BIOMARKERS OF EXPOSURE TO FOODBORNE AND ENVIRONMENTAL CARCINOGENS: ENTEROSORBENT INTERVENTION IN A HIGH RISK POPULATION

A Dissertation

by

NATALIE MALEK JOHNSON

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

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Toxicology

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Approved by:

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ABSTRACT

Biomarkers of Exposure to Foodborne and Environmental Carcinogens: Enterosorbent Intervention in a High Risk Population. (August 2010)

Natalie Malek Johnson, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Timothy D. Phillips

The need to assess human exposures to foodborne and environmental carcinogens, particularly in populations at high risk for cancer and disease, has led to the development of chemical-specific biomarkers. Sensitive biomarkers for aflatoxin and polycyclic aromatic hydrocarbons (PAHs) have been useful in providing information on population exposure and reducing associated public health impacts. Aflatoxins are fungal metabolites found in a variety of foods. Among these toxins, aflatoxin B_1 (AFB_1) is the most predominant and hepatocarcinogenic. Acutely, AFB_1 can cause disease and death, necessitating safe and effective intervention strategies. Inclusion of NovaSil (NS) clay in the diet represents a practical, sustainable approach. NS has been shown to prevent aflatoxicosis in multiple animal species by binding aflatoxins in the gastrointestinal tract, reducing toxin bioavailability. Co-exposure to PAHs, hazardous environmental contaminants, has been shown to increase the risk for hepatocellular carcinoma (HCC). Therefore, objectives of this research were to utilize biomarkers to assess aflatoxin and PAH exposures in susceptible populations in Ghana and the U.S. and to evaluate the safety and efficacy of NS intervention in Ghana (a population at risk for aflatoxicosis).

After 3-month intervention with 3.0g NS/day, median aflatoxin M_1 (an AFB_1 metabolite) was significantly reduced (up to 58%) compared to the placebo group. Furthermore, no significant differences were found in levels of nutrient minerals between NS and placebo groups at baseline and 3-months suggesting NS can be used to effectively sorb AFB_1 without affecting serum concentrations of important minerals. PAH biomarker results showed participants in Ghana were significantly exposed to high levels of PAHs based on the presence of 1-hydroxypyrene (1-OHP) in the majority of urines (98.9%). NS treatment had no effect on 1-OHP levels, further confirming the preferential binding of aflatoxins by NS. U.S. population data from a Hispanic community in Texas with an elevated incidence of HCC demonstrated a lower percentage and level of aflatoxin and PAH biomarkers. Aflatoxin M_1 excretion, however, was associated with increased consumption of certain foods prone to aflatoxin contamination; thus, some individuals may be more vulnerable to exposure and associated interactions that increase the risk for HCC (e.g., PAHs or hepatitis infection).

DEDICATION

This work is dedicated to my family. To my husband, James, whose love, assurance, and willingness to drive many miles has been a source of strength throughout my graduate education. To my Mom and Dad for their hard work, consistent prayers, and support for my education. My Mom's ability to excite students about the scientific method as a middle school teacher and my Dad's skill for solving problems has greatly influenced my love for science. To my sisters, Kate, Elissa, and Laura, and brothers, Jacob and Jeb, for their support, love, and friendship. Finally, to the research team and individuals from the communities within the Ejura-Sekyedumase District, who are my extended family, for their hospitality and participation that made this research possible.

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I. INTRODUCTION

In the past century, great strides have been made in the understanding of chemical carcinogenesis, expanding the field from a basic knowledge that chemicals were able to cause cancer to the development of technologies to assess human exposures and study molecular pathways (Loeb and Harris 2008). Much of what we know today can be attributed to initial research findings from epidemiological studies and basic research focusing on two groups of hazardous contaminants known as aflatoxins (AFs) and polycyclic aromatic hydrocarbons (PAHs). For instance, Yamagiwa and Ichikawa first experimentally induced cancer by applying coal tar to rabbits, confirming Pott's past observation of elevated cancer in chimney sweeps occupationally exposed to coal tar (Pott 1775; Yamagiwa and Ichikawa 1918). Within the next fifteen years, scientists identified PAHs as the carcinogenic material in coal tar and isolated benzo[*a*]pyrene, a model carcinogenic PAH (Kennaway 1930; Cook et al. 1933). In the 1960s, aflatoxin B_1 (AFB_1) was shown to be carcinogenic in rats (Barnes and Butler 1964). Following a compilation of model laboratory and epidemiology studies AFB_1 was established as a group 1 carcinogen (carcinogenic to humans) (IARC 1993). The vast amount of research on AFs and weight of evidence largely based on human exposure science makes AF a 'template' for environmental carcinogen research (Eaton and Groopman 1994).

Overall, advancements in chemical carcinogenesis spurred the development of chemical-specific biomarkers, giving scientists the ability to assess human exposures to

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This dissertation follows the style of Food Additives and Contaminants.

important foodborne/environmental carcinogens such as AFs and PAHs. Importantly, biomarkers can be used to identify populations at risk for exposures. Often, the incidence and impact of exposures are elevated in developing countries and underserved rural or metropolitan communities. For instance, people living in poverty are most likely to consume AF-contaminated foods, and therefore suffer the most severe effects, including disease and even death following acute exposure (Lewis et al. 2005). AF contamination in food products constitutes a serious burden in the developing world where a lack of untainted food supplies and poverty present a major and persistent challenge (McAlpin et al. 2002; Shephard 2003). In a world where the population of undernourished people surpasses one billion and an uncountable number of individuals daily face food insecurity (Fedoroff et al. 2010), detoxification strategies or approaches to remedy adverse effects from exposure to contaminated foods is of critical necessity. Furthermore, interactions with other environmental contaminants or biological factors have been shown to increase individual vulnerability to AF exposure. A classic example is the synergistic interaction between hepatitis B virus and AFB_1 in the development of hepatocellular carcinoma (HCC) (Ross et al. 1992; Henry et al. 2002). Epidemiological evidence has also suggested that concurrent exposure to additional environmental carcinogens, for example PAHs, can increase the risk of HCC development (Wu et al. 2007). While the amount and source of environmental PAH exposure varies widely from country to country, developing countries have been shown to account for the higher percentages of PAH emissions, for instance in the case of benzo[*a*]pyrene (Zhang and Tao 2008). Thus, the implementation of biomonitoring in populations at high risk for

cancer and disease may play an important role in providing information to help reduce the negative public health impacts of exposures.

1.1 Occurrence of aflatoxins

In the past five decades, over 8,500 articles have been published describing the occurrence and effects of aflatoxins (AFs). In turn, AFs have become one of the most well-known classes of mycotoxins, secondary metabolites of fungal growth. AF-contamination has had a major impact on food and feed industries worldwide presumably even before the original isolation of AF in the 1960s as the etiologic factor associated with turkey 'X' disease (Blount 1961). AFs are produced primarily by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Busby and Wogan 1984; Kurtzman et al. 1987). They have been characterized as unavoidable contaminants in a variety of commodities, mainly grain and nut crops. These most notably include maize, peanuts, cottonseed, and tree nuts (CAST 2003). AF production can occur in the field and in storage. Pre-harvest fungal contamination is influenced primarily by temperature and moisture and is favored when growth conditions range between 24 – 35ºC at moisture contents between 12 – 20% (Wilson and Payne 1994). Additional factors that increase crop susceptibility include plant genotype (Mehan et al. 1986), insect damage to the plant (Lynch and Wilson 1991), and drought (Sanders et al. 1993). Periods of drought in conjunction with high temperatures can greatly increase the production of AFs since damaged crops have increased infection sites that are vulnerable to aggressive wound pathogens like *A. flavus* (Diener et al. 1987). Therefore, populations living in areas with these environmental conditions may be at increased risk for AF exposure. In regions

between the latitudes 40º N and 40º S, termed the 'hot zone,' an estimated 4.5 billion people living in developing countries are chronically exposed to AFs (Williams et al. 2004). Additionally, post-harvest contamination is largely dependent on the adequate drying and storage of crops, i.e., relative humidity <85% (Wilson and Abramson 1992). The significant economic investment of drying/storage facilities may be not be feasible in developing countries. Poor housing conditions for crops that generate areas of local condensation (e.g., insect respiration, rodent activity, roof leaks, etc.) provide ideal conditions for *Aspergillus* growth and ensuing AF production (Wilson and Payne 1994). In addition to the occurrence of AFs in crops, lactating humans and animals have been shown to excrete toxic AF metabolites in milk (aflatoxin M_1) after consuming AFB_1-contaminated foods or feeds (Rodericks and Stoloff 1977; Sieber and Blanc 1978; El Nezami et al. 1995; Navas et al. 2005; Galvano et al. 2008). Subsequently, AF-contamination may occur in milk and dairy products (Jones 1995). The young are especially at risk due to their reliance on milk as a major source of nutrition.

1.2 Chemistry of aflatoxins

Upon isolation and identification of naturally-occurring AFs, the components were subdivided into two groups, B and G, due to their blue and green fluorescence, respectively, under ultraviolet light (Hartley et al. 1963). Major members of the AF group include four bisfuranocoumarin metabolites B_1, B_2, G_1, and G_2 (Figure 1), with AFB_1 being the most toxic and usually the most predominant (Jones 1977). These main congeners: AFB_1 ($C_{17}H_{12}O_6$), AFB_2 ($C_{17}H_{14}O_6$), AFG_1 ($C_{17}H_{12}O_7$), and AFG_2 ($C_{17}H_{14}O_7$) have molecular weights of 312, 314, 328, and 330, respectively.

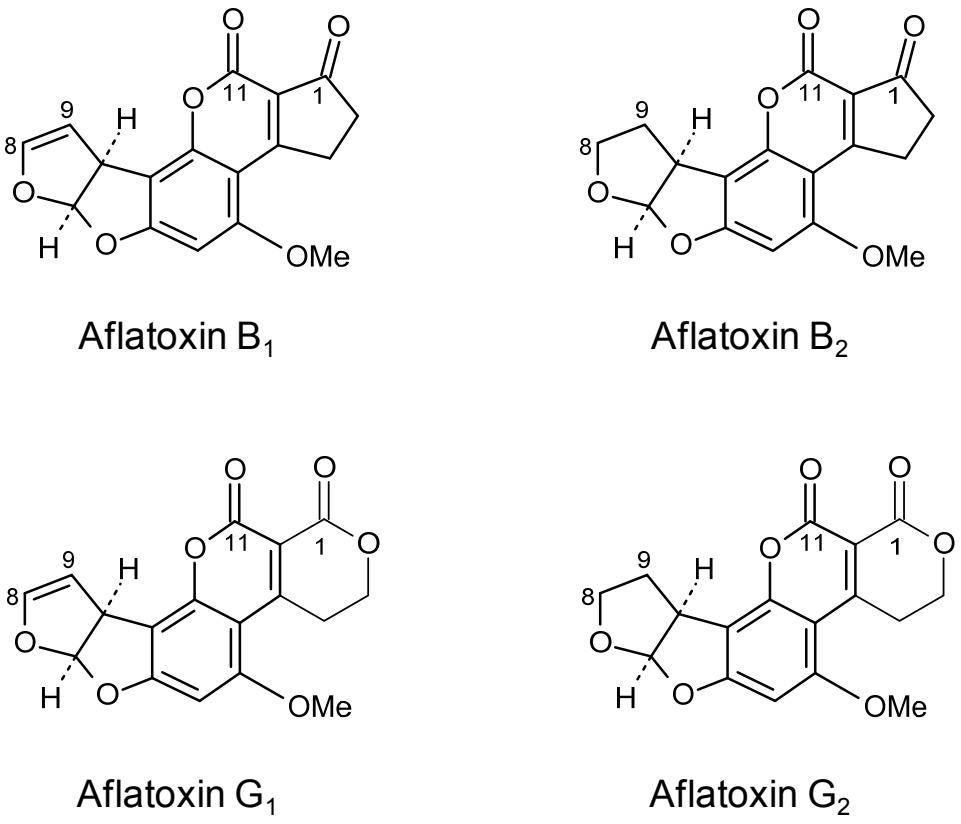

Figure 1. Chemical structures of commonly occurring aflatoxins B_1, B_2, G_1, and G_2. The series nomenclature denotes characteristic fluorescence emission under UV light: (B) blue or (G) green fluorescence.

Hydrogenation of AFB_1 and AFG_1 in the laboratory yields the less carcinogenic species AFB_2 ($C_{17}H_{14}O_6$), and AFG_2 ($C_{17}H_{14}O_7$), which lack the double bond at the C8,9 position (van der Merwe et al. 1963). When in the presence of inorganic acids, acid-catalyzed hydration of the C8,9 double bond in AFB_1 and AFG_1 results in the hemiacetal forms, AFB_{2A} and AFG_{2A}, respectively (Ciegler and Peterson 1968; Pohland et al. 1968). In various animal species, AFB_1 and AFB_2 can be hydroxylated at the 9a position to yield AFM_1 and AFM_2, which were originally designated as M due to their discovery in milk (Holzapfel et al. 1966). Importantly, all of the AFs are heat stable, surviving temperatures up to melting points which range from 237 to 299°C, depending on the species (Jones 1977). Therefore, treatment of contaminated foods according to normal food processing practices is not sufficient for decontamination. Under the alkaline conditions employed during nixtamalization (the preparation of maize in lime solution containing calcium), hydrolysis of the lactone ring occurs (Jones 1977). Disruption of the compound's aromaticity renders it undetectable by common analytical methods that rely on fluorescence for detection; however, this reaction is reversible in acidic conditions (De Iongh et al. 1962). Therefore, reports originally describing the process of nixtamalization as a detoxification method should be carefully evaluated since the original structure of the toxin is restored in the acidic conditions of the stomach (Price and Jorgensen 1985). "Detoxification" by ammoniation has been utilized in California and Arizona to decrease parent AF levels in cottonseed intended for dairy cows (Park et al. 1988). These methods usually are non-reversible, as they proceed at elevated temperatures and pressures, and decarboxylation follows hydrolysis of the lactone ring.

The reaction may proceed further to yield AFD_1 and a product with a molecular weight of 206 (Kiermeier and Ruffer 1974). Due to the potential of detoxification products to remain toxic and carcinogenic, the U.S. Food and Drug Administration (FDA) has not approved this strategy.

1.3 Toxicology of aflatoxins

Among the AFs, the most prevalent congener, AFB_1, is also the most toxic (Carnaghan et al. 1963). Wogan et al. (1971) reported median lethal dose (LD_{50}) values of 0.73, 1.18, 1.76, and 2.83 mg/kg body weight for AFB_1, AFG_1, AFB_2, AFG_2, respectively, in a sensitive duckling model. In addition to AFB_1, Holzapfel et al. (1966) showed that ducklings were extremely sensitive to AFM_1, an AFB_1 metabolite, with LD_{50} values ranging from 12 to 16 μg/animal. AFB_1 is also the most carcinogenic of the AFs (Wogan and Newberen 1967); thus, the majority of discussion will focus on the toxicology of AFB_1. The most common route of exposure is oral via consumption of contaminated foods, although occupational exposure to inhaled AFB_1 in contaminated grain dust may also occur (Sorensen et al. 1981). Animal studies have shown that approximately 50% of an orally administered dose of AFB_1 is rapidly absorbed from the duodenal region of the small intestine (Coulombe and Sharma 1985). AFB_1 is concentrated in the liver (and to a lesser extent in the kidney) via the portal vein (Wilson et al. 1985). Metabolism of AFB_1 yields a variety of metabolites, many of which have been used as biological markers of exposure (Wild and Turner 2002). Figure 2 illustrates AFB_1 metabolism by various phase I and phase II enzymes and highlights metabolite biomarkers in the blood and urine.

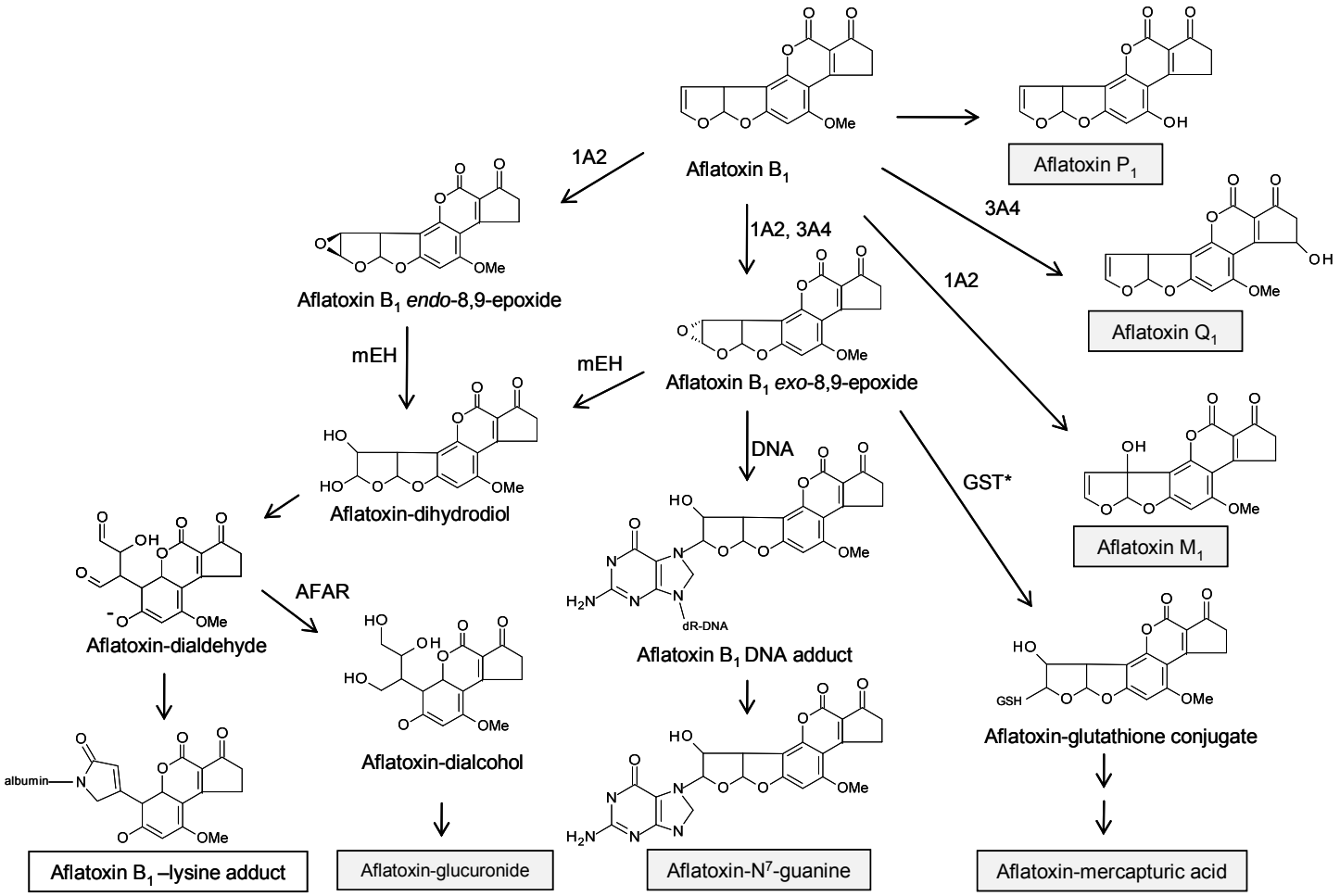

Figure 2. Metabolism of aflatoxin B_1 by phase I and phase II enzymes. Phase I enzymes include CYP 3A4 and 1A2. Detoxification of epoxide metabolites occurs via microsomal epoxide hydrolases (mEH) and glutathione-S-transferases (GST). *GSTs also conjugate the *endo*-epoxide. Aflatoxin aldehyde reductase (AFAR) acts upon the AF-dialdehyde. Biomarkers are highlighted in blood (white box) and urine (gray box). Adapted from Wild and Turner 2002.

Biotransformation of AFB_1, predominantly by human cytochrome P450 (CYP) enzymes 3A4 and 1A2 in the liver primarily leads to formation of the AFB_1 *exo*-8,9-epoxide over the *endo*-8,9-epoxide (Gallagher et al. 1994; Guengerich et al. 1998). The highly unstable *exo*-epoxide is the ultimate AF carcinogen and forms covalent DNA adducts, primarily at the N-7 position of guanine residues (Essigman et al. 1983; Gopalakrishnan et al. 1990). This adduct can either undergo cleavage (to be excreted in the urine as aflatoxin-N^7-guanine) or rearrange to a foramidopyrimidine (FAPY) adduct (Croy and Wogan 1981). Ultimately, this adduct triggers a G → T transversion, which may result in genetic alterations that enhance malignant transformation of affected cells (Smela et al. 2001). The epoxide ring in the *endo* isomer (unlike the *exo*-form) is positioned above the plane, in *trans* to the 5a and 9a protons, hindering its reaction with guanine (Raney et al. 1993). Other metabolic pathways, in addition to expoxidation, involve the direct hydroxylation of AFB_1 by CYP enzymes. Biotransformation by CYP 3A4 and 1A2 yields AFQ_1 and AFM_1 metabolites respectively, which are excreted in urine (Coulombe 1993). Additionally, O-demethylation of AFB_1 produces the urinary metabolite AFP_1 (Eaton et al. 1994). All three of these hydroxylation products have been identified in human urine (Groopman et al. 1985).

Detoxification pathways may reduce DNA adduct formation, and an important mechanism is through the conjugation of reactive epoxide metabolites to glutathione (GSH) by glutathione S-transferase (GST) enzymes (Raney et al. 1992). Upon conjugation with GSH, peptidases and acetylases, in order to salvage amino acid residues, convert the AF-GSH conjugate to AF-mercapturic acid, which is excreted in

urine (Wild and Turner 2002). Hydrolysis of the AFB_1-8,9-epoxide by microsomal epoxide hydrolases (mEHs) has also been shown; however, there is competition from rapid non-enzymatic hydrolysis (Johnson et al. 1997; Guengerich et al. 1998). In either case, hydrolysis yields a dihydrodiol that undergoes base-catalyzed ring opening rearrangement to form a dialdehyde (Roebuck et al. 1978). This product can be further metabolized by AF aldehyde reductase, which reduces the dialdehyde to a dialcohol that is then glucuronidated prior to urinary excretion (Hayes et al. 1993). Alternatively, the dialdehyde can covalently react with proteins (e.g., albumin) by binding primary amines through a Schiff base mechanism (Sabbioni et al. 1987). For instance, the AFB_1-lysine albumin adduct, a metabolite in peripheral blood, consists of an AF metabolite bound to the epsilon amino group in lysine.

The carcinogenicity and toxicity of AFB_1 in various animal species is largely dependent on variances in metabolism. For instance, an almost 100,000-fold difference in susceptibility to AF-induced hepatomas is evident in more sensitive trout and rat species compared to resistant mice strains. At oral doses as high as 10,000 ppb mice do not develop liver cancer; whereas, levels as low as 15 ppb can produce a significant increase in liver tumors in rats (Wogan 1973). Differences in GST expression account for most of the variation between species. For instance, mice constitutively express an *alpha*-GST isoform (mGST A3-3), which has a high catalytic activity toward the AFB_1 8,9-epoxide; thus, mice conjugate this epoxide up to 50 times faster than rats (Eaton and Gallagher 1994). Conversely, rats express a related form of GST with much less activity, increasing the lifetime of the ultimate carcinogen. Furthermore, mice readily conjugate

the *exo*-epoxide with GSH while rat and human *alpha*-GSTs do not readily conjugate the *exo*- or *endo*-epoxide (Wang et al. 2002). Human μGSTs can conjugate the epoxide; however, the *endo* isomer is preferred (Wang et al. 2000). Therefore, it is suggested that humans may be as sensitive to AF-induced hepatomas as rats.

1.4 Biomarkers of exposure to aflatoxins

In order for a toxicant to cause an effect, there must first be an exposure. Early work estimating human exposure to AFs relied on measurements from contaminated foods. However, due to the large variability associated with measuring AF levels in bulk food matrices (Whitaker and Park 1994) and reliance on individual dietary recall, the application of AF measurement in body fluids via immunochemical methods became a more useful method to assess biologically relevant exposure. AF-specific biomarkers currently used as measures of individual exposure include AFB_1 metabolites and AFB_1-macromolecular adducts (i.e., AFM_1 in urine and AFB_1-lysine albumin adduct in serum) (Groopman et al. 1994, 1996; Wang et al. 2001a).

1.4.1 Aflatoxin M_1 metabolite in urine

AFM_1, a major oxidative metabolite of AFB_1, is a reliable biomarker reflective of internal dose. The excretion of AFM_1 represents recent exposure (i.e., within 24 to 48 hours) (Groopman et al. 1988; Cheng et al. 1997). Using a monoclonal antibody immunoaffinity/high performance liquid chromatography (HPLC) method, Groopman et al. (1992) reported that in rats dosed with AFB_1, AFM_1 accounted for the highest percentage of total urinary AF metabolites (34.5), followed by AF-N^7-guanine (9.6), AFP_1 (8.0), AFQ_1 (1.8), and AF-dihydrodiol (1.5). Furthermore, AFM_1 was found to be

an excellent marker linearly associated with AFB_1, whereas AFP_1 was not found to be linear with excretion. AFM_1 is also the major AFB_1 metabolite in monkeys after i.v. dosing (0.3 mg AFB_1/kg) and oral administration of [^{14}C]AFB_1 (0.4 or 0.015 mg AFB_1/kg) (Dalezious et al. 1973; Wong and Hsieh 1980). Urinary AFM_1 has been further characterized and validated in multiple human populations, largely in Asia and Africa (Table I). Campbell et al. (1970) originally reported AFM_1 as the major hydroxylated AFB_1 metabolite in urine samples from Filipinos exposed to peanut butter containing high levels of AFB_1 (~0.5 mg/kg). The predominance of AFM_1 in urine was confirmed in a large number of samples collected from individuals in Zimbabwe (Nyathi et al. 1987). Moreover, Zhu et al. (1987) measured AFM_1 in a Chinese population frequently exposed to dietary AFs and found a positive correlation (r = 0.65) between AFB_1 intake and AFM_1 excretion over a three day period. Approximately 1.2 to 2.2% of AFB_1 from the diet was excreted as urinary AFM_1, similar to excretion rates reported to range from 1 to 4% (Campbell et al. 1970; Nyathi et al. 1987). In addition to measuring exposure, AFM_1 detection has aided in identifying populations at risk for hepatocellular carcinoma (HCC). For instance, the risk of HCC development was shown to be significantly increased (by 4.4-fold) in individuals with detectable urinary AFM_1 levels (Qian et al. 1994). In agreement with these data, Sun et al. (1999) reported a 3.3-fold increase in the relative risk of HCC in subjects with detectable AFM_1 in a cohort of Chinese men chronically infected with hepatitis B virus (HBV). Importantly, findings confirmed that AF exposure accounted for a substantial portion of HCC risk in individuals with chronic HBV.

Table I. Documented ranges of urinary aflatoxin M_1 levels in humans.

Country	Exposure rate (n)[a]	Reported range	Reference
Indonesia	38% (68 adults)	0 – >1000 pg/100 ml urine	Tsuboi et al. 1985
Philippines	27% (52 adults)	0 – 968 pg/100 ml urine	Tsuboi et al. 1985
Japan	8% (40 adults)	0 – 66 pg/100 ml urine	Tsuboi et al. 1985
Nigeria	9% (161 adults)	0.89 ng/100 ml urine[b]	Bean et al. 1989
China (Shanghai)	21% (317 males)	0.17 – 5 .2 ng/ml urine	Qian et al. 1994
China (mainland)	64% (138 males)	0 –108 ng/12h urine	Cheng et al. 1997
Taiwan	66% (32 males)	0 – 17 ng/12h urine	Cheng et al. 1997
Sierra Leone	41% (134 boys)[c]	0.5 – 374 ng/ml urine	Jonsyn-Ellis 2000
Sierra Leone	44% (110 girls)[c]	2.3 – 34 ng/ml urine	Jonsyn-Ellis 2000
Sierra Leone	43% (97 boys)[d]	0.1 – 35 ng/ml urine	Jonsyn-Ellis 2000
Sierra Leone	59% (93 girls)[d]	0.3 – 124 ng/ml urine	Jonsyn-Ellis 2000
China (Guangxi)	89% (27 adults)	0.9 – 3569.7 ng/24h urine	Wang et al. 2001b
Ghana	91% (91 adults)	0.01 – 17.24 ng/ml urine	Jolly et al. 2006
Egypt	8% (50 children)	5.0 – 6.2 pg/ml urine	Polychronaki et al. 2008
Guinea	64% (50 children)	8.0 – 800 pg/ml urine	Polychronaki et al. 2008

[a]Total number of people in study; [b]Average concentration; [c]Dry season; [d]Rainy season.

1.4.2 Aflatoxin B_1-lysine albumin adduct in serum

The AFB_1-lysine albumin adduct (or AF-albumin adduct) in serum represents a chronic biomarker of biologically effective dose reflecting integrated exposures over two to three months due to the 21-day half-life of serum albumin (Sabbioni et al. 1990; Wang et al. 1996a). In humans, approximately 2% of ingested AFB_1 has been reported to be covalently bound to serum albumin (Gan et al.1988), similar to the percentage (1-3%) observed in rats dosed with AFB_1 (Wild et al. 1996). Like AFM_1, positive correlations between AFB_1 intakes and AF-albumin adduct levels have been observed from several regions of the world (Gan et al. 1988; Wild et al. 1990). Furthermore, epidemiological studies have shown a relationship between AF-albumin adduct levels and increased HCC risk in China (Groopman et al. 1996; Kuang et al. 1996; Wang et al. 2001b), Taiwan (Chen et al. 1996; Wang et al. 1996b; Lunn et al. 1997), and Africa (Allen et al. 1992). The AF-albumin adduct has also been used as a biological response indicator of advanced liver disease in hepatitis C virus patients in Taiwan (Chen et al. 2007) and aflatoxicosis in Africa (Azziz-Baumgartner et al. 2005).

1.5 Consequences of aflatoxin exposure

Adverse effects due to animal and human AF exposure have resulted in significant economic, agricultural, and public health consequences. Human health hazards may come in the form of primary or secondary mycotoxicoses, depending on if the exposure is from direct consumption of AF-contaminated foods or from animal by-products, such as AF metabolites in milk (Samson 1992). The dose and duration of exposure affect the resulting consequences. Short-term high dose exposure results in

acute toxicity, usually in the form of liver cirrhosis, which may cause illness or possibly death, whereas chronic sublethal doses can have nutritional and immunological consequences (Williams et al. 2004). All doses can have a cumulative effect on cancer risk (Roebuck and Maxuitenko 1994).

1.5.1 Acute toxicity

Acute exposure to high levels of AFB_1 via the diet causes disease (aflatoxicosis) and death in animals and humans. Signs of aflatoxicosis usually include acute hepatic necrosis, bile duct proliferation, edema, and lethargy (Cullen and Newberne 1994). The sensitivity of various avian species has resulted in notable outbreaks in the poultry industry. Historically, the epizootic outbreak in Britain in 1960, which resulted in the death of over 100,000 turkeys, emphasized the significant impact of the AF problem (Lancaster et al. 1961). Subsequently, a number of studies determined a range of acute toxicities for AFB_1 in multiple animal species. Susceptible species such as duck (0.36 mg/kg), rabbit (0.30 mg/kg), and trout (0.81 mg/kg) have much lower LD_{50} values compared to more resistant species such as chickens (18.0 mg/kg) and mice (60 mg/kg) (Wogan 1973; Cullen and Newberne 1994). Nonetheless, at high enough concentrations AFB_1 acts as an acute poison targeting the liver. Massive hepatotoxicity (necrosis and hemorrhage) has been identified in the wake of AF outbreaks in the poultry and livestock industries (Osweiler and Trampel 1985; Choudary 1986; Van Halderen et al. 1989). In addition, acute aflatoxicosis in dogs, due to contamination of commercially available feeds, has been documented in Texas (Garland and Reagor 2001) and the eastern U.S (Stenske et al. 2006). In western India, an epizootic outbreak in dogs

preceded human deaths from consumption of heavily molded corn (van Rensburg 1977; Shank 1981). Corn containing 6 to 16 mg AF/kg corn was consumed in over 200 villages, and mortalities occurred in 100 of 400 patients examined. Evidence of clinical aflatoxicosis has also been reported in Taiwan and Uganda (Shank 1977, 1981), characterized by abdominal pain, vomiting, pulmonary edema, fatty liver infiltration, and liver failure (Ngindu et al. 1982). Recently in Kenya, 125 deaths out of 317 cases of acute aflatoxicosis from January to July, 2004 was linked to the consumption of meals prepared from locally grown and poorly stored maize contaminated with AF levels as high as 8,000 ppb (Azziz-Baumgartner et al. 2005; Lewis et al. 2005). This severe outbreak reaffirmed the AF problem as a contemporary issue, especially in populations living in conditions that may necessitate the consumption of lower quality foodstuffs.

1.5.2 Growth retardation and impact on vitamins and nutrients

A strong relationship between AF exposure and growth impairment has been established in many studies with domestic animals. For instance, significantly decreased body weight gains and/or reductions in feed conversion efficiencies have been reported in Leghorn and broiler chicks (Kubena et al. 1990, 1993b; Huff et al. 1992), turkey poults (Kubena et al. 1991), weaning piglets (Lindemann et al. 1993; Marin et al. 2002), and growing barrows (Harvey et al. 1989, 1994a) and lambs (Harvey et al. 1991a) following exposure to AF-contaminated feed. A similar effect has been shown in humans during periods of development. For instance, Gong et al. (2002) demonstrated a correlation between decreased z-scores (reflective of growth stunting and underweight) and serum AF-albumin adducts in children from Benin and Togo. *In utero* AF exposure

has also been shown to cause growth faltering in infants in The Gambia, signifying the vital impact of early life exposure (Turner et al. 2007). In addition to growth suppression, the impact of AF on vitamins and micronutrients represents a major public health concern, particularly in developing countries where malnutrition or nutritional deficiencies are common. Work in animal models and human populations have indicated that AFs may affect important fat-soluble vitamins A, D, and E. For example, hepatic vitamin A concentrations in broiler chicks were decreased with increasing concentration of AFs (0, 500, and 2000 ppb) (Reddy et al. 1989) and significantly reduced following dosing with 5000 ppb AFB_1 (Pimpukdee et al. 2004). Additionally, Harvey et al. (1994b) reported AF exposure in growing barrows resulted in decreased retinol concentrations; authors suggested that AF exposure may exacerbate vitamin A deficiencies. Human vitamin A modulation as a function of AF exposure has been somewhat inconsistent, although the literature is limited. Earlier work showed no relationship between vitamin A and AF-albumin adduct levels in Gambian children (Turner et al. 2003); however, recent work in an adult Ghanaian population illustrated a strong correlation between AF–albumin adduct levels and decreased serum vitamin A (Tang et al. 2009). Further work is needed to clarify the role of AFs in reducing vitamin A levels in humans. Moreover, the use of AF-albumin adducts as a measure of biological response for vitamin A should also be further assessed. Similarly, vitamin D and E levels are affected by AF exposure. For example, vitamin D concentrations in broiler chicks were significantly reduced following 5-day treatment with 1000 ppb AFs (Glahn et al. 1991). An inverse relationship between AF exposure and levels of serum vitamin E has also

been shown in growing barrows (Harvey et al. 1994b) and in the same Ghanaian population, wherein Tang et al. (2009) showed a correlation between decreased serum vitamin E levels in individuals with high AF-albumin adducts. Since vitamins A, D, and E play import roles in immune system competence, nutritional interference of AFs may impact normal, healthy immune function. Likewise, multiple micronutrients are critical to health and immunity. Reports in laboratory and farm animals have shown AF exposure interferes with the nutrition of iron (Fe), zinc (Zn), and selenium (Se) (Harvey et al. 1988; Dimri et al. 1994; Kalorey et al. 1996; Mocchegiani et al.1998; Hegazy and Adachi 2000; Williams et al. 2004). For example, Mocchegiani et al. (1998) documented reduced levels of serum Zn (56.0 μg/dL versus 119.2 μg/dL in controls) in piglets exposed to AFB_1 *in utero*. Consequently, the cellular immune status of exposed piglets was impaired, mainly due to decreased activated thymulin. A similar relationship between decreased concentrations of serum Zn and immune system impairment has been shown in rats in rats following AF exposure (Doyle et al. 1977; Ikegwuonu et al. 1985). Furthermore, Hegazy and Adachi (2000) demonstrated an analogous effect of AFs on Se levels and lowered immunity. In humans, AF-albumin levels have been show to be inversely correlated with Se concentrations in Chinese men (Chen et al. 2000). Although the role of AF exposure on Zn and Fe is not certain in humans, animal data suggest AFs may promote deficiencies, particularly in the young.

1.5.3 Immunosuppression

Cell culture work and animal models have demonstrated that AF is an immunotoxic agent that primarily suppresses cell-mediated immune responses and

phagocytic cell function (Bondy and Pestka 2000). While a limited number of studies to date have assessed the immunologic affect of AFs in humans, three recent studies conducted in West Africa have shed some light on the role of chronic AF exposure on the human immune system. For instance, Turner et al. (2003) reported decreased secretory immunoglobulin A levels in saliva of Gambian children with detectable AF-albumin adduct levels. Results from an adult population in Ghana demonstrated a marked effect on cellular immune system components and functions in participants with high AF-albumin adduct levels (Jiang et al. 2005). Authors hypothesized that changes in immunological parameters as a result of AF exposure may increase individual susceptibility to infection. This is in concordance with previous animal work showing decreased resistance to infectious diseases (Hamilton and Harris 1971; Edds et al. 1973; Wyatt et al. 1975; Cysewski et al. 1978; Joens et al. 1981) and induced reactivation of chronic infection (Venturini et al 1996; Kubena et al. 2001) following AF exposure. Further work by Jolly and co-workers investigated the possible interaction of AF and the human immunodeficiency virus (HIV) on immune suppression (Jiang et al. 2008). In this report, high AF-albumin adduct levels were associated with lower perforin expression on CD8+ cells among all patients regardless of HIV status. However, HIV+ patients with high AF-albumin adducts had significantly lower percentages of CD4+ T-regulatory-cells, naive CD4+ T-cells, and B-cells compared to HIV+ participants with low AF-albumin adducts. The immunotoxic effects of AFs in humans should be further explored due to the paucity of data and the important role that AFs may play in modulating susceptibility to infectious disease.

1.5.4 Carcinogenicity

Perhaps one of the most recognized consequences of chronic AF exposure in humans is development of hepatocellular carcinoma (HCC), which is the most common form of primary liver cancer and represents the third leading cause of cancer deaths worldwide (600,000 deaths annually) (Parkin et al. 2001). Liu and Wu (2010) assessed the global burden of HCC attributable to AF exposure and found that of the 550,000-600,000 new HCC cases worldwide per year, AF may play a causative role in up to 155,000 cases (28.2% of all global HCC cases). The prevalence of HCC is substantially higher in developing versus developed countries. Wild and Hall (2000) estimated that 80% of all HCC cases occur in developing countries. Historically, studies conducted in Asian and African populations demonstrated a positive correlation between HCC incidence, largely based on cancer registry data, and high AFB_1 intake, calculated from analysis of foods consumed. For example, Bulatao-Jayme et al. (1982) showed that the relative risk (RR) for liver cancer when consuming a high level of AFs plus a minor amount of alcohol was 17.5, as compared with a RR of 3.9 when alcohol consumption was heavy and AF intake was low. Based on these types of epidemiological data and a large body of work in experimental animals, AFB_1 was designated as a known human carcinogen (group 1) by the International Agency for Research on Cancer in 1993 (IARC 1993). A serious confounder in these studies however, was the high rate of hepatitis B virus (HBV) in these populations. In areas of high HCC incidence, such as sub-Saharan Africa, China, and Southeast Asia, HCC occurrence is closely related to HBV infection (Turner et al. 2002). Later studies involving the measurement of AF-

specific molecular biomarkers (i.e., AFM_1 and AF-albumin adduct) and careful consideration of HBV status helped elucidate the role of AFs and HBV in HCC development. For instance, Ross et al. (1992) demonstrated a strong interaction between serological markers of chronic HBV infection, i.e., the HBV surface antigen (HBsAg), and urinary AF metabolites in liver cancer risk. In addition, HCC risk was remarkably elevated (RR = 59.4) in HBsAg+ individuals who excreted elevated levels of urinary AF metabolites when compared with HbsAg+ individuals with non-detectable AF exposure (RR = 7.3) (Qian et al. 1994). Subsequent findings, described previously in this section, strengthened the conclusion that AF was a causative agent for HCC and co-infection with HBV significantly increased HCC risk (Sun et al. 1999). Experiments in HBV-transgenic mice and woodchucks infected with woodchuck hepatitis virus recapitulated the synergistic effect seen in epidemiological studies (Sell et al. 1991; Bannasch et al. 1995). Although the mechanisms of interaction between AF and HBV in increasing hepatocarcinogenicity are not certain, a variety of reasons have been proposed including: 1) chronic HBV infection may induce activation of AFB_1 to the mutagenic epoxide, thereby increasing the opportunity for mutation; 2) the generation of reactive oxygen species and inflammation resulting from HBV infection may have promoter-like activity, increasing the chance of clonal expansion of mutated cells; and 3) HBV may suppresses DNA repair mechanisms that may select for carcinogenesis from AFs (reviewed by Kew 2003). A recent study indicated HBV infection significantly contributes to oxidative stress, shown by the oxidative stress biomarker 8-OHdG, in a population chronically exposed to AFs, which may contribute to HCC (Liu et al. 2008).

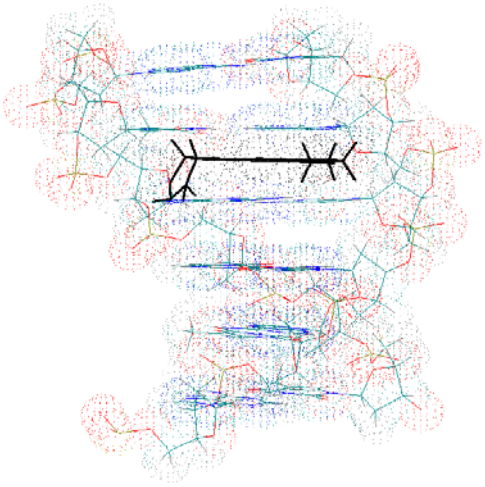

Figure 3. Molecular model of aflatoxin B_1 intercalated between strands of DNA.

1.5.5 Biochemical mode of action

The ability of AFB_1 to act as an initiator stems from its bioactivation to a direct-acting mutagen. Essigmann et al. (1977) first identified the most frequent AF-DNA adduct, the 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB_1 adduct, modeled above (Figure 3). Importantly, AFB_1 disrupts several genes involved in the progression of cancer. In rats dosed with AFB_1, a high expression of the activated forms of c-H-ras and c-myc proto-oncogenes are observed in hepatic tumors (McMahon et al. 1987). Furthermore, in cultured human hepatocytes AFB_1 produces a G:C to T:A mutation in the third base of codon 249 in the p53 tumor suppressor gene (Aguilar et al. 1993). This mutation has been identified in tumors at a high frequency in populations exposed to high levels of dietary AFs (Greenblatt et al. 1994); whereas, few such mutations have been observed in HCC of patients residing in regions of low AF exposure. This mutational hot-spot has been recognized as a 'molecular fingerprint' linking initial AFB_1 genotoxicity to the ultimate progression to HCC. Benzo[*a*]pyrene (B*a*P), a model carcinogenic PAH,

induces tumor formation using metabolic pathways similar to that observed for AFB_1; B*a*P is metabolized to an epoxide (B*a*P 7,8-diol-9,10-epoxide) which forms DNA adducts primarily at nitrogen residues of guanine bases, which is an initial step for the malignant transformation of cells (Ross and Nesnow 1999; Miller and Ramos 2001). DNA adducts resulting from AFB_1 and B*a*P are illustrated in Figure 4. In addition, B*a*P-induced G:C to T:A transversions in nucleotide residues of the p53 gene have been identified in human lung cancers (Puisieux et al. 1991). Thus, due to similarities of B*a*P and AFB_1 in initiating cancer, it was hypothesized that populations frequently exposed to dietary AFs may be affected by a concurrent exposure to PAHs.

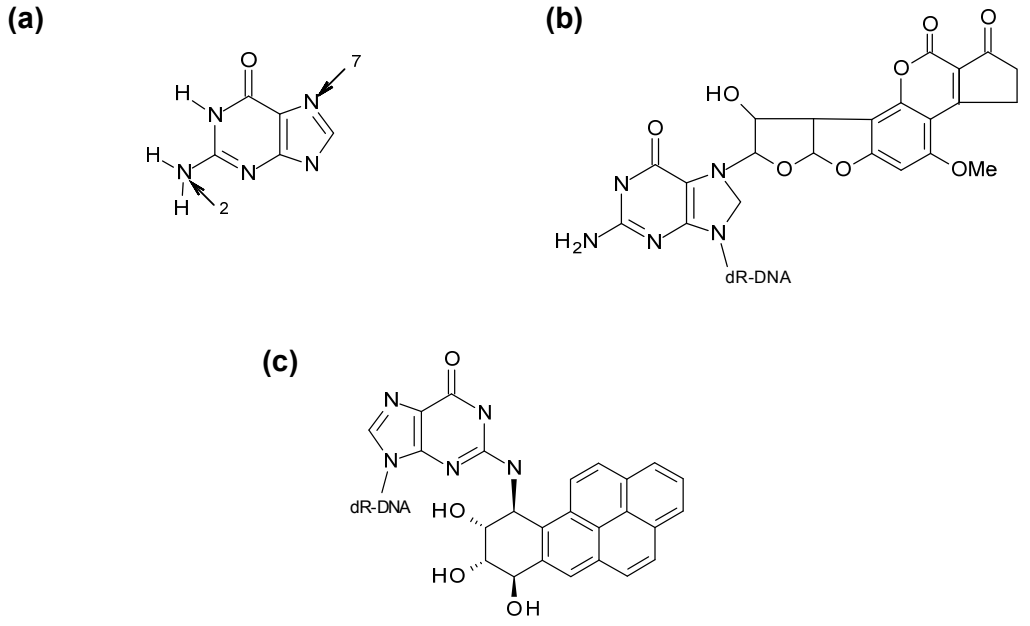

Figure 4. Similarity of primary aflatoxin B_1 and benzo[*a*]pyrene DNA adducts at nitrogen residues of guanine bases. AFB_1 and B*a*P primarily alkylate DNA at the N-7 and N-2 positions of guanine (a) to yield the principal adducts shown in (b) and (c), respectively.

1.6 Occurrence of polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs), also known as polynuclear aromatic hydrocarbons, are a class of unavoidable environmental pollutants produced from the incomplete combustion of organic material (Samanta et al. 2002). PAHs represent a group of several hundreds of chemical compounds composed of fused aromatic rings. Representative PAH structures are illustrated in Figure 5. Collectively, PAHs may be produced from natural and anthropogenic activities; complex mixtures have been shown to occur in the environment (e.g., forest fires, urban air, automobile exhaust, and tobacco smoke); in foods (e.g., charbroiled and smoked foods); and in industrial settings (e.g., during processing of coal and coal coking and in aluminum and asphalt production plants) (Nikolaou et al. 1984). In many developing countries, especially in rural areas, indoor wood burning cook stoves or open fires are often employed as the primary means of cooking and heating. According to a World Health Organization report assessing the global burden of disease (WHO 2008), breathing smoke from traditional cook stoves and open fires may have caused as many as 1.96 million premature deaths worldwide in 2004. Although multiple components make up the list of harmful constituents in smoke (e.g., carbon monoxide, particulate matter, volatile organic compounds, etc.), it has been well documented that the combustion of wood and other fuels for cooking constitutes a significant source of PAHs (Knight and Humphreys 1985; Lioy and Greenberg 1990). This suggests that individuals continuously exposed to smoke from these cooking methods may be prone to increased PAH exposure.

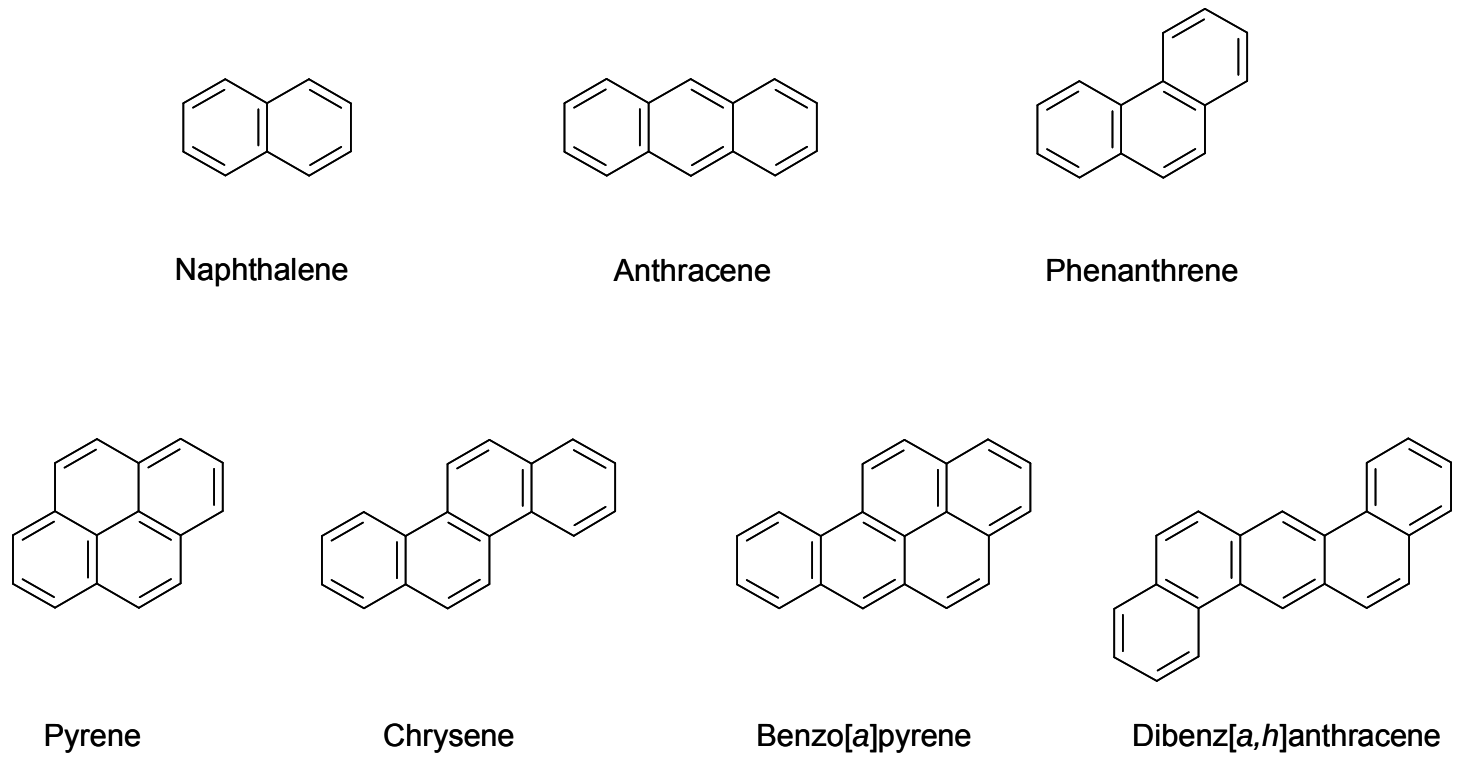

Figure 5. Chemical structures of representative PAH compounds. PAHs vary based on the number (i.e., molecular weights) and arrangement (i.e., linear, angular, clustered) of rings. Delocalization of pi-electrons leads to the resonance stabilization.

1.7 Chemistry of PAHs

PAHs are planar, lipophilic, non-polar molecules composed of two or more benzene rings that share two or more carbon atoms (Vollhardt 1987). PAH compounds and their chemistry vary based on the number (i.e., molecular weights) and arrangement of rings (i.e., linear, angular, clustered). Various chemical characteristics of representative PAH compounds are illustrated in Table II. Larger molecular weight compounds are generally less water soluble and less volatile. Partition coefficients between octanol and water (log K_{OW}) are high, which greatly influences their bioaccumulation. The well-characterized PAH, benzo[*a*]pyrene (B*a*P), consists of five fused rings (Figure 5) and has a molecular weight of 252 (Klaunig and Kamendulis 2007); B*a*P has a log K_{OW} of 5.97 cm^3/g (HSDB 2000). Also shown in Figure 5, pyrene, a lower molecular weight PAH (MW = 202), consists of four aromatic rings and has a log K_{OW} of 4.88 cm^3/g (Lide 1998). Both of these representative PAHs fluoresce at characteristic wavelengths and have strong UV absorptions. For instance, the absorption maxima (and extinction coefficient) for pyrene in cyclohexane are as follows: 273 nm (log ε = 4.77), 306 nm (log ε = 5.07), 320 nm (log ε = 4.51), and 335 nm (log ε = 4.78) (Lide 1998). Delocalization of pi-electrons leads to the resonance stabilization of PAH compounds. While their aromaticity decreases reactivity, PAHs are not completely unreactive. For instance, PAHs can be degraded by photochemical oxidation, biodegradation by microorganisms, and metabolism in higher biota (discussed in detail in the next section) (Douben 2003).

Table II. Chemical characteristics of PAH compounds.

PAH compound	No. of rings	MW	S^a (g/m^3 or mg/L)	Log $(K_{OW})^b$
Naphthalene	2	128	31	3.37
Phenanthrene	3	178	1.1	4.57
Anthracene	3	178	0.045	4.54
Pyrene	4	202	0.132	5.18
Benz[*a*]anthracene	4	228	0.011	5.91
Chrysene	4	228		5.86
Benzo[*b*]fluoranthene	5	252	0.0015	5.80
Benzo[*k*]fluoranthene	5	252	0.0008	6.00
Benzo[*e*]pyrene	5	252	0.004	
Benzo[*a*]pyrene	5	252	0.0038	6.04
Dibenz[*a*,*h*]anthracene	5	278	0.0006	6.75

[a] Water solubility; [b]Octanol–water partition coefficient; Table adapted from Douben 2003.

1.8 Toxicology of PAHs

Most exposure to PAHs occurs via the airways, skin and digestive tract. While metabolic activation primarily occurs in the liver, the slow absorption through most epithelial cells can lead to the induction of CYP enzymes at the site of entry, i.e., through activation of the aryl hydrocarbon receptor (AhR) (Swanson 2004). This uneven dose distribution may explain the high propensity of PAHs to act as carcinogens at the sites of entry (IARC 2010). B*a*P is one of the most comprehensively studied PAHs; due to the vast amount of research conducted, the majority of discussion will focus on the toxicology of this specific PAH. B*a*P is readily absorbed following oral, dermal, and inhalation routes of exposure (ATSDR 1990). In rats dosed with 250 mg B*a*P in the diet, 60% of the toxin was absorbed across the gastrointestinal tract; furthermore, absorption was shown to be enhanced when administered in a lipophilic vehicle (Ekwall et al. 1951). Following dermal administration in several animal models, the applied dose permeated the skin at levels ranging from 0.1% in guinea pigs to 10% in mice (Kao et al. 1985). After inhalation, B*a*P was rapidly distributed to several tissues, with highest levels found in the liver, esophagus, small intestine, and blood 30 min after exposure (Sun et al. 1982; Weyand and Bevan 1986). The metabolism of B*a*P and pathways leading to tumorigenesis have been well-characterized by multiple investigators (Conney 1982). Initially, B*a*P is metabolized by CYP enzymes (mainly 1A1, 1A2, and 1B1) to several arene oxides, i.e., 1,2-, 2,3-, 4,5-, 7,8-, 9,10-, or 11,12-oxide. These products may rearrange to phenols or undergo catalysis by epoxide hydrolases to yield the corresponding dihydrodiols. Alternatively, CYPs may directly hydroxylate B*a*P yielding

metabolites like the phenol 6-hydroxyB*a*P, which can be further oxidized to the 1,6-, 3,6-, or 6,12-quinones. Collectively, these phenol, quinone, and dihydrodiol metabolites may be detoxified by phase II enzymes via conjugation to glucuronides, sulfates, or glutathione adducts. Alternatively, dihydrodiols may undergo further oxidative metabolism to yield highly reactive diol epoxides. This pathway leads to the ultimate carcinogenic metabolite, B*a*P-7,8-diol-9,10-epoxide (BPDE), illustrated in Figure 6 (Yang et al. 1976; Lowe and Silverman 1994).

The predominant isomer (+)-anti-BPDE has been shown to possess the highest tumor-inducing activity of all the BPDE analogs (Kapitulnik et al. 1978). In mice treated with B*a*P, (-)-syn-BPDE, and (+)-anti-BPDE, 14, 12, and 100% of mice developed pulmonary tumors with 0.15, 0.13, and 7.67 tumors/mouse, respectively (Buening et al. 1978). *In vitro* studies in a variety of mammalian cells have shown BPDE preferentially binds deoxyguanosine at the N-2 position (Meehan et al. 1977; Meehan and Straub 1979). Unless adducts are efficiently removed by DNA repair, they may trigger G → T transversions similar to the AFB_1 adduct (Miller and Ramos 2001). The 'bay region' theory developed by Lehr and Jerina (1977) indicated that bay region diol-epoxides have an increased stability owing to stabilization from angularly fused benzene rings which allows sufficient life-time for the direct alkylating agent (i.e., C10) to reach biological nucleophiles. Identification of similar regions in other carcinogenic PAHs, like the carbonium ion at C1 in the bay region of benz[*a*]anthracene-3,4-diol-1,2-epoxide, helped confirm this theory. B*a*P excretion is predominantly hepatobiliary followed by elimination in the feces (USEPA 2006). Lower molecular weight PAHs have been

shown to be excreted in the urine more often than the feces, perhaps owing to their increased water solubility (Singh and Weyand 1994).

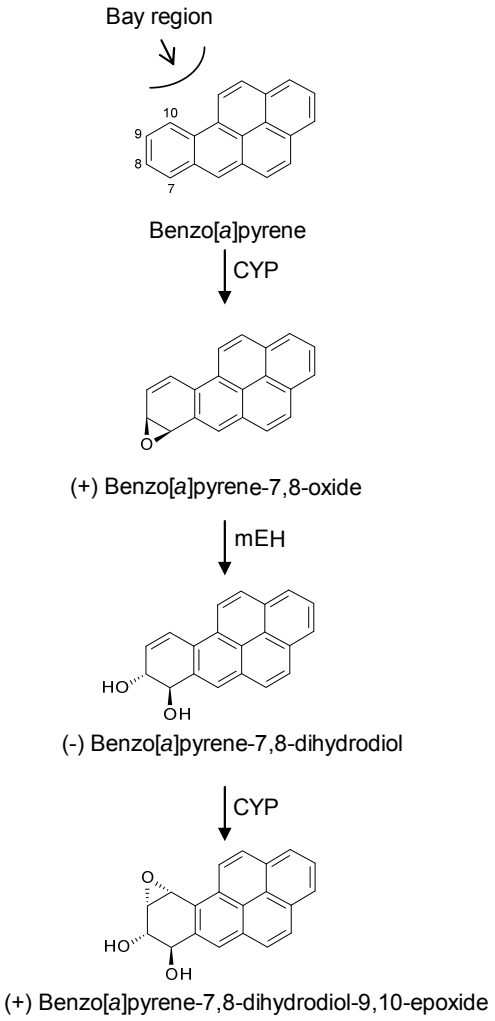

Figure 6. Metabolism of benzo[a]pyrene to ultimate carcinogen. Metabolism by cytochrome P450 (CYP) and microsomal epoxide hydrolase (mEH) enzymes yield B*a*P-7,8-diol-9,10-epoxide. Adapted from Klaunig and Kamendulis 2007.

1.9 Biomarkers of exposure to PAHs

PAH exposure levels classically have been estimated through air sampling. Much like assessing AF exposure through food measurements, this method does not supply information regarding internal dose. Furthermore, air monitoring only estimates respiratory intake; therefore, the use of molecular markers in biological fluids is useful in quantifying dose and biologically relevant exposure.

1.9.1 1-hydroxypyrene metabolite in urine

The urinary metabolite 1-hydroxypyrene (1-OHP) has been widely accepted as a dosimeter of PAH exposure due to the high percentage of pyrene in most PAH mixtures (Bouchard and Viau 1999; Hansen et al. 2008). Similar to B*a*P, pyrene is easily absorbed and distributed throughout the body and undergoes phase I metabolism by hepatic CYP enzymes. Pyrene is almost exclusively metabolized to 1-OHP and is largely conjugated to glucuronic acid or sulfate prior to excretion in human urine (Strickland et al. 1994). Since the majority of 1-OHP is excreted as a conjugated product (Law et al. 1994), methods of detection require enzymatic hydrolysis to yield the hydroxylated metabolite. Jongeneelen et al. (1985, 1987) originally developed a method to measure urinary 1-OHP and showed excretion was dose-dependent suggesting its use as a biomarker of PAH exposure. Since, increased levels of 1-OHP excreted in human urine significantly correlate with levels of PAH exposure by inhalation (Ny et al. 1993), oral (Buckley and Lioy 1992) and dermal routes (Viau et al. 1995). 1-OHP excretion represents recent PAH exposure with half-life values reported to range from 24 to 48 hours, 6 to 35 hours, and 16 to 20 hours (Jongeneelen et al. 1990; Buchet et al. 1992). Hence, 1-OHP is

regarded as a short-term biomarker of exposure. Moreover, 1-OHP has been extensively characterized and validated in many human populations both environmentally and occupationally exposed (Jacob and Seidel 2002). Urinary 1-OHP concentrations as high as 100 μmol/mol creatinine have been detected in workers exposed to exceedingly high levels of PAHs, but values less than 0.1 μmol/mol creatinine are generally observed in individuals not occupationally exposed. Background levels of 1-OHP in non-smokers and smokers vary from country to country, as illustrated in Table III. Cooking culture and behavior influence urinary 1-OHP excretion. For instance Viau et al. (2000) measured 1-OHP levels in two populations in Burundi, a central African country. The reported range in individuals living in traditional rural houses (0.26 – 15.62 μmol/mol creatinine) on average was 30 times higher than levels measured in people living in the capital (0.009 – 0.17 μmol/mol creatinine). It was concluded that high doses of PAHs were mainly generated by the unventilated burning of wood in traditional homes. Similar findings have been shown in a Chinese population, where the widespread use of coal burning is common (Zhao et al. 1995). While researchers consider urinary metabolites of additional PAHs to be valid, e.g., naphthalene, phenanthrene, and benz(a)anthracene (Grimmer et al. 1993, Hansen et al. 1994, Lintelmann et al. 1994, Popp et al. 1997), 1-OHP remains the most reliable and comprehensively studied biomarker in the urine.

Table III. Background 1-hydroxypyrene levels in non-occupationally exposed humans from various countries.

Country	1-OHP levels (μmol/mol creatinine)[a]	
	Non-smokers	Smokers
Canada	0.07 (95)	0.13 (45)
China	0.68[b] (74)	0.76[b] (84)
Denmark	< 0.07 (27)	< 0.07 (76)
Germany	0.04 (90)	0.12 (49)
Italy	0.08 (19)	0.13 (22)
Japan	0.06[b] (7)	0.12[b] (10)
Netherlands	0.17 (14)	0.51 (28)
Sweden	0.03 (48)	0.09 (10)
Thailand	0.91[b] (6)	3.03[b] (7)
U.S.	0.27 (10)	0.76 (11)
	Residing in capital	Residing in rural area
Burundi	0.05[c] (18)	1.50[c] (32)*

[a] Data are median levels unless otherwise specified (number of people in study); [b]Arithmetic mean concentration; [c]Geometric mean concentration; *In most studies, the 1-OHP level in smokers is significantly greater when compared with non-smokers (excluding China); however, in Burundi there was no significant difference between smokers and non-smokers. Thus, these data are presented together un-stratified. Sources: Levin 1995; Viau 2000; Chetiyanokornkul et al. 2004.

1.10 Consequences of PAH exposure

1.10.1 Non-carcinogenic effects

Adverse effects due to PAH exposure can be divided into carcinogenic and non-carcinogenic effects. In terms of non-carcinogenic outcomes, the liver, kidney, and vascular system are often the most affected (Klaassen 2007). It has also been documented that PAHs cause cellular and humoral immunotoxicity *in vitro* and *in vivo* (White 1986). The immunological effects of PAHs appear to be biologically relevant, as researchers have shown a strong correlation between immunosuppression due to PAHs and decreased resistance to infectious agents or transplantable tumor cells in animal models (Ward et al. 1984). Moreover, complex effects on human lymphocytes have been documented; the high sensitivity of cells suggests human immunity may be adversely affected by PAH exposure (Mudzinski 1993; Davila et al. 1996; Burchiel and Luster 2001). Currently, little is known about the immunotoxic effects of PAHs in humans. In an epidemiologic study in coke oven workers in Poland, Szczeklik et al. (1994) noted that workers who were highly exposed to PAHs exhibited a marked depression of mean serum IgG and IgA and a trended decrease in IgM and increase in IgE. Karakaya et al. (2004) showed a statistically significant inhibition in T-lymphocyte proliferative responses of asphalt and coke oven workers compared to controls. Significantly higher natural killer cell activities were observed solely in the asphalt workers (shown to be more highly exposed than controls but less exposed than coke oven workers based on 1-OHP values). Thus, authors concluded (in agreement with previous literature) that chronic exposure to PAHs at different levels may alter immune responses in different

ways. Overall, it is apparent that PAH exposure may have important consequences in humans in regards to immunity and should continue to be monitored.

1.10.2 Carcinogenic effects

Despite the lack of direct experimental evidence of PAH carcinogenicity in humans, occupational studies have indicated a strong correlation between PAH exposure and the incidence of lung, skin, and bladder cancers (Jacob and Seidel 2002). For example, increased cancers of the lung and other tissues (e.g., scrotum, bladder, kidney and colon) have been observed in workers employed in coke production and coal gasification (Lloyd 1971; Doll et al. 1972; Mazumbar et al. 1975; Redmond et al. 1976, 1981; Chau et al. 1993; Costantino et al. 1995). Furthermore, elevated rates of skin cancer have been observed in workers chronically exposed to coal tar, coal tar pitch, or creosote (wood preserving materials shown to be predominantly composed of PAHs) (Malaiyandi et al. 1982; Karlehagen et al. 1992). Thus, complex mixtures of PAHs have been identified as carcinogenic to humans (IARC 2010). PAHs containing more than three aromatic rings account for 70–90% of the total carcinogenic effect (IARC 1973, 1983; WHO 1998), and the USEPA (2006) has classified the following seven PAHs as probable human carcinogens: benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]- and benzo[*k*]-fluoranthene, dibenz[*a,h*]anthracene, indeno[1,2,3-*cd*]pyrene, and chrysene. Environmental PAH exposure, which is largely variable, has been harder to link to carcinogenic effects, although causal relationships do exist. A major source of environmental exposure is from environmental tobacco smoke (ETS). ETS exposure has been correlated with an increased risk of lung cancer (Pershagen 1994), and PAHs are

likely to be among the carcinogenic agents responsible. In addition, urban air pollution, which is a complex mixture of chemicals including PAHs, appears to be associated with an increased risk of lung cancer in humans (reviewed by Pershagen and Simonato1993; Hemminki and Pershagen 1994).

To date, little research has investigated the carcinogenic effect of environmental PAHs on human HCC. Chen et al. (2002) showed higher levels of PAH-albumin adducts in non-tumor tissues of HCC patients in Taiwan compared to controls. In a long-term follow up study in the same area, an increased risk of HCC was associated with higher levels of PAH-albumin adducts; HCC risk was highest among participants who were also exposed to high levels of AFs, based on the AF-albumin adduct, and chronically infected with HBV (Wu et al. 2007). Thus, enhanced hepatocarcinogenicity of AFs or HBV due to PAH exposure may be probable. The molecular pathogenesis of HCC involves multiple genetic aberrations in the control of hepatocyte proliferation, differentiation, and death (Mínguez et al. 2009). Effects on hepatocyte proliferation have been observed in animals following PAH exposure. For instance, cell proliferation (determined by organ weight and mitotic index) was investigated in rats dosed with fluorene (Danz et al. 1991). Results demonstrated that liver weight increased in a dose-dependent manner to 20% over control values, and the mitotic index of the hepatocytes was increased by 6-fold after 48 hours. Additionally, Ah-responsive strains of mice (C57BL/6, C3H/HeN, BALB/cAnN) orally administered B*a*P (120 mg/kg/day) in their diet for 6-months exhibited a 13% increase in relative liver weights (Robinson et al. 1975). Thus, a possible mechanism may be through the effect of PAHs on hepatic

growth. In addition, liver injury resulting from hepatitis infection may lead to enhanced cell proliferation; as a result, mutated cells from carcinogenic PAH insult may undergo clonal expansion. While prevention of HBV infection is promising, as vaccination is becoming more widespread, this is still a long-term strategy in many low and middle income countries where HBV continues to persist (Hall and Wild 2003). In addition, no vaccine is currently available for other hepatitis viruses, namely hepatitis C virus (HCV), which accounts for many HCC cases worldwide. Therefore, a reduction in PAH and AF exposures may aid in reducing the overall burden of HCC, particularly in populations with high rates of hepatitis. Due to the severe negative health effects associated with these foodborne/environmental exposures (both carcinogenic and non-carcinogenic), innovative detoxification strategies have been developed. One such approach that has shown promise for the management of AFs as a sustainable public health intervention is clay-based enterosorption. Although no interventions have been reported for PAHs using enterosorbent strategies, some reports have described interference with PAH gastrointestinal absorption using organic materials (discussed below).

1.11 Detoxification of aflatoxins by clay-based enterosorption

The inclusion of various binding agents or "detoxifying clays" in the diet has been given considerable attention as a strategy to reduce foodborne exposures to mycotoxins. For centuries, humans and animals have been reported to eat clay minerals, a process known as geophagy (Carretero 2002). In the Amazon of Southeastern Peru, macaws have been reported to flock to riverbanks daily to eat clay from distinct strata. The birds' diet largely consists of seeds that contain toxic alkaloids, which have been

proposed to be sorbed by the clay (Munn 1994). The consumption of edible clays by people in various regions of the world (e.g., China and many South American and African countries) is common and considered culturally acceptable (Johns and Duquette 1991; Diamond 1999). Even in the U.S., people have reportedly ingested clay for a variety of reason, including as a means of reducing symptoms of morning sickness during pregnancy (Ferguson and Keaton 1950; Loggi et al. 1992). Thus, the addition of non-nutritive clay minerals in the diet (given the safety and efficacy) represents a practical approach for reducing AF exposure from contaminated foods. Using multiple animal models, Phillips et al. (1995, 2002, 2008) has shown that NovaSil (NS) clay, a dioctahedral smectite, can prevent the adverse effects of exposure to dietary AFs by reducing toxin uptake and bioavailability.

1.11.1 Smectite clay mineral structure and chemistry

The ability of AFs to be adsorbed onto clay surfaces is largely dependent on the clay mineral structure. Multiple studies have confirmed that the active ingredient for AF binding is smectite clay (Phillips et al. 2002; Kannewischer et al. 2006; Marroquin-Cardona et al. 2009). The clay fraction of soils is dominated by phyllosilicate minerals (including smectite clays) with high surface areas and unique cation exchange properties (Schulze 1989). In general, phyllosilicates are composed of layer-lattice silicates with repeating layers of tetrahedral and octahedral sheets. In the tetrahedral layer, each SiO_4 tetrahedron (Si^{4+} held in tetrahedral coordination by four O^{2-} ions) shares three O^{2-} ions with three adjacent tetrahedra. The distinct layers of phyllosilicates form due to the ability of the apical oxygens (unbound O^{2-} ions) to replace hydroxyl (OH-) groups in the

octahedral layer and coordinate metal cations. Depending on if divalent (e.g., Mg^{2+}) or trivalent (e.g., Al^{3+}) cations are held in corrdination with oxygens and OH^- groups in the octahedral layer, the arrangement is termed trioctahedral or dioctahedral, respectively (since to balance the charge divalent cations must fill three of three available sites and trivalent cations need only fill two of three spaces). Condensation of layers may be in a 1:1 proportion (i.e., kaolinites) or 2:1 proportion (i.e., smectites, vermiculites, mica) where the octahedral layer is sandwiched between two tetrahedral layers (Schulze 1989).

Many phyllosilicates possess unique cation exchange properties due to isomorphous substitutions of cations that results in a permanent net negative charge, i.e., Al^{3+} for Si^{4+} in the tetrahedral layer or Mg^{2+} for Al^{3+} or Fe^{3+} in the octahedral layer (McBride 1989). To balance the resulting charge, cations such as Na^+ and Ca^{2+} are attracted to the negative region between the layers (the interlayer). The amount of charge is dependent on the amount of isomorphous substitution. For instance, smectites have an intermediate charge per formula unit of 0.25 to 0.6, affording smectites characteristic swelling properties as interlayer cations are subject to layers of hydration (Bohn et al. 1979). Montmorillonites are a subclass of smectites that contain isomorphous substitution in the octahedral sheets. These clays are naturally abundant and have surface areas as high as 800 m^2/g making them ideal sorbent materials (Borchardt 1989). NS clay is a naturally occurring calcium montmorillonite (dioctahedral smectite), modeled in Figure 7. Given its GRAS status (generally recognized as safe) NS has commonly been used as an anti-caking agent in animal feeds at levels up to 2.0%.

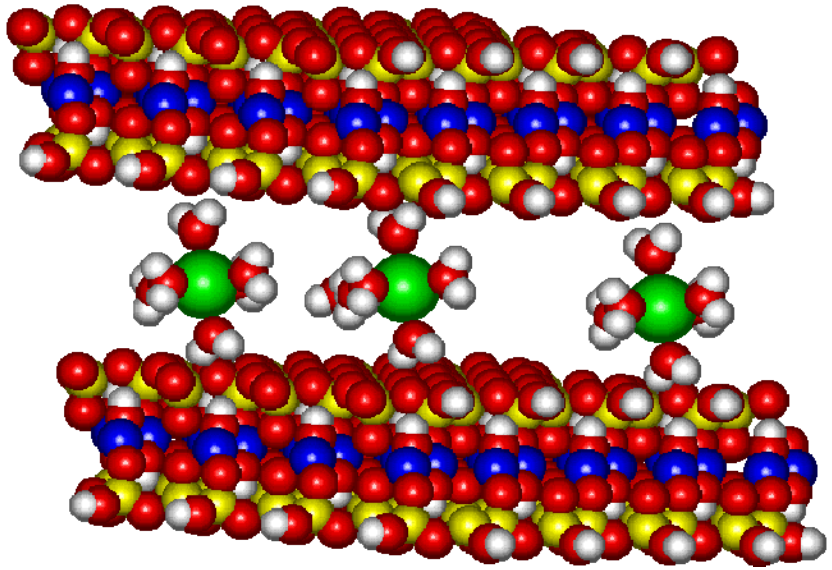

Figure 7. Molecular model of smectite clay structure. This schematic representation shows hydrated calcium (green) as the predominant interlayer cation. Molecular modeling studies have illustrated AFB_1 is predominantly bound within the interlamellar region of NS clay, a dioctahedral smectite. Key: Si^{4+} (yellow) compose the tetrahedral layer and Al^{3+} (blue) ions are coordinated in the octahedral layer. O (red) and H (white).

Langmuir isotherm equation: $$q = Q_{max} \times \left(\frac{K_d \times C_w}{1 + K_d \times C_w} \right)$$

Figure 8. Langmuir mathematical equation. This equation describes the amount of ligand adsorbed (q), the maximum amount of ligand that could be sorbed by a given weight of sorbent under optimum experimental conditions (Q_{max}), the equilibrium concentration of ligand in solution (Cw), and the equilibrium constant (K_d) which depicts sorbent affinity.

1.11.2 NovaSil clay research

Several *in vitro* studies have assessed the sorption of AF onto the surface of NS clay (previously referred to as HSCAS in the literature) (Grant and Phillips 1998; Phillips 1999). Isothermal data fit to the Langmuir equation (Figure 8) demonstrate high capacity (Q_{max}) and high affinity (K_d) characteristics for AFB_1 sorption onto NS. The calculated enthalpy of sorption (ΔH_{ads}) reported at –40 to –50 kJ/mol indicates multiple sites on the surface of NS clay may act to chemisorb AFB_1. Additionally, molecular modeling studies and experiments comparing isothermal data from heat-collapsed NS illustrate AFB_1 is predominantly bound within the interlamellar region of NS clay, most likely in a planar orientation. Figure 9 illustrates the notably lower capacity (94% less) for heat collapsed NS (0.0267 mol/kg) versus unmodified NS (0.456 mol/kg). Importantly, isothermal analyses have shown that NS tightly and preferentially sorbs AFB_1 and similar analogs that contain an intact β-dicarbonyl system. The partially positive (i.e., electron poor) carbons comprising the dicarbonyl system (C1 and C11) have been shown to be essential for sorption.

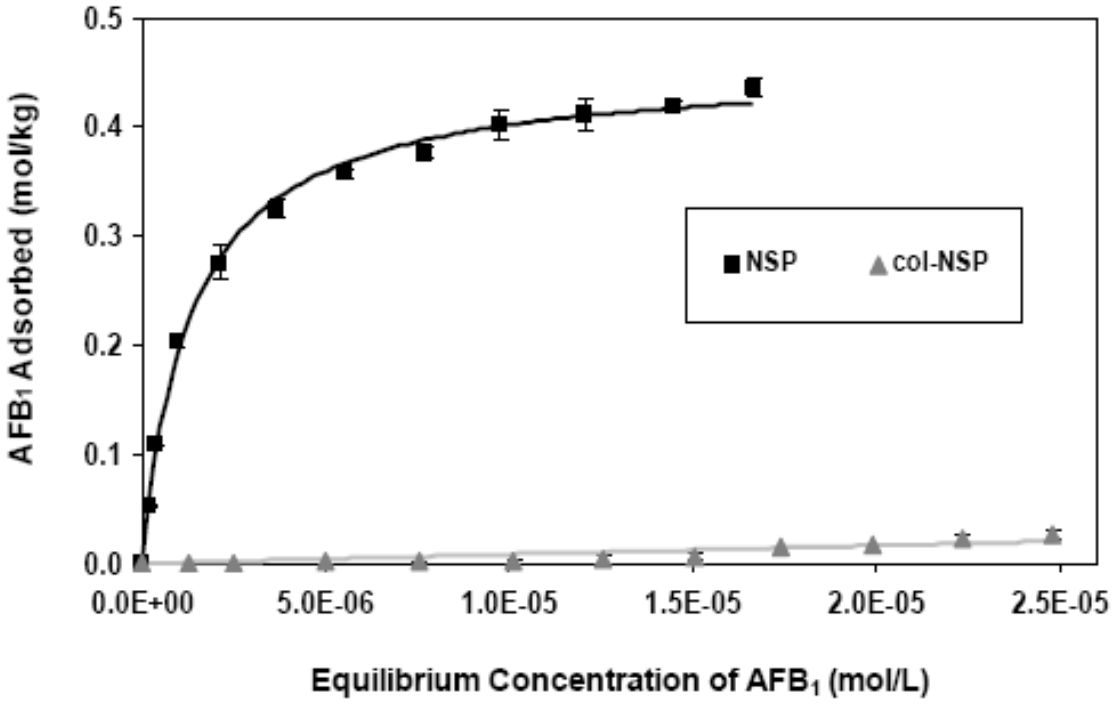

Figure 9. Isothermal plots of untreated versus collapsed NS clay. Isothermal sorption plots demonstrate the notably lower capacity (Q_{max}) for collapsed NS (col-NSP, gray) compared to untreated NS (NSP, black). Computer-generated isotherm data were fitted using the Langmuir model. The solid lines show the curve fits whereas the experimental data are represented by the average data points ± SEM. Adapted from Afriyie-Gyawu 2004.

A large volume of scientific literature has indicated that dietary inclusion of NS clay (or HSCAS) is safe and effective for reducing AF exposure in multiple animal models (Table IV). In chicks dosed with [^{14}C]AFB_1, NS at 0.1 and 0.5% markedly diminished radioactivity in the blood and hepatic tissues of treatment groups compared to controls suggesting NS may reduce AF adsorption and distribution to target organs, thereby decreasing AF bioavailability *in vivo* (Davidson et al. 1987). Furthermore, NS addition to the diet has been shown to prevent the adverse effects of AFs in a variety of species including chickens (Phillips et al. 1988; Kubena et al. 1990, 1993b, Pimpukdee et al. 2004), turkey poults (Kubena et al. 1991), pigs (Harvey et al.1989, 1994a; Lindemann et al. 1993), lambs (Harvey et al.1991a), mink (Bonna et al. 1991), and pregnant rodents (Mayura et al. 1998). Notably, NS (at 0.5%) protected poultry against 7.5 ppm AF in the diet, signifying it may be used to reduce exceptionally high levels of AFs, such as those observed in outbreak situations (Phillips et al. 1988). Dietary NS inclusion has also reduced AFM_1 residues in milk of dairy cows and goats and urine of rats, turkeys, and dogs indicating its ability to reduce AF biomarkers exposure (Harvey et al. 1991b; Smith et al. 1994; Sarr et al. 1995; Edrington et al. 1996; Mayura et al. 1998; Bingham et al. 2004). While NS intervention has clearly demonstrated efficacy for AFs, the same effectiveness has not been shown for other mycotoxins (e.g., zearalenone, deoxynivalenol, T-2 toxin, ochratoxin A, cyclopiazonic acid, and ergotamine). The adverse effects of these toxins were not prevented upon NS inclusion in the diet signifying the preferential binging of AFs by NS (Bursian et al. 1992; Chestnut et al. 1992; Huff et al. 1992; Kubena et al. 1993a; Dwyer et al. 1997).

Table IV. Comparison of *in vivo* studies with NS clay

Species	AF in feed	% NS in feed (duration)	Toxicity from NS	Major effects of NS clay reported in study	Reference
Chicken	Yes	0.5 (28 d)	None	Growth inhibition diminished	Phillips et al. 1988
Chicken	Yes	0.1/0.5 (24 h)	None	Reduced bioavailability of AF to the liver	Davidson et al. 1987
Chicken	Yes	0 – 1.0 (21 d)	None	Feed conversions improved; growth inhibition diminished	Doerr 1989
Chicken	Yes	1.0 (21 d)	None	Growth inhibition completely prevented	Ledoux et al. 1999
Chicken	Yes	0.5 (21 d)	None	Decreased growth inhibitory effects	Huff et al. 1992
Chicken	Yes	0.5 (21 d)	None	Diminished growth inhibition	Kubena et al. 1990
Chicken	No	0.5/1.0 (14 d)	None	NS did not impair phytate or inorganic phosphorous utilization	Chung and Baker 1990
Chicken	No	0.5/1.0 (14 d)	None	NS did not impair utilization of riboflavin, vitamin A, or Mn	Chung et al. 1990
Chicken	Yes	0.125 – 0.5 (21 d)	None	Protected against vitamin A depletion in the livers of chicks exposed to aflatoxins	Pimpukdee et al. 2004
Chicken	No	0.5 (19 d)	None	Did not affect growth performance or tibial mineral concentrations of chicks	Southern et al. 1994
Turkey	Yes	0.5 (21 d)	None	Decreased mortality	Kubena et al. 1991
Turkey	Yes	0.5 (21 d)	None	Decreased urinary excretion of AFM_1	Edrington et al. 1996
Pig	Yes	0.5 (42 d)	None	Diminished growth inhibition	Lindemann et al. 1993
Pig	Yes	0.5/2.0 (28 d)	None	Diminished growth inhibition, hepatic lesions and immunosuppression	Harvey et al. 1988
Pig	Yes	0.5/2.0 (28 d)	None	Decreased growth inhibition; prevention of serum effects and hepatic lesions	Harvey et al. 1989
Pig	Yes	0.5 (35 d)	None	Growth inhibitory effects reduced	Schell et al. 1993
Dog	Yes	0.5 (48 h)	None	Significantly reduced the bioavailability of aflatoxins and urinary AFM_1	Bingham et al. 2004
Lamb	Yes	2.0 (42 d)	None	Diminished growth inhibition and immunosuppression	Harvey et al. 1991a
Cow	Yes	0.5/1.0 (28 d)	None	Reduction of AFM_1 in milk	Harvey et al. 1991b
Goat	Yes	1.0/2.0/4.0 (12 d)	None	Reduction of AFM_1 in milk	Smith et al. 1994
Rat	Yes	0.5 (48 h)	None	Decreased urinary excretion of AF metabolites (M_1 and $_{P1}$)	Sarr et al. 1995
Rat	Yes	0.5 (21 d)	None	Significant prevention of maternal and developmental toxicity	Mayura et al. 1998
Rat	No	0.25 – 2.0 (6 mo)	None	No adverse effects including vitamin utilization	Afriyie-Gyawu et al. 2005a

While the potential affect of NS on bioavailability of PAHs from the gastrointestinal tract has not been investigated, it is not likely that NS would be effective based on the previous specificity data and the importance of a charged/partially charged moiety for NS adsorption (Phillips 1999, Phillips et al. 1995). Alternatively, clays exchanged with various cationic surfactants (organoclays) have been shown to be effective sorbents for a variety of PAHs in solution (Smith and Jaffe 1994; Xu et al. 1997). *In vitro* findings have indicated that organoclay-composites, specifically cetylpyridinium exchanged low-pH montmorillonite clay (CP-LPHM), have a high capacity for contaminants found in aqueous wood preserving waste (Ake et al. 2003; Wiles et al. 2005). Authors proposed that inclusion of CP-LPHM may be an effective strategy for groundwater remediation of high concentrations of PAHs, in particular high molecular weight and carcinogenic PAHs. Regrettably, the use of organoclays as enterosorbents in the diet is not a prudent strategy since these clays exhibited toxicities in initial safety studies in animals (Afriyie-Gyawu et al. 2005b).

In all of the short-term animal studies with NS clay, no observable adverse effects were noted following dietary ingestion (Phillips 1999; Phillips et al. 2002). These data suggest long-term use is feasible. Hence, Afriyie-Gyawu et al. (2005a) investigated the safety of dietary NS inclusion (up to 2%, w/w) in a chronic study in Sprague-Dawley rats. After 6.5-months of treatment, there were no dose-dependent or NS-related adverse effects on body weight gains, relative organ weights, histological appearance of major organs, or hematological and serum biochemistry parameters. As a result of the extensive safety data in animal models, it was hypothesized that NS may be safe and

beneficial for humans, particularly in populations at high risk for aflatoxicosis. As precursor to a human intervention study, a short-term adverse events trial was conducted to evaluate the safety and tolerance of NS capsules in healthy human volunteers (n = 50). After two weeks of capsule ingestion, NS (up to 3.0 g/day) was considered safe for long-term intervention studies based on physical examination results, biochemistry, hematology, and selected micronutrient parameters which did not differ significantly compared to baseline levels at the start of the study (Wang et al. 2005). This study provided the basis to conduct the first Phase IIa human intervention trial in the Ashanti Region of Ghana, of which the main objectives of this research focus.

1.12 Research objectives

Since AF contamination of foods is a long-standing, seemingly inextricable problem (CAST 1989; Phillips 1999; Phillips et al. 2002), there is a need for safe and effective interventions. A lack of untainted foods represents a serious burden in many parts of the world; thus, detoxification strategies should be economically viable and culturally acceptable in addition to being safe and effective. Inclusion of NS clay in the diet meets the above mentioned requisites and represents a practical, effective approach to diminish or block exposure to AFs. Still, long-term use in humans requires careful observation of safety parameters and interactions with micronutrients. In addition to dietary AF exposure, populations may be at increased risk for cancer and disease due to supplementary factors such as PAH exposure. Measurement of molecular dosimeter biomarkers offers a way to identify populations that are vulnerable to both AF and PAH exposures. Consequently, biomonitoring is important for comparing public health risks

from foodborne/environmental exposures across populations, especially in developing countries and underserved rural or metropolitan communities where the incidence and impact of these exposures are elevated. Studies in this dissertation focus on the measurement of biomarkers with the following specific objectives:

1) to evaluate the effectiveness of NovaSil (NS) clay to act as an enterosorbent for AFs in humans after 3-mo intervention in a population chronically exposed to AFs (i.e., the Ashanti Region of Ghana) by measuring urinary AFM_1 as an indicator of intervention efficacy;
2) to investigate interactions of NS with important nutrient minerals by measuring serum mineral concentrations before and after NS intervention;
3) to determine PAH exposure in this population shown to be at risk for aflatoxicosis by measuring 1-OHP in urine samples and to verify if a relationship between AF and PAH exposures exists; of additional interest is to investigate the possible consequences of enterosorbent intervention on PAH exposure;
4) to assess AF and PAH exposure in a vulnerable U.S. population with an elevated incidence of HCC; to investigate dietary factors that may contribute to increased AF exposure; and to evaluate other factors that may contribute to this increased HCC incidence, namely the prevalence of hepatitis infection in the population.

II. NOVASIL CLAY INTERVENTION IN A GHANAIAN POPULATION AT HIGH RISK FOR AFLATOXICOSIS: REDUCTION OF URINARY AFLATOXIN M_1 BIOMARKER*

2.1 Introduction

Given the safety and efficacy of NovaSil (NS) clay, as demonstrated in a variety of animal models, it was hypothesized that NS-based intervention may benefit humans who are frequently exposed to high levels of AFs and at risk for aflatoxicosis. The previously conducted Phase I adverse events trial (Wang et al. 2005) provided the basis for our first objective, to evaluate the efficacy of NS clay for reducing biomarkers of AF exposure (i.e., AFM_1) in a clinical intervention trial. In order to achieve this aim, we conducted a 3-month double-blind, placebo-controlled Phase IIa intervention trial in the Ashanti Region of Ghana, a West African country. Previous reports have indicated that AF contamination of foods, particularly maize and groundnuts constitutes a major food safety problem in Ghana. For instance, Jesperson et al. (1994) reported that in 15 out of 16 market samples of kenkey, a traditional food made from fermented maize dough, the total AF levels ranged from 6.15 to 196.10 ppb, with a mean value of 50.88 ppb. An additional study indicated high levels of AF contamination in groundnut samples from local markets in ten regions, with levels ranging from 5.7 to 22,168 ppb (Awuah and

* Parts of the data reported in this chapter are reprinted with permission from "NovaSil clay intervention in Ghanaians at high risk for aflatoxicosis: I. Study design and clinical outcomes" by Afriyie-Gyawu E, Ankrah NA, Huebner HJ, Ofosuhene M, Kumi J, et al. 2008a. Food Addit Contam 25:76–87, Copyright [2008] by Taylor & Francis; "NovaSil clay intervention in Ghanaians at high risk for aflatoxicosis: II. Reduction in biomarkers of aflatoxin exposure in blood and urine" by Wang P, Afriyie-Gyawu E, Tang Y, Johnson NM, Xu L, et al. 2008. Food Addit Contam 25:622–634, Copyright [2008] by Taylor & Francis.

Kpodo et al. 1996). These levels reported are well above action levels for AFs permitted in foods for human consumption, which range from 0.5 – 50 ppb depending on the country and food matrix (Adams and Motarjemi 1999). The Ejura-Sekyedumase district (ESD), one of the 21 districts in the Ashanti Region, was chosen as the study site based on previously reported biomarker levels confirming this population was at high risk for AF exposure and disease. In this report, Jolly et al. (2006) detected the AFB_1-lysine albumin adduct in 100% of 140 blood samples and the AFM_1 metabolite in 91.2% of 91 urine samples. Moreover, high AFM_1 levels were measured in the urine of most study participants (mean ± SD = 1,800.14 ± 2602.01 pg AFM_1/mg creatinine). Hence, the ESD comprised a well-defined study population known to be at high risk for aflatoxicosis.

2.2 Materials and methods

2.2.1 Chemicals and reagents

Authentic AFM_1 reference standards were purchased from Sigma Chemical Co. (St. Louis, MO). UV-visible spectrophotometry (350 nm; ε=18,815 $M^{-1}cm^{-1}$) was used to verify the concentration of AFM_1 stock solutions. All of the experiments were done using filtered and deionized water (18.2 MΩ·cm) (Millipore, Milford, MA) and HPLC-grade solvents. Preparative monoclonal antibody columns (AflaTest® WB) were purchased from Vicam (Watertown, MA). All other chemicals and reagents used were obtained commercially at the highest purity available.

2.2.2 Procurement of test article (NovaSil clay)

NS clay was obtained from Engelhard Chemical Corporation (Iselin, NJ). Prior to the intervention study, NS was evaluated for potential environmental contaminants

including polychlorinated dibenzo-p-dioxins/furans (PCDDs/PCDFs) and heavy metals to ensure their levels met U.S. and international standards. Evaluation of NS for the U.S. Environmental Protection Agency (USEPA) priority dioxins/furans was performed by Columbia Analytical Services (CAS), Inc. (Houston, TX). Standardized procedures were used for sample preparation, cleanup, and analysis with high resolution capillary column gas chromatography/high resolution mass spectrometry (USEPA Method 1613B). NS was further analyzed for heavy metals, according to EPA, by CAS, Inc. (Kelso, WA). Metal analysis procedures followed standard USEPA protocols (e.g. 200.8, 6010B and 7471A). Following analyses, NS was determined to contain acceptable levels of contaminants and was sent to College Pharmacy (Colorado Springs, CO) for encapsulation under sterile conditions in a setting with good manufacturing practices.

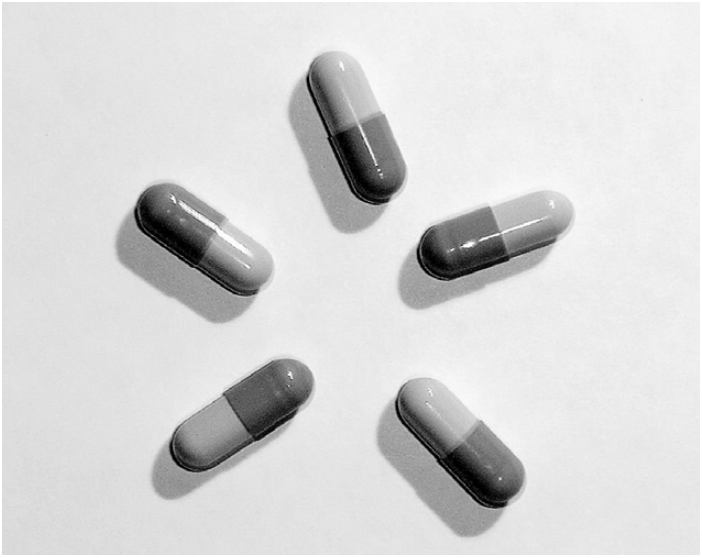

Figure 10. Capsules used in clinical intervention trial in Ghana. Treatment doses were 0, 1.5, or 3.0 g NS/day. All capsules were the same size, shape and color to ensure participants were blinded to respective dose groups.

Capsules, shown in Figure 10, were formulated to contain 500 mg NS (high dose), 250 mg NS:250 mg placebo (low dose), or only placebo (microcrystalline cellulose) based on previous dosimetry protocols (Wang et al. 2005). Isothermal adsorption analysis confirmed the lack of binding capacity for AFs by the placebo (Figure 11). All capsules were sterilized by electron beam irradiation at the National Center for Electron Beam Food Research, Texas A&M University (TAMU). A target dose range of 8.2 – 9.4 KGy was applied and followed protocols similar to those used for sterilizing foods in the U.S.

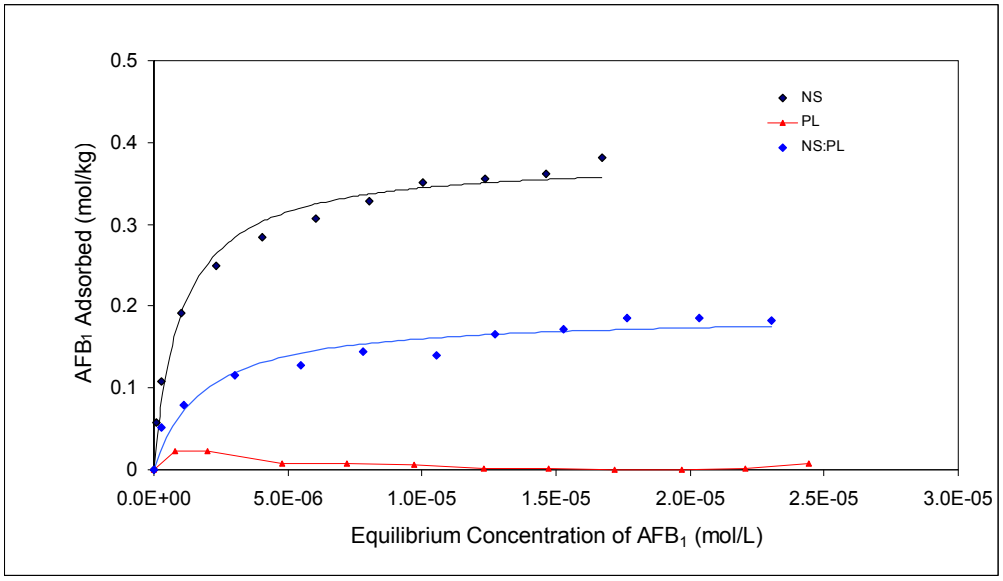

Figure 11. Isothermal plots of NS clay versus placebo (cellulose). Computer-generated isotherm data were fitted using the Langmuir model. Solid lines indicate the best curve fits whereas the experimental data are represented by the average data points. Plots demonstrate the notably lower capacities for the PL (red) and NS:PL mixture (blue) compared to NS (black). These data confirmed the lack of binding capacity for AFB_1 by the PL.

2.2.3 Overall study design and procedures

The overall study design followed the guidelines for a randomized, double-blind, placebo controlled Phase II clinical trial. The study protocol was approved by the Institutional Review Board (IRB) at TAMU and Noguchi Memorial Institute for Medical Research (NMIMR) IRB for Ethical Clearance at The University of Ghana. Screening of volunteers was initiated in September, 2005. The trial started in December 2005 and was completed in April, 2006 (including 1-month post trial follow-up). Figure 12 shows the overall study design and sample collection procedure. Briefly, 180 participants were recruited from a total of 507 screened volunteers. This sample size was chosen based on the standard 100 to 300 subjects required by the U.S. NIH Guidelines for Phase II clinical studies. Individuals (males and females) who qualified as study participants met the following criteria: signed informed consent, age 18-58 yr, healthy status based on physical examination, normal ranges of hematological parameters and liver and renal function indicators, no history of chronic disease(s), no use of prescribed medications for chronic or acute illness, non-pregnant and/or non-breastfeeding females, intake of corn and/or groundnut-based foods at least 4 times a week, and serum AF-albumin adduct levels > 0.5 pmol AFB_1/mg albumin. Participants were randomly assigned to one of three groups: high dose (HD), low dose (LD), or placebo (PL) and took two capsules containing either 500 mg NS, 250 mg NS, or placebo three times per day (before meals and with at least 100 ml of water). In total, the HD and LD groups received 3.0 g NS/day and 1.5 g NS/day, respectively.

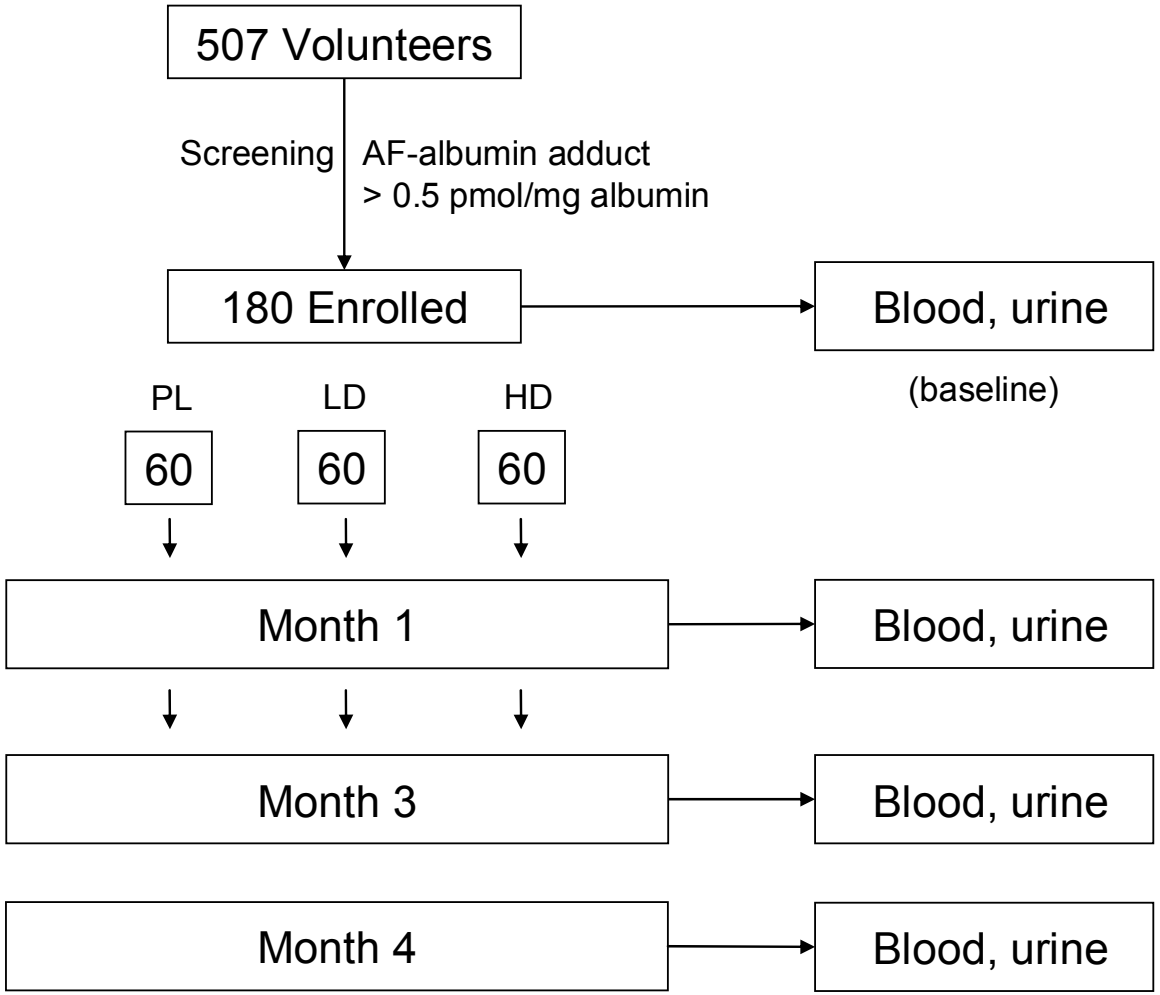

Figure 12. Overall study design and sample collection procedure for the Phase IIa clinical intervention trial in Ghana.

The high dose, 0.25% NS (w/w), represented the minimal effective dose (MED) shown to significantly reduce the effects of AFs in animals (Pimpukdee et al. 2004). Furthermore, no adverse effects were demonstrated in experimental animals dosed at levels approximately eight times higher (Afriyie-Gyawu et al. 2005a). To ensure maximum compliance to the defined treatment regimens and participant well-being, trained study monitors delivered the capsules daily, witnessed ingestions, and recorded any symptoms that subjects might have experienced. Physical examinations were performed monthly to evaluate the general health status of study subjects. Blood and urine samples were collected from each study participant at the beginning of the study (baseline), at 1- and 3-months of intervention, and 1-month following completion of the trial. Aliquots of the first urines collected were stored in polypropylene tubes and shipped to TAMU where they were kept frozen at -80°C until urinary analysis. Serum, plasma and blood cells were immediately separated and transported to Texas Tech University (Lubbock, TX) where they were stored at -80°C until biomarker analysis.

2.2.4 Urinary aflatoxin M_1 analysis

Urinary AFM_1 levels were analyzed with immunoaffinity column purification followed by HPLC with fluorescence detection developed by Groopman et al. (1992) with modifications of Sarr et al. (1995) and Wang et al. (1999). Urine samples were thawed and centrifuged at 500 × g for 5 min to remove particulate matter. Each sample (5.0 ml urine) was subsequently adjusted to an acidic pH with 0.5 ml of 1.0 M ammonium formate (pH 4.5). The volume was increased to 10 ml with deionized water, and samples were loaded on immunoaffinity columns at a flow rate of approximately 0.3

ml/min. After washing, the purified AF fraction was eluted with 80% methanol and dried under N_2 for analysis using a Waters HPLC system (Waters Corporation, Milford, MA). Excitation and emission parameters were set at 365 and 425 nm, respectively. A 250 mm x 4.6 mm LiCrospher RP-18 endcapped column with a pore size of 100 Å and a particle size of 5 μm (Alltech Associates, Deerfield, IL) was used to resolve AF metabolites. The mobile phase consisted of 22% ethanol in water buffered with 20 mM ammonium formate (pH 3.0). Chromatographic separation was achieved by isocratic elution of the mobile phase for 25 min. Samples (100 μl) were injected on the column at an elution rate of 1.0 ml/min. The limit of detection (LOD) for this method was 0.5 pg AFM_1/ml of urine. AFM_1 quantification was based on peak area and retention time compared to external standards injected daily. Urinary creatinine (mg/dL) was measured at St. Joseph's Regional Health Laboratory in order to adjust for variation in urine dilution.

Authentic AFM_1 standard (5 μg) was initially dissolved in acetonitrile (2 cc) as a stock solution and was then diluted in 80% methanol/20mM ammonium formate (pH 3.0) (1:1, v/v) to serve as an external standard. The machine was calibrated daily by injecting multiple aliquots of AFM_1 standard solution. Calibration curves were linear over the tested range of 1 – 5 ng ($r^2 = 0.9998$) for the instrument. The AFM_1 peak was detected at a retention time averaging 15.4 min (Figure 13), and its identity (from standard injections and representative participant samples) was verified with mass spectrometry (Texas Veterinary Medical Diagnostic Laboratory, College Station, TX) (Figure 14).

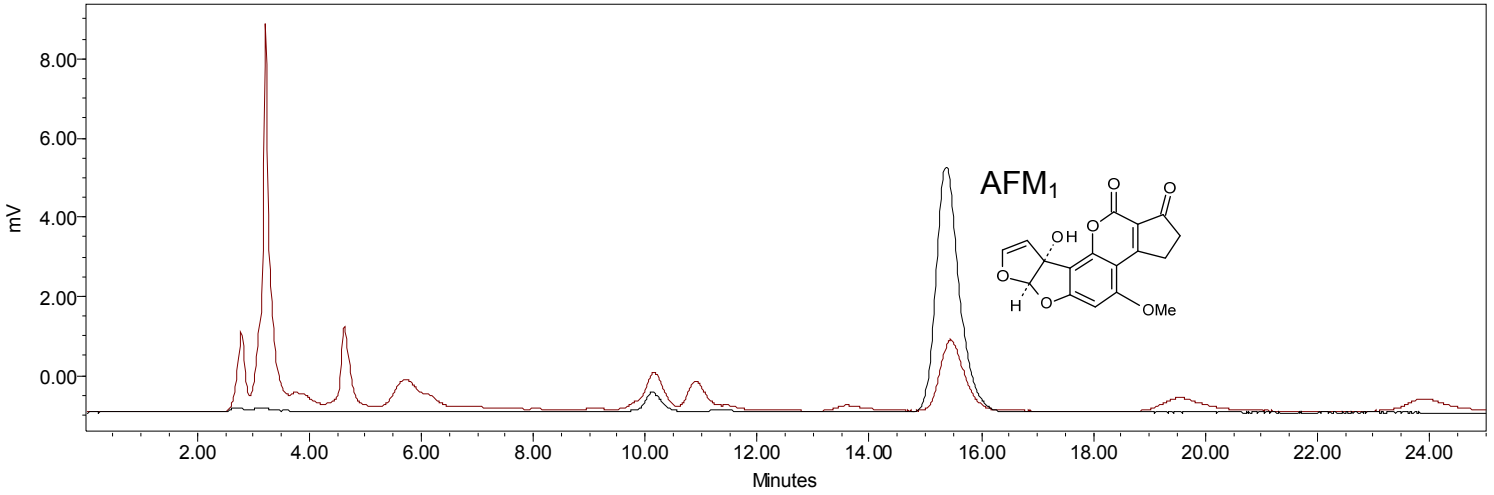

Figure 13. Representative HPLC chromatograph showing urinary aflatoxin M_1 metabolite peaks. A representative sample collected from a study participant at baseline is shown in red. Peak areas were used for quantification of AFM_1 in participant samples as compared to external AFM_1 standards injected daily, for instance, a 50 ng/ml AFM_1 standard peak is shown in black.

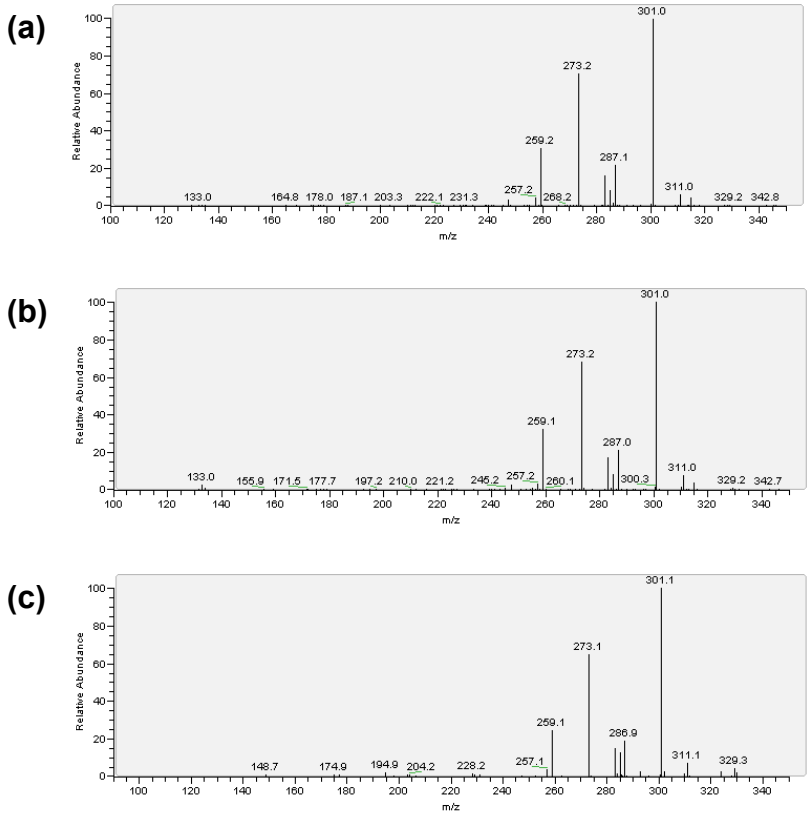

Figure 14. Representative chromatograms confirming AFM_1 structure. Following HPLC separation, the identity of AFM_1 was verified in a 10 ppb reference standard in acetonitrile (a), a 10 ppb reference standard in blank urine (b), and a representative urine sample using mass spectrometry.

2.2.5 Statistical analysis

Median, mean, standard deviation (SD) and range were calculated for concentrations of urinary AFM_1 standardized to urinary creatinine. AFM_1 levels below the LOD were assigned a value of half the LOD for statistical analysis. All data generated were analyzed with SPSS software version 15.0 (Chicago, IL). To assess the efficacy of NS intervention, statistical evaluation focused on comparisons among different treatment levels and different time points. Since urinary AFM_1 levels were not normally distributed, the Kruskal-Wallis test or Wilcoxon rank sum test were used for comparisons. To evaluate the effect of dose-time interactions on NS treatment, a nonparametric mixed-effect model was applied, as described by Brunner et al. (2002). This ranking is based on a method in the repeated measurement design, in which the significance of dose, time, and dose–time effects are determined by a non-parametric marginal effects approach to derive the analysis of variance-type statistics and establish the distribution of the estimated relative effects when the null hypothesis is not true. A *p*-value ≤ 0.05 (two-tailed) was considered significant.

2.3 Results

2.3.1 Dioxins/furans and metal analyses in NovaSil clay

Among the USEPA priority PCDDs/PCDFs, 1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin (HpCDD) and 1,2,3,4,6,7,8,9-octachlorodibenzo-p-dioxin (OCDD) were the only contaminants present above the limits of detection (LODs = 1.11 parts per trillion, ppt for HpOCDD and 1.91 ppt for OCDD). The mean concentrations of HpCDD and OCDD in NS (1.0 g) were 4.42 and 23.74 ppt, respectively. The toxic (or TCDD) equivalency

(TEQ) values of these dioxins were calculated to be 0.0442 and 0.00237 ppt for HpCDD and OCDD, respectively, with a combined TEQ of 0.0466 ppt. Thus, the HD treatment (3.0 g NS/day) provided a TEQ of 0.1397 pg/day. According to standards set by the World Health Organization (WHO), the tolerable human intake (THI) of TCDD is 2.3 pg/kg BW/day, which is calculated to be 161 pg/day for a 70 kg man and 138 pg/day for a 60 kg woman (developed by WHO, reported by Van den Berg et al. 1998). Based on these values, the TEQ from 3.0 g NS would be considerably lower (by ~1000 times) than the daily WHO-THI standards. In addition, all metals calculated to be in 3.0 g NS were considerably lower than the JECFA standard recommended values (Table V).

Table V. Trace metal levels calculated in NS clay.

Trace metal	Concentration in NS (mg/kg)	Amount in 3.0 g NS (mg)[a]	JECFA value (mg/day)[b]
Arsenic (As)	2.23	0.0067	0.150
Cadmium (Cd)	0.260	0.0008	0.060
Chromium (Cr)	1.12	0.0034	0.250
Cobalt (Co)	1.36	0.0041	0.016
Lead (Pb)	10.3	0.0310	0.210
Mercury (Hg)	ND	ND	0.043
Molybdenum (Mo)	0.150	0.0005	0.110
Nickel (Ni)	2.82	0.0085	0.300
Selenium (Se)	0.500	0.0016	0.057
Strontium (Sr)	1430	4.2900	5.000
Zinc (Zn)	66.9	0.2008	45.00

[a]Derived dose of metals in 3.0 g NS (assuming bioavailability of total metal concentration); [b]Tolerable daily human intake of metals from foods based on Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommendations (1998). ND = non-detectable.

2.3.2 Participant adherence to the study protocol and sample collection

A total of 180 participants were selected from 507 screened volunteers based on predetermined inclusion criteria. Three of the selected subjects (1 from the LD group and 2 from the PL group) were removed from the study prior to intervention since two females became pregnant and one male opted out because of a new job. Therefore, 177 individuals comprised our study population. Table VI delineates the demographic characteristics of the subjects enrolled in the study.

Table VI. Demographic characteristics of study participants in Ghana.

Demographic characteristics	Treatment group		
	Placebo	Low dose	High dose
Participants	58	59	60
Gender			
Male	37	31	34
Female	21	28	26
Community			
Dromankoma	20	12	13
Ejura Group	9	8	10
Nkwanta	5	8	6
Hiawoanwu	12	15	18
Kasei	3	5	3
Kotokoli Line	9	11	10
Age (year)[a]	36.5 ± 10.8	37.3 ± 11.8	38.6 ± 13.0

[a] Data are presented as mean ± SD.

A total of 162 subjects (91.5%) completed the 3-month trial. Data representing study completion and participant compliance are summarized in Table VII. The overall adherence (number of times capsules were taken) among the participants, whether or not they completed the study, was over 97%.

Table VII. Participant compliance and completion of treatment regimen.

	Treatment group			
	Placebo	Low dose	High dose	Overall
Participants				
Started	58	59	60	177
Completed (3 months)	55	53	54	162
Completion (%)	94.8%	89.8%	90.0%	91.5%
Treatment regimen				
Times Capsule taken	15035	14697	14847	44579
Times Capsule missed	220	543	390	1153
Total reported	15255	15240	15237	45732
Adherence (%)	98.6%	96.4%	97.4%	97.5%

2.3.3 Urinary aflatoxin M_1 levels

Among the four time points of sample collection, ≥ 90% of urine samples were collected from participants, indicating valid sample numbers for final statistical evaluation (Table VIII). A total of 624 urine samples over the study period were analyzed for AFM_1. About 87% of the samples had detectable levels and no significant difference was found in detection rate among three study groups. Mean ± SD and median levels of AFM_1 at baseline and following intervention are presented in Table IX.

Table VIII. Sample information for each collection time in each treatment arm in NS intervention study in Ghana.

Treatment group	Number of urine samples collected[a]			
	Baseline	1-month	3-month	4-month
Placebo	53 (4)	52 (4)	54 (8)	55 (7)
Low dose	53 (9)	53 (9)	51 (9)	43 (2)
High dose	53 (6)	52 (6)	53 (10)	52 (10)

[a] Valid number of samples collected from study participants at each time point (non-detectable AFM_1).

Table IX. Levels of urinary aflatoxin M_1 in study participants in Ghana

Treatment group	Aflatoxin M_1 levels (pg/mg creatinine)[a]			
	Baseline	1-month	3-month	4-month
Placebo	53.42 644.22 ± 2026.53 (0.89 - 13297.67)	24.58 94.71 ± 160.13 (1.66 - 798.11)	52.38 181.26 ± 675.90 (2.02 - 5006.37)	17.32 56.84 ± 110.14 (0.04 - 529.41)
Low dose	45.54 183.58 ± 334.96 (0.66 - 1547.39)	34.19 202.06 ± 639.73 (0.46 - 4338.52)	51.17 307.08 ± 1248.35 (1.59 - 8878.78)	32.87 358.59 ± 1594.00 (2.87 - 10510.81)
High dose	60.27 256.30 ± 615.17 (0.69 - 3901.90)	20.99 175.09 ± 818.41 (0.70 - 5882.71)	21.61* 67.31 ± 102.54 (0.80 - 411.68)	12.22 70.39 ± 155.68 (0.39 - 873.72)

[a]Data are presented as median, mean ± SD (detectable range). * $p < 0.05$ as compared with placebo and low dose groups.

2.3.4 Effect of NovaSil intervention on aflatoxin M_1 levels

Since the data were highly variable, non-parametric analysis was applied for statistical evaluations. The distribution of urinary AFM_1 levels in the three treatment arms throughout the study duration is shown in Figures 15 and 16. No significant differences were found in median AFM_1 levels among the three study groups at baseline ($p = 0.2485$). Furthermore, no significant differences were found in median AFM_1 levels among the three groups 1-month after the NS intervention ($p = 0.3342$). Statistically significant differences were observed after 3-months of NS intervention ($p = 0.0445$). While the median AFM_1 level was comparable between the PL group and the LD group ($p = 0.3951$), a reduction rate of 58.7% in the median AFM_1 level was found between the HD group and the PL group ($p = 0.0391$) at this time point. A reduction rate of 57.8% in median AFM_1 was also found between the HD group and the LD group ($p = 0.0219$). Significant differences in median AFM_1 levels ($p = 0.0024$) were also found among the three study groups 1-month post intervention, but was mainly due to higher AFM_1 levels in the LD group. As shown in Figure 16, significant decreases in AFM_1 levels were seen in the HD group over the 4-month study period, showing a significant time effect ($p = 0.009$). Although a significant time effect was also noticed in the PL group ($p = 0.002$), levels of AFM_1 were highly variable, as shown by higher median levels at baseline and 3-months compared to the lower median levels at 1-month and 4-months. No significant time effect for AFM_1 levels was found in the LD group over the 4-month study period ($p = 0.3277$).

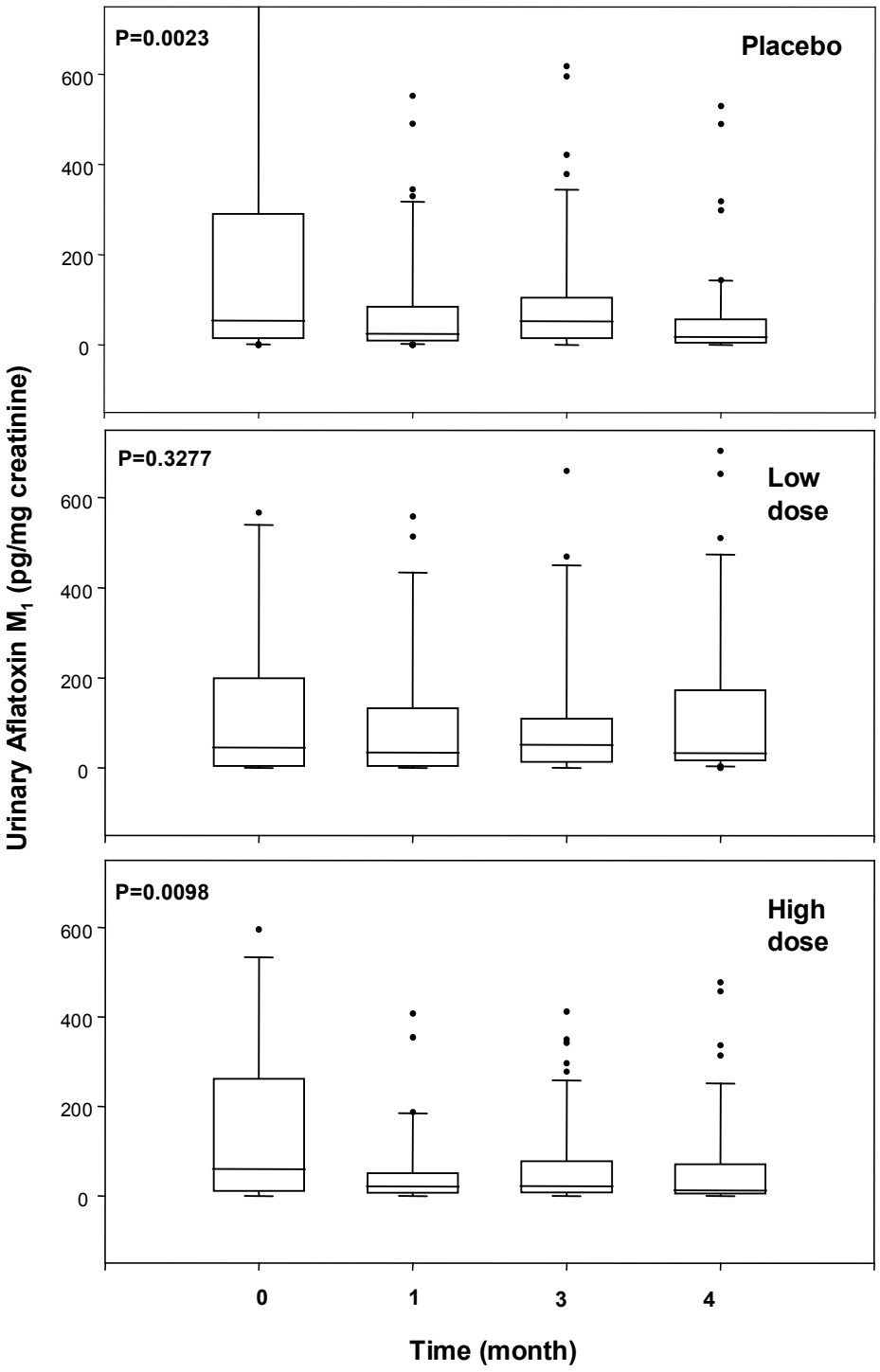

Figure 15. Time effects of NS intervention on AFM_1 levels over the study duration. The box represents values ranging from 25 to 75 percentile of the total samples, the line within it indicating the median value. The bars on both sides of a box represent values ranging from 5 to 25 percentile and from 75 to 95 percentile, respectively.

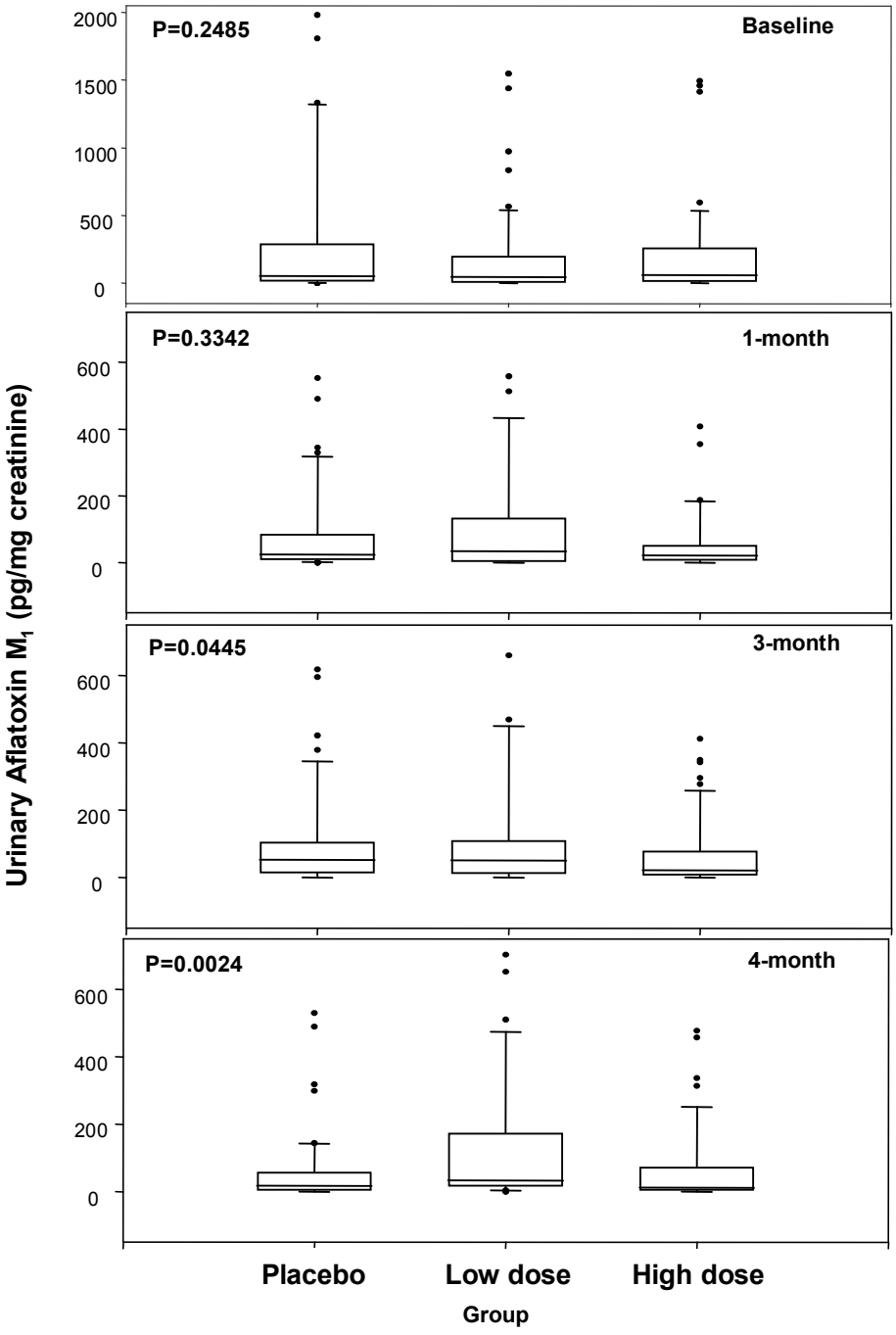

Figure 16. Dose effects of NS intervention on AFM_1 levels over the study duration. The box values ranged from 25 to 75 percentile of the total samples, the line within it indicating the median value. The bars on both sides of a box represent values ranging from 5 to 25 percentile and from 75 to 95 percentile, respectively.

Importantly, non-parametric mixed-effect model analysis showed a significant dose-time interaction (p = 0.0495) delineating a reduction in urinary AFM_1 levels attributable to NS intervention.

2.4 Discussion

It has been well documented that AFs are ubiquitous, naturally-occurring contaminants in a variety of food products. Avoiding consumption of AF-contaminated foods is one of the most fundamental approaches for reducing exposure; however, this approach is not feasible for many populations in developing countries where food security is an issue. Thus, a need for viable intervention strategies to manage AF-contaminated diets persists. Safety and efficacy are the two most important criteria for assessing potential therapeutic and/or clinical intervention agents. The safety of NS has been well documented in animals at levels up to 2.0% in the diet (Phillips et al. 2002, 2006; Afriyie-Gyawu et al. 2005a). Details on the safety of NS intervention in this 3-month trial have been published separately (Afriyie-Gyawu et al. 2008a). Safety data demonstrated daily administration of 1.5 g and 3.0 g NS clay did not produce any dose-dependent adverse effects or differences in hematological parameters, liver and kidney function or electrolytes. The primary aim of this objective was to determine the efficacy of NS for reducing AF exposure in humans. In this study, high levels of urinary AFM_1 were observed in study participants at baseline (before NS intervention) confirming previous data from four of the six communities that comprised our study population (Jolly et al. 2006). In that report levels of AFM_1 were highly variable, similar to this study in which AFM_1 values ranged form undetectable to 13.3 ng/mg creatinine.

Variations in urinary AFM_1 levels have also been observed in other human populations in Africa and China (Allen et al. 1992; Diallo et al. 1995; Jonsyn-Ellis et al. 2000; Wang et al. 2001b). This may be attributed to the short-term nature of the AFM_1 metabolite (i.e., 24 to 48 hours), as well as genotypic or phenotypic variations in AF-metabolizing enzymes. A significant time-dependent effect over the course of the study period (i.e., the 3-month intervention and 1-month post-intervention) was observed in two of the three study groups, e.g., the HD and PL groups, which may reflect variations in seasonal AF exposure levels. Nevertheless, findings from this study showed that administration of NS at 3.0 g/day over a period of 3-months significantly reduced AFM_1 levels (Figure 15). Furthermore, a significant dose-time interaction demonstrated a reduction in urinary AFM_1 levels attributable to NS intervention. This confirms earlier work showing NS inclusion in the diet reduced levels of AFM_1 in the milk of dairy cows (Harvey et al. 1991b) and diary goats (Smith et al. 1994) and in the urine of rats (Sarr et al. 1995; Mayura et al. 1998), turkeys (Edrington et al. 1996), and dogs (Bingham et al. 2004). The reduction rate of up to 58.7% in the median AFM_1 level of the HD treatment group versus the PL group is comparable with an overall 55% reduction observed in median urinary levels of AFB_1-N^7-guanine (a short-term biomarker like AFM_1) after 3-month intervention with chlorophyllin (Egner et al. 2001). In addition, our reduction rate was similar to that observed after 5-weeks of weekly administration of 500 mg oltipraz, a chemopreventive agent, which resulted in a 51% reduction of urinary AFM_1 levels (Wang et al. 1999). However, in that study no significant differences in AFM_1 levels were observed in the arm receiving 125 mg of oltipraz daily. Unlike the enterosorption

strategy which reduces the external dose of toxin prior to adsorption and metabolism, chemoprevention involves a reduction in the biologically effective dose of toxin to cells by modulating cellular metabolism. Hence, a difference was observed between intermittent, high-dose and sustained low-dose oltipraz treatment. In our study, a concurrent reduction in serum AFB_1–lysine albumin adduct levels (measured at Texas Tech University) was observed 3-months after NS intervention (Wang et al. 2008). This signifies that intervention with NS clay can effectively reduce AF exposure from contaminated diets, as represented by AF-specific biomarkers in both the blood and urine. This is the first study to show NS is an effective enterosorbent in the diet of humans, suggesting its potential use in populations at high risk for aflatoxicosis.

In addition to being safe and effective, strategies should be sustainable, economically practical, and cultural acceptable. Clay-based enterosorption with NS represents a sustainable strategy for the reduction of AF exposure, evidenced by participants' excellent adherence to the treatment regimen (over 97%) and number of subjects completing the study (>90%) (Table VII). Additionally, geophagy (clay-eating) is common in cultures of sub-Saharan Africa. Women often consume clay during pregnancy, reportedly at high levels that may range from 30 to 50 g/day (Wiley and Solomon 1998; ATSDR 2000). Thus, NS inclusion in the diet is considered culturally acceptable and in the future may be an important vehicle for the protection of developing fetuses and young infants against AFs. This Phase IIa intervention trial provides the basis for future long-term (phase IIb or III) studies to evaluate the safety and efficacy of

NS as an enterosorbent therapy for acute AF exposure and for the prevention of chronic AF-induced disease or cancer.

III. NOVASIL CLAY INTERVENTION IN A GHANAIAN POPULATION AT HIGH RISK FOR AFLATOXICOSIS: LACK OF INTERACTION WITH SERUM NUTRIENT MINERALS*

3.1 Introduction

Given the anti-nutritional effects of AFs in animals and humans, it is important that enterosorbent strategies aimed at managing AF-induced disease do not significantly interfere with physiological levels of important vitamins or micronutrients. This is especially important in developing countries where individuals may be chronically exposed to uncontrolled amounts of AF and nutritional deficiencies are common (Williams et al. 2004). Dietary inclusion of NS clay (also referred to as HSCAS) has been shown to be safe in over 30 animal models (Table IV). Studies have also indicated that NS addition does not affect levels of vitamins or micronutrients (except in one case in poultry which suggested Zn utilization may be slightly impaired). Here, authors reported that inclusion of HSCAS at 0.5 or 1.0% for 14 days in the diet of broiler chicks did not impair utilization of vitamin A, riboflavin, P or Mn; however, total tibia Zn was reduced in the presence of 1.0% clay indicating Zn may be affected, albeit only slightly, at the highest level of addition (Chung et al. 1990; Chung and Baker 1990). Conversely, Southern et al. (1994) investigated the influence of HSCAS inclusion at 0.5% in chicks fed nutrient-deficient diets and found no effect on tibia mineral concentrations, including

* Reprinted with permission from "NovaSil clay does not affect the concentrations of vitamins A and E and nutrient minerals in serum samples from Ghanaians at high risk for aflatoxicosis" by Afriyie-Gyawu E, Wang Z, Ankrah NA, Xu L, Johnson NM, et al. 2008b. Food Addit Contam 25:872–884, Copyright [2008] by Taylor & Francis.

Zn, Mn, P, and Ca. Still, Chung's finding that Zn may be possibly affected at high levels of montmorillonite inclusion called for the careful observation of the impact of NS on micronutrients. Thus, future *in vivo* studies investigated serum or tissue concentrations of vitamins and minerals following NS treatment. For instance, Pimpukdee et al. (2004) confirmed earlier findings showing NS treatment alone at three different levels (0.125, 0.25, and 0.5%) did not affect vitamin A levels. Moreover, NS (as low as 0.25%) preserved the levels of hepatic vitamin A in groups exposed to high levels of AFB_1 (5 mg/kg), verifying that NS can ameliorate the adverse effects of AF exposure. Further experiments in Sprague-Dawley (S-D) rats demonstrated dietary inclusion of NS clay did not affect any serum vitamin or micronutrient levels. For example, in dams fed NS throughout pregnancy at levels up to 2.0% (w/w), no significant changes occurred in trace metal bioavailability in a variety of maternal and fetal tissues (Wiles et al. 2004). Additionally, in a chronic study S-D rats fed NS at levels ranging from 0.25 – 2.0% (w/w) over 6.5-months did not exhibit any significant dose-dependent differences in concentrations of hepatic vitamin A and E or serum levels of Fe, Zn and vitamins A and E compared to controls (Afriyie-Gyawu et al. 2005a). Based on the safety of NS shown in these S-D rat studies and in a phase I adverse events trial, the first phase IIa clinical intervention trial with NS clay was conducted (described in detail in section II). The high dose (HD) group in this study received 0.25% NS in the diet, representing the minimally effective dose. Of note, no interactions with vitamins or nutrients have been cited at this level in animal models. Therefore, the primary aim of this portion of the study was to investigate interactions of NS with important nutrient minerals by measuring serum

concentrations before and after clinical intervention. In addition, levels of non-nutrient minerals were also evaluated.

3.2 Materials and methods

3.2.1 Samples, chemicals, and reagents

Selected serum samples collected previously from participants in the Phase IIa intervention study were shipped to TAMU frozen and stored at -80°C. Sera from 60 individuals in the HD (n = 29) and PL (n = 31) groups collected at baseline and 3-months after intervention were available for mineral analysis. Therefore, concentrations of nutrient and non-nutrient minerals were determined in a total of 120 samples (60 baseline; 60 3-month). Sample digestion and trace element analysis was conducted at the Trace Element Research Laboratory at TAMU (College Station, TX). This laboratory supplied standard reference materials (Seronorm, Billingstad, Norway) to ensure batch-to-batch consistency. All other chemicals and reagents were purchased commercially at the highest degree of purity available.

3.2.2 Analysis of nutrient and non-nutrient minerals in serum

Approximately 0.45 g of each sample was mixed with 200 µl of concentrated nitric acid in a 15 ml centrifuge tube and heated overnight at 90°C. After samples were cooled, 100 µl of 30% H_2O_2 was added and samples were heated at 70°C for 1 hour. Following cooling, concentrated hydrochloric acid (50 µl) was added and samples were heated at 70°C for 1 hour and cooled. Samples were brought to a final volume of 10 ml with purified water. Mercury (Hg) concentrations were determined by cold vapor atomic absorption (CVAA) using an M-7500 (Cetac Technologies, Omaha, NE) with stannous

chloride as a reductant. Aluminum (Al), barium (Ba), beryllium (Be), calcium (Ca), cobalt (Co), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), molybdenum (Mo), sodium (Na), phosphorus (P), sulfur (S), strontium (Sr), titanium (Ti), vanadium (V), and zinc (Zn) were determined with an inductively coupled plasma- optical emission spectrometer (ICP-OES) using a CirOS (Spectro Analytical Instruments, Fitchburg, MA) with axial viewing and ytterbium (Yb) as an internal standard. Silver (Ag), arsenic (As), cadmium (Cd), chromium (Cr), manganese (Mn), nickel (Ni), lead (Pb), selenium (Se), and thallium (Tl) were determined with an inductively coupled plasma-mass spectrometer (ICP-MS) using an Elan 6100 (Perkin-Elmer, Wellesley, MA) with As, Cr, Mn, and Se acquired in DRC mode, and bismuth (Bi), gallium (Ga), and rhodium (Rh) as internal standards. In addition to blanks, spiked blanks, duplicate samples, and spiked samples, results were validated by preparing and analyzing standard reference materials with each batch of samples.

3.2.2 Statistical analysis

All data sets generated were stored in an Excel database and analyzed with SPSS software version 15.0 (Chicago, IL). Mean, median, and standard deviations (SD) were calculated for concentrations of serum minerals, which were grouped into nutrient or non-nutrient minerals. Comparison of the mineral components in the HD and PL groups at baseline and 3-months following intervention was performed using a student's t-test or the Wilcoxon rank sum test depending on normality. To evaluate the effects of dose, time, and the dose–time interactions of NS treatment on levels of the trace elements, a

non-parametric mixed-effect model was applied as previously described (Brunner et al. 2002). A p-value of ≤ 0.05 (two-tailed) was considered statistically significant.

3.3 Results

A total of 120 serum samples, collected from participants in the HD and PL groups at baseline and 3-months were used for the mineral analyses. The serum levels of 30 minerals were grouped into two categories, nutrient (15) and non-nutrient (15), and are represented in Tables X and XI. In the gender-stratified nutrient category (Table X), baseline levels of each of the 15 minerals measured in the HD and PL groups were not significantly different in male and female participants. Furthermore, no statistically significant differences were found in levels of these nutrient minerals between the HD and PL groups at the end of the 3-month intervention trial. With respect to the 15 non-nutrient minerals analyzed (Table XI), there were no significant differences in any mineral at the baseline between HD and PL groups. After 3-month NS intervention, only levels of strontium (Sr) in serum were significantly different between the PL and HD groups ($p < 0.001$) in both males and females. In the 3-month samples, serum Sr levels were significantly higher in the HD group (113.65 ± 28.00 μg/L) as compared with the PL group (83.55 ± 39.90 μg/L) in males and in females with levels in the HD group (116.40 ± 24.26 μg/L) versus the PL group (90.47 ± 25.68 μg/L). No significant gender difference in serum Sr levels was found. Gender-adjusted non-parametric mixed-effect model analysis showed a significant dose ($p = 0.0214$), time ($p = 0.0000$), and dose*time ($p = 0.0000$) effect for serum Sr indicating increased levels may be attributable to NS intervention. No other significant dose*time effects were noted for minerals.

Table X. Serum levels of nutrient minerals in study participants in Ghana at baseline and 3-months of intervention.

Nutrient minerals	Baseline				3-Months			
	Male		Female		Male		Female	
	Placebo	High dose	Placebo	High dose	Placebo	High dose	Placebo	High dose
Ca (mg/L)	90.28 ± 4.28	93.84 ± 4.58	93.18 ± 4.25	90.29 ± 5.48	93.72 ± 7.58	97.00 ± 4.79	95.63 ± 6.42	93.90 ± 5.72
Cu (mg/L)	1.12 ± 0.19	1.32 ± 0.27	1.41 ± 0.30	1.66 ± 0.42	1.14 ± 0.16	1.27 ± 0.25	1.38 ± 0.22	1.36 ± 0.16
Fe (mg/L)	1.29 ± 0.42	1.23 ± 0.30	1.45 ± 1.14	1.36 ± 0.43	1.40 ± 0.57	1.33 ± 0.32	1.23 ± 0.32	1.31 ± 0.51
K (mg/L)	220.42 ± 23.00	219.10 ± 25.62	224.83 ± 27.89	207.70 ± 17.58	189.00 ± 19.38	195.32 ± 17.65	196.67 ± 24.08	190.00 ± 16.69
Mg (mg/L)	18.72 ± 1.20	18.57 ± 1.68	18.09 ± 1.62	17.59 ± 1.75	19.85 ± 1.97	19.32 ± 1.31	19.42 ± 1.71	19.08 ± 2.06
Na (mg/L)	3097.90 ± 71.61	3130.53 ± 61.87	3121.67 ± 79.07	3100.00 ± 95.92	3160.00 ± 164.76	3205.79 ± 64.32	3184.17 ± 168.12	3185.00 ± 141.52
P (mg/L)	106.54 ± 6.89	107.51 ± 15.07	117.96 ± 13.90	105.35 ± 12.38	112.02 ± 13.24	115.98 ± 19.67	121.66 ± 16.13	111.50 ± 11.72
S (mg/L)	1197.90 ± 60.33	1244.74 ± 76.26	1241.67 ± 97.50	1216.00 ± 78.34	1253.68 ± 136.27	1287.37 ± 73.09	1303.33 ± 112.44	1268.00 ± 81.62
Zn (mg/L)	1.37 ± 0.57	1.21 ± 0.12	1.20 ± 0.28	1.36 ± 0.65	1.38 ± 0.40	1.38 ± 0.33	1.31 ± 0.23	1.25 ± 0.54
Co (µg/L)	1.36 ± 0.78	1.30 ± 0.85	1.06 ± 0.38	1.77 ± 1.71	1.65 ± 1.07	1.35 ± 0.88	1.21 ± 0.47	0.95 ± 0.20
Cr (µg/L)	7.84 ± 11.89	4.66 ± 0.48	6.12 ± 2.62	8.31 ± 10.41	6.50 ± 3.92	6.19 ± 3.61	4.68 ± 0.52	7.25 ± 3.50
Mn (µg/L)	8.35 ± 8.28	4.64 ± 4.42	4.95 ± 5.59	4.38 ± 2.14	9.13 ± 13.90	5.37 ± 3.39	2.94 ± 0.87	3.77 ± 1.51
Mo (µg/L)	14.97 ± 5.06	17.04 ± 3.94	17.07 ± 4.22	16.59 ± 5.84	23.73 ± 11.58	20.40 ± 6.48	23.52 ± 18.74	21.32 ± 9.12
Ni (µg/L)	18.47 ± 2.61	16.25 ± 2.98	17.61 ± 4.24	21.18 ± 17.80	19.39 ± 5.59	19.08 ± 9.06	16.14 ± 2.94	16.51 ± 3.36
Se (µg/L)	111.87 ± 2 0.01	113.94 ± 23.67	123.13 ± 23.50	122.17 ± 20.96	122.80 ± 22.18	121.33 ± 26.28	128.92 ± 16.40	128.87 ± 28.71

Note: Data represent the mean ± SD of the serum levels of trace elements. After 3-months of NS intervention, no significant differences were found in the HD group compared to PL group in both male and female participants ($p > 0.05$).

Table XI. Serum levels of non-nutrient minerals in study participants in Ghana at baseline and 3-months of intervention.

Non-nutrient minerals	Baseline				3-Months			
	Male		Female		Male		Female	
	Placebo	High dose	Placebo	High dose	Placebo	High dose	Placebo	High dose
Ag (µg/L)	0.23 ± 0.02	0.22 ± 0.01	0.25 ± 0.07	0.23 ± 0.02	0.33 ± 0.48	0.22 ± 0.01	0.22 ± 0.01	0.24 ± 0.06
Al (µg/L)	146.84 ± 63.24	120.58 ± 55.05	105.42 ± 43.48	157.90 ± 122.94	133.53 ± 66.54	134.58 ± 71.70	98.17 ± 38.00	153.80 ± 111.88
As (µg/L)	8.64 ± 1.39	9.27 ± 1.77	8.38 ± 1.01	8.91 ± 1.28	8.78 ± 2.28	8.98 ± 1.28	8.09 ± 1.09	8.30 ± 1.21
Ba (µg/L)	79.74 ± 11.90	83.16 ± 10.32	74.75 ± 17.22	81.20 ± 24.54	122.05 ± 24.42	112.74 ± 18.13	108.42 ± 44.53	119.30 ± 51.27
Be (µg/L)	1.13 ± 0.10	1.11 ± 0.03	1.10 ± 0.02	1.12 ± 0.03	1.11 ± 0.05	1.10 ± 0.03	1.10 ± 0.03	1.19 ± 0.29
Cd (µg/L)	0.63 ± 0.16	0.78 ± 0.60	0.71 ± 0.20	0.67 ± 0.30	0.72 ± 0.36	0.74 ± 0.58	0.69 ± 0.17	0.63 ± 0.17
Hg (µg/L)	5.63 ± 0.50	5.55 ± 0.16	5.48 ± 0.08	5.59 ± 0.16	5.55 ± 0.24	5.18 ± 0.16	5.51 ± 0.14	5.95 ± 1.43
Li (µg/L)	22.63 ± 1.86	22.16 ± 0.60	21.91 ± 0.29	22.40 ± 0.70	22.21 ± 0.98	22.00 ± 0.67	21.92 ± 0.67	23.90 ± 5.69
Pb (µg/L)	17.53 ± 7.02	15.12 ± 8.57	13.70 ± 6.03	18.32 ± 13.04	15.67 ± 4.39	14.05 ± 4.84	18.02 ± 18.43	12.10 ± 6.00
Sb (µg/L)	1.13 ± 0.10	1.11 ± 0.03	1.10 ± 0.02	1.12 ± 0.03	1.14 ± 0.17	1.10 ± 0.03	1.10 ± 0.03	1.19 ± 0.29
Sr (µg/L)	70.15 ± 16.48	68.01 ± 16.54	75.70 ± 27.45	75.68 ± 12.33	83.55 ± 39.90	113.65 ± 28.00**	90.47 ± 25.68	116.40 ± 24.26**
Ti (µg/L)	112.68 ± 9.87	111.10 ± 3.11	109.67 ± 1.50	111.80 ± 3.33	110.84 ± 4.68	110.37 ± 3.18	110.25 ± 2.80	118.90 ± 28.61
Tl (µg/L)	0.23 ± 0.03	0.22 ± 0.01	0.26 ± 0.09	0.26 ± 0.09	0.30 ± 0.35	0.22 ± 0.01	0.23 ± 0.04	0.24 ± 0.06
U (µg/L)	0.28 ± 0.21	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.23 ± 0.04	0.24 ± 0.06
V (µg/L)	11.27 ± 0.99	11.11 ± 0.31	10.97 ± 0.15	11.18 ± 0.33	11.08 ± 0.47	11.04 ± 0.32	11.03 ± 0.28	11.89 ± 2.86

Note: Data represent the mean ± SD of the serum levels of trace elements. After 3-months of NS ingestion, only Sr levels were significantly higher ** $p < 0.001$ in the HD versus PL groups in both male and female participants.

3.4 Discussion

In the past, it has been suggested that the positive charge deficiencies on phyllosilicate clays create potential for sorption of positively charged or cationic compounds, including minerals (Theng 1974). In fact, this mechanism may be partly responsible for the adsorption of AFB_1 onto NS surfaces. Conversely, interference with micronutrients may hinder the use of enterosorption for the reduction of AFs. Therefore, to further ensure the safety of NS administration, we investigated the potential for NS to interfere with vitamins and minerals in participants in our clinical trial in Ghana. Details on the effect of NS intervention on serum vitamin A and E levels have been described separately (Afriyie-Gyawu et al. 2008b). Data demonstrated that daily administration of 1.5 g and 3.0 g NS clay did not produce any dose-dependent adverse effects or differences in concentrations of these two important vitamins. Results from this portion of the study clearly illustrate that administration of NS clay at the high dose level over a period of 3-months did not affect the serum concentrations of nutrient minerals, evidenced by comparable levels of these parameters among the HD and PL groups in males and females (Table X) at both time-points. Importantly, these findings confirm the results of our previous short-term human study (Wang et al. 2005) and numerous animal studies (Wiles et al. 2004; Afriyie-Gyawu et al. 2005a). Furthermore, concentrations of non-nutrient minerals, with the exception of Sr, did not differ significantly between groups at baseline or 3-months with NS treatment. Overall, these findings reflect the purity of the test agent and our efforts to minimize potential environmental toxicants and non-nutrient minerals in NS. For instance, priority metals were either below the LOD for

the analytical procedures used, or present in the HD at levels 7- to 240-fold lower than the JECFA standards (Table V). Data also suggest that mineral components in NS are largely unavailable for absorption. However, serum Sr levels were statistically increased in the HD group compared to the PL group at 3-months of NS intervention in both genders, implying bioavailability of this mineral. These data are in agreement with previous Phase I results which showed an apparent dose-dependent increase in serum Sr after 2-week NS intake in participants from the U.S. (Wang et al. 2005). Since clinical reference ranges are currently not available for serum Sr, it is challenging to interpret the significance of this effect on a clinical basis. Sr is naturally present in water and foods, such as cereals, grains and seafood at levels up to 25 mg/kg (Cabrera et al. 1999). Naturally-occurring Sr, other than its radioactive isotope, is considered non-hazardous. Sr is similar to Ca in that these minerals are absorbed in the GI tract, concentrated mainly in the bone, and primarily excreted in the urine (Cohen-Solal 2002). In a clinical trial for treatment of osteoporosis, up to 680 mg Sr/day did not result in significant adverse effects in human volunteers (Meunier et al. 2004). In the present study, the amount of Sr calculated to be in 3.0 g NS (the HD level) was equal to 4.29 mg/day. This is considerably lower than levels found in certain foods and drastically lower than levels administered in the osteoporosis intervention trial. Thus, it is not anticipated that this level would be of any clinical significance to participants. Still, in future studies we intend to closely monitor physiological levels of Sr in addition to vitamin and mineral concentrations. Overall, these results in combination with to findings presented in Section II indicate that NS intervention (up to 3.0 g/day) is safe and effective, supporting

the prospect of applying NS for the protection humans who are either acutely or chronically exposed to high levels of AFs in their diet.

IV. ASSESSMENT OF PAH EXPOSURE IN A GHANAIAN POPULATION AT HIGH RISK FOR AFLATOXICOSIS*

4.1 Introduction

In the Ashanti Region of Ghana, our previous biomarker data have shown individuals are at high risk for aflatoxicosis based on levels of AF exposure biomarkers measured in the blood and urine (Jolly et al. 2006; section II). It is also well-known that chronic AF exposure significantly contributes to the risk of developing liver cancer (Wogan 1992; IARC 1993), and in West Africa HCC risk is significantly increased by chronic infection with hepatitis B virus (HBV) (Kao and Chen 2002). While population-based cancer morbidity and mortality data in Africa on the whole are scarce to non-existent, as early as the 1950s, researchers noted primary liver carcinoma was common in West African troops (Findlay 1950; Edington 1956). Since Edington's report, cancer mortalities were largely undocumented in Ghana until a recent review article by Wiredu and Armah (2006). In this report authors reviewed cancer mortality patterns in Ghana based on autopsies and hospital records from the previous 10 years and showed liver cancer was the leading cause of cancer death in men and third leading cause in women. This is consistent with reports from other geographic regions where chronic AF exposure and HBV infection persist, particularly in China, Taiwan, and other parts of Africa (Kirk et al. 2006). Since it has been proposed that a concurrent exposure to PAHs

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may interact with known risk factors for HCC, e.g., AF and HBV (Wu et al. 2007), it was of interest to determine PAH exposure in our well-defined study population in Ghana. Although some published studies have determined PAH concentrations in soil and water matrices (Essumang et al. 2006; Tano-Debrah et al. 2007), no direct biomarker measurements for human exposure to PAHs have been reported in Ghana to our knowledge. Therefore, the main objectives of this portion of the study were to assess overall exposure to PAHs by measuring urinary 1-hydroxypyrene (1-OHP) and to determine if there was a relationship between AF and PAH exposures. Since concurrent exposures may affect HCC risk, strategies that reduce AFs and PAHs are highly desirable for populations at risk for both agents. Among various strategies, clay-based enterosorption by NovaSil (NS) appears to be safe and effective for the reduction of AFs (Afriyie-Gyawu et al. 2008a, 2008b; Wang et al. 2008). The enterosorption strategy would not be expected to reduce PAH exposure from inhalation; however, the potential affect of NS on the bioavailability of PAHs from the gastrointestinal tract has not been investigated. Thus, a secondary objective was to determine the effect of NS versus PL treatment on 1-OHP levels in urine samples collected from the NS intervention trial.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Authentic 1-OHP reference standard was obtained from the Midwest Research Institute Chemical Carcinogen Reference Standard Repository (Kansas City, MO). UV-visible spectrophotometry was used to verify the concentration of 1-OHP stock solutions. Sep-Pak C18 cartridges were purchased from Waters (Milford, MA), and β-

glucuronidase from Helix pomatia (type HP-2S) were obtained from Sigma Chemical Co. (St. Louis, MO). All of the experiments were done using filtered and deionized water (18.2 MΩ.cm) (Millipore, Milford, MA) and HPLC-grade solvents. All other chemicals and reagents were purchased commercially at the highest degree of purity available.

4.2.2 Study design

The study protocol (previously described in detail in section 2.2.3) was approved by the Institutional Review Boards at Texas A&M University and Noguchi Memorial Institute for Medical Research in Ghana for Ethical Clearance. To delineate contributing factors to PAH exposure, participants were further classified as smokers or non-smokers. Trained study monitors collected blood and urine samples from each participant at specified time points (Figure 12). Aliquots of the first urines collected were stored separately (~4.5 ml) in polypropylene tubes for 1-OHP analysis and shipped to TAMU where they were kept frozen at -80°C.

4.2.3 Urinary 1-hydroxypyrene analysis

Urinary 1-OHP levels were measured using an HPLC-fluorescence method based on a procedure described by Gardiner et al. 1992. Briefly, urine samples (4.0 ml) were adjusted to pH 5.0 with an equal volume of 1.0 M acetate buffer (pH 5.0). β-Glucuronidase (50 µl) from Helix pomatia possessing sulfatase activity was added, and samples were incubated for 4 hours at 37°C while gently shaking in a water bath. The hydrolyzed samples were passed through primed Sep-Pak C18 columns on a vacuum manifold followed by sequential washing steps of 3.0 ml deionized H_20 followed by 3.0

ml 50% MeOH in water. 1-OHP was eluted from the column with 100% MeOH, and the eluates were evaporated to dryness under N2 gas and reconstituted in MeOH. The analyses were conducted using an HPLC system with fluorescence detection (Waters, Milford, MA). Excitation and emission parameters were set at 240 and 388 nm, respectively. Aliquots of the extracts were injected and analyzed using a 125 x 4.6 mm Spherisorb ODS2 HPLC column (Waters) with a particle size of 3 µm. The mobile phase was comprised of 75% MeOH in water, and chromatographic separation was achieved by isocratic elution at a flow rate of 1.1 ml/min for 15 min. The 1-OHP peak was detected at a retention time averaging 6.2 min (Figure 17), and the limit of detection was approximately 0.25 nmol/L of urine. Urinary concentrations of 1-OHP were expressed as µmol/mol creatinine in order to correct for variations in urine dilution.

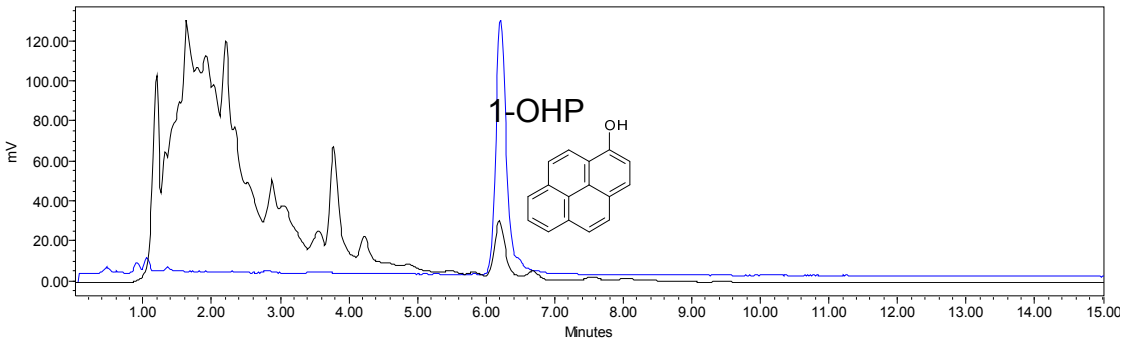

Figure 17. Representative HPLC chromatograph showing urinary 1-hydroxypyrene metabolite peaks. The 1-OHP metabolite in a participant sample collected at baseline (black) is compared to a 10 ng/ml 1-OHP standard solution (blue). Peak areas were used for quantification of 1-OHP in urine samples collected from at baseline and 3-months of intervention.

Blank urines spiked with 1-OHP standard in the range of 0.01 – 2 ng were prepared, incubated and extracted as described in the procedure minus the enzyme addition to generate a six-point calibration curve that was linear over the tested range ($r^2 = 0.9904$) for the instrument. The repeatability precision was 7.0% and recoveries averaged 99%. The machine was calibrated daily by injecting multiple aliquots of 1-OHP reference standard, which was prepared bi-weekly and stored in the dark at 4°C.

4.2.4 Statistical analysis

Data generated from HPLC analysis were transferred into an Excel database for management. Mean, median, standard deviation (SD) and range were calculated for concentrations of 1-OHP. Statistical analyses were done using SPSS software version 15.0 (Chicago, IL). Correlation analysis was done to examine the relationship of AF and PAH exposures (i.e., AF-albumin adduct compared to 1-OHP levels); *p*-values were generated based on Spearman correlation coefficients. To show the consequence of NS ingestion on 1-OHP levels, statistical evaluation focused on the comparisons among different treatment groups at baseline and 3-months following intervention. Since the parameters were not normally distributed, the Kruskal-Wallis test or Wilcoxon rank sum test was used to compare the differences among and between treatment groups at each time point. To evaluate the effect of dose and time interactions, a nonparametric mixed-effect model was applied as previously described (Brunner et al. 2002). A *p*-value of $\leq$ 0.05 (two-tailed) was considered significant.

4.3 Results

4.3.1 Urinary 1-hydroxypyrene levels

Overall, a total of 279 urine samples collected at baseline (n = 121) and 3-months (n = 158) were available for 1-OHP analysis. Out of the total urines, 98.9% showed detectable levels of 1-OHP. Median, mean ± SD, and the range of 1-OHP levels at both time points are presented in Table XII; participants are further delineated as smokers or non-smokers. Only 9 of the 177 study participants were known tobacco-smokers. In non-smokers, levels of 1-OHP were detected in 98.2% (111/113) and 99.3% (148/149) of the samples collected at the beginning of the study (baseline) and following 3-months intervention, with median values of 0.64 and 0.69 μmol/mol creatinine, respectively. Of the participants classified as tobacco-smokers, 1-OHP was detected in 100% of urine samples from baseline (8/8) and 3-months (9/9). Median levels of 1-OHP did not differ significantly in participants classified as smokers compared to non-smokers in samples measured at baseline ($p = 0.098$) or after 3-months of intervention ($p = 0.822$).

Table XII. Levels of urinary 1-OHP in study participants in Ghana.

	1-OHP levels (μmol/mol creatinine)[a]	
Smoking status	Baseline	3-months
Non-smokers	0.64, 0.80 ± 0.68 (0.01 – 3.37)	0.69, 1.08 ± 1.09 (0.06 – 5.11)
Smokers	0.42, 0.42 ± 0.25 (0.10 – 0.78)	0.61, 1.00 ± 0.81 (0.32 – 2.37)

[a]Data are presented as median, mean ± SD (detectable range).

4.3.2 Relationship between 1-hydroxypyrene and AF-albumin adduct levels

Correlation analysis was done to examine the relationship between AF exposure (measured by the AF-albumin adduct) and 1-OHP levels; results are illustrated in Figure 18. No significant correlations were found between levels of the AF-albumin adduct and 1-OHP at baseline (Figure 18a CC=0.080, p=0.394) or after 3-months of intervention (Figure 18b CC=0.029, p=0.717); CC represents Spearman correlation coefficients.

4.3.3 Effect of NovaSil intervention on 1-hydroxypyrene levels

The distribution of urinary 1-OHP levels in participants at baseline and after 3-months of intervention is illustrated in Figure 19. There were no significant differences in median 1-OHP levels among the three study groups at baseline (p = 0.890) or after 3-months of intervention (p = 0.384). The median 1-OHP levels were comparable between the PL and LD group in urine samples collected at baseline (p = 0.820) and 3-months (p = 0.186). Additionally, no significant differences were found in median levels of 1-OHP between the PL and HD group at baseline (p = 0.843) or after 3-months (p = 0.325). Furthermore, no significant time effect for 1-OHP levels were found in the HD (p = 0.373), LD (p = 0.113) or PL (p = 0.758) groups over the 3-month study period (Figure 20). Non-parametric mixed-effect model analysis did not show a significant dose-time interaction (p = 0.436) delineating that there was not a reduction in urinary 1-OHP levels due to the NS intervention. A trend for reduction in 1-OHP levels in participants in the PL group was observed after 3-month intervention; median 1-OHP values were 0.66 μmol/mol creatinine at baseline and 0.58 μmol/mol creatinine after 3-months. However, this difference was not statistically significant.

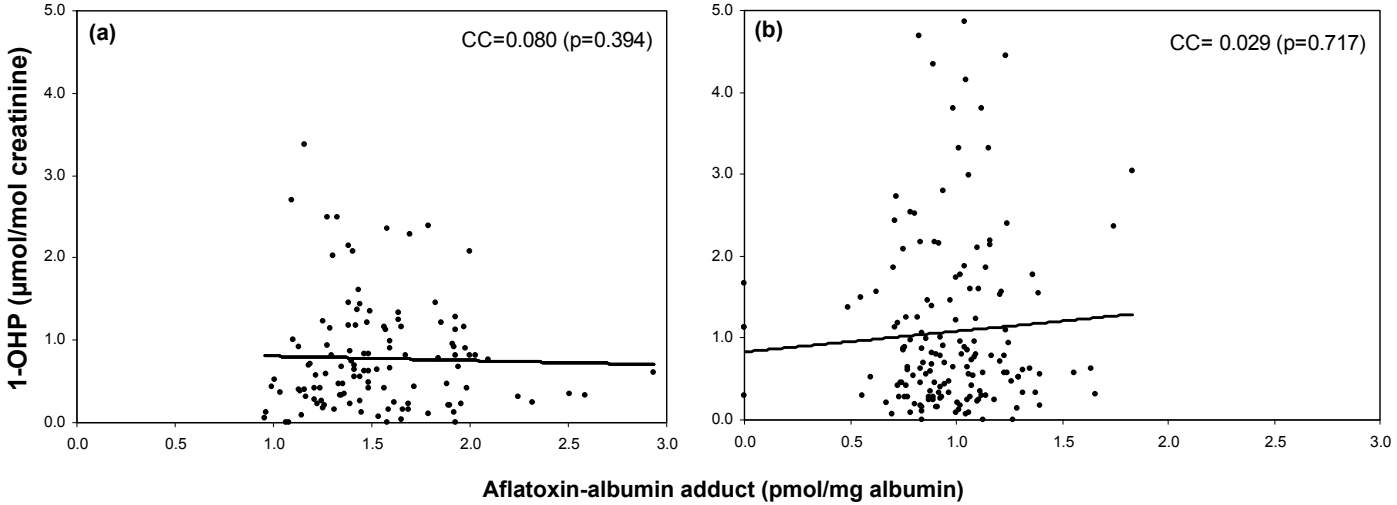

Figure 18. Correlation analyses between 1-OHP and AF-albumin adduct levels in study participants in Ghana. No significant correlations were found between AF-albumin adduct and 1-OHP levels at baseline (a) or after 3-months of intervention (b); *p*-values were generated based on Spearman correlation coefficients (CC).

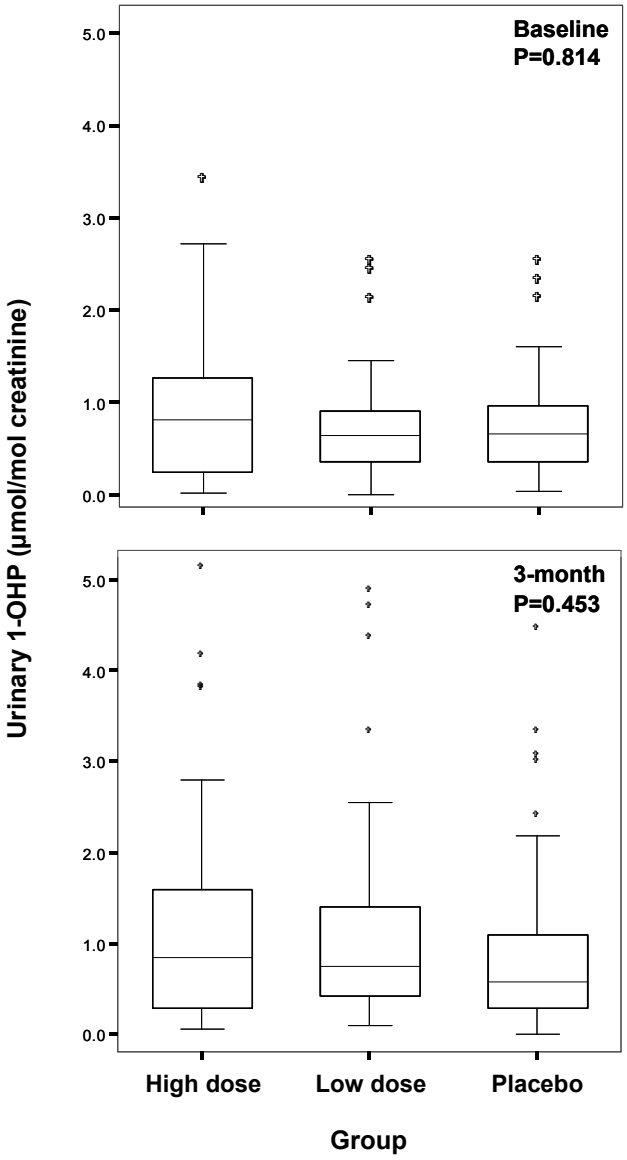

Figure 19. Dose effects of NS intervention on 1-OHP levels over the study duration. The box represents values ranging from 25 to 75 percentile, the line within indicates the median value. The bars on both sides of the box represent values ranging from 5 to 25 percentile and from 75 to 95 percentile, respectively.

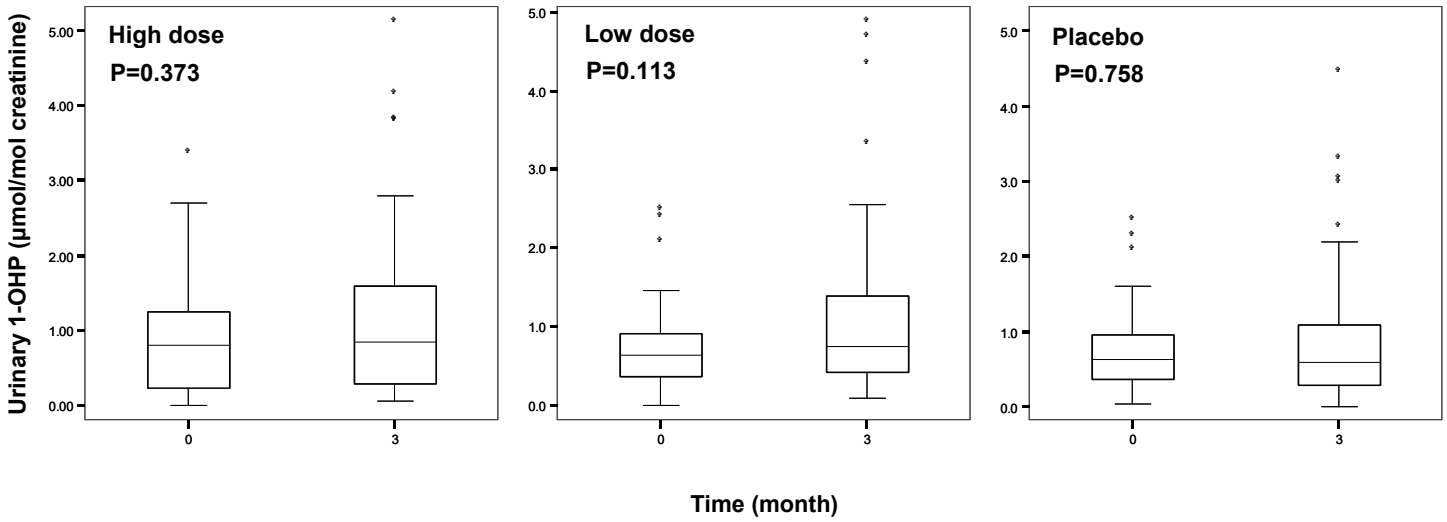

Figure 20. Time effects of NS intervention on 1-OHP levels over the study duration. The box plots show distributions of 1-OHP levels in each group at baseline (0-month) and after 3-months of intervention. The box represents values ranging from 25 to 75 percentile, the line within it indicating the median value. The bars on both sides of the box represent values ranging from 5 to 25 percentile and from 75 to 95 percentile, respectively. No significant time effect for 1-OHP levels were found in any of the treatment arms over the course of the study period.

4.4 Discussion

Findings from this portion of our study illustrated that the majority of study participants in Ghana had a measurable exposure to PAHs (Table XII). Data further demonstrated 1-OHP levels did not vary significantly between participants classified as smokers and non-smokers. The influence of cigarette smoke on 1-OHP levels has yielded differing results in previous work. When individuals were exposed to background levels of environmental PAHs, the consequence of smoking resulted in increased excretion of 1-OHP (Levin et al. 1995; Viau et al. 1995). Conversely, at higher exposure levels, smoking failed to produce any differences in urinary 1-OHP levels, compared to not smoking (Buchet et al. 1992; Bouchard and Viau 1999; Viau et al. 2000). In this study, smoking did not show an effect on 1-OHP levels possibly because environmental exposure was predominant. In addition, of the small percentage classified as smokers in our study population (only 9 subjects), details such as amount and frequency of smoking was unknown. This information would be of interest in future studies. Nonetheless, 1-OHP levels measured in this Ghanaian population were higher than those previously recorded for non-smoking individuals in numerous countries (Table III). For instance, the median 1-OHP level measured in non-smokers at baseline in our study population (0.64 μmol/mol creatinine) was considerably higher than median 1-OHP concentrations documented in non-smokers in the U.S. (0.27 μmol/mol creatinine), Canada (0.07 μmol/mol creatinine), and other Western European countries (Levin 1995). Alternatively, our results were remarkably similar to reported levels of 1-OHP measured in non-smokers in a Chinese population (median = 0.68 μmol/mol

creatinine) and comparable, albeit lower, to individuals living in traditional houses in rural Burundi (geometric mean = 1.50 μmol/mol creatinine) (Zhao et al. 1995; Viau et al. 2000). In both reports, authors proposed the increased levels of 1-OHP excretion may be attributable to the broad use of coal or wood burning for cooking and heating purposes. In fact, the use of indoor wood/coal burning fireplaces and stoves are employed as the primary means of cooking and heating in many developing countries, particularly in rural areas. It has been documented that the combustion of wood and other fuels for cooking constitutes a significant source for PAH exposure (Lioy and Greenberg 1990) and, the subsequent consumption of grilled foods has been linked to increased levels of 1-OHP in the urine (van Maanen et al. 1994). The elevated level of 1-OHP measured in our study population and knowledge of cooking culture in this rural area of Ghana largely suggests that inhabitants are exposed to high doses of PAHs from the indoor preparation of foods by using wood as fuel, socializing by fires, and the ingestion of smoked foods.

Another possible route of PAH exposure in our study population may be through the ingestion of dried/smoked maize. Maize if often dried directly on hot asphalt roads, suggesting an additional mode of PAH contamination. The direct drying and smoking of this staple crop is regularly employed as the first step in food preparation or as a way to decrease mold contamination in storage or repel insects. AF-contaminated maize constitutes a major food safety problem in Ghana. Since exposure to both AFs and PAHs may result in an even greater risk of significant negative health impacts, we investigated the correlation between 1-OHP and AF-albumin levels as a means of indirectly linking a

high intake of AF-contaminated food with high PAH exposure. Although 1-OHP is considered a short-term biomarker of exposure, measurements at both time-points in our study population did not show a significant time-effect. Thus, PAH exposure may be assumed to be relatively continuous over this time period. Correlation analyses showed that there was not a significant linear correlation between urinary 1-OHP levels and serum AF-albumin adducts levels at baseline or 3-months (Figure 18). This is likely due to multiple and different pathways of exposure to these compounds. Ultimately, subjects exposed to both PAHs and AFs, even if the exposure levels are independently distributed, may be at increased risk for disease or possibly HCC.

A second objective of this portion of our clinical intervention study was to determine the consequence of NS treatment on urinary 1-OHP. To date, no clinical interventions have been reported for PAHs using enterosorbent strategies. Results from our study illustrated that NS-treatment did not significantly alter 1-OHP levels after 3-months of intervention. This may be due to variation in the route of exposures, i.e., inhalation versus ingestion. The enterosorption strategy would not be expected to reduce exposure from air. Moreover, the structural characteristics of PAH compounds did not predict sorption in the gastrointestinal tract; thus, data further confirm the specificity of NS for the enterosorption of AFs Although not statistically significant, we observed a trend for reduction in 1-OHP levels in participants in the PL group: median reduced from 0.66 μmol/mol creatinine at baseline to 0.58 μmol/mol creatinine after 3-months. The placebo material used in the intervention study was composed of microcrystalline cellulose. Previous work has shown that cellulose (or fiber in general) may alter the

entrerohepatic recycling of 1-OHP. For instance, Viau et al. (2004) noted a trend in the reduction of urinary 1-OHP excretion in rats fed pyrene and diets containing fiber. Additionally, bulk cellulose in the diet was shown to affect the recovery of 1-OHP from the urine. Further research is warranted to test cellulose and similar materials for their ability to affect toxin adsorption and disposition following oral exposures.

In summary, this study illustrates that 1-OHP may be utilized to identify populations vulnerable to PAH exposure. A population living in a rural area of Ghana frequently exposed to AFs was shown to be highly exposed to PAHs; however, the exposure levels were independently distributed. Future research should focus on this population to better comprehend the sources of PAH exposure. Additionally, long-term biomarkers, like the PAH–albumin adduct could be utilized in prospective studies to further investigate associations between PAH exposure and its public health impact. Other strategies for the reduction of PAHs besides clay-based enterosorption are warranted in this population, as is further investigation to evaluate if the combined decrease of exposures to AFs and PAHs may reduce the incidence of liver cancer or additional disease.

V. ASSESSMENT OF AFLATOXIN AND PAH EXPOSURE BIOMARKERS IN A U.S. POPULATION WITH A HIGH INCIDENCE OF HEPATOCELLULAR CARCINOMA

5.1 Introduction

Hepatocellular carcinoma (HCC) is the most common and widespread form of primary liver cancer (Parkin et al. 2001). Approximately 600,000 deaths annually are attributed to this malignancy, making HCC the third leading cause of cancer mortality worldwide (Kew 2002). Historically, it has been estimated that the majority of HCC cases occur in developing countries (Wild and Hall 2000); however, an increased incidence of HCC in many developed countries, including the United States, has been recognized over the recent decades (El-Serag and Mason 1999). The state of Texas in particular has been shown to have the highest HCC mortality rate in the U.S. (Devesa et al. 1999). A Hispanic population residing within several zip codes in a community in Bexar County, TX has been disproportionately affected by a high incidence of HCC (ATSDR 2001). Age-adjusted cancer incidence rates from 2002-2006 (Table XIII) demonstrate that Hispanics in Bexar Co. have an increased HCC incidence rate (16.5) compared to Hispanics in Texas (10.9); rates per 100,000 (Texas Cancer Registry 2009). Notably, the HCC incidence rate for Hispanics living in Bexar Co. is considerably higher than all races in Bexar Co. (10.0) and all races in Texas (5.8). Hispanic males in Bexar Co. were shown to have the highest incidence rate during this time period at 27.1. Thus we were interested in exploring factors that may contribute to HCC in this community.

Table XIII. Age-adjusted HCC incidence rates in Texas and Bexar County.

Texas					
All races and ethnicities			Hispanic ethnicity		
Male	Female	Both	Male	Female	Both
9.6[a] (9.3 – 9.9)	2.7 (2.5 – 2.8)	5.8 (5.7 – 6.0)	17.1 (16.2 – 18.0)	5.8 (5.3 – 6.2)	10.9 (10.4 – 11.4)
Bexar County					
All races and ethnicities			Hispanic ethnicity		
Male	Female	Both	Male	Female	Both
16.3 (14.8 – 17.8)	5.0 (4.3 – 5.8)	10.0 (9.2 – 0.8)	27.1 (24.2 – 30.2)	8.4 (7.0 – 9.9)	16.5 (15.0 -18.0)

[a]Rates are per 100,000 and age-adjusted to the 2000 U.S. standard population (95% confidence intervals). Date prepared by the Texas Department of State Health Services, Cancer Epidemiology and Surveillance Branch, Texas Cancer Registry.

Multiple factors including diet, environment, lifestyle, health status, gender, and genetics have been shown to play a role in the etiology of HCC. In section I, this report described the importance of dietary AF exposure as a major risk factor for HCC, particularly in developing counties. However, numerous individuals in underserved populations, even in developed areas, face food insecurity which may necessitate the consumption of lower quality foods and thereby increase the likelihood of AF exposure. For instance, corn intended for animal feeds (which have much higher allowable AF action levels) has been rumored to be consumed by some individuals living in low socioeconomic conditions. Furthermore, individuals may be at increased risk for HCC due to additional biological factors, namely hepatitis virus and/or concurrent PAH exposure. While HBV has been shown to be endemic to parts of the world with high HCC cases, the frequency of HBV infection in the U.S. is far lower. Conversely, an association between HCV infection and HCC incidence has been demonstrated in the U.S., particularly in Texas (Davila et al. 2004). Records from University of Texas (U.T.) M.D. Anderson Cancer Center have shown that more than 50% of HCC cases observed in Texas could be attributed to HCV infection (Hassan et al. 2002). In recent work, Chen et al. (2007) showed AF-albumin adduct levels were associated with advanced liver disease in HCV patients in an endemic area in Taiwan, suggesting AFB_1 may also interact with HCV. Hoque et al. (1999) previously demonstrated the presence of AF-albumin adducts in a small number of HCC patients (5/5 sera samples) registered at the U.T.M.D. Anderson Cancer Center, prompting the question 'does AFB_1 play a role in the etiology of HCC in the U.S.?' While it is established that a viral-chemical interaction

exists between hepatitis and AFB_1, the possible contribution of AFs in the human diet has not yet been assessed in Bexar Co. Due to the disproportionate occurrence of HCC observed in an underserved community in this county, an environmental health study was conducted as a preliminary survey to assess AF and PAH biomarkers of exposure; investigate dietary factors that may contribute to increased AF exposure; and to determine other factors that may contribute to increased HCC incidence, namely the prevalence of hepatitis infection in the population. Additionally, it was important to evaluate biomarker levels as compared to levels in a high risk population (i.e., the Ashanti Region of Ghana) as a way of relating domestic and international foodborne and environmental exposures.

5.2 Materials and methods

5.2.1 Participant recruitment and sample collection

Study participants were recruited from three zip codes in the San Antonio metropolitan area of Bexar Co., where the incidence of liver cancer has been shown to be significantly elevated. These zip codes encompass nearly 11% of Bexar Co.'s population, and residents are predominantly Hispanic (90.2%). A total of 186 participants were recruited at the San Antonio Metropolitan Health District (SAMHD) from October 2007 to May 2008. Volunteers (males and females) who qualified as study participants met the following criteria: 1) at least 18 years of age and 2) a minimum of two years residency (within the last 12 months) in one of the three specified study zip codes. The study protocol was approved by the Institutional Review Board at TAMU, and all participants were provided written informed consent, as well as oral explanation

of the study protocol prior to beginning the study. Upon enrollment, SAMHD public health officials administered a questionnaire (in English or Spanish) and collected demographic information through in-person interviews. Biological samples, including venous blood and urine, were collected, separated, and stored frozen until serum and urinary biomarker analyses at the University of Georgia (Athens, GA) and TAMU, respectively. Following sample collection, it was noted that two participants did not meet the eligibility criteria concerning residency, and data collected from these subjects were not included. Thus, a total of 184 participants comprised our study population.

5.2.2 Chemicals and laboratory analyses

Authentic AFM_1 and 1-OHP standards were purchased from Sigma Chemical Co. (St. Louis, MO) and the Midwest Research Institute Chemical Carcinogen Reference Standard Repository (Kansas City, MO), respectively. Blood specimens collected from study participants were analyzed for complete blood count, HBV surface antigen (HBsAg) and anti-HCV antibodies at SAMHD according to standard laboratory operating procedures.

5.2.3 Urinary aflatoxin M_1 and 1-hydroxypyrene analyses

Urinary AFM_1 levels were analyzed using immunoaffinity column purification followed by HPLC with fluorescence detection (previously described in detail in Section II). Urinary 1-OHP levels were also measured with an HPLC-fluorescence method (as described in Section IV). Quantification of AFM_1 and 1-OHP were based on peak area and retention times as compared to external standards run daily. The limit of detection for urinary AFM_1 and 1-OHP using these methods was 0.5 pg/ml and 0.25 nmol/L of

urine, respectively. Creatinine concentrations were measured at St. Joseph's Regional Health Center Laboratory in order to correct for variations in urine dilution.

5.2.4 Statistical analysis

Median, mean, standard deviation (SD) and detectable range were calculated for concentrations of all biomarkers measured. Statistical analyses were done using SPSS software version 15.0 (Chicago, IL). For comparisons, student's t or Wilcoxon rank sum tests were used as appropriate to examine differences between biomarker data. Chi-square tests were performed to examine demographic data and variables assessed by the questionnaire. Crude odds ratio estimates for the relationship between various dietary factors and AF biomarkers were determined by generating 2 x 2 contingency tables. A *p*-value ≤ 0.05 (two-tailed) was considered significant.

5.3 Results

5.3.1 Demographics of study participants and hepatitis virus prevalence

Table XIV provides the descriptive characteristics and HBV and HCV status in the study population. Slightly more than one fourth (26.6%) of the participants were male and 73.4% were female. The average participant age was 48 (median: 49; range: 18 – 83 years). The majority of participants (97.3%) were of Hispanic ethnicity; the remaining percentage of the study population (1.1 and 1.6%) was Native American and African American, respectively. Serum analysis at SAMHD included screening for HBsAg and anti-HCV antibodies. None of the participants were HBsAg+, whereas 7.1% (13/184) of the study population was anti-HCV+.

Table XIV. Demographic characteristics and HBV/HCV serology in Bexar County study participants

Demographic characteristics	n (%)
Gender	
Male	49 (26.6)
Female	135 (73.4)
Ethnic group	
Hispanic	179 (97.3)
African American	2 (1.1)
Native American	3 (1.6)
Age (year)[a]	48.3 ± 16.1
Hepatitis Status	
HBsAg+[b]	0 (0.0)
Anti-HCV+[c]	13 (7.1)

[a] Data are presented as mean ± SD; [b]Hepatitis B virus surface antigen; [c]Antibodies to hepatitis C virus.

5.3.2 Urinary aflatoxin M_1 and 1-hydroxypyrene levels

Urinary AFM_1 was detectable in 11.7% of samples analyzed (n = 179) with the average level in the detectable group at 223.85 ± 250.56 pg/mg creatinine (median: 141.53; range: 1.89 – 935.49 pg/mg creatinine). Characteristics described above did not differ significantly among the participants in the AF-detectable and non-detectable groups. Of the samples available for 1-OHP analysis (n = 160), 51.2% of 125 non-smokers and 100% of 35 tobacco-smokers had detectable 1-OHP levels with median excretion values of 0.01 and 0.17 μmol/mol creatinine, respectively. Moreover, there was a significant difference between mean 1-OHP levels measured in non-smokers (0.07 ± 0.13 μmol/mol creatinine) and smokers (0.26 ± 0.33 μmol/mol creatinine) ($p < 0.01$). While a slight lack of concordance between the total number of study participants and amount of samples analyzed for AF and PAH biomarkers arose due to unforeseeable events during sample collection and transfer, adequate amounts of samples were analyzed to validate statistical analysis.

5.3.3 Food consumption questionnaire

A primary aim of the questionnaire administered at SAMHD was to investigate dietary factors that could be related to increased AF exposure in the study population. Results from questions on food consumption showed that > 98% of participants reported that they ate commodities prone to AF-contamination (e.g., corn, nuts, rice and a variety of corn/peanut-based foods) at varying frequencies. A large percentage of the population (44.8%) consumed corn tortillas frequently (3-14 times per week); the majority (58.2%) ate > 2 tortillas at each time of consumption. When food consumption was examined

according to urinary AFM_1 detection, the amount of corn tortillas ($p = 0.009$), rice ($p = 0.037$), and nuts ($p = 0.033$) consumed was found to be significantly associated with AFM_1 excretion (Table XV).

5.3.4 Comparison of biomarker levels to a high-risk population

Biomarker results were compared to levels measured in our well-characterized study group in the Ashanti Region of Ghana, which was determined to be a population at high risk for both AF and PAH exposures. The distribution of urinary AFM_1 and 1-OHP in both populations is illustrated in Figure 21. Of the urines collected in Ghana at baseline (n = 159), 88.1% had detectable AFM_1 levels (section II). The median level of the three study groups (49.57 pg/mg creatinine) was significantly higher than the median level calculated for 179 participants from Bexar Co. (0.02 pg/mg creatinine) ($p < 0.001$); non-detectable values were assigned half the LOD for statistical analysis. However, the median level detected in 140 positive Ghanaian individuals (68.07 pg/mg creatinine) did not differ significantly compared to the median level detected in 21 positive participants from Bexar Co. (141.53 pg/mg creatinine) ($p = 0.707$). In samples collected at baseline and measured for 1-OHP, the vast majority of Ghanaian participants (98.2% of 113 non-smokers) had detectable 1-OHP levels (mean ± SD = 0.80 ± 0.68 μmol/mol creatinine). This level was significantly higher than the average level detected in non-smokers in Bexar Co. (mean ± SD = 0.07 ± 0.13 μmol/mol creatinine) ($p < 0.001$). Overall, the median 1-OHP level for non-smokers observed in Ghana (0.64 μmol/mol creatinine) was over 60-times higher than the median level in Bexar Co. (0.01 μmol/mol creatinine).

Table XV. Food consumption in Bexar County study population by distribution of aflatoxin M_1 biomarker.

Type of Food	Amount of food consumed[a]	Aflatoxin M_1[n (%)][b]	
		Detectable	Non-detectable
Corn	< ½ cup (1 ear)	2 (10.5)	28 (18.9)
	≥ ½ cup (1 ear)	17 (89.5)	120 (81.1)
Corn tortillas	< 1 tortilla	2 (11.8)	2 (1.4)
	≥ 1 tortilla	15 (88.2)*	142 (98.6)
Corn bread/muffins	< 1 piece (muffin)	1 (12.5)	12 (13.0)
	≥ 1 piece (muffin)	7 (87.5)	80 (87.0)
Corn chips	< 1 cup (10 chips)	5 (26.3)	44 (30.3)
	≥1 cup (10 chips)	14 (73.7)	101 (69.7)
Rice	< ½ cup	0 (0.0)	31 (19.9)
	≥ ½ cup	18 (100.0)*	125 (80.1)
Peanut butter	< 1 tablespoon	4 (25.0)	17 (19.3)
	≥ 1 tablespoon	12 (75.0)	71 (80.7)
Nuts	< ¼ cup	2 (12.5)	54 (39.7)
	≥ ¼ cup	14 (87.5)*	82 (60.3)

[a]Amount of food consumed at each time of consumption; [b]Numbers within subgroups differ slightly from the total number of samples analyzed for AFM_1 due to missing responses; *$p \leq 0.05$ in comparison of distribution in AFM_1-detectable and AFM_1-non-detectable groups in Fisher exact test.

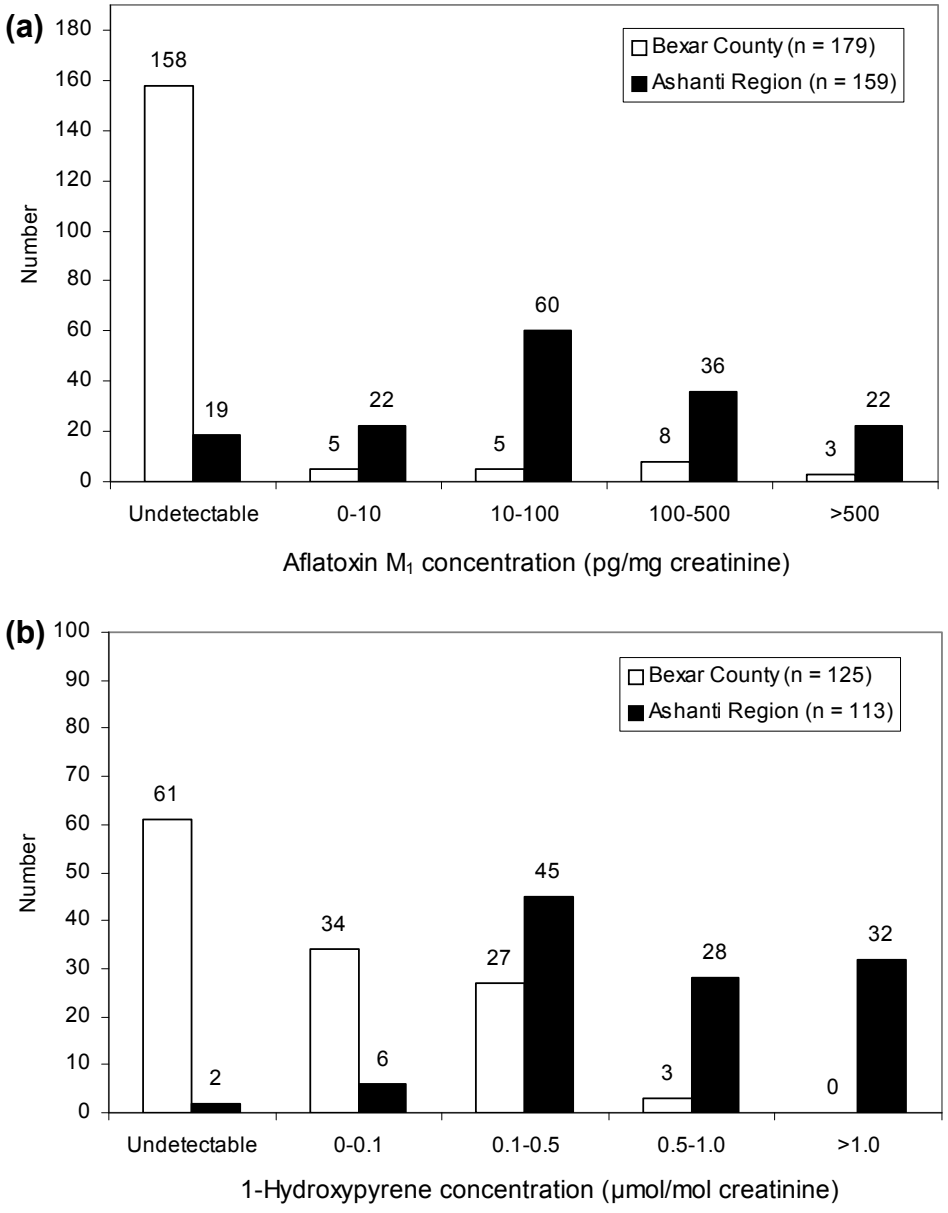

Figure 21. Distribution of urinary AFM_1 and 1-OHP in study participants in Ghana and the U.S. AFM_1 levels in study participants from Bexar Co. were detected at a lower percentage (11.7% versus 88.1%) and overall level compared to Ghana (a). Additionally, 1-OHP concentrations in non-smoking participants from Bexar Co. were detected at a lower percentage (51.2% versus 98.2%) and level compared to the average level measured in Ghana (b).

5.4 Discussion

Areas located between latitudes 40° N and S (which includes the Southern U.S.) encompass populations at risk for chronic AF exposure based on suitable temperature, humidity and vulnerability of staple commodities for mycotoxin contamination (Williams et al. 2004). Since contamination has been reported in foods from Texas and surrounding areas such as Mexico, particularly after periods of drought (Torres et al. 1995; Wood 1992), our primary objective was to assess AF exposure in a predominantly Hispanic population in Bexar Co. with an increased incidence of HCC. In this study, urinary AFM_1 data revealed that the majority of participants had a non-detectable AF exposure; this was confirmed by serum AFB_1-lysine adduct data, wherein only 20.7% of the study population exhibited low levels of detectable AF-adducts (Qian et al. 2009). While the correlation between serum and urine AF biomarkers was not significant, results indicated that some individuals showed low levels of chronic exposure whereas others exhibited low to moderate to high short-term AF exposure. This may be due to the differences in half-lives of the two biomarkers. The AF-albumin adduct has a longer *in vivo* half-life reflecting integrated exposures over weeks to months compared to AFM_1 excretion representing recent exposure (i.e., 24 to 48 hours) (Wang et al. 1996b). Perhaps in populations with sustained AF exposure at elevated levels it is more likely that these biomarkers would have a better correlation, as evidenced previously in Ghana (Jolly et al. 2006). While the measurable exposure to AFs at our site in Bexar Co. was relatively low, it appeared that an increased consumption of certain food products, namely, corn tortillas, nuts and rice, were associated with the detection of AFM_1 in the

urine. AFs have frequently been detected in corn and groundnuts, and contamination of rice has occasionally been reported (Tanaka et al. 2007). Borrud et al. (1989) reported that the intake patterns of Mexican Americans demonstrated an adherence to traditional Mexican food items, for instance corn tortillas. Several survey studies in Mexico have shown corn tortillas to be highly contaminated with AFs (Carvajal et al. 1987; Yepiz and Rosas 1994). This commodity may represent a possible source of AF exposure, in addition to other corn-based products (e.g., corn masa and tamales and other foods made using corn masa) when consumed in high amounts. Further work delineating the source of corn (e.g., store bought, home-grown crops, human food grade quality, etc.) may be of importance since low socioeconomic conditions may necessitate the use of lower quality foods. Importantly, no tolerable daily intake has been set for AFB_1, as determined for other (less carcinogenic) mycotoxins. However, the FDA has set an action level of 20 ppb in foods intended for human consumption, which corresponds to ~30 μg AFs/day. Assuming a urinary excretion rate of 2 – 5% (Cheng et al. 1997) and an estimated metabolic rate of 1500 ml urine/day, daily AFB_1 consumption can be calculated from AFM_1 values. It was estimated that the mean AFM_1 excretion in Bexar Co. corresponds to an average daily consumption ranging from 9.8 – 24.6 μg AFB_1/day. Although the estimated average daily AFB_1 intake is below 30 μg, individuals in the 75th and 95th percentiles may have estimated daily AFB_1 consumptions ranging from 15.3 – 38.2 and 49.2 – 122.9 μg/day, respectively. Thus, human health hazards associated with such AF exposure over time cannot be ruled out.

When compared to a population in a developing country (i.e., Ghana), findings demonstrated that participants in Bexar Co. overall had a lower exposure to AFs. By comparison, the percentage and level of AFM_1 were lower than those observed in baseline samples collected in the Ashanti Region prior to a clinical intervention trial (Figure 21a). It can be estimated that the mean AFM_1 excretion in this 'high-risk' population (making the same assumptions as above) corresponds to an average daily AFB_1 consumption ranging from 17.3 – 43.4 μg/day, with maximum levels reaching as high as >547 μg/day. Therefore, it is clear that this population in rural Ghana is at high risk for aflatoxicosis. Proper production, storage and processing of foods and effective enforcement of regulations contribute to reduced AF exposure in developed countries. The lack of food security in sub-Saharan Africa stresses the need for intervention strategies to reduce these high level exposures.

Of additional concern in Bexar Co. was assessing co-exposure to PAHs, which may increase the risk for HCC in the presence of AFs and hepatitis virus infection (Wu et al. 2007). Findings from this portion of our study illustrated that all study participants classified as tobacco-smokers had detectable levels of urinary 1-OHP, whereas approximately half of non-smoking participants did not show a measurable exposure to PAHs. Data further demonstrated a significant difference in mean 1-OHP concentrations when participants were stratified by smoking status. This is in agreement with previous work showing statistically significant increases in 1-OHP excretion in tobacco-smokers exposed to background levels of environmental PAHs (Levin et al. 1995; Viau et al. 1995). Conversely, in our population in Ghana smoking failed to produce any

differences in urinary 1-OHP levels, compared to not smoking, indicating a predominant environmental PAH exposure. Moreover, 1-OHP levels measured in Bexar Co. were considerably lower than those previously recorded in Ghana (Figure 21b). Findings in this U.S. population were comparable or lower than those previously recorded for non-smoking individuals in numerous developed countries (Table III). Overall, results suggest that non-tobacco smokers in Bexar Co. are not at high risk for PAH exposure, based on this short-term biomarker. Further work assessing chronic PAH exposure may be warranted.

An additional objective of our environmental health study in Bexar Co. was to determine the prevalence of HBV and HCV since hepatitis virus infection clearly contributes to the overall burden of HCC. Previous findings from a study in a Texas male prison population indicated that inmates who were older, Hispanic, and infected with HCV or HBV had elevated rates of both HCC prevalence and mortality (Baillargeon et al. 2009). While no participants in our study population were positive for the HBsAg, 7.1% were positive for HCV. HBV infection is closely linked to HCC in developing countries; however, its impact may be far less in areas of the U.S. where HBV vaccination is common. In contrast, the prevalence of HCV is of significant importance, especially since no vaccination is currently available. The HCV positivity rate in this Bexar Co. community (7.1%) was higher than the overall prevalence in Texas reported to be 1.79% (varying from 1.25 – 2.63% across Texas counties) (Yalamanchili et al. 2005). Thus, the implementation of biomonitoring and intervention strategies, particularly in vulnerable individuals, at high risk for HCV-induced HCC, may play an

important role in reducing the overall negative public health impact of dietary AF exposure.

In conclusion, results from our environmental health study showed a significant association of increased consumption of certain foods and the excretion of AFM_1 in a minority population in Texas. In addition, the HCV positivity rate is comparatively high in this community and warrants considerable attention. Biomarkers measured in this study reflect current exposures to AFs and PAHs. The development of HCC is a multistage process that involves many factors over an extended period of time. The increased incidence of liver cancer observed in Bexar Co. presumably was initiated by exposures occurring 20-30 years ago. The primary goal of this pilot study was to gain insight into the current public health of this underserved community and gather information to support further exploration of potential factors that can contribute HCC risk. A limitation of our study was the uneven recruitment of females due to the lack of male participation. Since the incidence of HCC in Hispanic males in Bexar Co. has been shown to be significantly elevated, further work is warranted to reflect the participant demographic that is apparently the most vulnerable to HCC development. Additionally, proper control populations in the U.S. are needed to better elucidate the role of AFs in populations at risk for HCC.

VI. SUMMARY

Chronic exposure to dietary AFs is a major risk factor for human hepatocellular carcinoma development. At high levels, AFs can cause disease (aflatoxicosis) and death. Therefore, the significant negative health impact of AFs necessitates safe and effective intervention strategies to reduce biological exposure in humans. This has been especially apparent in developing countries and the poorest populations like those in sub-Saharan Africa where the likelihood and resulting impact of exposure is elevated. The recent outbreak in Kenya, which resulted in 125 deaths following acute exposure to high levels of AFs, reaffirmed AF exposure as a critical issue requiring attention. NovaSil (NS) clay, a dioctahedral smectite, has been shown to prevent aflatoxicosis in multiple animal species by preferentially binding AFs in the gastrointestinal tract and reducing toxin bioavailability. Given the safety of NS, demonstrated in a variety of animal models and in a short-term human study, a Phase IIa clinical intervention trial was conducted in the Ashanti Region of Ghana. Previous biomarker data showed this population was at risk for aflatoxicosis based on high levels of aflatoxin M_1 (AFM_1) in the urine and aflatoxin B_1-lysine albumin adducts in the blood of study participants. Thus, the specific aims of this study were to evaluate the ability of NS to reduce AF biomarkers of exposure (objective 1) and to investigate interactions of NS with important nutrient minerals (objective 2) following intervention. After 3-months of NS capsule ingestion at the high dose level, median AFM_1 levels were significantly reduced (up to 58%) when compared to levels in the placebo group. In addition, there was a concurrent reduction in AF-

albumin adduct levels after 3-month intervention at this minimally effective dose level. This was the first study to show NS can effectively reduce AF exposure from contaminated diets of humans, signifying its potential use in populations at high risk for aflatoxicosis. Furthermore, no significant differences were found in levels of nutrient minerals between NS-treated and placebo groups after clinical intervention, suggesting NS can be used to effectively sorb dietary AFs without affecting serum concentrations of important nutrient minerals. Of the 30 nutrient and non-nutrient minerals measured, only levels of serum strontium (Sr), a non-nutrient mineral, were affected by NS dietary inclusion. Results from a clinical trial in osteoporosis patients indicated Sr supplementation, at a level over 150-times higher than the estimated amount of Sr that could be supplied from NS clay, did not produce any adverse effects in study participants. Therefore, findings do not suggest physiological levels of Sr would be significant enough to yield a negative effect from this low level in NS clay. In fact, Sr could be considered a potential benefit in the diet since it may act like Ca in regards to its concentration in the bone. Since NS is a natural product, considerable efforts were made to ensure its purity prior to the intervention. For instance, all potential contaminants (i.e., dioxins and heavy metals) were either below the LOD or present in the high dose at levels considerably lower than the current WHO/JECFA standards. In the future, the purity and batch-to-batch consistency of the test agent should continue to be closely monitored. It is also important that any strategy for the reduction of AFs is both sustainable and culturally acceptable. NS meets both of these criteria, as it is common practice in many parts of sub-Saharan Africa, including Ghana, for humans to

consume ‘edible’ clays. As a rationale to affirm sustainability of this strategy, work is ongoing to identify clays in Ghana similar to NS for subsequent human studies. Overall, findings provide the basis for future long-term studies to evaluate the safety and efficacy of NS as an enterosorbent therapy for acute AF exposure and for the prevention of chronic AF-induced disease or cancer. In the future, inclusion of NS clay in the diet may become a readily available technology that constitutes an economically feasible therapy for use in developing countries to possibly save human lives by reducing disease and preventing death in outbreak situations.

Of additional concern were other environmental carcinogen exposures that could contribute to or enhance AF-induced disease. Recent epidemiological data indicated an increased HCC risk was found among participants co-exposed to high levels of AFs and polycyclic aromatic hydrocarbons (PAHs). It was postulated that our study population in Ghana (at risk for aflatoxicosis) may be concurrently exposed to PAHs from a variety of environmental sources. Although sources of PAH exposure may be familiarized with images of industrial manufacturing or polluted urban air, there is a great opportunity for exposure in rural areas of developing countries since approximately 90% of these populations (totaling over 50% of the world populace) burn biomass as an energy source. Subsequently, a third objective of this study was to determine PAH exposure (previously unknown in this rural population) by measuring 1-hydroxypyrene (1-OHP) in urine samples collected from the NS intervention study. Results showed participants were significantly exposed to PAHs based on the presence of 1-OHP in the majority of urines collected (98.9%) and high levels measured at baseline and 3-months (median

excretion values were 0.64 and 0.69 μmol/mol creatinine, respectively). The potential effect of enterosorption on the bioavailability of PAHs from the gastrointestinal tract had yet to be investigated. Thus, a supplementary objective was to compare 1-OHP values in NS and placebo groups. Findings demonstrated treatment with NS clay had no effect on 1-OHP levels, further confirming the preferential binding of AFs by NS. A trend for the reduction in median 1-OHP levels in the placebo group was observed following 3-month intervention. However, since the placebo consisted of cellulose, i.e., bulk fiber, the effect was likely due to the alteration in enterohepatic recycling of 1-OHP. Furthermore, the cooking culture of this population implied a predominant exposure from preparing foods and socializing by fires; thus clay-based enterosorption would clearly not have an effect on PAH exposure via inhalation routes. Future strategies to reduce exposure from this route would be of great benefit. For instance, in a previous study in Mexico, researchers showed 1-OHP levels were significantly reduced following an intervention aimed at reducing indoor smoke exposure by introducing new stoves with metal chimneys that expelled smoke outdoors (Torres-Dosal et al. 2008). Furthermore, cleaner cook stoves implemented in homes in India have shown great promise to reduce human exposure to contaminants in smoke, i.e., PAHs (Adler 2010). Likewise, an initiative in Ghana to employ cleaner cook stoves, mainly powered by propane, is being considered by the Ghanaian government. The success of this initiative, particularly in remote rural areas, like the Ejura-Sekyedumase district, will depend on the cost to the consumer and cultural acceptance of new cooking methods to ensure long-term viability. Overall, biomarker data from this study illustrated a population at high risk for aflatoxicosis was at a parallel

risk for PAH exposure. Further investigation is warranted to evaluate if the combined decrease of exposures to AFs and PAHs may reduce the incidence of liver cancer or additional disease.

While it has been reported that the majority of liver carcinomas occur in developing countries, the incidence of HCC in the U.S. has increased over the recent decades. In order to compare domestic and international foodborne and environmental exposures, an environmental health survey was conducted in Bexar County, Texas (objective 4) in a Hispanic community shown to have an elevated incidence of HCC. In this study, the incidence and level of AF and PAH exposures were lower than data observed in our study population in Ghana; however, some participants were exposed to moderate to high levels of dietary AFs, based on elevated AFM_1 values. Additionally, the increased consumption of certain foods prone to AF contamination (i.e., corn tortillas, nuts, and rice) was associated with AFM_1 excretion. Thus, some individuals may be more vulnerable to exposure and associated interactions with other biological factors, namely HCV. The positively rate for HCV in this population was considerably higher than recorded prevalence rates in other areas of Texas. It is well-established that HBV infection increases the carcinogenic potency of AFB_1 (reported to be ~30 times higher in HBsAg+ individuals versus HBsAg- individuals). A correlation between AF exposure and advanced liver disease has also been demonstrated in HCV positive individuals. Therefore, reducing AF exposure in populations with a high prevalence of HBV or HCV may have a substantial impact on reducing liver disease and HCC rates.

In summary, the ability for researchers to monitor and quantify human exposure to carcinogens stems from the development and validation of chemical-specific biomarkers. An important application of biomonitoring can be to identify populations at risk for environmental exposures, as shown in this work. In regards to the further advancement of the field of exposure science, it has been suggested that the human 'exposome' needs to be sequenced to fully understand the extent and effect of human exposures. The application of this 'omics' discipline, arising from the sequencing of the human genome published nearly a decade ago, may provide a breath of knowledge to aid in further identifying what exposures exist and possibly means to negate them. Overall, from basic research stemming from the discovery of AFs to more recent epidemiological work strengthening correlations between exposures and human health effects, AF research has become a model for how fundamental toxicological science can translate into public health protection.

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