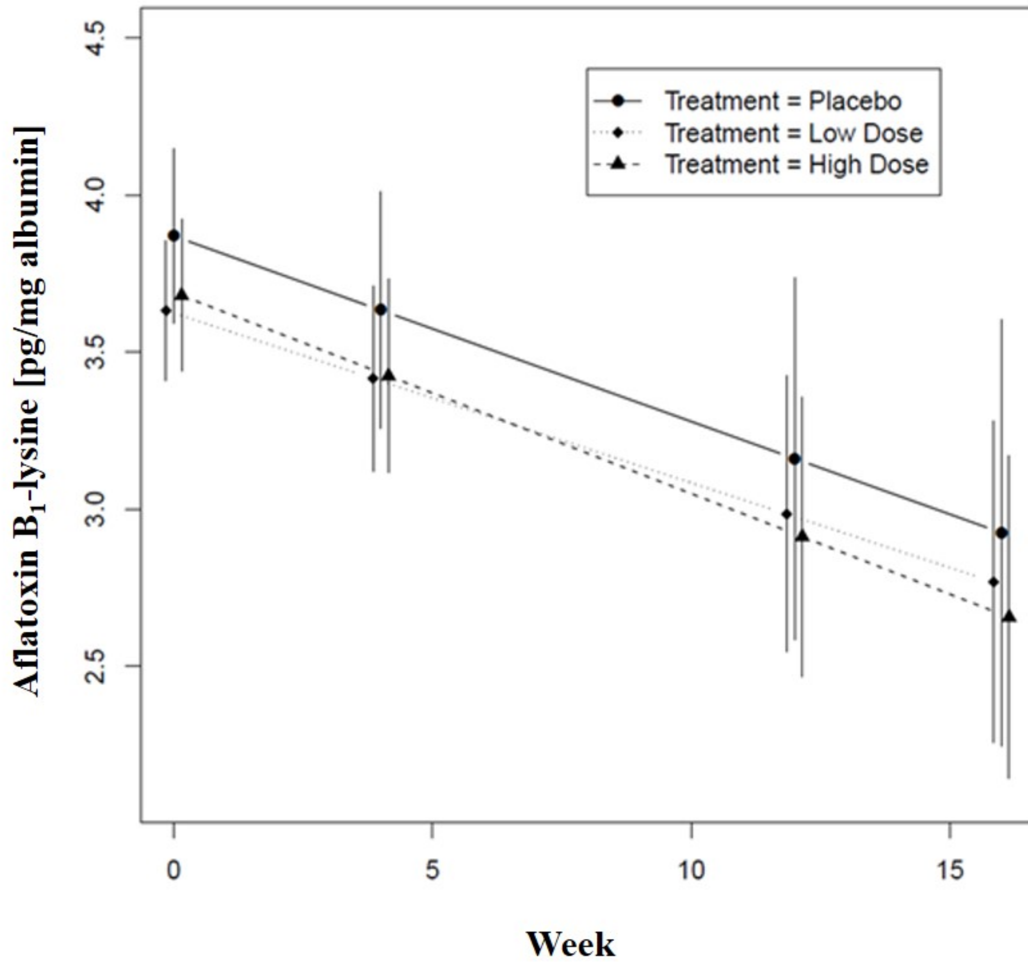

Figure 20. Log mixed-effect regression model showing estimated AFB_1-lys adduct. AFB_1-lys adduct (pg/mg albumin) levels for each treatment group are represented over time.

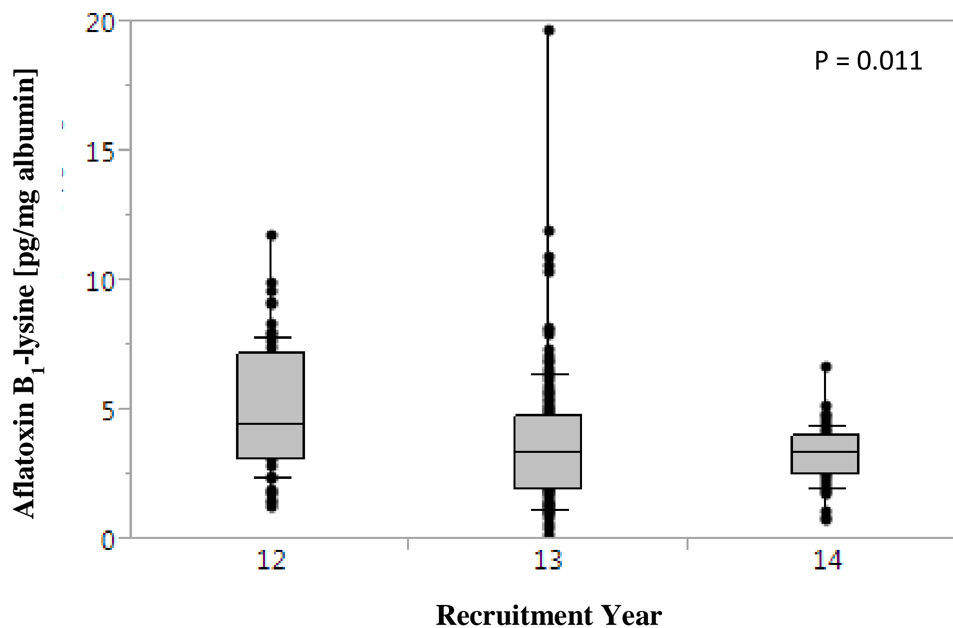

Figure 21. Distribution of AFB_1-lys adduct by recruitment year. Mean AFB_1-lysine adduct (pg/mg albumin) levels from the year 2010 were significantly higher than levels measured at 2013 and 2014.

Table 16. Change in AFM_1 (pg/mg crt) over time in urine from San Antonio study participants.

	Placebo	**Low Dose**	**High Dose**
Week	**Mean ± s.d.**	**Mean ± s.d.**	**Mean ± s.d.**
0	6.43 ± 35.62	7.18 ± 27.28	6.42 ± 25.01
4	0.47 ± 1.53	21.27 ± 101.09	51.77 ± 300.18
12	0.89 ± 2.37	8.87 ± 58.75	1.12 ± 4.02
16	1.32 ± 3.46	2.56 ± 10.78	0.30 ± 1.16

5.3.3 Trial efficacy—aflatoxin M_1

In Table 16, mean values for AFM_1 are shown for the treatment groups at baseline (0 weeks), the first follow-up (4 weeks), the second follow-up (12 weeks), and 4 weeks after discontinuation (16 weeks). Due to the low incidence at baseline, treatment effect analyses were only conducted in those who had detectable levels of AFM_1 at screening. AFM_1 positive results at screening were evenly distributed but resulted in poor sample sizes: Placebo (n=11), Low Dose (n=8), and High Dose (n=7). There were no significant differences in mean or median AFM_1 at the baseline among treatment groups (26.26, 3.06; 21.16, 2.75; 22.621, 2.65 pg AFM_1/mg Crt for the Placebo, Low dose, and High dose group, respectively). However, a dose-response trend was observed with average AFM_1 levels decreasing with treatment, and rebounding by month 4 (Figure 22). By month 3, no detectable levels of AFM_1 were observed in the High Dose group with average levels in Low Dose and Placebo of 0.61 and 2.07 pg AFM_1/mg crt, respectively.

5.3.4 Adverse events and serum biochemistry

Participants were encouraged to report all self-suspected adverse effects to the study monitors in person, or by phone at any time. A variety of AEs were reported throughout this study for all treatment groups, including the Placebo and both doses of ACCS100. Most of the AEs that were reported were graded as "mild" (Grade 1) and were gastrointestinal in nature, including indigestion/heartburn, nausea, constipation, diarrhea, flatulence, abdominal discomfort, and bloating (data not shown). For Grade 1 symptoms, a significant difference was observed between the Placebo group and the Low dose ($p = 0.0014$) and High dose ($p = 0.0044$) groups. However, most of these events were reported

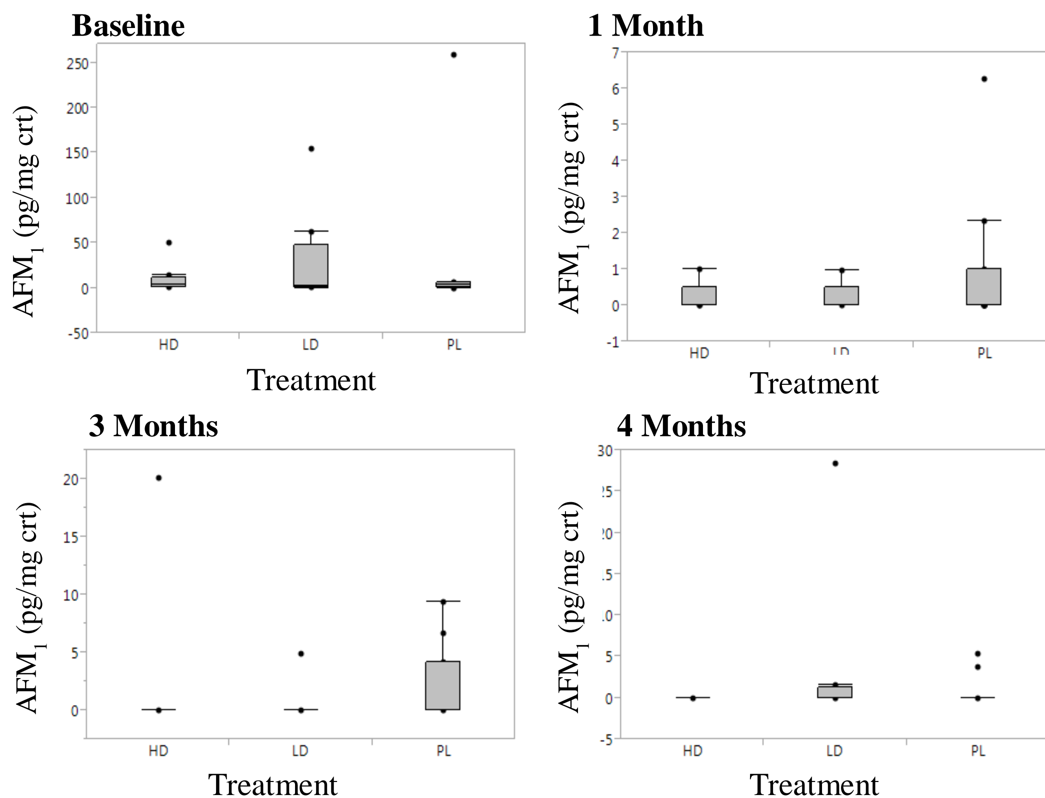

Figure 22. AFM_1 distribution within groups over the study period. Urine was collected at each study visit. The box values ranges from 22 to 75 percentiles of the total samples, and the line within it indicated the median value. The bars on both sides of a box represent values ranging from 5 to 25 and from 75 to 95 percentiles, respectively.

Table 17. Hematological analysis and serum biochemistry analysis in San Antonio study participants.

Hematology	Placebo		Low Dose		High Dose		
	Visit 1	Visit 4	Visit 1	Visit 4	Visit 1	Visit 4	Reference Range
WBC (x10E3/uL)	7.0 ± 1.5	6.9 ± 1.5	7.1 ± 1.9	7.0 ± 2.0	6.7 ± 1.9	6.5 ± 1.5	3.4-10.8
RBC (x10E6/uL)	4.7 ± 0.4	4.6 ± 0.4	4.7 ± 0.3	4.6 ± 0.3	4.6 ± 0.4	4.6 ± 0.4	3.8-5.8
Hemoglobin (g/dL)	14.2 ± 4.1	13.7 ± 1.1	13.6 ± 1.2	13.4 ± 1.2	13.8 ± 1.5	13.8 ± 1.4	11.1-17.7
Hematocrit (%)	39.9 ± 5.8	40.8 ± 2.9	40.5 ± 3.0	40.2 ± 2.8	41.3 ± 3.8	41.1 ± 3.7	34.0-46.6
MCV (fL)	87.6 ± 4.7	88.1 ± 4.3	87.0 ± 4.9	87.0 ± 4.9	89.2 ± 4.9	88.8 ± 4.2	79.0-97.0
MCH (pg)	29.4 ± 1.9	30.7 ± 8.8	29.1 ± 2.1	29.1 ± 2.2	29.8 ± 2.0	29.6 ± 2.3	26.6-33.0
MCHC (g/dL)	33.6 ± 1.0	33.5 ± 1.1	33.5 ± 1.0	33.4 ± 1.1	33.4 ± 1.1	33.6 ± 0.9	31.5-35.7
RDW (%)	13.9 ± 0.9	14.0 ± 0.8	14.2 ± 0.9	14.3 ± 1.0	13.9 ± 0.8	14.1 ± 1.4	12.3-15.4
Platelets (x10E3/uL)	270.1 ± 59.4	268.6 ± 53.0	277.6 ± 79.6	277.6 ± 81.1	276.6 ± 59.4	261.8 ± 59.2	150-379
Neutrophils (%)	58.4 ± 8.3	59.5 ± 8.6	59.0 ± 9.3	59.9 ± 7.3	56.4 ± 9.1	56.8 ± 8.6	40-74
Lymphocytes (%)	31.6 ± 7.3	31.1 ± 7.7	31.6 ± 7.2	31.2 ± 6.3	33.1 ± 8.3	33.1 ± 7.9	14-46
Monocytes (%)	7.1 ± 2.0	6.6 ± 2.0	6.4 ± 1.9	6.3 ± 1.7	6.9 ± 2.1	6.5 ± 2.0	4-12
Eosinophils (%)	2.4 ± 1.3	2.3 ± 1.5	2.2 ± 2.4	2.2 ± 1.7	2.9 ± 2.1	3.0 ± 1.6	0-5
Basophils (%)	0.3 ± 0.5	0.4 ± 0.5	0.4 ± 0.5	0.5 ± 0.5	0.6 ± 0.8	0.6 ± 0.5	0-3
Neutrophils, abs (x10E3)	4.1 ± 1.2	4.1 ± 1.2	4.3 ± 1.5	4.2 ± 1.6	3.8 ± 1.5	3.7 ± 1.1	1.4-7.0
Lymphocytes, abs (x10E3)	2.3 ± 0.7	2.1 ± 0.7	2.2 ± 0.7	2.1 ± 0.6	2.2 ± 0.7	2.1 ± 0.6	0.7-3.1
Monocytes, abs (x10E3)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.1-0.9
Eosinophils, abs (x10E3)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.0-0.4
Basophils, abs (x10E3)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.0-0.2
Immature Grans (%)	0.0 ± 0.0	0.0 ± 0.1	0.3 ± 2.0	0.0 ± 0.1	0.2 ± 0.6	0.0 ± 0.0	0-1
Immature Grans, abs (x10E3)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0-0.1

Serum Biochemistry	Placebo		Low Dose		High Dose		
	Visit 1	Visit 4	Visit 1	Visit 4	Visit 1	Visit 4	Reference Range
Glucose, Serum (mg/dL)	93.5 ± 28.6	98.7 ± 35.7	92.9 ± 13.8	94.5 ± 22.9	100.8 ± 33.4	103.8 ± 44.9	65-139
BUN (mg/dL)	13.2 ± 3.7	13.2 ± 3.3	12.9 ± 4.4	12.8 ± 3.5	16.0 ± 16.3	14.0 ± 4.7	5-27
Creatinine, Serum (mg/dL)	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.57-1.27
eGFR If NonAfrican Am (mL/min/1.73^2)	100.2 ± 18.3	98.8 ± 17.2	122.2 ± 143.9	100.2 ± 24.1	100.0 ± 14.1	104.3 ± 16.0	>59
eGFR If African Am (mL/min/1.73^2)	115.7 ± 20.9	114.1 ± 19.8	117.9 ± 23.1	117.1 ± 23.5	115.5 ± 16.4	120.2 ± 18.5	>59
BUN/Creatinine Ratio	17.1 ± 5.1	17.3 ± 4.7	17.0 ± 4.9	17.0 ± 4.7	17.5 ± 5.1	18.9 ± 5.4	9-23
Sodium, Serum (mmol/L)	139.4 ± 2.2	139.0 ± 2.3	139.3 ± 2.3	138.9 ± 2.0	139.1 ± 2.7	138.5 ± 2.9	134-144
Potassium, Serum (mmol/L)	4.1 ± 0.4	4.1 ± 0.4	4.1 ± 0.3	4.1 ± 0.4	4.2 ± 0.4	4.2 ± 0.3	3.5-5.2
Chloride, Serum (mmol/L)	102.1 ± 2.2	101.0 ± 2.2	102.0 ± 2.0	102.3 ± 1.8	101.2 ± 2.5	101.2 ± 2.7	97-108
Carbon Dioxide, Total (mmol/L)	22.4 ± 1.9	23.0 ± 2.1	22.0 ± 1.8	22.2 ± 2.1	22.8 ± 2.8	22.5 ± 2.3	18-29
Calcium, Serum (mmol/L)	9.4 ± 0.3	9.4 ± 0.4	9.4 ± 0.3	9.3 ± 0.4	9.5 ± 0.4	9.4 ± 0.4	8.7-10.2
Protein, Total, Serum (g/dL)	7.0 ± 0.4	7.0 ± 0.4	7.1 ± 0.4	6.9 ± 0.4	7.2 ± 0.4	7.0 ± 0.5	6.0-8.5
Albumin, Serum (g/dL)	4.5 ± 0.7	4.4 ± 0.3	4.4 ± 0.2	4.3 ± 0.3	4.5 ± 0.3	4.3 ± 0.3	3.2-4.6
Globulin, Total (g/dL)	2.6 ± 0.4	2.6 ± 0.4	2.7 ± 0.4	2.6 ± 0.3	2.7 ± 0.4	2.6 ± 0.4	1.5-4.5
A/G Ratio	1.7 ± 0.3	2.0 ± 2.0	1.7 ± 0.3	1.7 ± 0.3	1.7 ± 0.4	1.7 ± 0.3	0.7-2.0
Bilurubin, Total (mg/dL)	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.4 ± 0.3	0.4 ± 0.2	0.4 ± 0.2	0.0-1.2
Alkaline Phosphatase, S (IU/L)	78.7 ± 26.3	75.7 ± 26.8	73.4 ± 17.7	69.2 ± 14.2	78.5 ± 23.1	77.0 ± 24.0	39-142
AST (SGOT) (IU/L)	22.8 ± 10.1	22.8 ± 9.8	20.0 ± 5.9	20.6 ± 8.0	23.1 ± 10.1	21.8 ± 8.2	0-40
ALT (SGPT) (IU/L)	26.7 ± 20.5	26.9 ± 26.3	21.9 ± 11.0	23.2 ± 15.6	23.9 ± 14.5	24.6 ± 15.2	0-44
Bilirubin, Direct (mg/dL)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.0-0.4

by only 1-3 participants in the study. Grade 4 symptoms included anxiety (from 2 different participants in the Placebo and Low dose groups), and one report of temporary colitis symptoms from a participant in the Low dose group. However, the participant experiencing colitis did not feel these symptoms were related to the ingestion of the study medication, and this was verified by magnetic resonance imaging. All three participants who experienced Grade 4 events were removed from the study and, upon further scrutiny, their symptoms were determined to not be life threatening as the definition states. Importantly, all AEs reported were determined to be unrelated to the study agent; no participants were hospitalized throughout the trial, and no grade 5 events occurred.

Hematological and blood serum analysis (Table 17) revealed no significant differences or dose-dependent effects in any of the parameters tested at visit 1 (baseline) or visit 4. Protein values decreased after treatment for all groups ($p = 0.003$), including the Placebo group, but remained within normal levels. Analysis of serum chloride levels indicated a significant difference between treatment arms ($p = 0.03$). Upon further post hoc analysis, the values in the Placebo group were not significantly different from either the Low dose or High dose ACCS100 groups at Visit 1 or 4.

5.3.5 Dietary survey

Dietary survey information (data not shown) was completed for the 234 enrolled participants. Analysis of dietary survey information revealed that a majority of the study population consumed Mexican foods once to five times a week. Corn is eaten less than once a week and equal to about 1 ear or ½-1 cup. Similarly, corn products are consumed less than once a week and equal to 1 or more items greater than or equal to half

a cup. Frequency of corn tortilla consumption was evenly distributed with 90 participants reporting consumption of one to two tortillas less than once a week. Rice was reported at a frequency of 1-5 times a week with ½ to 1 cup of rice. Consumption of 1 to 8 tablespoons of peanut butter less than once a week was reported by the majority. Similar trends were observed for peanut consumption. About half of the study population consumed 10 to 25 tortilla chips once to 5 times per week. About a quarter of the study population consumes 4 to 8 ounces of a corn and rice drink (horchata) less than once a week.

When responses were compared to AFB_1-lys adduct, there was a significant association between detectable adduct levels and rice corn drink ($p = 0.0483$). When responses were compared to AFM_1, there was a significant association between detectable levels and the frequency ($p = 0.0238$) and amount ($p = 0.0338$) of corn tortilla chips consumed.

5.4 Discussion

AFB_1 has been implicated as a major factor in the etiology of HCC (IARC, 1993; IARC, 2002) and end-stage liver disease (Kuniholm *et al.*, 2008). The incidence of HCC in the United States has steadily increased over time (El-Serag and Mason, 1999), with the State of Texas reporting the highest mortality rate in the country (Devesa *et al.*, 1999). In South Texas, the incidence of HCC for Hispanics is considerably higher than all other races (Ramirez *et al.*, 2012). Multiple factors may be attributed to higher incidence including diet, environment, lifestyle, hepatitis B or C infection status, and possibly genetic susceptibility. Additionally, as climate warms and weather patterns become less predictable, countries such as the United States may become more vulnerable to

environmental contaminants. Due to increased temperatures and droughts which encourage *Aspergillus* growth, good agricultural practices are not be sufficient to prevent AF contamination in the food supply; it has been postulated that contamination may become widespread in areas such as the US Midwest that were previously unaffected (Cotty and Jaime-Garcia, 2007). Exposure to AFs in many parts of the developing world is well above that observed in the United States, where levels in the food supply are commonly regulated. As a result, the U.S. public is less aware of AF contamination and its associated adverse health effects. Importantly, we observed a prevalence of AF exposure approximately three times higher than reported in our previous survey in Bexar County (Johnson *et al.*, 2010). Thus, strategies to educate and mitigate chronic exposures to AFs in the U.S. are warranted.

The current study was based on a similar 3-month efficacy trial conducted in Ghana, a country with historically high AF exposure, where the levels of AFB_1-albumin adduct at three months were significantly decreased in both the Low dose and the High dose groups compared with levels in the Placebo group (Wang *et al.*, 2008). Importantly, baseline levels in Ghana (1.52 pmol/mg alb or 474.2 pg/mg alb) were almost 100 times higher than those observed in Texas (0.013 pmol/mg alb or 4.04 pg/mg alb). In Ghana, the reduction rates of AFB_1-lys for the Low and High dose groups were 22.3 and 22.4% at one month of intervention and 42.8 and 40.2% at three months of intervention, respectively. In the current study, a reduction rate of 36 and 20% at 1 month after the intervention and 33 and 24% at 3 months after was observed in the Low dose and High

dose treatment group, respectively. Findings from these studies suggest that low dose usage of ACCS100 could significantly protect against AF-derived adverse health effects. Previous studies suggest that no safe level for AF exists due to its lack of a threshold for dose-dependent adduct formation (Appleton *et al.*, 1982; Buss *et al.*, 1990; Lutz *et al.*, 1980; Phillips, 1999; Wild *et al.*, 1986). However, Johnson *et al.* (2014) recently demonstrated complete protection from AF-induced liver tumors with a 66% reduction in urinary AFB_1-N^7-guanine through the use of CDDO-IM, compared to 96% HCC incidence in the AFB_1 treatment group. This study proposes that a threshold for AF-induced cancer may exist. Similarly, ACCS100 reduces the formation of DNA adducts by decreasing bioavailability of the toxin and may also be effective in protection against HCC, and as therapy during emergency outbreaks of aflatoxicosis. In this study, we found that the ACCS100 was well tolerated because of the limited incidence of high grade AEs. Also, none of these were deemed to be attributable to the test article.

Results presented here are similar to our previous 3-month study in Africa where AFB_1-lysine adduct levels were decreased over time in all three groups, including placebo. This suggests that subjects may have adjusted their intake of foods associated with AF exposure. This is further supported by the fact that adduct levels never returned to baseline after a month of no treatment, suggesting a behavioral change. The baseline assessment included a dietary questionnaire that focused on foods that are associated with AF exposure. Such awareness could have altered subject behavior by decreasing consumption of specific foods associated with AF exposure. This type of behavior is largely

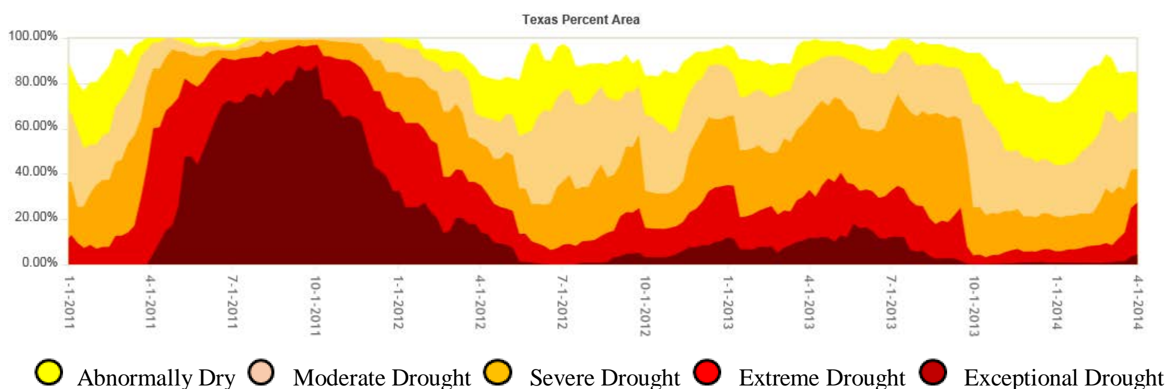

Figure 23. Percent area of Texas in drought from January 2011 to April 2014. Source: National Drought Mitigation Center, 2016

unavoidable and has been observed in other clinical trials since investigators are required to explain detailed study protocols, potential risks and benefits to study participants.

Another possibility for the downward trend observed in all three treatment groups is the overall decrease in AF exposure during the 3-year study period as demonstrated by baseline AFB_1-lys adduct levels distributed by the year collected (Figure 23). This could be explained by the onset of drought that was experienced in Texas and the Southern Plains in 2011, which was recorded as one of the hottest and driest summers in history (National Weather Service, 2016). Figure 23 shows the percent of area in Texas experiencing drought from 2011-2014 highlighting the record-breaking 2011-year and the subsequent return to average levels. Therefore, due to the relatively stability of AFB_1-lys adduct biomarker and possibility of commodities harvested in 2011 and sold in 2012 (i.e., corn meal), the significantly higher biomarker levels measured in 2012 from the current study could also be a reflection of the 2011 drought. Baseline adduct levels and the percentage

of Texas in drought both decreased in 2013 and 2014. This trend could explain the decrease observed over the study period in all three groups, including placebo, as a factor of time and overall decrease in exposure.

Unlike the intervention trial in Ghana, AFB_1-lys levels in San Antonio were not decreased in a dose-dependent manner. This may have occurred for a variety of reasons including: 1) considerably lower AF exposures in the U.S. (which makes it harder to detect substantial reductions over the course of the study); 2) sub-optimal subject adherence; and 3) possible differences in dispersion and reactivity of clay from capsules containing high versus low doses. Although the self-reported adherence rate for the current study was satisfactory, this may not have translated to actual compliance. This could also explain why we did not observe a dose-dependent response in the High dose group. In comparison to our previous 3-month intervention trial in Ghana, where maximum compliance was achieved through the daily in-home monitoring of capsule distribution and ingestion, the current study relied on participant reports collected during monthly visits. With this design, total compliance was difficult to assess because participants may forget about doses taken or missed (Claxton *et al.*, 2001); however, in-the-field daily monitoring by study personnel was not practical for this study, and therefore self-reporting was the only option. It is also possible that rolling enrollment could have led to an uneven distribution of AF exposure in study participants, however, it is the most common practice for community-based intervention studies. Nevertheless, the randomization procedure used produced an even distribution of the adduct levels at the baseline in the three treatment groups.

Analysis of the urinary AFM_1 data for participants with detectable levels at screening (n=26) revealed a dose-dependent reduction trend of the metabolite biomarker (Fig 21). It is important to note that the sample size in this analysis is very small and therefore statistically invalid. However, this observation raises the potential benefit for the use of both long- and short-term biomarkers of exposure for intervention recruitment since the 26 participants represented in this data were positive for AFM_1 and AFB_1-lys (required for enrollment). This "double positive" situation could occur when an individual has a history of exposure (AFB_1-lys) and is currently exposed (AFM_1) therefore representing an optimal candidate for a dietary intervention.

Based on the fact that ACCS100 was well tolerated in the majority of participants and no significant changes in serum biochemistry or hematology were detected in any treatment group, we postulate that this AF-reduction strategy was safe for a period of three months in this population. This conclusion agrees with our findings from other clay-based clinical intervention trials in humans and animals. Thus, long-term use of CM clay (i.e., UPSN, ACCS100) at low doses may provide a viable strategy to reduce dietary AFB_1 bioavailability in populations exposed to this carcinogen. Studies are warranted to further establish dosimetry in humans.

6. SUMMARY

AFs are known human carcinogens that contaminate dietary staples such as corn and peanuts. AFs have also been demonstrated in animal models to compromise the immune system, interfere with protein metabolism and block the adsorption of micronutrients and their effect have also been confirmed in human epidemiology studies. The contamination levels of AFs in food vary with seasons, but the problem is largely driven by drought stress in the field and poor storage conditions post-harvest. Unfortunately, in many developing countries, the primary food source is derived from corn and/or peanut product, leading to the estimation that ~4.5 billion people living between 40° north and south of the equator are chronically exposed to AFs (Williams *et al.*, 2004). Furthermore, HBV is prevalent in regions afflicted with AF exposure and HBV and AF co-exposure has a synergistic effect in causing HCC. This has been observed in western Africa and southeast Asia where AF and HBV have been implicated as major risk factors for the development of HCC. Recently, a population in south Texas with a high incidence for HCC was surveyed for AF exposure (Johnson *et al.*, 2010) and AFB_1-lys adduct was detected in 20.6% of the samples with a high hepatitis C virus positivity (7.1%) was observed. The study also suggested that although AF levels were significantly lower than those reported in Africa, individuals consuming higher amounts of foods prone to AF contamination may be more vulnerable to exposure and interactions with other environmental/biological factors.

What was once considered a public health issue for developing countries with inadequate agricultural technology and food insecurity and now is emerging as a global issue due to increased exposure as a result of prolonged drought and unpredictable climates. Strategies to reduce exposure include investments in production, drying, and storage facilities, to education on properly sorting and washing contaminated food. Thus far, these strategies have not proven to be sustainable in the developing world. Furthermore, due to the heterogeneous nature of AF in grains, they often enter the food chain undetected regardless of national GDP. As a result, interventions with clay binders have been introduced to high-risk populations to mitigate AF exposure. It should be noted that acceptance by the study subjects and successful delivery of the active compound is critical to achieve confident efficacy values. Therefore, many factors including culture, economics and logistics should be considered when implementing such intervention.

The objective of the first study was to determine the efficacy of a calcium montmorillonite clay (UPSN) in binding AF when delivered as a food additive. UPSN must be stable in complex, acidic mixtures that are often exposed to heat during the process of fermented gruel preparation, which is commonly consumed in sub-Saharan Africa. Therefore, the objective of the present study was to test the ability of UPSN to sorb AF while common cooking conditions were applied. The process of fermentation, heat treatment, acidity, and processing time were investigated with and without UPSN. Analyses were performed using the field-practical Vicam assay with HPLC verification. My data demonstrated that UPSN significantly reduced AF levels (47-100%) in cornmeal, regardless of processing conditions. Among each cooking process element tested, time

appeared to be the most important factor in UPSN efficacy. The greatest reductions in AF concentrations were reported in samples that were allowed to incubate (with and without fermentation) for 72 hr. These data suggest that the addition of UPSN in foods where a higher incidence of AF contamination occurs would be a sustainable approach to reduce the exposure to this food toxin.

Maize is frequently consumed in the Hispanic community, however very little is known about exposure to combinations of AF and FB, a frequent co-contaminant of maize, in Mexico. Initial work in animals with co-exposure to AF and FB have demonstrated either additive or synergistic effects in cellular toxicity and development of HCC. Recent studies have demonstrated that both AF and FB were associated with growth stunting. Therefore, investigation of co-exposure in Mexico, would have important implications for public health and possibly identify populations in need of intervention. The objective of this co-exposure study was to assess exposure through AF and FB biomarker analyses in spot urine samples from individuals recruited in the metropolitan area of Monterrey, Mexico. Participant urine samples were tested positive for AFM_1 in 69% of the study population which represents the AFB_1 consumed in the 24-48 hr prior to sample collection. Similarly, the urinary FB_1 biomarker was detected in 71% of samples and is also indicative of recent exposure. Out of 106 urine samples, 55% contained both AFM_1 and FB_1. It should be noted that AFM_1 levels were considerably lower than those observed in Ghana (Obuseh *et al.*, 2010). However, many variables must be considered before stating that this study population represents a low AF exposure community. Samples should be taken at various points throughout the year to identify any seasonal variation in AF exposure in

a region and more information regarding maize handling and storage should be considered. FB_1 concentrations were similar to those reported in Guatemala. However, no NOAELs or LOAELs exist for FB; thus the data are insufficient to make a conclusion from these exposure levels. Unlike parts of sub-Saharan Africa and southeast Asia, peanuts are not commonly consumed in Mexico; thus, maize is the likely source of exposure. Not surprisingly, analysis of the dietary survey revealed associations between corn tortillas and mycotoxin status, with greater than 90% of the study population consuming two or more in one serving. However, statistical significance was only reached when asked about the quantity and not frequency, thus exposing a weakness of dietary surveys and the importance of reliable biomarkers of exposure. The role for nixtamalization in the reduction of AF and FB was not addressed in this study, but should be explored in future studies to determine exposure risk in Mexico and Latin America. Overall, the high frequency of exposure to AF and FB demonstrates a potentially significant public health risk and more information is required before mitigation therapy with CM clay is suggested in this region.

Unlike Mexico, eastern Kenya has a history of AF outbreaks and aflatoxicosis-related deaths. In 2004, consumption of AF-contaminated foods resulted in 125 deaths in this region. Due to poverty, food scarcity, and drought, intervening with a CM to prevent bioavailability of foodborne toxins is an attractive strategy. This strategy has proven to be effective in reducing AF biomarkers of exposure in Ghanaian adults and children at doses up to 2.0% and 1.5% w/w, respectively. Despite the apparent effectiveness of ACCS100, there is a need to establish feasibility and efficacy in Kenya. There are differences between

Ghana and Kenya (e.g., different cultures, ethnicities, diets, institutional policies, infrastructure, etc.) that could affect the efficacy, palatability, and acceptability of ACCS100. Thus, the study's objectives were to assess these qualities of ACCS100 in a high-risk population. Previous work has demonstrated the successful use of the short-term biomarker, AFM_1, in a cross-over study design. This biomarker is a preferred endpoint, as it is field practical and is cost and time-efficient compared to serum analysis, which require months-long intervention trials. Although AFM_1 excretion positively correlates with AFB_1 intake, the day to day variability complicates statistical analyses. Daily urine samples and pooling weekly data in a cross-over design has been demonstrated to negate that variability. Therefore, the objective of the Kenya study was to determine the efficacy, acceptability, and palatability of a calcium montmorillonite (ACCS100) to reduce biomarkers of exposure when delivered as a mixture in water in a Kenyan population. Urinary AFM_1 was reduced by 46.6% within seven days in the ACCS100 treated group when pooled and compared to placebo. This is similar to the 55% reduction observed by Mitchell et al. (2013). Furthermore, the cross-over design clearly showed the switch from placebo to ACCS100. Seven days of ACCS100 treatment also reduced the long-term exposure biomarker, AFB_1-lys, further supporting the reduced bioavailability of AFB_1 by calcium montmorillonite clay. This is an especially interesting finding since previous studies utilizing this biomarker and clay report a stable half-life of albumin indicating a biomarker representative of a month of exposure and intervention trials that require one month to observe significant decreases. The current study showed a reduction in AFB_1-lys levels after seven days of treatment and two weeks between sampling. This short

duration of efficacy could be due to the significantly lower AFB_1-lys levels reported in this study population compared to those collected during outbreak times. Further studies are warranted to confirm the stability of albumin (lysine) as a biomarker of long-term exposure and/or appropriate interpretation of results from studies completed in less than 20 days (half-life of albumin). The data showed the reduction of AF biomarkers within a week. The intervention strategy is potentially applicable in an outbreak situation. However, this strategy should be validated at higher exposure levels to confirm efficacy.

In addition to the threat of acute exposure to AF, chronic exposure can increase the risk of developing HCC. In the United States, South Texas currently has the highest incidence of HCC, a disease that disproportionately affects Hispanic populations in the region. AFB_1 is present in a variety of foods in the U.S., including corn and corn products. In a randomized double-blind placebo controlled trial, the effects of a three-month administration of ACCS100 on AF exposure biomarkers and serum biochemistry were evaluated in 234 healthy men and women residing in Bexar and Medina County, Texas. Participants recruited from 2012–2014 received either a Placebo, 1.5 g, or 3 g ACCS100 each day for three months, and no treatment during the 4th month. Adverse event rates were similar across treatment groups; no significant differences were observed for serum biochemistry and hematology parameters. Differences in levels of AFB_1-lys adduct at 1, 3, and 4 months were compared between Placebo and active treatment groups. Although AFB_1-lys adduct levels were decreased by month 3 for both treatment groups, the Low dose was the only treatment that was significant ($p = 0.0005$). The return to baseline at month 4 supported the efficacy of ACCS100. A possible association with drought was

also observed as baseline AFB_1-lys levels were significantly higher in participants recruited in 2012, the year following the worst drought recorded in Texas. This finding highlights the vulnerability of populations in developed nations possibly due to effects of an unpredictable climate.

In summary, AF and FB exposure is a global and unavoidable public health concern. Moreover, it is a dynamic threat with the magnitude seemingly associated with climatic conditions and food insecurity, making prevention of exposure a difficult and moving target. Biomarkers are important tools for monitoring exposure. However, because modulators of metabolism can effect biomarker levels and measurements, more than one biomarker should be measured to evaluated exposure risk. The use of CM clay such as ACCS100 may be a viable strategy to reduce dietary AFB_1 bioavailability during AF outbreaks and can serve as a therapeutic option in populations chronically exposed to this carcinogen. Furthermore, CM clay delivery can be tailored to the recipient population (i.e., capsule, mixed in water, or added to food) allowing for increased acceptability and adherence to the treatment regimen which would translate to optimal protection.

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