ESTIMATING THE GLOBAL BURDEN OF AFLATOXIN-ATTRIBUTABLE LIVER CANCER RISK

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Background: Over 4 billion people worldwide are exposed to dietary aflatoxins, which can cause liver cancer (hepatocellular carcinoma, HCC) in humans independently and interact with chronic hepatitis B virus (HBV) infection to increase the cancer risk. However, the global burden of HCC from aflatoxin exposure remains unclear.

Objectives: We sought to determine 1) the global burden of HCC attributable to aflatoxin exposure; 2) the population-attributable risk (PAR) of HCC from aflatoxin in high exposure areas; 3) the quantitative model of effects between aflatoxin exposure and HBV infection in increasing liver cancer risk.

Methods: We first conducted a quantitative cancer risk assessment, for which we collected global data on foodborne aflatoxin levels, consumption of aflatoxin-contaminated foods, and HBV prevalence. Aflatoxin's cancer potencies for HBV+ and HBV- individuals, and uncertainty in all variables, were considered in calculating the global burden of aflatoxin-related HCC. Then, we conducted a meta-analysis on the eligible studies identified by literature search. Summary odds ratios (ORs) of aflatoxin-related HCC with 95% confidence intervals were calculated in HBV+ and HBV- individuals, as well as the general population. We calculated the PAR of

aflatoxin-related HCC for each study as well as the combined studies, accounting for HBV status.

Results: 25,200-155,000 HCC cases worldwide may be attributable to aflatoxin exposure. Most cases occur in sub-Saharan Africa, Southeast Asia, and China, where populations suffer from both high HBV prevalence and largely uncontrolled dietary aflatoxin. In these areas, the PAR of aflatoxin-related HCC with 95% CI was estimated at 17% (14-19%) for overall population, and higher in HBV+ (21%) than HBV- (8.8%) populations. If the one study that contributed most to heterogeneity in the analysis is excluded, the summarized OR of HCC with 95% CI is 73.0 (36.0-148.3) from the combined effects of aflatoxin and HBV, 11.3 (6.75-18.9) from HBV only, and 6.37 (3.74-10.86) from aflatoxin only. The PAR of aflatoxin-related HCC increases to 23% (21-24%).

Public Health Significance: Aflatoxin may play a causative role in 4.6-28.2% of all global HCC cases. In high exposure areas, aflatoxin exposure may multiplicatively interact with HBV to induce HCC and attribute to 14-19% of liver cancer cases.

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PREFACE

I want to thank Dr. Felicia Wu for leading me to the area of risk analysis, providing her invaluable advices in mycotoxin research and general research methods. In past 4 years, not only I learned skills and professional knowledge, I also learned the persistent spirit and upbeat attitude in exploring unknown science world from her. I believe the experiences I have had in PhD training with Dr. Wu at University of Pittsburgh will have positive impacts in my career for lifetime.

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1.0 INTRODUCTION

Aflatoxins are secondary metabolites produced primarily by the fungi *Aspergillus flavus* and *A. parasiticus,* which can infect crops such as maize, peanuts, cottonseed, and tree nuts. Aflatoxins contaminate the diets of a large proportion of the world's population. Exposures are highest in tropical and sub-tropical regions where the fungus is prevalent, maize and peanuts are staples in the diet, and food storage conditions are sub-optimal [1,2].

Aflatoxin is one of the most potent naturally occurring human hepatocarcinogens known. The International Agency for Research on Cancer (IARC) has classified "naturally occurring mixes of aflatoxins" as a Group 1 known human carcinogen [3]. Abundant epidemiological evidences suggest that high aflatoxin exposure interacts with chronic hepatitis B virus (HBV) infection to increase liver cancer (hepatocellular carcinoma, HCC) risk in individuals with both risk factors [4-8]. More recently, toxicological models for the mechanism of the synergism of these two risk factors have emerged [9-11], and are summarized in Wild and Gong [12]. Unfortunately, both high aflatoxin exposure and chronic hepatitis B are prevalent in many parts of the developing world, particularly in sub-Saharan Africa and Asia. It was estimated that 4.5 billion persons worldwide suffer from uncontrolled aflatoxins from dietary food [1]. However, the global burden of HCC cases attributed to aflatoxin, individually and in conjunction with HBV infections, remained undefined. As previously reported by the Joint Food and Agriculture

Organization/World Health Organization Expert Committee on Food Additives (JECFA), the population attributable risk (PAR) fraction of aflatoxin as risk factor of liver cancer in Europe and the United States is "limited or none," while in Africa and Asia, it is "not quantified" [13].

An important input to decision-making and planning processes in health is a consistent and comparative description of the burden of diseases from the associated risk factors. Indeed, the Aflatoxin Workgroup [2] identified four issues that warrant immediate attention, including the following two: 1) quantifying human health impacts and burden of disease due to aflatoxin exposure, and 2) compiling an inventory, evaluating the efficacy, and disseminating results of ongoing intervention strategies.

In this study we aimed to address these two crucial issues. We applied two different approaches to estimate the burden of aflatoxin-attributable liver cancer risk, at global level and in high exposure areas. In the first approach, we compiled available information on aflatoxin exposure and HBV prevalence from multiple nations in a quantitative cancer risk assessment, to estimate the number of HCC cases attributable to aflatoxin worldwide per year. In the second approach, we searched the literature, and selected cohort or case-control studies with reported odds ratios (ORs) or relative risks (RRs) of aflatoxin in relation to liver cancer, in areas with high aflatoxin exposures. By combining the relevant odds ratios (ORs) and relative risks (RRs) from these studies, we conducted meta-analyses to calculate population-attributable risk (PAR) of aflatoxin-related HCC in the population overall, as well as in HBV+ and HBV- populations. The model of combined effects between two risk factors – aflatoxin exposure and chronic HBV+, was also evaluated.

This study is the first attempt to utilize the quantitative risk assessment and systematic approach to estimate the global burden of liver cancer attributable to aflatoxin exposure. This

study also examined the quantitative mode of combined effects between aflatoxin exposure and chronic hepatitis B from a meta-analysis. These numbers allow for a comparison of the relative importance of aflatoxin and chronic HBV infection as risk factors of liver cancer for various regions of the world. If the major contributor of aflatoxin-related hepatocellular carcinoma is the interaction with HBV infection, HBV vaccination may be sufficient to reduce the incidence of hepatocellular carcinoma associated with aflatoxin exposure [14].

1.1 HUMAN TOXICOLOGY OF AFLATOXIN

1.1.1 AFLATOXINS' DISCOVERY AND METABOLISM IN HUMANS

The first discovery of aflatoxins was due to the "turkey X" disease, an epidemic occurring in London in late 1950's and early 1960's, involving deaths of numerous turkey poults and ducklings which were fed with diets containing groundnuts imported from South America [15]. Aflatoxins were discovered as the causative agents for this disease. Aflatoxins are a group of approximately 20 related fungal metabolites. There are four major aflatoxins known as B_1, B_2, G_1 and G_2, plus two additional metabolic products, M_1 and M_2, found in milk and milk products [3]. Aflatoxins B_2 and G_2 are the dihydro- derivatives of the parent compounds B_1 and G_1 [16]. They were so named because of their strong blue or greenish-yellow fluorescence in ultraviolet light [17]. These properties facilitated the rapid development of methods in the early 1960s for monitoring grains and other food commodities for the presence of the toxins. The aflatoxins occur mostly in developing countries in tropical and sub-tropical areas between 40º N latitude

and 40º S latitude when the temperatures are between 24 and 35 °C and the moisture content exceeds 7% [1]. They accumulate during the post-harvest storage under conditions that promote fugal growth.

Humans are exposed to aflatoxins by consuming commodities contaminated during growth, harvest or storage. The levels of aflatoxin in the grain products may vary from less than 1 µg/kg to greater than 12,000 µg/kg [18]. In developed countries, harmful aflatoxin exposure has been mostly eliminated, thanks to the establishment of regulatory limits on traded foods, the enforcement of these limits through food monitoring, and the implementation of optimal drying and storage practices [2]. However, it is difficult to apply these strategies in developing countries because of differences in food production, such as the prominence of subsistence farming in developing countries. Furthermore, these countries often lack the resources, technology, and infrastructure necessary for routine food monitoring as well as optimal drying and storage practices [19]. And they often export the grain with best quality but keep the worst ones for their own consumption. In a recent outbreak of aflatoxicosis in Kenya, 55% of sampled maize products in local market had aflatoxin levels greater than the Kenyan regulatory limit of 20 ppb, 35% had levels > 100 ppb, and 7% had levels > 1,000 ppb [19]. Generally, diets may contain AFB_1 and AFB_2 in concentration ratios of 1.0 to 0.1 and if all four aflatoxins occur, AFB_1, AFB_2, AFG_1 and AFG_2 are presenting at the proportion of 1.0:0.1:0.3:0.03 [17]. Among all the strains, aflatoxin B_1 is the most abundant, toxic and most potent as a carcinogen [20].

After dietary intake to human body, AFB_1 is metabolized, mainly in the liver by cytochrome P450, to AFB_1-8,9-exo-epoxide and AFB_1-8,9-endo-epoxide. AFB_1-8,9-exo-epoxide is much more toxic and active because it binds to DNA to form the predominant 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy AFB_1 (AFB_1-N^7-Gua) adduct [21], or binds to serum albumin to form long-

lived lysine adducts [22]. In addition, the epoxide can be conjugated by certain glutathione S-transferases (GSTs) and then further metabolized to form aflatoxin-mercapturic acid detoxification product [23]. The most prevalent mutation with AFB_1-N^7-Gua was demonstrated to be G to T transversions targeted to the site of the original adduct [24]. There are other metabolites formed from AFB_1, including AFM_1, AFQ_1 and AFP_1 [25]. These metabolites and other naturally occurring aflatoxins, AFG_1, AFG_2 and AFB_2, are less mutagenic, carcinogenic and toxic than AFB_1, because they are poorer substrates for epoxidation and the AFB_1-8,9 exo-epoxide intercalates more readily into DNA [21,25]. Figure 1 illustrated the metabolism of aflatoxin B_1 leading to reactive metabolites and biomarkers (adapted from Wild and Turner [25])

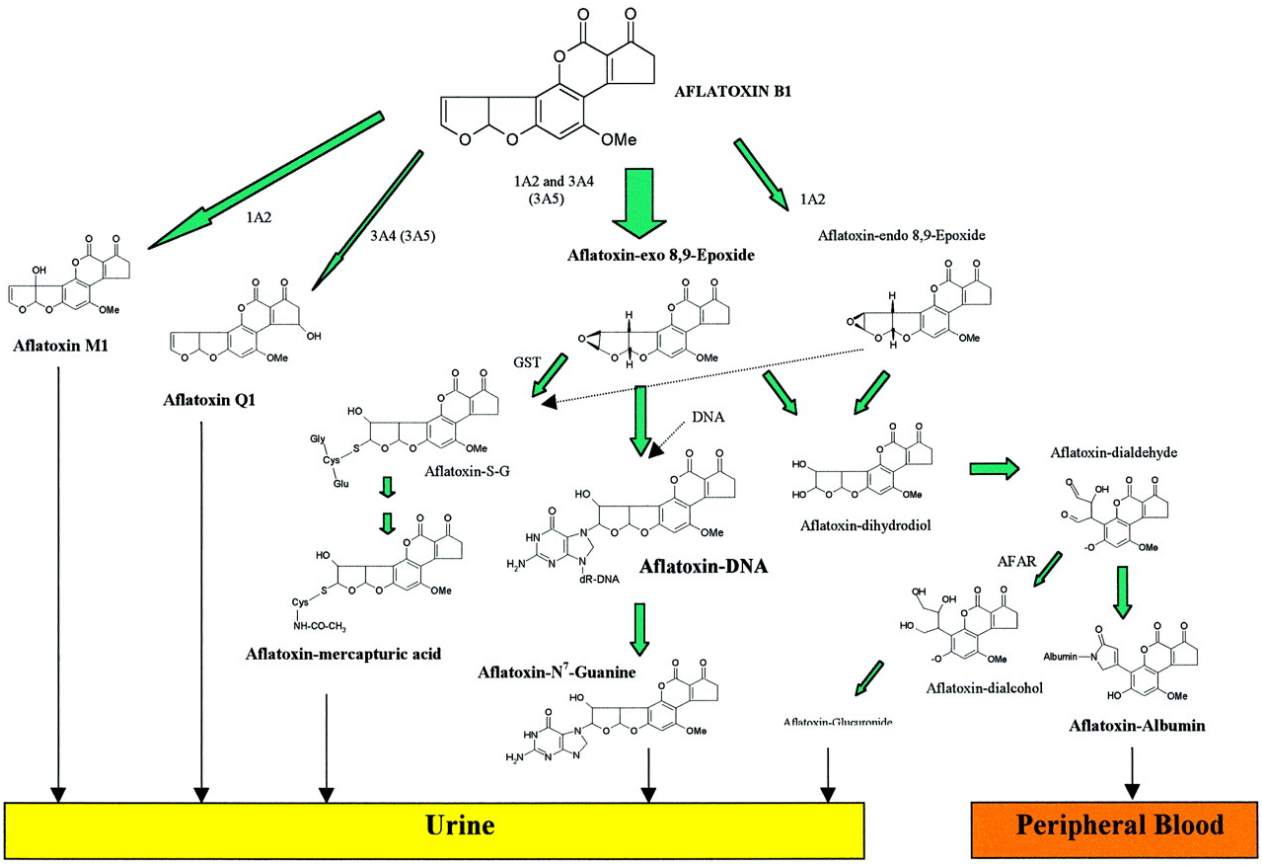


Figure 1. Principle metabolism of aflatoxin B_1 leading to reactive metabolites and biomarkers

(Figure is adapted from Wild C and Turner P, Mutagenesis 2002;17:471-481[21])

1.1.2 AFLATOXIN AND HEPATOCELLULAR CARCINOMA

Since their identification, 50 years of investigation on aflatoxins' toxicity has revealed diverse adverse health effects related to dietary food contamination with aflatoxins, including acute aflatoxicosis, liver cancer, stunting in children, and immunosuppression effects. The link between aflatoxin exposure and both acute aflatoxicosis and liver cancer are well established [4,6,26-30], whereas the association of exposure with stunting in children [31], immunosuppression [32,33] remains tenuous but interesting and become increasing focus in studies.

HCC is the third leading cause of cancer deaths worldwide with 749,000 cases and over 695,000 deaths every year [34]. 85% of the cases occur in developing countries. The regions of high incidence are Eastern and South-Eastern Asia, Middle and Western Africa where the incidence ranges from 10 to 100 cases per 100,000 per year [34]. In developed countries, the rate of liver cancer incidence is much lower (1-10 cases/100,000/yr). Major risk factors for HCC include virus infection (HBV, HCV), alcoholism, smoking, environmental contaminants exposure such as arsenic and dietary aflatoxin, and the newly emerging risk factors in western developed countries, diabetes and obesity [35]. Among all the risk factors, chronic HBV infection (determined by positive detection of hepatitis B surface antigen (HBsAg) twice 6 months apart), representing 300-400 million carriers worldwide, attribute to 54% of the global burden of liver cancer cases [36], and most of the cases occur in developing countries. Alcohol consumption and tobacco smoking are also clearly recognized as environmental risk factors of HCC, but the mechanisms of these factors, individually and in conjunction with viral infections are not well defined. In addition, HCC development varies depending on factors as the age, gender and host

genetic differences of the infected patients (reviewed in [37]). It is also notable that chronic HBV or HCV infection rates show marked geographical differences which remain largely unexplained.

Dietary aflatoxin is believed to play a major role in high HCC incidence in the developing world in Africa and Asia, because the occurrence of HCC and the high chronic HBV infection rates were observed coincident with the high, ubiquitous aflatoxin exposure in these areas. Early studies began in 1960s to investigate a possible ecological association between aflatoxin exposure and liver cancer [30]. However, they were hindered by lack of the measurement of individual aflatoxin exposure and not taking into account HBV infection [12]. Now, HCC as a result of chronic aflatoxin exposure has been well documented. The liver carcinogenicity of aflatoxin has been obtained from studies in many species of animals, including rodents, nonhuman primates and fish (reviewed in [17]). In humans, the improvement of exposure assessment using biomarkers and the availability of prospective cohort studies in Asia revealed the significant interaction effects between aflatoxins and chronic HBV infection in inducing liver cancer [4,28,37,38]. The risk of liver cancer in individuals exposed to chronic HBV infection and aflatoxin is up to 30 times greater than the risk in individuals exposed to aflatoxin only [18]. Aflatoxin also appears to have a synergistic effect on HCV-induced liver cancer[37,39,40], although the quantitative relationship is not as well-established as that for aflatoxin and HBV in inducing HCC.

Chronic liver injury and regenerative hyperplasia resulting from HBV infection are critical to the development of liver cancer [41]. However, the model of interaction between aflatoxins and HBV infection in liver cancer etiology has not been well understood yet. One possible mechanism is that aflatoxin-induced DNA adduct are fixed as mutations because of the HBV-related increase in cell proliferation and hyperplasia, thus promoting the mutant cells

expansion. Another possible mechanism is that HBV could predispose hepatocytes to the carcinogenic action of aflatoxins by altering the hepatic expression of aflatoxin metabolism enzymes and consequently the extent to which aflatoxins bind to DNA [42].

Acute aflatoxicosis, characterized by jaundice, ascites, vomiting, abdominal pain, hepatitis and even death [43], has been described in humans exposed to 1-7 mg of aflatoxin daily for approximately 1-3 weeks [12]. There have been sporadic historical reports of human aflatoxicosis in the developing world, including India, Kenya and Malaysia [26,44-46]. A more recent aflatoxicosis outbreak, occurred in Kenya 2004, resulted in 125 deaths and 317 cases [41](mortality 39%) and the extent of contaminated maize consumption (geometric mean > 350 ppb) was associated with the risk of aflatoxicosis [26].

Aflatoxin and immunosuppression in humans has been relatively less well-characterized, but could in fact have enormous significance from a global health perspective [1]. Several recent human studies have shown evidence of immunomodulation [32,33,47] and this may be one explanation for the stunted growth in children that appears to follow a dose-response relationship with aflatoxin exposure [31,33,48], though the actual outcomes of such immunomodulation have yet to be characterized in humans. Another explanation may be altered intestinal integrity [49].

In this study we focus on the liver carcinogenicity of aflatoxins, because this is the most extensively studied feature of aflatoxin toxicology in humans with well-established data to conduct the risk assessment.

1.1.3 AFLATOXIN BIOMARKERS

Biomarkers of exposure, internal dose and the biologically effective dose of aflatoxins are most commonly used for assessing exposure or intervention in epidemiological studies. A biomarker of exposure refers to measurement of the specific interactive products in human body compartment or fluid and indicates the presence and magnitude of current and past exposures. Biomarkers of an internal dose and a biological effective dose for aflatoxins reflect the presence and magnitude of a biological response to aflatoxin exposure [17]. The knowledge of aflatoxin metabolism and carcinogenesis in humans provides the basis for the development of aflatoxin biomarkers, and the use of biomarkers to measure exposure and determine risk.

Urinary measures of AFM_1, AFB_1-mercapturic acid and serum aflatoxin-albumin adducts are used as biomarkers of internal dose [21,50,51], while AFB_1-N_7-guanine in urine serves as a biomarker of biologically effective dose [17]. The measurement of aflatoxin-DNA and protein adducts are of major interest because they are derived from the carcinogenic species aflatoxin-8,9-exo-epoxide [52]. Measurements of aflatoxin biomarkers in urine reflect recent exposure, while aflatoxin-albumin adducts in serum reflect cumulative exposures over the past 2-3 months [53]. Indeed, studies conducted in China and The Gambia, both areas with high incidences of HCC, determined that the levels of urinary aflatoxin biomarkers (aflatoxin M_1 and aflatoxin-N^7-guanine) followed a dose-response relationship with dietary aflatoxin intake [50,54]. Similarly, significant association between dietary aflatoxin intake and aflatoxin-albumin levels was observed [51,55]. More recently, a positive correlation has been shown between population estimates of aflatoxin exposure and the proportion of HCC patients with TP53 249^{ser} mutation [5]. The TP53 249^{ser} mutation occurrence is common in HCC patients in geographic regions with

high aflatoxin exposure, such as Qidong, China and The Gambia, Africa [41]. However, this mutation is rare in areas with low aflatoxin exposure such as Europe and the United States. This finding suggested that the detection of TP53 249^{ser} mutation may be used as a biomarker of an early neoplastic event, chronic exposure to aflatoxin or a combination of both. Indeed, this finding has led to the application of this marker in population-based studies [5,56-58].

2.0 GLOBAL BURDEN OF AFLATOXIN-INDUCED HEPATOCELLULAR CARCINOMA: A RISK ASSESSMENT

The Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization (FAO) and World Health Organization (WHO) undertook an aflatoxin-HCC risk assessment in 1998, to estimate the impact on population liver cancer incidence by moving from a hypothetical total aflatoxin standard of 20 ng/g to 10 ng/g [20,59]. Assuming that all food that contained higher aflatoxin levels than the standard was discarded, and that enough maize and nuts would remain to preserve consumption patterns, JECFA determined that HCC incidence would decrease by about 300 cases per billion people per year, if the stricter aflatoxin standard were followed in nations with HBV prevalence of 25%. However, in nations where HBV prevalence was 1%, the stricter aflatoxin standard would only save 2 HCC cases per billion people per year. This assessment associated HCC risk with particular doses of aflatoxin; however, these doses do not correspond with actual doses in food in different parts of the world, and the two hypothetical values for HBV prevalence, 1% and 25%, were not intended as a complete global assessment.

Shephard 2008 [48] estimated population risk for aflatoxin-induced HCC in select African nations; we expand this to include the rest of the world. We also briefly describe interventions

that have been developed to either reduce aflatoxin directly in food, or to reduce adverse health effects caused by aflatoxin.

2.1 METHODS

We analyzed extensive datasets by nation or world region: food consumption patterns (specifically of maize and peanuts), aflatoxin levels in maize and peanuts, HBV prevalence, and population size, in order to perform a quantitative cancer risk assessment for aflatoxin-related liver cancer. Risk assessment is the process of estimating the magnitude and the probability of a harmful effect to individuals or populations from certain agents or activities. Four steps are involved in estimation of the risk: hazard identification, dose-response analysis, exposure assessment, and risk characterization [60].

2.1.1 HAZARD IDENTIFICATION

Hazard identification is the process of determining whether exposure to an agent can increase the incidence of a particular health condition. Aflatoxin exposure is associated with an increase in incidence of HCC in humans and sensitive animal species [18]; indeed, IARC has classified aflatoxin B1 (AFB1) as a Group 1 carcinogen [3].

2.1.2 DOSE-RESPONSE ANALYSIS

This second risk assessment step involves characterizing the relationship between the dose of an agent – in this case, aflatoxin - and incidence of HCC. Because of the synergistic impact of aflatoxin and HBV in inducing HCC, the assessment must be done separately for populations with and without chronic HBV infection. Though chronic HCV infection may also have synergistic effects with aflatoxin in inducing HCC, this was not included because: 1) there is much less overlap worldwide between aflatoxin and HCV exposures in general; 2) chronic HCV infection usually occurs later in life while chronic HBV infection occurs much earlier; hence, the time of overlapped exposure is less significant for aflatoxin and HCV (Dr. John Groopman, personal communication); and 3) much less is known about the quantitative relationship of aflatoxin and HCV in inducing HCC.

For cancer risk assessment, it is traditionally assumed that there is no threshold of exposure to a carcinogen below which there is no observable adverse effect [61]. Cancer potency factors are estimated from the slope of the dose-response relationship, assumed to be linear, between doses of the carcinogenic agent and cancer incidence in a population. The JECFA aflatoxin risk assessment had selected two different cancer potency factors for aflatoxin: 0.01 cases per 100,000 per year per ng/kg bw/day aflatoxin exposure for individuals without chronic HBV infection, and 0.30 corresponding cases for individuals with chronic HBV infection. This was based on one cohort study that estimated cancer potency in both HBsAg+ (HBV surface antigen: a biomarker of chronic HBV infection) and HBsAg- individuals [30] as well as other human studies that assessed cancer potency in either HBsAg+ or HBsAg- individuals. We used these same potency factors for this risk assessment. Because only one of the studies [30]

specifically assessed cancer potency in both cohorts, there may be considerable uncertainty associated with these potency factors. However, several epidemiological studies confirm that aflatoxin's cancer potency is about 30 times greater in HBV+ compared with HBV- individuals [5,6,62].

2.1.3 EXPOSURE ASSESSMENT

Exposure assessment involves estimating the intensity, frequency, and duration of human exposures to a toxic agent. Specifically, we sought to determine how individuals' exposure to aflatoxin increases their risk of HCC, which is depending on whether they are chronically infected with HBV. Aflatoxin exposure is a function not only of aflatoxin concentrations in maize and nuts, but also of how much of these foodstuffs individuals consume in different parts of the world.

Aflatoxin exposure assessment has evolved significantly over the last two decades, largely due to the characterization of biomarkers for both aflatoxin exposure and effect [63]. Prior to these biomarkers, the primary way by which aflatoxin exposure was estimated was to observe how much maize and nuts people consumed on average by world region; and to measure or assume aflatoxin levels in these foods. However, by measuring biomarkers such as aflatoxin-albumin adducts in serum or aflatoxin-N^7-guanine in urine, it is possible to improve estimations of aflatoxin exposure and of how much has been biotransformed to increase cancer risk [18].

As data do not exist on aflatoxin biomarker levels in most parts of the world, we estimated average aflatoxin exposure or contamination levels in the maize and nuts in different world regions by collecting data on estimated HBV prevalence in these countries, maize and nut

consumption patterns in different world regions, and. Where aflatoxin exposure data were not already estimated, we used food consumption patterns and aflatoxin contamination levels to estimate exposure. The studies estimating HBV prevalence were based on HBsAg detection in males and females in both urban and rural settings across all age groups. Data on maize and peanut consumption in different parts of the world are adapted from the WHO Global Environment Monitoring System (GEMS) Food Cluster Diet database [64]. Aflatoxin exposure data in different nations were estimated from multiple different sources through literature searches.

2.1.4 RISK CHARACTERIZATION

This final step of risk assessment integrates dose-response and exposure data to describe the overall nature and magnitude of risk. For our study, this final step consisted of quantifying, across the globe, the burden of aflatoxin-related liver cancer. For each nation, we estimated total number of individuals with or without chronic HBV by multiplying prevalence by population size. To estimate aflatoxin-induced HCC cases per 100,000 in these two populations (with and without chronic HBV infection), we multiplied the corresponding cancer potency factor by aflatoxin exposure estimates. Then we multiplied these values by the nations' HBV+/HBV- population sizes, divided by 100,000, to derive total number of aflatoxin-induced HCC cases in each nation. We summed across all world regions to arrive at an estimate for global burden of aflatoxin-induced HCC.

The risk characterization model:

Population liver cancer risk caused by aflatoxin = Potency of aflatoxin * Average aflatoxin daily intake*population

In which, Potency = [Potency factor of AF for HBV+ persons] * [% of HBV+ persons]

+ [Potency factor of AF for HBV- persons] * [% of HBV- persons]

2.2 RESULTS

2.2.1 THE PREVALENCE OF CHRONIC HBV INFECTION BY WORLD REGION

Table 1 lists the age-adjusted prevalence of chronic HBV infection by world region, as measured by the seroprevalence of HBsAg in different parts of the world. Though there is uncertainty and variability in these different estimates, all data are from literature published on or after 2000, to ensure HBV prevalence estimates that are as updated and relevant as possible. Countries are grouped by WHO designated regions [65]: Africa, Eastern Mediterranean, Europe, America, Southeast Asia, and Western Pacific Region. Some regions are further divided into subgroups, because of significantly varied aflatoxin exposure and HBV prevalence within the region.

Table 1. Estimates of HBV prevalence based on HBsAg Seroprevalence in select countries

WHO Region	Countries	Chronic HBV Prevalence (HBsAg Seroprevalence, range)
Africa	Dem. Repub. Congo	6-10% [66,67]
	Ethiopia	6-7% [68,69]
	The Gambia	15-20% [70,71]
	Kenya	11-15% [72,73]
	Mozambique	4.5-10.6% [74]
	Nigeria	13.2% [75]
	South Africa	3.3-10.4% [72,76]
	Tanzania	5-9% [77-79]
	Zimbabwe	10-15% [72,80]
	Others	9-20% [72,79,81]
North America	Canada	1-2% [82,83]
	United States	0.3- 2% [65,84,85]
Latin America	Argentina	0.8-1.1% [86,87]
	Brazil	2.1-3.4% [86,87]
	Mexico	<0.3% [88]
	Others	0.5-3% [86,87]
Eastern Mediterranean	Egypt	2.2-10.1% [89-91]
	Iran	0.41-0.56% [92,93]
	Pakistan	3.3% [94,95]
	Sudan	6-26% [96]
	Others	0.65-10% [65,97-99]
South-East Asia	India	2.4- 4.7% [76,100,101]
	Indonesia	2.5-5% [102-104]
	Thailand	4.6-8% [76,105]
	Others	2-7% [65,106-108]
Western Pacific Region	Australia	<1% [109]
	China	8-10% [102,109]

Table 1 continued

	Malaysia	5% [102,109]
	Philippines	5-16% [109-111]
	Korea	4-5% [109,112]
	Others	1-10% [102,113,114]
Europe	Eastern Europe	2-7% [115,116]
	Southern Europe	2-7% [65,117,118]
	Western Europe	0.5-1% [65,119,120]

2.2.2 THE MAIZE AND PEANUT CONSUMPTION PATTERN IN SELECTED COUNTRIES OF THE WORLD

Table 2 contains calculations of maize and peanut consumption in select countries of the world. The GEMS/Food Consumption Cluster Diets database divides countries of the world into thirteen groups based upon diets [64]. For each group cluster, the GEMS/Food Consumption database has estimated the amount of cereals, nuts and oilseeds consumed. We thus estimated average maize and nut consumption by individual country. There are limitations to these data because of the clustering into thirteen groups (which may lead to wide ranges among nations within a group), as well as variability in data quality regarding diet and aflatoxin exposure estimates.

Table 2. Maize and peanut consumption in select countries, adapted from GEMS/Food Cluster Diet database

WHO Region	Countries or Populations	Maize[i] (g/person/day)	Peanut[ii] (g/person/day)
Africa	Dem. Rep. Congo	57	52
	Ethiopia	83	13
	Gambia	57	52
	Kenya	248	11
	Mozambique	248	11
	Nigeria	57	52
	South Africa	248	11
	Tanzania	248	11
	Zimbabwe	248	11
North America	Canada	86	17
	United States	86	17
Central and South America	Argentina	86	17
	Brazil	63	2
	Mexico	300	5
Eastern Mediterranean	Egypt	136	5
	Iran	32	2
	Pakistan	35	18
	Sudan	57	52
Southeast Asia	India	35	18
	Indonesia	35	18
	Thailand	35	18

[i] Including maize, flour and germ.

[ii] Including groundnuts in shell and shelled

Table 2 continued

Western Pacific Region	Australia	86	17
	China	35	18
	Malaysia	35	18
	Philippine	59	2
	Republic of Korea	59	2
Europe	Eastern Europe	32	2-10
	Southern Europe	148	7
	Western Europe	33	10

2.2.3 ESTIMATED AFLATOXIN EXPOSURE AND HCC INCIDENCE ATTRIBUTABLE TO AFLATOXIN BY WHO REGION

We estimated (based on the data in Tables 1 and 2) or found in the literature the average aflatoxin exposure in different world regions. These are listed in the middle column of Table 3. The rightmost columns of Table 3 contain calculations for the estimated incidence of aflatoxin-induced HCC, with and without the synergistic impact with HBV, in the corresponding populations of each nation and world region. Specifically, within each WHO-designated region, aflatoxin exposures in the most populous nations were found. The "in general" rows in Table 3 represent a small proportion of each region: nations in which aflatoxin data were not available, or very small nations. For these, we assumed a range for aflatoxin exposure that incorporated the ranges of the nations within the region for which aflatoxin data were found.

Table 3. Estimated HCC incidence attributable to aflatoxin by WHO region

WHO Region	Countries or Populations	Aflatoxin exposure (ng/kg body weight/day[a])	Estimated HCC incidence attributable to aflatoxin, HBsAg- (/100, 000/yr)	Estimated HCC incidence attributable to aflatoxin exposure, HBsAg+ (/100, 000/yr)
Africa	Dem. Rep. Congo	0.07-27 [b 121]	0.0007-0.27	0.02-8.10
	Ethiopia	1.4-36 [b 122]	0.01-0.36	0.42-10.8
	The Gambia	4-115 [48,123]	0.04 – 1.15	1.20 – 34.5
	Kenya	3.5 – 133 [48,123]	0.04 – 1.33	1.05 – 39.9
	Mozambique	39-180 [123]	0.39-1.80	11.7 – 54.0
	Nigeria	139-227 [b 124,125]	1.39-2.27	41.7-68.1
	South Africa	0-17 [123,126]	0-0.17	0-5.10
	Tanzania	0.02-50 [b 121]	0.0002-0.50	0.06-15.0
	Zimbabwe	17.5-42.5 [20]	0.18-0.43	5.25-12.8
	In general [c]	10-180 [48,123]	0.10-1.80	3.0-54.0
North America	Canada	0.2-0.4[d 127]	0.002 – 0.004	0.06-0.12
	United States	0.26 [20]	0.003	0.08
	In general [c]	0.26-1	0.003-0.01	0.08-0.3
Latin America	Argentina	0-4 [b 128,129]	0-0.04	0-1.20
	Brazil	0.23-50 [b 3,130-132]	0.002-0.50	0.07-15.0
	Mexico	14-85 [b 133-135]	0.14-0.85	4.20-25.5
	In general [c]	20-50	0.20-0.50	6.0-15.0
Eastern mediterranean	Egypt	7-57 [b 136]	0.07-0.57	2.1-17.1
	Iran	5-8.5 [b 137,138]	0.05-0.09	1.50-2.55
	Pakistan	7-50 [b 139]	0.07-0.50	2.10-15.0
	Sudan	19-186 [28]	0.19-1.86	5.70-55.8
	In general [c]	10-80	0.10-0.80	3.00-24.0

Table 3 continued

South-East Asia	India	4-100 [140]	0.04-1.00	1.20-30.0
	Indonesia	9-122 [b 3,141,142]	0.09-1.22	2.7-36.6
	Thailand	53-73 [123,143b]	0.53-0.73	15.9-21.9
	In general [c]	30-100	0.30-1.00	9.00-30.0
Western Pacific Region	Australia [iii]	0.15 -0.18 [16,144]	~0.002	~0.05
	China	17-37 [b 6,145-147]	0.17-0.37	5.10-11.1
	Malaysia	15-140 [b 3,148]	0.15-1.4	4.5-42
	Philippine	44-54 [b 3,148,149]	0.44-0.54	13.2-16.2
	Republic of Korea	1.2-6 [62,150]	0.01-0.06	0.36-1.80
	In general [c]	15-50 (except Australia & New Zealand)	0.15-0.50	4.5-15.0
Europe	Eastern Europe	3.5-4 [e 151]	0.04	~1.20
	Southern Europe	0-4 [f 152,153]	0-0.04	0-1.20
	Western Europe	0.3-1.3 [154]	0.003-0.01	0.09-0.39
	In general [c]	0-4	0-0.04	0-1.2

[a] Assuming 60 kg bodyweight per individual

[b] Aflatoxin exposure was estimated by multiplying aflatoxin concentrations in staple foods by consumption rates of those foods [64].

[c] "In general" line summarized the aflatoxin exposure estimates, the estimated HCC incidence attributable to aflatoxin in HBV+ and HBV- individuals for all other countries classified in the same WHO region.

[d] 1-2 ng/kg bw/day was measured in children's diets; here we assume the adult daily aflatoxin intake is 20% of children's.

[e] Average daily aflatoxin intake was estimated based on AFB1contamination levels in Czech maize, multiplied by average daily maize consumption in Eastern Europe.

[f] Average daily aflatoxin intake was estimated based on AFB1contamination levels in maize of North Italy and Turkey, multiplied by average daily maize consumption in Southern Europe.

2.2.4 ESTIMATED GLOBAL BURDEN OF HCC CASES ATTRIBUTABLE TO AFLATOXIN EXPOSURE IN HBSAG+ AND HBSAG- POPULATION

These data provide the necessary information to calculate the total estimated cases of aflatoxin-induced HCC cases annually, worldwide. Populations for each relevant nation and world region are listed in Table 4. Accounting for chronic HBV infection prevalence as shown in Table 1, and the risk incidence estimates for HBV+ vs. HBV- individuals in Table 3, the numbers of cases of aflatoxin-induced HCC can be estimated in each world region. These are then summed to produce a global estimate of the number of annual aflatoxin-induced HCC cases. Our estimate is that anywhere from **25,200-155,000** annual HCC cases worldwide may be attributable to aflatoxin exposure.

Table 4. Estimated global burden of HCC cases attributable to aflatoxin exposure in HBsAg+ and HbsAg- population

WHO Region	Countries or Populations	Population [155]	HCC cases attributed to aflatoxin, HBsAg-	HCC cases attributed to aflatoxin, HBsAg+
Africa	Dem. Rep. Congo	68 million	1-173	1-551
	Ethiopia	85 million	11-288	21-643
	Gambia	1.7 million	1-17	3-117
	Kenya	38 million	11-450	44-2,270
	Mozambique	21 million	73-361	111-1,200
	Nigeria	149 million	1,800-2,940	8,200-13,400
	South Africa	48 million	0-79	0-255
	Tanzania	41 million	1-195	1-554
	Zimbabwe	13 million	19-50	68-249
	Total Region	755 million	2,150-9,300	9,230-50,600
North America	Canada	33 million	1	1
	United States	300 million	8	1-5
	Total Region	333 million	9	2-5
Central and South America	Argentina	40 million	0-16	0-5
	Brazil	190 million	4-930	3-969
	Mexico	109 million	152-924	14-83
	Total Region	562 million	589-2,980	84-2,060
Eastern Mediterranean	Egypt	81 million	51-452	37-1400
	Iran	66 million	33-56	4-9
	Pakistan	172 million	116-832	119-851
	Sudan	41 million	58-717	140-5,950
	Total Region	569 million	446-3,720	341-13,200
South-east Asia	India	1.15 billion	438-11,200	331-16,200
	Indonesia	237 million	203-2,820	160-4,340
	Thailand	63 million	307-439	461-1,100

Table 4 continued

	Total Region	~1734 million	1,740-17,300	1,460-27,600
Western Pacific Region	Australia	21 million	0-1	0-1
	China	1.3 billion	1,990-4,430	5,300-14,400
	Korea	50 million	5-29	6-45
	Malaysia	28 million	40-372	63-588
	Philippine	90 million	333-462	594-2,330
	Total Region	~ 1740 million	2,710-6,510	6,310-21,200
Europe	Eastern Europe	290 million	94-114	61-244
	Southern Europe	144 million	0-56	0-121
	Western Europe	183 million	5-24	1-7
	Total Region	617 million	99-184	62-372
Total (World)		6.28 billion	7,700-40,000	17,500-115,000
Total annual HCC cases attributable to aflatoxin worldwide		**25,200-155,000**		

Figure 2 illustrates the distribution of total HCC cases attributable to aflatoxin globally. The categories denote WHO world regions. Sub-Saharan Africa is the most important region as far as HCC cases attributable to aflatoxin; Southeast Asia and China (in the Western Pacific region) are also key regions where aflatoxin exposure is an important risk factor for HCC. Relatively fewer cases occur in the Americas, the Eastern Mediterranean, and Europe. Though Australia and New Zealand are grouped with the Western Pacific Region, these nations also have low aflatoxin-induced HCC incidences.

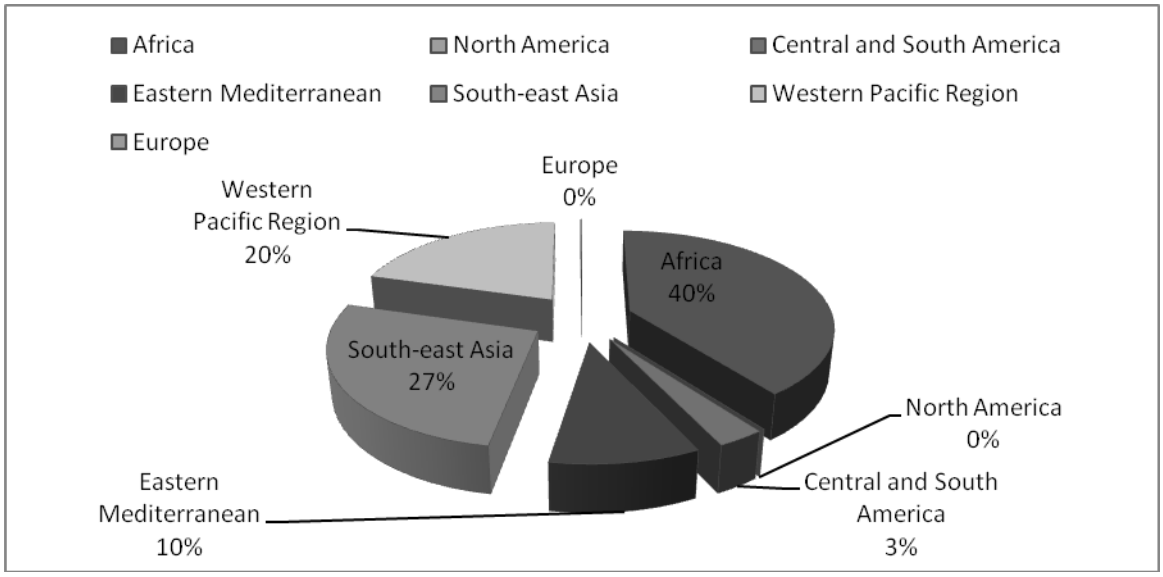


Figure 2. Distribution of HCC cases attributable to aflatoxin in different regions of the world

2.2.5 THE REDUCED GLOBAL BURDEN OF AFLATOXIN-INDUCED LIVER CANCER UNDER A HYPOTHETICAL SCENARIO OF SUCCESSFUL HBV VACCINATION PROGRAM WORLDWIDE

Vaccination is the most effective public health intervention to reduce the global incidence of hepatitis B. In 1991, the WHO recommended that all countries introduce a policy of universal hepatitis B vaccination by 1997 to prevent and control on a global scale HBV infection and ultimately, the incidence of HCC [156]. In countries with high (HBsAg > 8%) or intermediate disease endemicity (HBsAg 2%-8%), the most effective strategy is to incorporate the vaccine into the routine immunization schedule for newborn infants (<24h). Since 1997, substantial progress has been made in implementing this recommendation. In 2008, 177 WHO member states have introduced hepatitis B vaccine in routine infant immunization and 82 countries provided vaccine for infant at birth [157]. Because the aflatoxin cancer potency increases 30 times when the exposure is in conjunction with chronic hepatitis B, the decreasing chronic hepatitis B incidence will also substantially reduce the aflatoxin-attributable HCC incidence in the long term. As the effectiveness of massive vaccination will be seen in the next 20-30 years, we assumed a hypothetical scenario that universal infant HBV vaccination program was provided globally and successfully reduced chronic HBV infection prevalence to lower than 2% worldwide. We then estimated the global burden of HCC cases that aflatoxin will be attributable, individually and in conjunction with chronic HBV infection. We assume the food consumption pattern, the aflatoxin daily intake and the population remain the same as that we applied in the previous calculation for estimating the global burden of aflatoxin-induced liver cancer.

Our estimates were presented in Table 5. With ≤ 2% HBsAg seroprevalence worldwide, the annual number of aflatoxin-related HCC cases will be **13,420-65,400**, a reduction of 50%.

Table 5. The estimated reduced global burden of HCC cases attributed to aflatoxin

WHO Region	Countries or Populations	Population	Estimated HCC cases attributed to aflatoxin, HBsAg-	Estimated HCC cases attributed to aflatoxin, HBsAg+
Africa	Democratic Republic of Congo	68 million	0-180	0-110
	Ethiopia	85 million	12-300	7-184
	Gambia	1.7 million	1-19	0-12
	Kenya	38 million	13-495	8-303
	Mozambique	21 million	80-370	49-227
	Nigeria	149 million	2,030-3,315	1,243-2,029
	South Africa	48 million	0-80	0-49
	Tanzania	41 million	0-201	0-123
	Zimbabwe	13 million	22-54	14-33
	Total Region	755 million	2,442-10,135	1,495-6,205
North America	Canada	33 million	1	0
	United States	300 million	8	1
	Total Region	333 million	9	1
Central and South	Argentina	40 million	0-16	0-4

Table 5 continued

America	Brazil	190 million	4-931	3-570
	Mexico	109 million	152-924	14-83
	Total Region	562 million	600-2,980	84-824
Eastern Mediterranean	Egypt	81 million	56-452	34-277
	Iran	66 million	33-56	4-7
	Pakistan	172 million	118-843	72-516
	Sudan	41 million	76-747	47-458
	Total Region	569 million	488-3,737	282-2,261
South-east Asia	India	1.15 billion	451-11,270	276-6,900
	Indonesia	237 million	209-2,834	128-1,735
	Thailand	63 million	327-451	200-276
	Total Region	~1734 million	1,822-17,338	1,115-10,615
Western Pacific Region	Australia	21 million	0	0
	China	1.3 billion	2,166-4,714	1,326-2,886
	Korea	50 million	5-29	3-18
	Malaysia	28 million	41-384	25-235
	Philippine	90 million	388-476	238-292
	Total Region	~ 1740 million	2,969-6,833	1,818-4,184
Europe	Eastern Europe	290 million	99-114	61-70

Table 5 continued

	Southern Europe	144 million	0-56	0-35
	Western Europe	183 million	5-24	1-4
	Total Region	617 million	104-194	62-109
Total (World)		6.28 billion	8,514-41,226	4,906-24,199
Total annual aflatoxin-induced HCC cases		**13,420-65,400**		

Note: when a worldwide universal HBV vaccination program is accomplished, we assume 2% HBsAg prevalence in areas of moderate to high HBV prevalence

2.3 DISCUSSION

Aflatoxin contamination of food is a serious global health problem, particularly in less developed countries. Though it has been known for several decades that aflatoxin causes liver cancer in humans, the exact burden of aflatoxin-related HCC worldwide has been unknown. This study represents a first step in attempting to estimate that burden. We find that at its lower estimate, aflatoxin plays a role in about 4.6% of total annual HCC cases; while at its upper estimate, aflatoxin may play a role roughly 28.2% of all HCC cases worldwide. This large range stems from the considerable uncertainty and variability in data on cancer potency factors, HBV prevalence, and aflatoxin exposure in different world regions. The most heavily afflicted parts of the world are sub-Saharan Africa, Southeast Asia, and China. It is notable that Mexico may bear high aflatoxin exposure from contaminated food, and the resulted 152-924 HCC cases per 100,000 per year indicated aflatoxin could be a significant factor of HCC incidences in this country, despite the fact that the chronic HBV prevalence is relatively low in this country (< 0.3%).

Aflatoxin exposure varied within and between populations. Developing countries in tropical and sub-tropical areas are nearly ubiquitously exposed to moderate-to-high levels of aflatoxin from contaminated food. Aflatoxin is a controllable risk factor in food; yet the parts of the world in which the risk is particularly high have limited resources to implement most aflatoxin control strategies. Much agricultural land in Africa and Asia lies in climatic regions favorable for *A. flavus* and *A. parasiticus* proliferation. Suboptimal field practices and poor

storage conditions make the crops vulnerable to fungal infection and subsequent aflatoxin accumulation. Maize and groundnuts, the two crops most conducive to *Aspergillus* infection, are staples in many African and Asian diets. Because the very poor in these regions cannot afford much variety in their food, these particular staples make up a significant portion of their diets, increasing aflatoxin exposure.

Even within the same population, aflatoxin exposure varied geographically. Studies have shown that rural populations generally have higher levels of AFB_1 adducts than urban dwellers in developing countries [158] because urban people consume more diversified diets than rural dwellers. In addition, there is a strong seasonal variation in AFB1 exposure which correlates with the food availability [159,160].

Patterns and acquisition of chronic HBV infection vary geographically. Horizontal transmission is predominant in West Africa where HBsAg prevalence in infants and young children can be between 15%-20% [161]. Parenteral, drug use and sexual contact are the common modes of HBV transmission in most Western countries. In contrast, perinatal transmission is the most important reason for chronic HBV acquisition in Southeast Asia and Western-Pacific countries. The chronic HBV prevalence is generally higher in rural than in urban areas, and higher in males than females in most places. Our collected data of HBsAg seroprevalence are presented as a range for countries/populations, to take into consideration these variations.

This analysis focuses on carcionogenic aflaoxin, individually and in conjunction with chronic HBV carriage, to define the global burden of HCC attributed to aflatoxin. However, the role of aflatoxin in conjunction with other carcinogens, such as alcohol consumption, and host genetic differences in contributing to HCC development, is still undefined.

Though many nations that suffer from both high aflatoxin exposures and high HBV prevalence have nominally established maximum allowable aflatoxin standards in food, there is little if any enforcement of these standards in many rural areas. Indeed, the food in subsistence farming and local food markets is rarely formally inspected. When trading with other nations, strict aflatoxin standards can even lead to large economic losses for poor food-exporting nations [162]. Subsistence farmers and local food traders sometimes have the luxury of discarding obviously moldy maize and groundnuts. But in drought seasons, oftentimes people have no choice but to eat moldy food or starve [163].

Multiple public health interventions exist by which to control aflatoxin or its burden in the body, to prevent HCC. These interventions are grouped into three categories: 1) Agricultural, 2) Dietary, and 3) Clinical. *Agricultural* interventions are methods or technologies that can be applied either in the field ("preharvest") or in storage and transportation ("postharvest") to reduce aflatoxin levels in food. Agricultural interventions can thus be considered "primary" interventions, because they effectively reduce aflatoxin in food before it reaches the consumer's plate. *Dietary* and *clinical* interventions can be considered "secondary" interventions. They cannot reduce actual aflatoxin levels in food, but they can reduce aflatoxin-related illness; either by reducing aflatoxin's bioavailability in the body or by ameliorating aflatoxin-induced damage. Because aflatoxin-mediated mutations may precede HCC by several years, the effects of reducing aflatoxin exposure on HCC incidence may take time to become apparent [164].

One highly effective clinical intervention to reduce aflatoxin-related HCC is vaccination against HBV. A regular practice now in the U.S. and other industrial nations, HBV vaccination in children is now becoming more common worldwide. Vaccinating children against HBV has, over the last 30 years, significantly decreased HBV infection in several regions including Europe

[165,166], Taiwan [167] and Thailand [168]. For example, in Taiwan, the nationwide infant vaccination program for hepatitis B launched in 1984 significantly reduced the prevalence of HBsAg from 14.3% in 1995 to 1.1% in 2009 [167]. This vaccine has been and will continuously have significant impacts on liver cancer incidence worldwide over time. In our hypothetical scenario of HBV vaccination being made instantaneously available worldwide, the estimated number of HCC cases attributable to aflatoxin in the next generation, absent other changes, would drop 42-53%.; because removing a possible synergistic impact between HBV and aflatoxin exposure would significantly reduce HCC risk. However, there are currently 360 million chronic HBV carriers worldwide and HBV vaccine is still not incorporated into many national immunization programs [169]. Thus measures to reduce food spoilage by fungi and the associated dietary exposure to aflatoxins are also a desirable public health goal.

Our study highlights the significant role of aflatoxin in contributing to global liver cancer burden. While it is impossible to completely eliminate aflatoxin in food worldwide, it is possible to significantly reduce levels and dramatically reduce liver cancer incidence worldwide. The interventions described are first steps in reducing aflatoxin-induced liver cancer, but the challenge remains to deliver these interventions to places of the world where they are most needed.

3.0 VALIDATION OF THE AFLATOXIN-INDUCED LIVER CANCER RISK ASSESSMENT MODEL – A CASE STUDY IN FUSUI, GUANGXI, CHINA

Previously, we developed a risk assessment model to estimate the liver cancer risk caused by aflatoxin dietary intake alone and in combination with chronic HBV infection status at global level. Variables in the model include chronic HBV infection prevalence, aflatoxin contamination levels in food and the food consumption data in multiple nations. To validate this model, we picked a specific area of China to examine how this model will perform when it is applied to a particular area instead of global scale. The process of model validation also allows us to to conduct the sensitivity analysis, to analyze how the inputs to each variable will influence the results. Sensitivity analysis is a systematic technique used to understand how risk-based decisions are dependent on variability and uncertainty in the factors contributing to risk. In this chapter, we assigned different values to the key variables in the model based upon the data from epidemiological studies conducted in this area. Then we compared the estimated disease burden from the model, to the actual epidemiology surveillance data in the area. We also compared the results to determine the importance of the variables in the model.

China was selected to be the particular country to conduct the validation analysis because: 1) China is among the countries with both high prevalence of aflatoxin exposure and chronic HBV infection rates; 2) epidemiological evidences indicated that the HBV prevalence

and aflatoxin exposure in China are decreasing in the past 30 years due to the nationwide infant vaccination program, as well as the noticeable change in diet style in the areas with high aflatoxin exposure [164]; 3) extensive human study data are available on the association between aflatoxin and liver cancer risk in China, so that we have relatively more data sources to conduct a sensitivity analysis compared to other affected countries/areas. In addition, we specifically picked the Fusui County to do analysis because other than the above reasons, the chronic HBV infection and dietary aflatoxin exposure are the two major risk factors for HCC in Fusui, China, while the hepatitis C virus infection is extremely uncommon this area.

The objectives of this case study are: 1) to assess the reliability of the conclusions and inferences drawn from the previous risk assessment model; 2) to identify the key variables that influence the liver cancer risk in the proposed risk assessment model; and 3) to assess the benefit of different interventions to reduce the liver cancer risk in the affected population.

3.1 METHODS

We collected food consumption data which reflect the changing of diet style in local population, the aflatoxin contamination level in consumed food, and the chronic HBV infection rates in Fusui, China, from 1970s to 2010s. We then calculated and compared the results derived from applying the changed values to the variables in the model.

3.1.1 IDENTIFICATION OF THE KEY VARIABLES IN THE RISK ASSESSMENT MODEL

In Chapter 2, we applied the below model to characterize the liver cancer risk attributable to aflatoxin:

Population liver cancer risk caused by aflatoxin = Potency of aflatoxin * Average aflatoxin B_1 daily intake*population

In which, Potency = [Potency factor of AF for HBV+ persons] * [% of HBV+ persons]

+ [Potency factor of AF for HBV- persons] * [% of HBV- persons]

We analyzed the quantifiable determinants and the underlying determinants for each variable in the model, presented in Table 6. To quantitatively validate the model, the average dietary aflatoxin B_1 intakes for the population of Fusui in different years were estimated based on the daily food intakes and concentration of AFB_1 detected in food samples. The age-adjusted HBV prevalences were also estimated and the percentage of HBV- persons was estimated as (1-[% of HBV+ persons]). We first adopted the potency factors of aflatoxin in HBV+ persons and HBV- persons from a multiplicative effects model developed by JECFA 1998 [20], as we previously did in the global cancer risk assessment, and we calculated the disease burden in different decades in the area. Next, we applied the aflatoxin potency factors, from an additive effects model of the aflatoxin and HBV infection in inducing liver cancer [170], and compared the estimated liver cancer burden with the results from multiplicative model for the same area in the same decade years.

Table 6. Variables to be considered in the validation analysis

Possible key variables	Quantifiable determinants	Underlying determinants
Average daily dietary aflatoxin intake	• corn and peanuts consumption amount • aflatoxin contamination levels • bodyweight	• gender difference (to be averaged) • detection limit of contamination
Population	• population growth rates	age and gender distribution (to be standardized)
Chronic HBV prevalence	• vaccination rates	gender and age difference (to be averaged)
Potency factor of aflatoxin	• multiplicative effects with HBV+ • additive effects with HBV+	The multiplicative effects model was developed from JECFA 1998 [20]; however, the aflatoxin potencies reflect additive effects were derived from a study conducted in 2009 [170].

3.1.2 QUANTIFICATION OF THE POSSIBLE KEY VARIABLES IN THE MODEL

Aflatoxin daily dietary intake

Corn and peanut oil were confirmed to be the major source of dietary AFB_1 exposure in Fusui County [54]. Since the implementation of an HCC prevention program in Fusui from the 1990s, local residents have gradually changed their main foods from corn to rice [171]. As the results, a decline of AFB_1 exposure in the local population was observed over the years. Table 7 summarized the changes of the diet style and the accordingly decreased AFB_1 exposure. In 1970s, almost 100% of daily food consumed by Fusui population was corn. 30 years later, the percentage of corn in total diet decreased to 48%, as more than half of daily food was replaced by rice.

Table 7. Aflatoxin B_1 daily dietary intake in population of Fusui County, Guangxi, China, 1980s-2010s

Year	Corn consumed (g/day)	Percentage of corn in daily food consumption (%)	Peanut oil consumed per day (g/day)	Aflatoxin B_1 Exposure in study population from dietary food (ng/kg bw/day)
1985	350-500 [50]	Almost 100% for average [50]	Not available	806 (men) [50] 1290 (women) [50] 967 (67-3683) for total AF [54]
1999	575 men 322 women [172]	76% for men, 73% for women [145]	18g[145]	233 (men) 133 (women) [145]
2010	250 (75-500) [173]	48% in average [173]	7g [173]	45(17–242) [173]

HBsAg+ prevalence and liver cancer incidence

To control the endemic hepatitis B in the country, the Chinese government has implemented infant vaccination with hepatitis B vaccine from 1992, with the first dose to be administered within 24 h of birth and subsequent doses at 1 and 6 months [174]. Indeed, a pilot study of the universal HBV vaccination program was initiated in 1986 in Long An county, located 90 miles away from Fusui, Guangxi province, and then spread to several other areas of China [175]. It consisted of routine immunization services to all newborns in those areas in order to investigate the efficacy of vaccination. in Long An county, it was reported that the HBsAg positive rate was 16.5% in 1985 and 7.5% in 2005 [175]. However, the most significant decrease was observed in generation < 20 yr old, who received the vaccination at the birth. Chronic HBV infection is still endemic in generations between 20 to 65 years old in the area. Table 8 listed the HBsAg prevalence and liver cancer incidence data in Fusui, from 1980s to 2010s. The HBsAg positivity prevalence in Fusui population, as of in 2000s, decreased more than a half compared to it was in 1980s, while the liver cancer incidence only decreased slightly. The discrepancy

between the declining HBsAg positive rates and the liver cancer incidence reflect the cancer latency factor, which means it will take 20-30 years to see the real benefit of reducing liver cancer incidences by vaccination program, because most of the HCC cases occur at age of 20 to 65 in China.

Table 8. Population, HBsAg prevalence and liver cancer incidence in Fusui, Guangxi, China, 1980s -2010s

Decades	Population	HBsAg positive (%)	Age-standardized liver cancer incidence (per 100,000/yr)
1980s	366,000	23.3% in men [176]	120 in men, 31 in women [30]
1990s	403,000	29% [171]	92-97 in men [171]
2000s	442,000	10.6 % [177]	109 in male, 27 in female [178]

Aflatoxin potency factors

Most of the observational studies revealed significant synergistic interactions between aflatoxins and chronic HBV infection in relation to liver cancer, with more than multiplicative effect being reported. In 1998, JECFA estimated aflatoxin potencies based upon the multiplicative effects, with 0.01 (0.002-0.03) cancers/year/100,000 per ng aflatoxin/kg bw per day in HBsAg- individuals and 0.3 (0.05-0.5) cancers/year/100,000 per ng aflatoxin/kg bw per day in HBsAg+ individuals [20]. However, in a recent follow-up of the cohort in Taiwan with more HCC cases, Wu et al [170] reported the combined effects of AFB_1 and HBV was consistent with an additive model rather than the multiplicative one. To evaluate how the inputs of potencies would affect the aflatoxin-attributable cancer burden, we estimated that the aflatoxin potency in HBsAg+ inviduals will be 0.1 cancers/year/100,000 per ng aflatoxin/kg bw per day, based upon the additive effect suggested by Wu et al.

3.2 RESULTS

The estimated liver cancer incidences and cancer burden attributable to aflatoxin for Fusui County, Guangxi, China from 1980s to 2010s were calculated. Results were presented Table 9 and 10. In 1980s, we estimated 103 liver cancer cases per 100, 000 HBsAg male carriers and 11 cases per 100, 000 HBsAg male non-carriers per year would be attributable to aflatoxin exposure in Fusui. 27% of the total liver cancer cases occurred in males of Fusui in 1980s could be related to aflatoxin exposures, according to the actual disease burden of 120 cases/100,000 in males. In contrast, under an additive effects model, 34 liver cancer cases per 100,000 HBsAg male carriers and 11 per 100,000 HBsAg male non-carriers per year were estimated to be attributable to aflatoxin, in 1980s in Fusui. Under additive model, 13% of the total liver cancer burden in males could be related to aflatoxin exposures in food.

In 1990s, the estimated HCC cases burden in males attributable to aflatoxin decreased to 28 (15%) under a multiplicative model, and it would be 13 cases (7%) under an additive model. In 2000s, the estimated HCC cases attributed to aflatoxin continued to drop to 11 (4%) under the multiplicative model and 2 (0.7%) under the additive model. However, the wide range reflected the variation of the estimates of dietary aflatoxin exposure in the local population solely relying on food data. In high exposed individuals, the liver cancer burden attributable to aflatoxin could still go up to 19% (under multiplicative model).

Table 9. Estimated liver cancer incidences attributed to aflatoxin in Fusui, China

Decades	Population	Estimated HCC cases attributed to aflatoxin, HBsAg- (per 100,000/yr)		Estimated HCC cases attributed to aflatoxin, HBsAg+ (per 100,000/yr)	
		Multiplicative effects model	Additive effects model	Multiplicative effects model	Additive effects model
1980s	366,000	11 in men	11 in men	103 in men	34 in men
1990s	403,000	3 in men, 2 in women	3 in men, 2 in women	41 in men, 23 in women	14 in men, 8 in women
2000s	442,000	2 (1-10)	2 (1-10)	6 (2-35)	2 (1-11)

Table 10. Estimated HCC burden attributed to aflatoxin in Fusui, China [a]

Decades	Actual liver cancer cases [b] (per year)	Estimated HCC burden attributed to aflatoxin per year, multiplicative effects model N (%)	Estimated HCC burden attributed to aflatoxin per year, additive effects model N (%)
1980s	220 in males	59 in males (27%)	29 in males (13%)
1990s	185-195 in males	28 in males (15%)	13 in males (7%)
2000s	241 in males, 60 in females [178]	11 (5-56) 4% (2-19%)	2 (1-10) 0.7% (0.3 – 3%)

([a]Assuming 50% of the population is male, [b] calculated from the actual liver cancer incidence in Fusui County)

3.3 DISCUSSION

The risk assessment model we applied to estimate the aflatoxin-attributable liver cancer burden can be considered as a prediction model. The estimated burden of disease will actually reflect the situation in the next 15-40 years following the epidemiological and food data were collected, which is the latency time for a patient with chronic hepatitis to progress to liver cancer. That is to say, the estimated aflatoxin-attributable liver cancer cases based upon the data of dietary aflatoxin exposure, chronic HBV infection rates and the local population in 1980s, will

be the aflatoxin-attributed cancer burden of Fusui County in 2000 -2020. Indeed, the 27% of liver cancer burden attributed from aflatoxin calculated on the basis of data from 1980s, was confirmed by the population attributable risk fraction of liver cancer from aflatoxin in a Guangxi study [179] conducted in 2000s, calculated by another approach described in Chapter 4.

In this validation analysis, the reduction of estimated aflatoxin-related liver cancer burden in Fusui, based upon data obtained for 1980s and 1990s, reflected the benefits of reduced dietary aflatoxin exposure in population (because the HBsAg+ prevalence remained almost the same in two decades). By changing the diet style from almost 100% to 75% corn, and reducing the aflatoxin contamination level in corn (from 120 ppb to 24 ppb [54,145]), the aflatoxin exposure for the local population was reduced 70-90% in 10-20 years. As a result, the estimated aflatoxin-attributed liver cancer burden dropped about 50%.

The reduction of estimated aflatoxin-related liver cancer burden in Fusui, based upon data obtained for 1990s and 2000s, reflected the benefits of both HBV vaccination and the reduced dietary aflatoxin exposure. By reducing the HBsAg+ prevalence from 29% in 1990s to 11% in 2000s, and at the same time, further reducing 70-80% of the aflatoxin exposure in local population, the estimated aflatoxin-attributed liver cancer burden dropped about 70%.

A lower estimated aflatoxin-attributed liver cancer burden under the additive model was observed when compared to the results from multiplicative model. However, the estimates from multiplicative model were close to the estimated results from a second approach based upon a study conducted in Guangxi, 2008 [179] (see Chapter 4). This indicates a better defined model of combined effects in multiplicative relationship between aflatoxin exposure and chronic HBV

infection, compared to additive model. However, a comprehensive, systematic review of the epidemiological studies is needed to examine the model of combination effects.

Overall, using the proposed risk assessment model, the estimated aflatoxin-attributable HCC cases in Fusui County in three decades were well within the actual disease burden of the local population. The numbers reflected reasonable percentages of the total incident HCC cases in different time period. This fact validated that the risk assessment model is a reliable tool to estimate the general HCC burden attributable to aflatoxin exposure, although the wide range around the estimates and the factor of cancer latency should be noted when the results are interpreted.

4.0 POPULATION-ATTRIBUTABLE RISK OF LIVER CANCER FROM AFLATOXIN EXPOSURE: A SYSTEMATIC REVIEW AND META-ANALYSIS

We previously reported an estimate of global burden of HCC attributable to aflatoxin. By compiling food consumption data and aflatoxin contamination data in various countries and regions, we estimated that 25,200-155,000 HCC cases worldwide, or 5-28% of all HCC cases, can be attributable to dietary aflatoxin [180]. However, the large range of the reported estimate reflects the limitations in acquiring accurate exposures solely from food consumption data, uncertainties in the nature of the dose-response curve, uncertainties in the mode of interation between aflatoxins and viruses, and incomplete data on the prevalence of HBV in different regions of the world.

In this context, the risk estimates of HCC from aflatoxin exposure presented as odds ratios, from population-based studies in which biomarkers of aflatoxin exposure and effect were measured will be helpful to address this knowledge gap. Furthermore, combining data from all eligible studies by meta-analysis has the advantage of reducing random error and obtaining more precise estimates of the association between the aflatoxin exposure, HBV infection and the HCC risk. Therefore, in this study, we systematically reviewed cohort or case-control studies in different world regions with reported odds ratios (ORs) or relative risks (RRs) of aflatoxin in

relation to liver cancer. By combining the relevant odds ratios (ORs) and relative risks (RRs) from these studies, we conducted meta-analyses to calculate population-attributable risk (PAR) of aflatoxin-related HCC in the population overall, as well as in HBV+ and HBV- populations. The meta-analysis also allowed us to compute the combined ORs of HCC from combined effects of dietary aflatoxin and HBV+, from aflatoxin only and from HBV+ only, and to determine the mode of interaction effects between two risk factors. In the context of our study, PAR of aflatoxin-related HCC is the proportion of HCC cases that could be avoided in a chosen population by reducing aflatoxin exposures (as measured by biomarkers) from detectable to undetectable levels.

4.1 METHODS

4.1.1 SEARCH STRATEGY AND STUDY ELIGIBILITY CRITERIA

We performed a literature search to May 13th, 2011, using the Medline/PubMed databases without restrictions using the following search terms: (aflatoxin) and (hepatitis B) and (liver cancer); (aflatoxin) and (hepatitis B) and (hepatocellular carcinoma). Additionally, we searched the reference lists from retrieved articles to identify further potentially relevant studies. This systematic review was planned, conducted and reported in adherence to PRISMA standards for reporting meta-analyses [181]-[182].

Studies were included in the systematic review if they met the following criteria: (1) case-control or cohort study design; (2) aflatoxin as the exposure of interest, measured by biomarkers and/or food surveys; (3) HBV as the viral infection of interest (HBsAg positivity as a marker of chronic HBV infection); (4) HCC as the outcome of interest; and (5) RR or OR estimates with 95% confidence intervals (CIs) (or data to calculate these) reported. If data were duplicated (same study cohort and same period) in more than one eligible study, we included the study with validated biomarkers or enough quantitative measurements of the association between aflatoxin exposure and liver cancer. If more than one article reported results of a study from different perspectives, such as follow-up in different time periods (at least 5 years apart) or different target populations (HBsAg+ individuals only vs. general population), we treated these articles as different studies and included them all.

4.1.2 DATA EXTRACTION

The following data were extracted from each study: authors, publication year, study design and sample size, study location, study period, participants' gender and age range, metric and range of aflatoxin exposure, estimated adjusted RRs/ORs, and variables adjusted for analysis. Because all identified studies are case-control designs except one cohort study, and because RR and OR can be used interchangeably when the disease is relatively rare (<15%; HCC rates are lower than this in the populations studied), we combined the RR from this study with the ORs from the case-control studies to calculate a summary OR. If aflatoxin exposure was measured using different biomarkers in the same study, we selected the ones reflecting consistent biomarkers amongst different studies (one OR per study was used).

4.1.3 STATISTICAL METHODS FOR META ANALYSIS

The ORs from the studies were first combined in a meta-analysis using a random-effects model, and then a fixed-effects model if heterogeneity in the study pool was insignificant [183]. The studies were categorized by the recruited population type: general populations, and HBV+ or HBV- populations. First, all the studies providing data for general populations (including both HBV+ and HBV- individuals) were combined, and ORs of aflatoxin-related HCC after HBsAg+ adjustment and ORs for combined (aflatoxin+HBV) effects were analyzed. Then the studies with data from HBV+ populations (and studies that recruited from the general population but separately estimated ORs in HBV+ populations) were combined; and the ORs for HBV+ populations only were estimated. We also combined the studies that separately estimated the ORs in HBV- populations. If the study examined the association between aflatoxin exposure and HCC in various exposure categories, we chose the ORs reflecting highest and lowest levels of aflatoxin exposure for the meta-analysis.

Heterogeneity amongst the studies was evaluated using the Cochran's Q value calculated from the Mantel-Haenszel method and the I^2 statistic [183]. We performed sensitivity analyses in which each study was in turn removed and the rest analyzed to evaluate if the results were significantly affected by one particular study. Publication bias was assessed by a funnel plot and associated statistical tests of asymmetry. All statistical analyses were performed with Comprehensive Meta-Analysis software Version 2.2.

4.1.4 STATISTICAL METHODS FOR PAR CALCULATIONS

We estimated PAR for aflatoxin-related HCC in two groups: 1) HBsAg- individuals, and 2) HBsAg+ individuals, within each study if the data were available. To estimate the PAR for aflatoxin-related HCC using the adjusted RRs or ORs, we used an attributable fraction calculation formula described by Eq. 1 [184]:

$$\mathbf{AF_{pop}} = \sum_{i=1}^{z} \mathbf{W_i} \frac{\mathbf{P_i(RR_i - 1)}}{\mathbf{1 + P_i(RR_i - 1)}} \quad \mathbf{Eq.\,1},$$

where $\mathbf{AF_{pop}}$ is aflatoxin attributable risk fraction in the population including exposed and unexposed individuals, P_i is the proportion of the population in stratum i that is exposed, and W_i is the proportion of diseased individuals (cases) in stratum i. RR_i is the adjusted RR or OR in stratum i. The weighted attributable risk fraction was calculated for aflatoxin in HBsAg+ and HBsAg- groups. We use adjusted OR_i in stratum i as an approximation of RR_i.

If the study assessed the quantitative relationship between aflatoxin and HCC risk at different exposure levels, an overall attributable fraction was calculated by summarizing the AF across different exposure levels. If the study did not evaluate the association between aflatoxin and liver cancer risk stratified by HBsAg positivity but provided the risk estimates adjusted by HBsAg positivity, we then use the calculation formula Eq. 2 below [184] to estimate the PAR of aflatoxin in related with liver cancer in general population adjusted by HBsAg positivity:

$$AF_{pop} = \frac{P_c(RR - 1)}{RR} \quad \text{Eq. 2},$$

where P_c is the proportion of cases exposed in the combined population based on detection limits for aflatoxin biomarkers in the studies, and HBsAg positivity-adjusted OR is

used as an approximation of RR. For each AF_{pop}, we calculated confidence intervals using the method described in Daly [185].

4.2 RESULTS

4.2.1 LITERATURE SEARCH

The step by step process of our literature search is presented in Figure 3. From 479 results, we excluded human cell line studies, animal studies, review articles, and studies without available abstracts. We read the titles and/or abstracts of all the remaining studies and chose the ones that investigated the association between aflatoxin (by biomarker or by food survey) and liver cancer risk. Any case-control, nested case-control and cohort studies for adults (>18 yrs) were included for this analysis provided that they met the criteria described above. 27 studies were thus selected. We also identified 3 relevant studies from the reference lists of the 27 selected studies. We then read the full text of these 30 studies to determine 1) if the authors provided quantitative measurements of the association between aflatoxin exposure and HCC risk, 2) if they analyzed the association between aflatoxin and HCC risk in HBV+ and HBV- individuals separately, 3) if HBsAg positivity was taken into consideration when the overall risk estimates were provided for the general population, and 4) if the individual articles were duplicated reports for one study population or they were follow-up studies after the first initiated

study. Six studies were excluded because they were duplicated reports from the same population in the same time period, and 7 studies were excluded because the quantitative measurements of association between aflatoxin exposure and HCC with HBsAg+ adjustment were not provided in the text. Thus, 17 studies were included for this systematic review and PAR analysis.

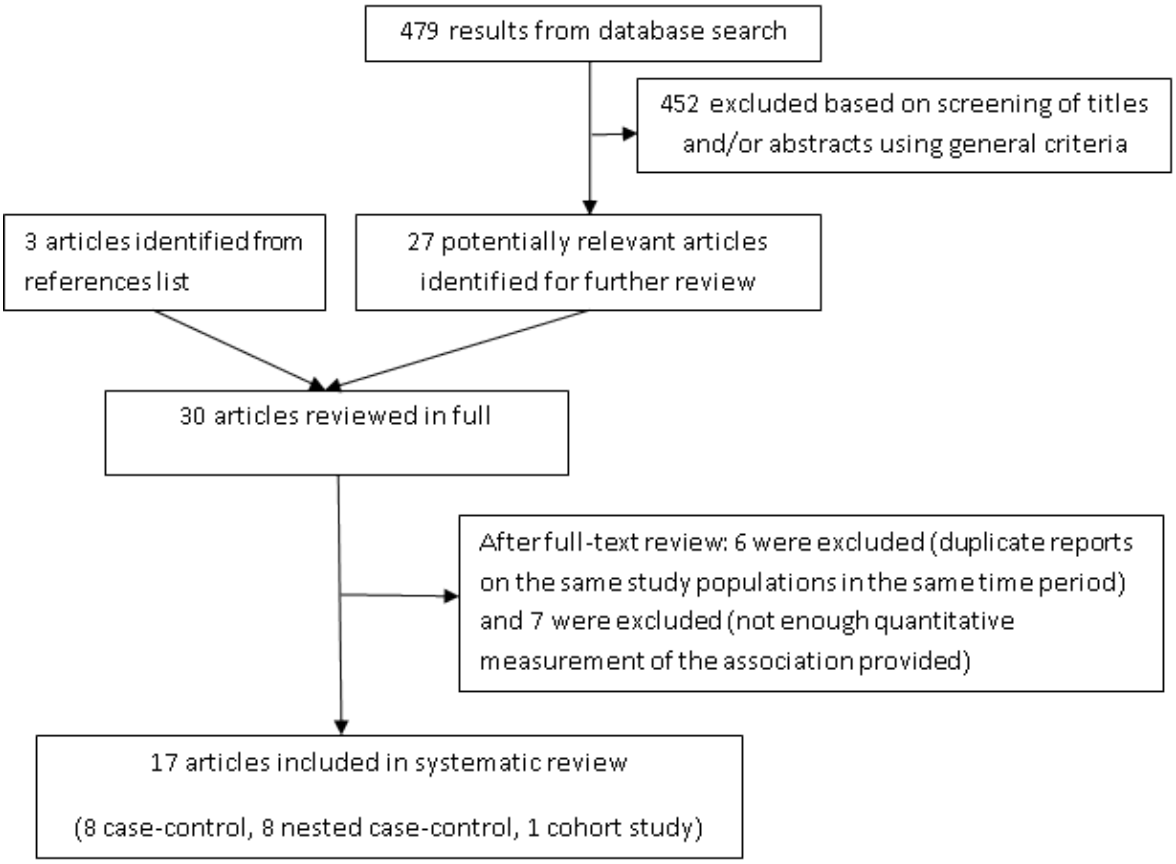


Figure 3. Selection of studies for inclusion in systematic review

4.2.2 STUDY CHARACTERISTICS

Table 11 provides an overview of the eligible studies, including the study year, the study population and sample size, the biomarker measured in the study, and the adjusted RRs or ORs for aflatoxin-related HCC provided by these studies.

The 17 studies [5-8,56-58,170,179,186-193] on aflatoxin exposure and liver cancer risk - 8 case-control studies, 8 nested case-control studies, 1 cohort study - were published between 1994 and 2009. There were 1680 HCC cases and 3052 controls in total. All the eligible studies were conducted in areas of the world where aflatoxin exposure was relatively high in the diet (until recently): China, Taiwan, and sub-Saharan Africa. 6 studies were conducted in China, 7 in Taiwan and 4 in sub-Saharan Africa. 14 out of 17 studies reported biomarker measurements for aflatoxin exposure, while the other 3 studies relied on food consumption data. Among the 14 studies that utilized biomarkers to measure aflatoxin exposure, 5 studies measured urinary aflatoxin biomarkers, including AFM_1 and AFB_1-N^7-Gua, 6 studies measured AFB_1-albumin adducts, 2 studies measured AFB_1-DNA adducts, and 3 studies measured TP53 249^{ser} mutation as the biomarker(s) of interest. Several studies included measures of more than one biomarker. 12 studies were conducted in both HBsAg+ individuals and non-individuals, with risk estimates that were adjusted for HBsAg positivity (9 studies), age (6 studies), gender (4 studies), tobacco smoking (4 studies), alcohol consumption (3 studies), anti-HCV (3 studies), and family liver disease history (3 studies). 5 studies were conducted in HBsAg+ individuals only, and the risk estimates were adjusted for cigarette smoking (2 studies), alcohol drinking (2 studies), age (2 studies) and gender (1 study).

Four studies reported results for one Taiwanese cohort from four different time periods[7,170,186,192] from 1980s to 2000s. To determine if all these studies should be included in the meta-analysis, we first examined the heterogeneity between the risk estimates provided by these studies. Because of the significant heterogeneity of aflatoxin exposures and HCC risk estimates in this cohort between the follow-up studies through the years, we treated these as independent studies in the analysis. In analyses that included only the most recent of all studies in a particular cohort, the results were nearly identical to those obtained when including all studies (Table 13). Two articles reported results from one case-control study in Sudan from different perspectives (risk estimates for the general population after adjustment of HBsAg+, and risk estimates for HBsAg+ or HBsAg- separately) [8,191]. Likewise, two articles reported results from a study in The Gambia with risk estimates for the general population after adjustment of HBsAg+, and risk estimates for HBsAg+ or HBsAg- separately [5,190].

Table 11. Characteristics of the eligible studies included in the systematic review / meta-analysis[iv,v]

No	Source	Location/ period	Sex	Age, yrs	No of Cases (% exposed)	No of Controls (% exposed)	Measure/Range of Exposure, detection limit	Adjusted ORs/RRs[vi]	Adjustment for Covariates
1	Qian et al., 1994 [6] (cohort of 18,244 middle-aged men)	China, 1986-1992	M	45-64	50 cases (36%)	267 matched controls (12%)	AFB_1-N^7-Gua adduct (detectable vs non-detectable, 0.07ng aflatoxins/ml urine	9.1 (2.9-29.2)	HBsAg positivity, cigarette smoking
					50 cases (72%)	267 matched controls (41%)	Multiple urinary biomarker (detectable vs non-detectable, 0.01fmol/µg)	5.0 (2.1-11.8)	
2	Chen et al., 1996 [186] (7 township cohort nested case-control study)	Taiwan, 1991-1992	F/M	36-65	20 cases (65%)	86 matched controls (37%)	AFB_1-albumin adducts (detectable Vs non-detectable, 0.01fmol/µg)	5.5 (1.2-24.5)	HBsAg, anti-HCV, family history of liver cancer cirrhosis
3	Chen et al., 1996 [187] (nested case-control in cohort of 4,841 male HBsAg individuals)	Taiwan, 1988-1992	M	30-65	32 cases (37.5% low exposure)	73 matched controls (33% low exposure)	AFB_1-albumin adducts (Low Vs Non-detectable, 0.01fmol/µg)	1.6 (0.6-4.0)	Cigarette smoking, alcohol consumption
					32 cases (19% high exposure)	73 matched controls (6.8% high exposure)	AFB_1-albumin adducts (High Vs Non-detectable, 0.01fmol/µg)	3.8 (1.0-14.5)	
4	Wang et al., 1996 [7] (7 township cohort nested case-control study	Taiwan, 1991-1995	F/M	30-64	52 cases (60%)	168 matched controls (37%)	AFB_1-albumin adducts (detectable vs non-detectable, 0.01fmol/µg)	1.6 (0.4-5.5)	HBsAg positivity
					38 cases (53%)	137 matched controls (45%)	Urinary aflatoxin metabolite (high vs low, 0.01fmol/µg)	3.8 (1.1-12.8)	

[iv] All the eligible studies were conducted in China (6), Taiwan (7), or sub-Saharan Africa (4). Fourteen studies reported biomarker measurements for aflatoxin exposure, while the other three studies relied on food consumption data. Twelve studies included both HBsAg+ and HBsAg- individuals, with risk estimates that were adjusted for HBsAg positivity (nine studies). Five studies were conducted in HBsAg+ populations only.

[v] Among the fourteen studies that utilized biomarkers, five measured urinary aflatoxin biomarkers, including AFM_1 and AFB_1-N^7-Guanine, six measured AFB_1-albumin adducts, two measured AFB_1-DNA adducts, and three measured TP53 249^{ser} mutations. Several studies included measures of more than one biomarker.

[vi] 15 out of 16 identified case-control studies provided matched ORs.

Table 11 continued

5	Zhang et al., 1997 [189] (Hospital-based case-control study)	China, 1994-1995	F/M	18-88	152 cases (33%)	115 non-hepatic patient controls (2%)	Corn consumption history from dietary questionnaire	(1:1 pair-matched) 16.44 (1.67-61.65)	HBV infection, individual history of liver diseases, family history of liver diseases, and peanut consumption
					152 cases (89%)	115 non-hepatic patient controls (49%)	Peanut consumption history from dietary questionnaire	3.51(1.45-8.47)	HBV infection, individual history of liver diseases, family history of liver diseases, and corn consumption
6	Yu et al., 1997 [188] (nested case-control of a cohort of 4841 male HBsAg individuals)	Taiwan, 1988-1994	M	30-65	42 cases (29%)	43 matched controls (14%)	AFB_1-N^7-gua, (below 0.21 ng/ml Vs 0.21-0.36 ng/ml, 0.05 ng aflatoxin/ml urine)	5.3(1.1-25.2)	Education level, ethnicity, habitual alcohol drinking and cigarette smoking status
					42 cases (14%)	43 matched controls (16%)	AFB_1-N^7-gua (below 0.21 ng/ml Vs >0.36 ng/ml, 0.05 ng aflatoxin/ml urine)	2.8 (0.6-12.9)	
					42 cases (24%)	43 matched controls (23%)	AFM_1 (below 1.61 ng/ml Vs 1.61-2.85 ng/ml, 0.05 ng aflatoxin/ml urine)	1.9(0.5-7.2)	
					42 cases (55%)	43 matched controls (35%)	AFM_1 (below 1.61 ng/ml Vs >2.85 ng/ml, 0.05 ng aflatoxin/ml urine)	6.0(1.2-29.0)	
7	Lunn et al., 1997[57] (case-control study)	Taiwan, 1984-1995	F/M		105 cases (80%)	37 controls (43%)	AFB_1-DNA adducts	Corrected OR: 3.9(1.4-11.5)	n/a
8	Kirk et al., 2000 [190] (case-control study)	The Gambia, 1997-1998	F/M	20-73	53 cases (36%)	53 matched controls (5.7%)	Ser-249 P53 mutation	16.4 (3.0-90.5)	Age, sex, recruitment site and HBsAg positivity
9	Sun et al., 2001 [192] (7 township cohort nested case-control study, HBsAg individuals)	Taiwan, 1991-1997	F/M	30-64	75 cases (64%)	140 matched controls (46%)	Aflatoxin-albumin adducts (detectable vs non-detectable, 0.01fmol/μg)	2.0 (1.1-3.7)	Sex, age and residence
10	Omer et al., 2001[191] (case-control study)	Sudan 1996-1998	F/M	20-70	115 cases	199 matched controls	Peanut butter consumption >300 g/mo Vs Peanut butter consumption <70 g/mo	3.3(1.4-8.1)	Age and hepatitis

Table 11 continued

11	Ming et al., 2002 [193] (Hospital-based cohort, 145 HBsAg individuals)	Qidong, China	M	27-74	31 cases	145 HBsAg+ carriers follow up	AFM_1 (>3.6 ng/l)	3.5(1.5-8.1)	Age, HCV, family history of HCC
12	Huang et al., 2003 [56] (case-control study)	Qidong, China	F/M	19-87	25 cases (40%)	30 controls (6.7%)	Ser 249 TP 53 mutation	22.1(3.2-91.7)	Sex, age, recruitment site and HBsAg positivity
13	Omer et al., 2004 [8] (case-control study)	Sudan, 1996-1998	F/M	20-70	114 cases (46%)	198 matched controls (26%)	Peanut butter consumption >300 g/mo Vs Peanut butter consumption <70 g/mo	n/a	age
14	Kirk et al., 2005 [5] (case-control study)	Gambia,	F/M		186 cases (40%)	348 matched controls (3.4%)	Ser-249 TP53 mutation	20.3 (8.19-50.0)	Adjusted for study group, season of recruitment and daily groundnut intake
15	Long et al., 2009 [179] (hospital-based case-control)	China, 2006-2008	F/M	12.1% <35, 77.8% 35-65, 10.1% >65	618 cases (28%)	712 matched control (29%)	AFB_1-adduct: Low (≤ 1·00 µmol/mol DNA) Vs Medium (1.01-2.00 µmol/mol DNA), (0.25µmol/mol DNA)	2.11 (1.54-2.90)	Age, sex, ethnicity, HBsAg, anti-HCV, and AFB_1 –exposure years
					618 cases (47%)	712 matched controls (17%)	AFB_1-adduct: Low (≤ 1.00 µmol/mol DNA) Vs High (≥2.01 µmol/mol DNA) (0.25µmol/mol DNA)	6.23 (4.48-8.67)	
16	Wu et al., 2009 [170] (7 township cohort nested case-control study)	Taiwan 1991-2004	F/M	30-64 yr	230 cases (93%)	1052 matched controls (95%)	AFB_1-albumin adduct(fmol/mg): Non-detectable Vs Detectable (0·01fmol/µg or 1fmol/ml)	0.99 (0.48-2.02)	HBsAg, anti-HCV, habitual smoking, alcohol drinking, BMI and the batch of aflatoxin biomarker assay
					230 cases (33%)	1052 matched controls (33%)	AFB_1-albumin adduct(fmol/mg): Below the mean (<59·8) Vs. Above the mean (≥59·8),	1.54 (1.01-2.36)	
					198 cases (88%)	904 matched controls (88%)	Urinary AFB_1 metabolites (fmol/ml): Non-detectable Vs Detectable (0.01fmol/µg or 1fmol/ml)	1.70 (0.89–3.25)	
					198 cases (57%)	904 matched controls (44%)	Urinary AFB_1 metabolites: Below the mean Vs Above the mean	1.76 (1.18-2.58)	

Table 11 continued

17	Szymanska et al., 2009 [58] (nested case-control study)	China, 1989-1998	M	30-59	126 cases (67%)	123 matched controls (68%)	AF-albumin Detectable Vs non-detectable (3 pg/mg)	0.90 (0.52-1.56)	In HBV individuals

4.2.3 RISK ESTIMATES FOR CALCULATION OF PAR OF AFLATOXIN-RELATED LIVER CANCER

To assess the effect of aflatoxin exposure on the relationship between HBV infection and HCC, data from eligible studies were extracted and stratified into two groups based on HBsAg status. Risk estimates of aflatoxin-related HCC in HBsAg+ and HBsAg- groups were calculated separately. Twelve studies estimated the overall ORs in the combined populations. Among them, six studies provided risk estimates in HBsAg+ and HBsAg- groups separately, while five studies provided risk estimates in HBsAg+ groups only. To analyze the population attributable risk of aflatoxin-related HCC in different studies, we calculated the percentage of exposed cases in the combined population (P_c) for eligible studies, the proportion of liver cancer cases in HBsAg+ groups (W_1) and the proportion of study population in this stratum exposed to aflatoxin, which is obtained from the percentage of detectable biomarkers in the HBsAg+ group (P_1), the proportion of liver cancer cases in HBV- group (W_2), and the proportion of the study population in this stratum exposed to aflatoxin (P_2). The results were presented in Table 12. We estimated the unadjusted ORs of aflatoxin in HBsAg+ group for those studies that did not stratify data by HBsAg positivity. Interestingly, the aflatoxin exposure prevalence was higher in HBsAg+ (P_1) than HBsAg- (P_2) individuals in 6 studies that analyzed the association in two groups separately.

Table 12. Risk estimates for calculation of liver cancer PAR from aflatoxin exposure from eligible studies

References	Exposure measurement	Proportion of exposed cases (P_c)	Proportion of HCC cases in HBsAg+ (W_1)	Proportion of aflatoxin exposed HBsAg+ (P_1)	Proportion of HCC cases in HBsAg- (W_2)	Proportion of aflatoxin exposed HBsAg- (P_2)	Adjusted RRs/ORs in HBsAg+	Adjusted RRs/ORs in HBsAg-
Qian et al., 1994[6]	urinary aflatoxin metabolites	36/(50+267)=0.114	32/63=0.51	30/63=0.48	18/254=0.07	115/254=0.45	8.76(2.80-27.4)[a]	3.4 (1.1-10.0)
Chen et al., 1996 [187]	high AFB_1-albumin level vs non-detectable	n/a	20/105=0.19	11/105=0.11	n/a	n/a	3.8(1.0-14.5)	n/a
	low AFB_1-albumin level vs non-detectable	n/a	26/105=0.25	36/105=0.34	n/a	n/a	1.6(0.6-4.0)	n/a
Chen et al., 1996 [186]	AFB_1-albumin	13/106=0.123	n/a	n/a	n/a	n/a	n/a	n/a
Wang et al., 1996 [7]	aflatoxin-albumin	31/(52+180)=0.134	40/64=0.625	36/64=0.563	8/161=0.05	53/161=0.33	2.8 (0.9-9.0)	0.3 (0-3.6)
	urinary aflatoxin metabolite	26/(38+137)=0.149	29/50=0.58	27/50=0.54	6/120=0.05	57/120=0.475	5.5 (1.3-23.4)	1.7 (0.3-10.8)
Yu et al., 1997 [188]	0.21-0.36 ng/ml (AFB_1-N^7-gua vs non-detectable)	n/a	36/86=0.42	18/86=0.21	n/a	n/a	5.3 (1.1-25.2)	n/a
	> 0.36 ng/ml (AFB_1-N^7-gua vs non-detectable)	n/a	30/86=0.35	13/86=0.15	n/a	n/a	2.8(0.6-12.9)	n/a
Lunn et al., 1997 [57]	AFB_1-DNA	84/(105+37)=0.59	79/88=0.90	67/88=0.76	26/54=0.48	33/54=0.61	1.69 (0.39-7.46)[b]	17.4(3.4-90.3)
Zhang et al., 1998 [189]	Corn consumption	50/(152+115) =0.187	n/a	n/a	n/a	n/a	n/a	n/a
	Peanut consumption	136/(152+115)=0.509	n/a	n/a	n/a	n/a	n/a	n/a
Kirk et al., 2000 [190]	TP53 Ser 249 mutation	19/(53+53)=0.179	n/a	n/a	n/a	n/a	n/a	n/a
Sun et al., 2001 [192]	AFB_1-albumin adducts	n/a	75/215=0.35	111/215=0.52	n/a	n/a	2.0(1.1-3.7)	n/a

Table 12 continued

Omer et al., 2001[191]	Peanut butter consumption >300 g/month	63/(85+104) =0.33	n/a	n/a	n/a	n/a	n/a	n/a
Ming et al., 2002 [193]	AFM_1	n/a	31/145=0.21	78/145=0.54	n/a	n/a	3.5 (1.8-8.1)	n/a
Huang et al., 2003 [56]	TP53 Ser 249 mutation	10/(25+30)=0.182	n/a	n/a	n/a	n/a	n/a	n/a
Omer et al., 2004 [8](Sudan)	Peanut butter consumption >300 g/month	53/(114+198)=0.170	29/38=0.76	30/38=0.80	38/131=0.29	74/131=0.56	1.19 (0.21-6.69)[c]	5.1(1.8-13.9)
Kirk et al., 2005 [5]	TP53 Ser 249 mutation	74/(186+348)=0.139	99/146=0.68	46/146=0.32	84/382=0.22	39/382=0.10	38.3 (5.08 – 289)[d]	13.2(4.99-35.0)
Wu et al., 2009 [170]	AFB_1-albumin adducts (above the mean vs below the mean)	75/(230+1052)=0.059	155/456=0.34	126/456=-0.28	75/826=0.09	293/826=0.35	1.43(0.76-2.71)	1.65(0.63-4.33)
	Urinary aflatoxin metabolites (above the mean vs below the mean)	113/(198+904)=0.103	143/411=0.348	209/411=0.509	55/691=0.08	299/691=0.433	1.19(0.72-1.98)	4.29(1.43-12.85)
Szymañska et al., 2009 [58]	AFB_1-albumin adducts	n/a	126/249=0.51	168/249=0.67	n/a	n/a	0.90 (0.52-1.56)	n/a
Long et al., 2009	AFB_1-DNA adducts medium vs low	172/1330 = 0.129	n/a	n/a	n/a	n/a	n/a	n/a
	AFB_1-DNA adducts high vs low	293/1330=0.220	n/a	n/a	n/a	n/a	n/a	n/a

([a,b,c,d,] Risk ratio estimates were unadjusted.)

4.2.4 AFLATOXIN EXPOSURE (DETECTABLE/HIGH VS. NON-DETECTABLE/LOW) AND HCC RISK, ANALYSIS SEPARATED BY HBSAG+ STATUS AND GEOGRAPHIC LOCATION

The association between aflatoxin exposure and liver cancer, independently or in conjunction with chronic HBV infection was analyzed from combining the eligible studies by HBsAg+ status and calculating the summary ORs (Table 13, forest plot Figure 4-7) in HBsAg+ individuals, HBsAg- individuals and general population. We also did meta-analysis for subgroups (China, Taiwan and Sub-Saharan Africa). In forest plot of figure 4 to figure 7, the squares and horizontal lines correspond to the study-specific OR and 95% CI; the box size is proportional to the meta-analysis study weight; the diamonds represent the combined OR and 95% CI. In summary, aflatoxin exposure is significantly associated with HCC risk, regardless of HBsAg status, with a summarized OR of 4.75 (2.78-8.11) from nine studies in the general population adjusted by HBsAg positivity, 2.39 (1.50-3.82) from eleven studies in HBsAg+ populations and 5.91 (3.66-9.55) from six studies in HBsAg- populations.

Aflatoxin Exposure and HCC Risk adjusted by HBsAg+

The adjusted (HBsAg positivity included) RRs/ORs for each study and all studies combined are shown in Figure 4. We combined nine studies which provided ORs adjusted by HBsAg positivity for analysis by the Comprehensive Meta-Analysis. Data from eight of these studies were eligible for the calculations. Other OR datasets were selected from the studies if associations were made using different biomarkers. When combining studies that provided a range of aflatoxin exposures, we chose the ORs corresponding to the highest and lowest

aflatoxin exposures in each study. The combined OR of HCC from the detectable/high vs. non-detectable/low aflatoxin exposures (adjusted by HBsAg positivity) is 4.75 (95% CI: 2.78-8.11). The heterogeneity between the studies is significant (Q=32.73, P<0.000, I^2=75.56). Because four out of the nine studies were a series of follow up studies from 1991 to 2004 as we previously described, we also analyzed the combined ORs by only including the most recent study Wu et al., published in 2009 and excluding the other three studies out of the pool. The recalculated combined OR of HCC from the detectable/high vs. non-detectable/low aflatoxin exposures (adjusted by HBsAg positivity) is 4.88 (95% CI: 2.69-9.10). The heterogeneity between the studies is still large (Q=32.55, P<0.000, I^2=81.57).

We also grouped the four follow up studies in Taiwan and taking the avergae effect of them to combine with studies from other areas. The recalculated combined OR of HCC from the detectable/high vs. non-detectable/low aflatoxin exposures (adjusted by HBsAg positivity) is 4.92 (95% CI: 2.74-8.82). The heterogeneity between the studies is still large (Q=29.48, P<0.000, I^2=79.65).

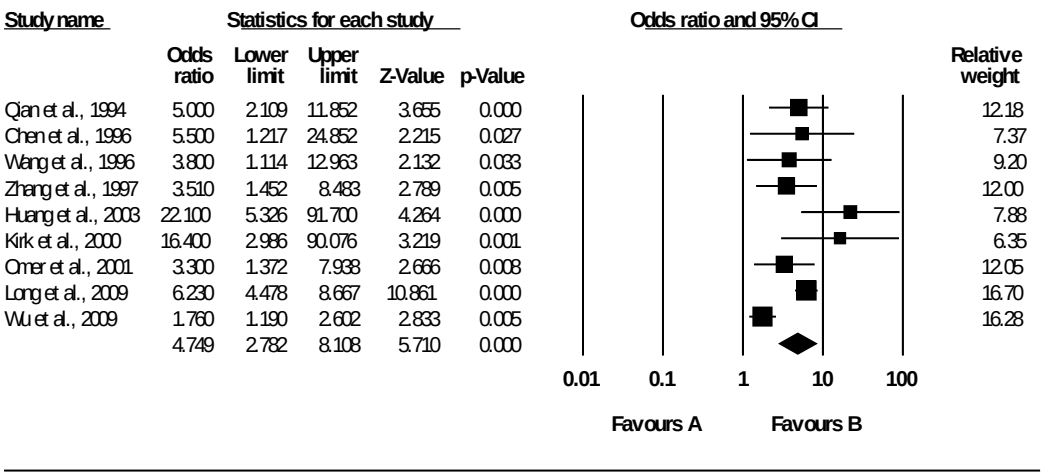


Figure 4. Odds ratios of liver cancer risk for aflatoxin exposure (detectable/high vs. non-detectable/low), adjusted by HBsAg status

Aflatoxin Exposure (Detectable vs Non-detectable) and HCC Risk in HBsAg+ Individuals

There were seven studies reported adjusted RRs/ORs on aflatoxin-related HCC risk in HBsAg+ individuals, and four studies did not provide the adjusted ORs of aflatoxin and liver cancer in HBsAg+ directly but the unadjusted ORs could be obtained from the data provided. We calculated the unadjusted ORs of aflatoxin and liver cancer risk in HBsAg+ individuals for each of these studies and combined them with ORs from the other seven studies with eligible data. Thus, eleven studies were combined, and the combined OR of liver cancer for the detectable/high vs non-detectable/low aflatoxin exposure in HBsAg+ individuals was 2.39 (95% CI: 1.50-3.82) with substantial heterogeneity (Q=27.99, P=0.002, I^2=64.27) (Figure 5). The analyses above included all follow-up studies within given study populations (described in previous paragraph). If only the most recent studies within each study population were included (Wu e al. [170] and Yu et al. [188]), the OR was 2.27 (95% CI: 1.24-4.14). The heterogeneity among these studies is statistically significant (Q=24.33, P=0.001, I^2=71.23). If we only combined studies with adjusted ORs, the combined OR was 2.10 (95% CI: 1.25-3.52) with Q=16.40, P=0.012, I^2=63.42

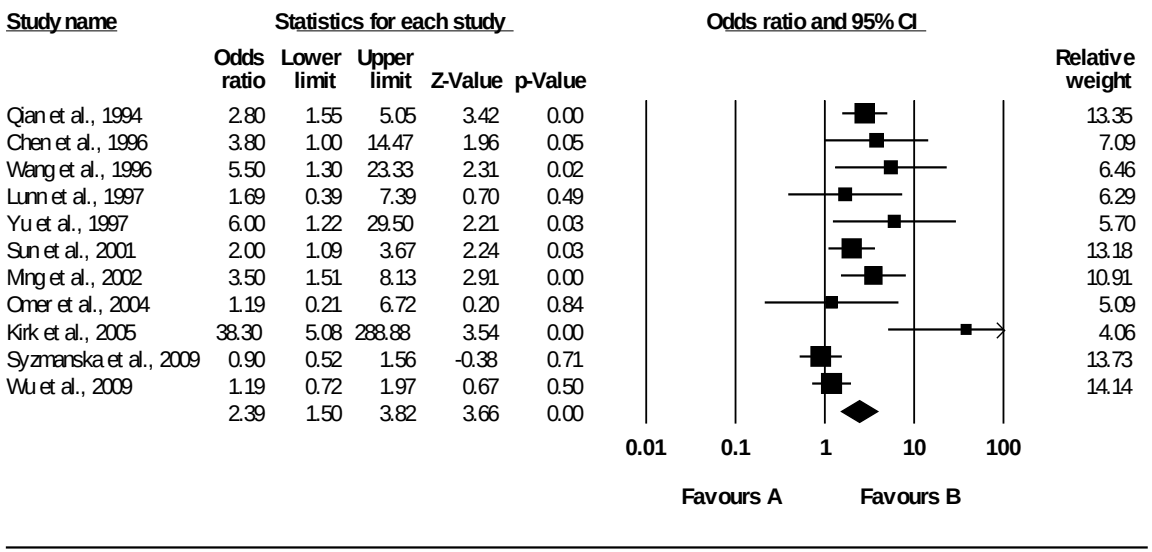


Figure 5. Adjusted odds ratios of liver cancer risk for the detectable/high vs. non-detectable/low aflatoxin exposure in HBsAg+ populations

Aflatoxin Exposure (Detectable vs. Non-detectable) and HCC Risk in HBsAg- Individuals

6 studies reporting RRs/ORs on aflatoxin exposure and liver cancer risk in HBsAg- individuals were combined. The reported RRs/ORs and the combined ORs are shown in Figure 6. The combined OR of liver cancer for the detectable vs. non-detectable aflatoxin exposure in HBsAg- individuals is 5.80 (95% CI: 3.17-10.59).The heterogeneity among these studies is insignificant (Q=7.51, P=0.185, I^2=33.42). We then assessed the association using fixed-effects model within this study pool because of the insignificant heterogeneity. The OR with fixed-effects model is 5.91 (95%: 3.66-9.55). If we analyze the combined studies which only include the most recent follow up study in the Taiwan cohort, Wu et al., published in 2009 [170], the OR will be 6.54 (95% CI: 3.62-11.82), The heterogeneity among these studies is insignificant (Q=5.51, P=0.24, I^2=27.49).

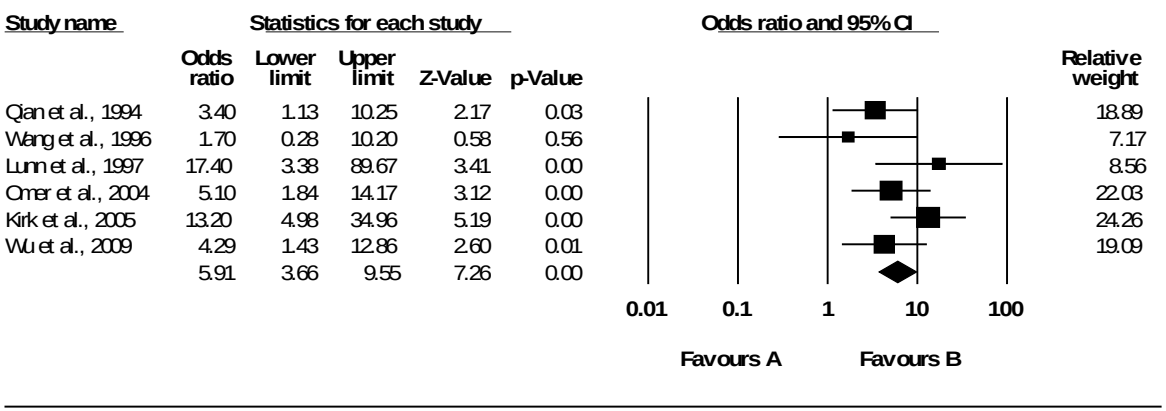


Figure 6. Adjusted odds ratios of liver cancer risk for the detectable/high vs. non-detectable/low aflatoxin exposure in HBsAg- populations

4.2.5 SENSITIVITY ANALYSIS

For the meta-analysis of aflatoxin-related HCC risk in the general population, our sensitivity analyses revealed that Wu et al. [170] was the most influential study in determining the summarized OR. After excluding this particular study, heterogeneity was significantly reduced (Q=8.40, P=0.30, I^2=16.66), and the summarized OR was 5.57 (3.78-7.79).

For the meta-analysis of aflatoxin exposure and HCC in HBsAg+ populations, our sensitivity analyses showed that two studies, Szymanska et al. [58] and Wu et al. [170], substantially influenced the summarized OR. After excluding the two studies, heterogeneity was significantly reduced (Q=11.16, P=0.19, I^2=28.29), and the summarized OR of HCC risk for detectable vs. non-detectable aflatoxin exposure in HBsAg+ individuals was 2.90 (2.09-4.01). These results suggest that the two studies that measured the association between HCC and aflatoxin exposure in the most recent years[58,170] appear to have significantly different results from relatively earlier studies.

For the 10 studies [6,7,56,57,170,179,186,189-191] associating aflatoxin and liver cancer in the general population, we assessed publication or other forms of selection bias by a funnel plot (Figure 8) and associated statistical tests of funnel plot asymmetry [194]. Seven studies are not included in this plot; five studied the association in HBsAg+ individuals only, and two are duplicate studies included in meta-analysis for different data extraction purposes, as explained in the Methods. The funnel plot provides little evidence of an important departure from symmetry, indicating that publication or other forms of selection bias were not a serious limitation in our

meta-analysis. This visual impression of symmetry was corroborated by the statistical tests of funnel plot asymmetry.

Table 13. Summary of combined odds ratios in the meta-analysis

Risk factor	Study Population	Study area (n of studies)	Cases/controls[vii]	Odds Ratio, 95% CI	Model	Heterogeneity
Aflatoxin only	General population with HBsAg+ adjustment	China (4) [6,56,179,189]	634 cases/913 controls	5.99 (3.70-9.69)	Fixed	Q=4.86, P=0.18, I^2=38.32
		Taiwan (3)[viii] [7,170,186]	198 cases/904 controls	2.01 (1.40-2.89)	Fixed	Q=3.19, P=0.20, I^2=37.29
		Sub-Saharan Africa (2) [190,191]	168 cases/252 controls	4.62 (2.12-10.08)	Fixed	Q=2·69, P=0·1, I^2=62·82
		Summary (9)	1000 cases/2069 controls	4.75 (2.78-8.11)	Random	Q=32.73, P<0·000, I^2=75.56
	General population with HBsAg+ adjustment after adjust heterogeneity	Summary (8) [5-8,56,179,186,189]	840 cases/1302 controls	5.72 (4.42-7.40)	Fixed	Q=8.40, P=0.30, I^2=16.66
	General population with HBsAg+ adjustment by only including Wu et al as follow-up for cohort in Taiwan	Summary (7) [5,6,8,56,170,179,189]	1000 cases/2069 controls	4.88 (2.62-9.10)	Random	Q=32.55, P<0.000, I^2=81.57
	General population with HBsAg+ adjustment by taking the average effect of the series of Taiwan Studies[ix]	Summary (7)	1000 cases/2069 controls	4.92 (2.74-8.82)	Random	Q=29.48, P<0.000, I^2=79.65
	HBsAg+ individuals	China (3) [6,58,193]	189 cases/268 controls	2.00 (0.84-4.75)	Random	Q=10·66, P=0·005, I^2=81·24
		Taiwan (6) [7,57,170,187,188,192]	254 cases/310 controls	1.81(1.29-2.56)	Fixed	Q=8.38, P=0.14, I^2=40.35
		Sub-Saharan Africa (2) [5,8]	128 cases/56 controls	6.48 (0.22-194)	Random	Q=6.54, P=0.01, I^2=84.71
		Summary (11)[x]	571 cases/634 controls	2.39 (1.50-3.82)	Random	Q=27.99, P=0.002, I^2=64.27

[vii] If there was a series of follow-up studies in the same cohort need to be combined, only the numbers of cases and controls from the largest follow-up study were counted, although different odds ratios from different follow-up studies were combined to assess the effect. All the cases and controls were only counted once, and as well as in calculations presented in Table 4 and 5.

[viii] This row shows the summary odds ratio of combing three follow-up studies in a Taiwan cohort in different years

[ix] The summary odds ratio obtained for the Taiwan cohort was used to represent the effect of all studies in this cohorts, and combine with other studies

[x] Seven studies (7, 15, 17, 21-22, 25-26) reported adjusted ORs on aflatoxin-related HCC risk in HBsAg+ individuals. Four studies (5-6, 8, 16) (including two studies conducted in Sub-Saharan Africa countries) did not provide adjusted ORs directly, but provided data to calculate the unadjusted ORs. We calculated the unadjusted ORs for each of these studies and combined them with ORs from other studies with eligible data, thus we can include the effects of studies in Sub-Saharan Africa population. In subgroup analysis, the large variation of summarized ORs of aflatoxin-related HCC in HBsAg+ individuals may be explained by combining the unadjusted ORs. The heterogeneity was significant when studies were combined to examine the association between aflatoxin exposure and HCC risk in the general population and in HBsAg+ individuals.

Table 13 continued

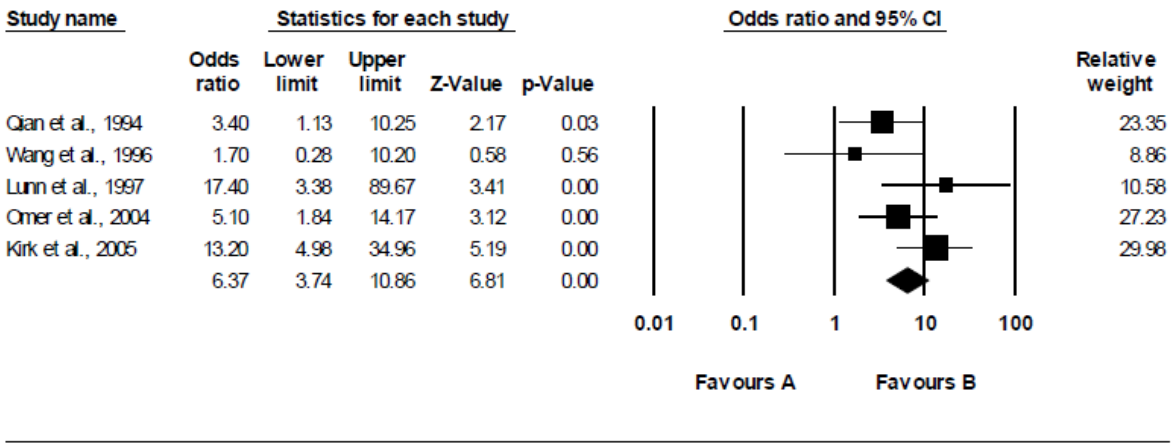
	HBsAg+ individuals after adjust heterogeneity	Summary (9) [5-8,57,187,188,192,193]	377 cases/383 controls	2.90 (2.09-4.01)	Fixed	Q=11.16, P=0.19, I^2=28.29
	HBsAg+ individuals by only including most recent follow-up studies in a cohort of Taiwan	Summary (8) [5,6,8,57,58,170,188,193]	571 cases/634 controls	2.27 (1.24-4.14)	Random	Q=24.33, P=0.001, I^2=71.23
	HBsAg+ individuals by only combing studies with adjusted ORs	Summary	332 cases/538 controls	2.10 (1.25-3.52)	Random	Q=16.40, P=0.012, I^2=63.42
	HBsAg+ individuals by taking the average effect of all follow-up studies in the same cohort [xi]	Summary (8)	571 cases/634 controls	2.35(1.38-3.99)	Random	Q=23·17, P=0·002, I^2=69.79
	HBsAg- individuals	China (1) [6]	18 cases/ 236 controls	3·4 (1·13-10·25)	/	/
		Taiwan (3) [7,57,170]	81 cases/664 controls	5·00 (2·22-11·28)	Fixed	Q=3·69, P=0·16, I^2=45·79
		Sub-Saharan Africa (2) [5,8]	122 cases/391 controls	8·40 (4·15-16·99)	Fixed	Q=8·40, P=0·19, I^2=42·63
		Summary (6)	221 cases/1291 controls	5·91 (3·66-9·55)	Fixed	Q=7·51, P=0·19, I^2=33·42
	HBsAg-individuals excluding Wu et al [170]	Summary (5)	172 cases/769 controls	6·37 (3·74-10·86)	Fixed	Q=7·11, P=0·13, I^2=43·71
HBV only	General population	Summary (6) [5-8,57,170]	244 cases/1072 controls	11·2 (7·48-16·7)	Fixed	Q=2·37, P=0·80, I^2=0·00
	General population after adjusted heterogeneity	Summary (5) [5-8,57]	171 cases/638 controls	11·3 (6·75-18·9)	Fixed	Q=2·36, p=0·67, I^2=0·00
Aflatoxin and HBV infection combined effects	General population	Summary (6) [5-8,57,170]	554 cases/1456 controls	54·1 (21·3-137·7)	Random	Q=13·65, p=0·02, I^2=63·36
	General population after adjust heterogeneity	Summary (5) [5-8,57]	452 cases/847 controls	73·0 (36·0-148·3)	Fixed	Q=3·48, p=0·48, I^2=0·00

[xi] The summary odds ratio obtained from different follow-up studies for the Taiwan cohort was used to represent the effect of all studies in this cohorts, and combine with other studies

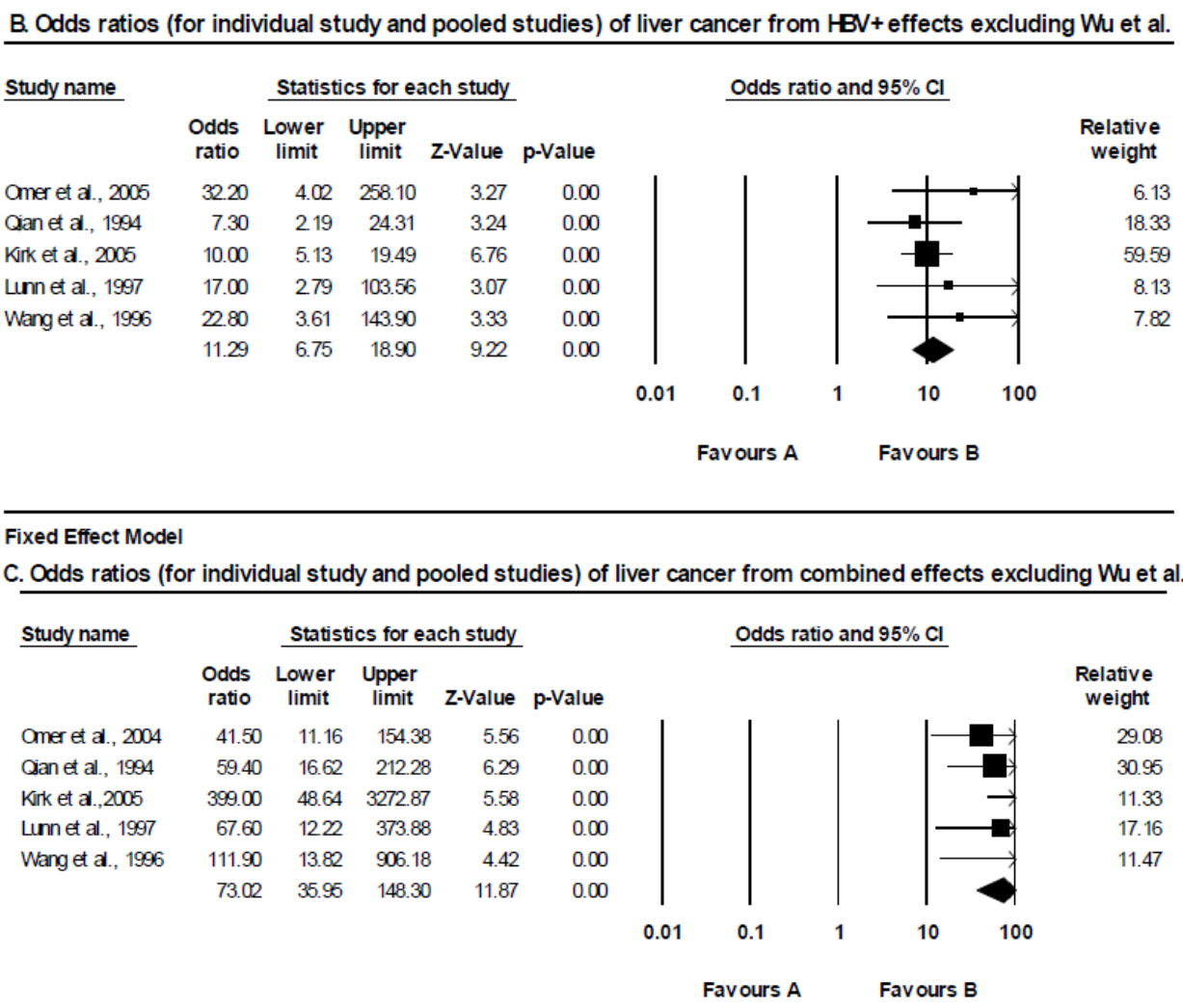
4.2.6 MULTIPLICATIVE MODEL OF EFFECTS BETWEEN AFLATOXIN EXPOSURE AND CHRONIC HBV INFECTION

The meta-analysis allowed us to quantitatively evaluate the model of effects between the two risk factors. The summary OR of six studies [5-8,57,170] reporting ORs of HCC risk from combination effects of aflatoxin exposure and HBV infection is 54.1 (21.3-137.7) with significant heterogeneity (Q=13.65, P=0.02, I^2=63.36). The summary OR of the same batch of studies on HCC risk from aflatoxin exposure only is 5.91 (3.66-9.55), while the summary OR on HCC risk from chronic HBV infection only is 11.2 (7.48-16.7), both with no significant heterogeneity. If we excluded Wu et al [170] which contributes to the heterogeneity, the summary OR for combined effects increased to 73.0 (36.0-148.3), 6.37 (3.74-10.86) for aflatoxin exposure only and 11.3 (6.75-18.9) for chronic HBV infection only (Figure 7). These numbers indicated an almost perfect multiplicative model of effects between aflatoxin exposure and chronic HBV infection in inducing HCC risk.

A. Odds ratios (for individual study and pooled studies) of liver cancer from aflatoxin exposure excluding Wu et al.



Fixed Effect Model



A. Forest plot of combined ORs with 95% CI for association between liver cancer and chronic HBV+ only, excluding Wu et al [170]; B. Forest plot of combined ORs with 95% CI for association between liver cancer and aflatoxin exposure only, excluding Wu et al [170]; C. Forest plot of combined ORs with 95% CI for association between liver cancer and the combination effects of two risk factors, excluding Wu et al [170]

Figure 7. Forest plot of combined ORs for association between liver cancer and two risk factors

4.2.7 POPULATION ATTRIBUTABLE RISK OF HCC FROM AFLATOXIN EXPOSURE IN EACH STUDY POPULATION

The PAR of aflatoxin-related HCC was calculated for each study population (Table 14). PAR is the proportion of the HCC cases that could be prevented by reducing aflatoxin exposures to "control" levels in each study. For example, HCC in the Chen et al [186] Taiwanese study population could be reduced by about 10% (2.5-12%) if dietary aflatoxin exposures in this population were reduced such that aflatoxin-albumin adduct levels were below 0.01 fmol/μg (detection limit in this study), or if dietary aflatoxin exposures could be decreased to below 4.3 ng/kg bw/day (biomarker detection limit extrapolated to dietary exposure). HCC in the study population of Shanghai males in Qian et al. [6] could be reduced by about 9.0% (5.9-10.4%) if aflatoxin exposures in this population were reduced to below 6 ng/kg bw/day: the average aflatoxin exposure level in the control group. Our results showed that the PAR of HCC caused by aflatoxin is higher in HBV+ populations than in HBV- populations.

In HBV+ populations in a Taiwanese cohort, the PAR for aflatoxin-related HCC is consistently decreasing, as indicated by a series of follow-up studies: 31% in 1980s [7], 12% in 1990s [192], and 3% in 2000s [170]. Overall, the PAR of aflatoxin-related HCC is decreasing in Taiwan in both HBV+ and HBV- individuals, from as high as 44% in 1990s [57] to 2% in 2000s[170].

The PAR of aflatoxin-induced liver cancer is changing over time, and varies by geographic regions; which may reflect differences in HBV prevalence, aflatoxin exposure across geographic regions, and changes in limits of detection. For example, the varying PAR estimates from six Chinese studies, Qian et al., Zhang et al., Sun et al, Huang et al, Long et al and

Szymanska et al [6,56,58,179,189,193] can be explained at least in part by these factors. These studies covered four different geographical areas of China: Shanghai, Henan, Qidong, and Guangxi. The Shanghai study indicated an 11% prevalence of detectable aflatoxin metabolites in urine samples, compared to 35% prevalence detected in the Guangxi study. This may reflect higher aflatoxin exposures in rural populations compared with the population of Shanghai. Correspondingly, the PAR of HCC from aflatoxin in Shanghai area is about 10%, while it is about 26% in Guangxi (Fusui area). In HBsAg+ individuals, the PAR of aflatoxin exposure for liver cancer risk is highest at 57% (16%-72%) in Qidong in Ming et al., 2002 [193], where there are some of the highest aflatoxin exposures in China [172]. In contrast, the PAR of aflatoxin-related HCC was nearly 0% as estimated from Szymanska et al 2009 [58]; possibly reflecting substantially reduced aflatoxin exposure from changing food patterns in the local population.

In the 1990s, the PARs calculated from studies utilized urinary aflatoxin metabolites or albumin adducts (three studies) with fairly consistent results ([6,7,195]. The PAR is about 10% in the general study populations adjusted by HBV status, 30-40% in HBV+ populations, and less than 5% in HBV- populations. In the early 2000s, the PARs of HCC from aflatoxin ranged from 0 to 63% in HBsAg+ groups, 2-20% in HBsAg- groups, and 2%-26% in general population. In two sub-Saharan African countries, aflatoxin exposure could contribute up to more than 60% of population risk of liver cancer in HBsAg+ individuals, but less than 20% in HBsAg- individuals [5,8,190,191].

Table 14. Population attributable risk of liver cancer caused by aflatoxin exposure in HBV+ populations, HBV- populations, and the general population

Studies	Exposure measurement	PAR for aflatoxin attributable HCC risk in HBsAg+	PAR for aflatoxin attributable HCC risk in HBsAg-	PAR for aflatoxin attributable HCC risk in general study population adjusted by HBsAg+
Qian et al., 1994 [6] (Shanghai, China)	Multiple urinary aflatoxin metabolites	40% (24% - 47%)[1]	3.6% (0.3% - 5.6%)	9.0% (5.9% -10.4%)
Chen et al., 1996 [186] (Taiwan)	AFB_1 albumin adducts	n/a	n/a	10% (2.5% - 12%)
Chen et al., 1996 [187] (Taiwan)	AFB_1 albumin adducts Low vs undetectable	4.2% (0-13%)	n/a HBV individuals only	n/a HBV individuals only
	AFB_1 albumin adducts High vs undetectable	4.5% (0-11%)	n/a HBV individuals only	n/a HBV individuals only
		Sum = 8.7% (0-24%)	n/a HBV individuals only	n/a HBV individuals only
Wang et al., 1996 [7] (Taiwan)	AFB_1 albumin adducts	31% (0-51%)	0 (0-2.3%)	5% (0-11%)
	Urinary aflatoxin metabolites	41% (8.1%-54%)	1% (0- 4.1%)	11% (1.4% - 13.7%)
Lunn et al., 1997 [57] (Taiwan)	AFB_1-DNA adduct	31% (0-75%)[2]	44% (29%-47%)	n/a
Yu et al., 1997[188] (Taiwan)	1.61-2.85 ng/ml AFM_1 vs non-detectable)	2.1% (0%-7.2%)	n/a HBV individuals only	n/a HBV individuals only
	> 2.85 ng/ml AFM_1 vs non-detectable)	19% (2.2%-25%)	n/a HBV individuals only	n/a HBV individuals only
		Sum = 21% (2.2% - 32%)	n/a HBV individuals only	n/a HBV individuals only
Zhang et al., 1997 [189] (Henan, China)	Corn consumption	n/a	n/a	17.5% (8%-18.4%)
	Peanut consumption	n/a	n/a	36% (16% - 45%)
Kirk et al., 2000 [190] (The Gambia)	Ser 249 TP53 mutation	n/a	n/a	17% (12%-18%)

Table 14 continued

Omer et al., 2001 [191] (Sudan)	Average peanut butter consumption	n/a	n/a	23% (11%-29%)
Sun et al., 2001 (Taiwan) [192]	AFB_1 albumin adducts	12% (1.7% - 20%)	n/a HBV individuals only	n/a HBV individuals only
Ming et al., 2002 [193] (Qidong, China)	AFM_1	57% (16%-72%)[3]	n/a	n/a
Huang et al. 2003[56] (Qidong, China)	Ser 249 TP53 mutation	n/a	n/a	17% (13%-18%)
Omer et al., 2004 [8](Sudan)	Average peanut butter consumption	5.4% (0-62%)[4]	20% (9.0%-25%)	n/a
Kirk et al., 2005 [5] (The Gambia)	Ser 249 TP53 mutation	63% (39%-67%)[5]	12% (6.3% -17%)	13% (12%-14%)[6]
Wu et al., 2009 [170] (Taiwan)	AFB_1 albumin adducts	3.7% (0-11%)	1.7% (0-4.6%)	2.1% (0.06%-3.4%)
	urinary aflatoxin metabolites	3.1% (0-11.7%)	4.7% (1.2%-6.7%)	4.4% (1.6%-6.3%)
Szymanska et al., 2009 [58] (Qidong, China)	AFB_1 albumin adducts	0 (0-14%)	n/a HBV individuals only	n/a HBV individuals only
Long et al., 2009[179] (Guangxi, China)	AFB_1-DNA adduct medium vs low	n/a	n/a	6.8% (4.5%-8.5%)
	AFB_1-DNA adduct high vs low	n/a	n/a	19% (17%-20%)
	Total	n/a	n/a	26% (22%-29%)

([1, 2, 4, 5] calculated from unadjusted ORs, [3] author estimated, [6] calculated from ORs unadjusted by HBsAg+)

4.2.8 POPULATION ATTRIBUTABLE RISK FRACTION OF AFLATOXIN-RELATED HCC IN COMBINED STUDIES

We combined the total number of exposed cases, the total number of HBsAg+ or HBsAg- individuals, and the total number of controls from all the eligible studies to calculate the PAR of liver cancer from aflatoxin by using the combined ORs in: 1) the general population adjusted for HBV status, 2) HBV+ populations, and 3) HBV- populations. The results are presented in Tables 15 and 16.

We combined all aflatoxin-exposed cases, HBV+ and HBV- individuals, and controls from all eligible studies to calculate the PAR of aflatoxin-related HCC by HBsAg status and world region (Tables 15, 16). The PAR of aflatoxin-related HCC in the general population after HBV adjustment is 17% (14-19%). Because the earlier sensitivity analysis demonstrated that the remaining studies after exclusion of Wu et al. [170] do not have statistically significant heterogeneity, we also calculated the PAR of aflatoxin-related HCC after exclusion of (30). The PAR increased to 23% (21-24%).

The PAR of aflatoxin-related HCC in the HBV+ population is 21% (10-29%). A separate calculation was performed excluding Szymanska et al [58] and Wu et al [170], the most influential studies indicated by the sensitivity analysis. The new PAR of aflatoxin-related HCC in the HBV+ population was 25% (18-30%). The PAR of aflatoxin-related HCC in HBV- populations is 8.8% (6.7-10%).

In subgroup, the aflatoxin exposure is a more important risk factor of liver cancer in China and Sub-Saharan African countries, compared to it is in Taiwan. The aflatoxin was

estimated to attribute 25% (95%CI: 22%-27% of liver cancer cases in general population of China, and 19% (95% CI: 13%-22%) in Sub-Saharan Africa. In Taiwan, the number was 5.2% (2.9%-6.7%).

Table 15. Estimated population attributable HCC risk from aflatoxin exposure in the general population by combining the eligible studies

Study population		Total exposed cases (n_1)	Total sample size (n_2)	P_c (n_1/n_2)	Summarized OR (95% CI)	PAR (95% CI)
General population adjusted by HBV status [6,7,56,170,179,186,189-191]	China	475	1588	0.299	5.99 (3.70-9.69)	25% (22%-27%)
	Taiwan	113	1102	0.103	2.01 (1.40-2.89)	5.2% (2·9%-6.7%)
	Sub-Saharan Africa	82	340	0.241	4.62 (2.12-10.08)	19% (13%-22%)
	Summary	670	3030	0.221	4·75 (2·78-8.11)	17% (14%-19%)
General population adjusted by HBV status after excluding Wu et al. 2009[170]	Summary	583	2103	0.277	5.72(4.42-7.40)	23% (21%-24%)

Table 16. Estimated population attributable HCC risk from aflatoxin exposure in HBV+ and HBV- populations by combining the eligible studies

Study population		Total HBsAg+ (or HBsAg-) (n_1)	Total HCC cases in HBsAg+ (or HBsAg-) (n_2)	Total exposed HBsAg+ (or HBsAg-) (n_3)	Proportion of HCC cases in HBsAg+ (or HBsAg-) (W_1)	Proportion of exposed HBsAg+ (or HBsAg-) (P_1)	Summarized OR (95%CI)	PAR (95% CI)
HBV+ population [5-8,57,58,170,187,188,192,193]	China	457	189	276	0.414	0.604	2.00(0.84-4.75)	16% (0-29%)
	Taiwan	564	254	314	0.450	0.557	1.81(1.29-2.56)	14% (6.3%-21%)
	Sub-Saharan Africa	184	128	76	0.696	0.413	6.48(0.22-194)	48% (0-69%)
	Summary[xii]	1205	571	666	0.473	0.553	2.39 (1.50-3.82)	21% (10%-29%)
HBV+ population after excluding Szymanska et al. 2009[58] and Wu et al[170]	China	208	63	108	0.303	0.519	3.01 (1.86-4.88)	16% (9%-20%)
	Taiwan	368	186	216	0.505	0.587	2.59 (1.63-4.13)	24% (14%-33%)
	Summary	760	377	400	0.496	0.526	2.90 (2.09-4.01)	25% (18%-30%)
HBV- population [5-8,57,170]	Taiwan	745	81	332	0.109	0.446	5.00 (2.22-11.28)	7% (3.8%-8·9%)
	Sub-Saharan Africa	513	122	113	0.238	0.220	8.40 (4.15 - 16.99)	15% (9.7% - 19%)
	Summary	1632	227	617	0.139	0.353	5.91 (3.66-9.55)	8.8% (6.7%-10%)

[xii] Studies (including two studies in Sub-Saharan Africa countries) with unadjusted ORs were also combined to calculate the overall PAR, thus the Sub-Saharan study population can be included.

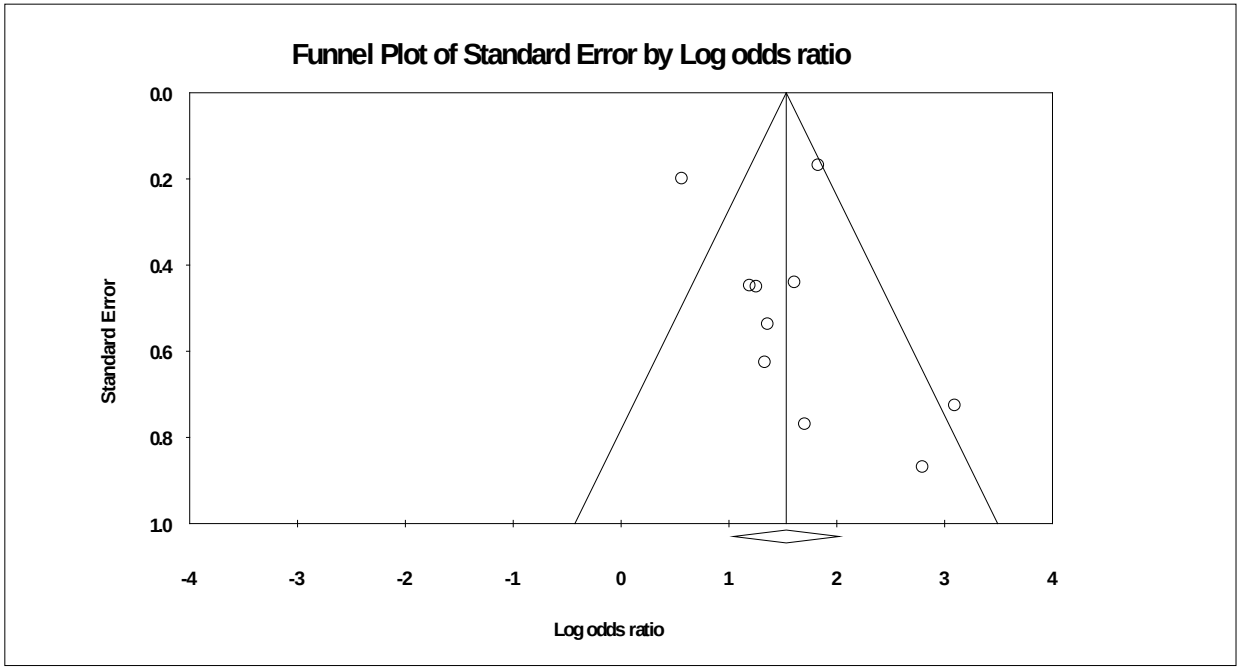


Figure 8. Funnel plot to assess possible publication or other selection bias for the association between aflatoxin exposure and liver cancer risk in general population.

No statistically significant asymmetry was found**.** Each circle represents 1 study. 10 studies [6,7,56,57,170,179,186,189-191] are eligible for this plot. 7 studies not included (5 only studied the association in HBsAg+ individuals, and 2 are duplicate studies included in meta-analysis for different data extraction purpose, as explained in the Methods section).

4.3 DISCUSSION

Aflatoxin exposure is significantly associated with HCC risk regardless of HBV status. Our meta-analyses show that in areas of high aflatoxin exposure and chronic HBV infection, aflatoxin exposure and HBV have a nearly perfectly multiplicative relationship in increasing HCC risk. In populations including both HBV+ and HBV- individuals in the geographic regions studied, the PAR of aflatoxin-related HCC was estimated at 17% (14-19%). This implies that if it were possible to reduce aflatoxin to below detectable limits in these regions, HCC incidence could be reduced by 14-19%. There are roughly 520,000 new HCC cases in China, southeastern Asia and sub-Saharan Africa each year [34]. If the PARs are generalized to these areas, the implication is that, by reducing aflatoxin in humans diets to below detectable levels, 72,800 to 98,800 new HCC cases could be prevented every year. If this PAR were generalized to regions of the world beyond Africa and Asia, the overall number of HCC cases (749,000 new cases per year (32)) that could be prevented by aflatoxin control would reach 105,000-142,000.

The PAR of aflatoxin-related HCC increases to 23% (21-24%), and heterogeneity amongst the studies decreases significantly, if one study [170] is excluded from the meta-analysis. However, this study is important because it suggests that aflatoxin exposure is decreasing over time in the Taiwanese (Penghu) population studied. Our PAR estimates for individual studies showed a decrease in PAR of aflatoxin-related HCC in the Penghu cohort in the last three decades. It is worth noting that in a 1970s food survey, over one-third of peanuts in Penghu were heavily contaminated by aflatoxins, with an average aflatoxin content of 167 μg/kg [196]. Mean

urinary aflatoxin in HCC patients in this cohort from was 219 µg/ml in 1991/1992 [7,186], and decreased to 0.017 µg/ml in HCC patients in the same cohort in 2004 [170]. Also, the HBV vaccination program in Taiwan has successfully reduced HBV prevalence, further reducing HCC risk [197].

In some parts of the world such as Taiwan, aflatoxin exposure is decreasing. In other parts of the world such as Africa, rural China, and Southeast Asia, there is little evidence that aflatoxin exposure is decreasing; in fact, two recent Kenyan events of extremely high aflatoxin levels in maize (in 2004-2005, and again in 2010) suggest the opposite. With climate change, aflatoxin contamination in food crops may become exacerbated due to conditions favoring proliferation of Aspergilli (35). Hence, further efforts to reduce aflatoxin-related disease are needed in high-risk areas of the world.

Aflatoxin exposure can be measured by dietary questionnaires, direct measurements in foodstuffs, or biomarkers. In the early years of investigating the association between aflatoxin and HCC development, measurement of aflatoxin exposure had several limitations. Dietary questionnaires are inadequate to measure the aflatoxin intake because the content of aflatoxin in individual foods, or even individual kernels of foods, can vary widely [198]; and recall bias of subject participants may further reduce accuracy of questionnaire methods.

In more recent studies, biomarkers of aflatoxin exposure and effect have become useful for more accurately determine the relevant exposures to aflatoxin that result in human disease.

The biomarkers AFB_1-N^7-guanine, AFB_1-albumin adduct, AFB_1-DNA adducts, and the TP53 249^{ser} mutations are all biomarkers of biological effects of aflatoxin [17]. Therefore, the P_c which is calculated as prevalence of aflatoxin exposure in population should be more properly interpreted as the prevalence of biological effects that are caused by aflatoxin in the study

population. This is not always directly correlated with actual aflatoxin exposure in the diet, due to individual differences in aflatoxin metabolism.

There are several limitations in this analysis. First, the epidemiological studies included were conducted in areas of the world with both high aflatoxin and HBV (Asia and sub-Saharan Africa). Thus, although these regions account for most of the aflatoxin-induced HCC cases worldwide (13), the estimated PAR is not necessarily applicable in areas with much lower aflatoxin exposures. Second, odds ratios from studies employing food surveys, exposure biomarkers and biological effect biomarkers were combined. This decreases the precision of the analysis, as different biomarkers have different detection limits and measure different endpoints, and food surveys are less precise than biomarkers for exposure estimation. Third, the PAR is meant to represent the proportion by which disease could be reduced if the risk factor in question were removed. It is not possible to instantaneously reduce aflatoxin to below detectable limits worldwide – rather, the PAR calculated is meant to estimate the burden of HCC caused by one risk factor (aflatoxin), and to project the extent to which the problem could be reduced in future generations if aflatoxin control strategies were widespread.

In summary, this study is the first to quantitatively evaluate the model of effects between aflatoxin and HBV in inducing liver cancer by combining results from multiple epidemiological studies. The range of PARs calculated in this analysis, 14-19% (21-24% excluding one study contributing to heterogeneity), is consistent with our previous report of 5-28% using a different methodology (quantitative cancer risk assessment) [180]. The PAR of aflatoxin-related HCC is higher in HBsAg+ populations than HBsAg- populations. In recent years, the PAR of aflatoxin-related HCC has shown a decreasing trend in areas such as Taiwan, indicating the benefits of reduced aflatoxin exposure and HBV prevalence by public health interventions.

5.0 CONCLUSIONS AND PUBLIC HEALTH SIGNIFICANCE

We adopted two methods to estimate the aflatoxin-attributable liver cancer risk in multiple countries/regions: 1) A risk assessment relying on food consumption and food contamination data to measure the exposure, and 2) a meta-analysis to pool the ORs from studies mainly relying on biomarker data to quantify the association. The overall range of PARs calculated from the two methods, 21-24% by meta-analysis excluding one study contributing to heterogeneity, and 5-28% using a quantitative cancer risk assessment[180], are consistent with each other (Table 17). They both presented a higher PAR in HBsAg+ individuals than in HBsAg- individuals. But the PAR calculated from the meta- analysis particularly reflected the disease burden in areas with high aflatoxin exposure, because the meta-analysis was dependent on the studies in these areas – China, Taiwan and Sub-Saharan Africa. Also, the estimates calculated by employing the meta-analysis has much narrower ranges compared to the ones from risk assessment, indicated the reduced uncertainties in exposure assessment, the dose-response relationship and the model of interaction effects between two risk factors (aflatoxin exposure and chronic HBV).

Table 17. Comparing PARs of liver cancer from aflatoxin exposure calculated by two methods

	Estimates from risk assessment model (Global burden)	Estimates from meta-analysis (Burden of areas with high aflatoxin exposure)
HBsAg-	5%-26%	8.8% (6.7% - 10%)
HBsAg+	11%-74%	25% (18%-30%)
Overall	5%-28%	23% (21%-24%)

While two analysis methods both aimed to address a significant public health question, each method has its own advantages and limitations. The wide range of risk estimates computed from risk assessment reflects the limitations in determining levels of aflatoxin exposure solely by food consumption and aflatoxin contamination data; however, food studies were best available data and easy-to-obtain sources, to help determining and communicating risk in vast majority of the countries of the world, and thus possible to conduct a disease burden assessment at global level. The assumptions of a uniformly multiplicative model of combined effects, and the incomplete data on the chronic HBV infection prevalence in different regions, also hindered the accuracy when the risk assessment results were extrapolated to specific countries or regions. From this point of view, the location-specific rates were employed to get the estimates for particular geographic areas.

Compared to risk assessment which relies on food data to measure aflatoxin exposure, the quantification of association between exposure and liver cancer risk is very much improved by pooling the ORs from studies using biomarker data to measure the exposure. The systematic approach is also helpful to get rid of random error and give reliable estimates on the strength of the association. However, there is limited number of eligible studies with accessible data. The

current meta-analysis is based upon studies from China, Taiwan, and two Sub-Saharan African countries. In addition, the meta-analysis is hindered by the heterogeneity and quality of studies.

This study answered an important public health question - the global burden of aflatoxin-attributable liver cancer cases. From our analysis, Aflatoxin may play a causative role in 4.6-28.2% of all global HCC cases. 25,200-155,000 HCC cases worldwide may be attributable to aflatoxin exposure. Most cases occur in sub-Saharan Africa, Southeast Asia, and China, where populations suffer from both high HBV prevalence and largely uncontrolled dietary aflatoxin. In these areas, the PAR of aflatoxin-related HCC is 23% (21-24%) overall, and is higher in HBV+ individuals (18-30%) than in HBV- individuals (6.7%-10%). After adjustment for heterogeneity, the combined OR of HCC from combination effects of aflatoxin and HBV+ is 73.0 (36.0-148.3), 11.3 (6.75-18.9) from HBV+ only and 6.37 (3.74-10.86) from aflatoxin exposure only, indicating an almost perfectly multiplicative model of effects between aflatoxin exposure and HBV+. HBV vaccination should be a very effective intervention strategy to reduce the cancer burden attributable to aflatoxin, and has demonstrated the impressive benefit, both in Fusui County, China and Taiwan.

Overall, the risk assessment model and meta-analysis are both useful tools to estimate the general HCC burden attributable to aflatoxin exposure. Together they not only help to provide policy makers and scientists straightforward information for risk communication and decision making, but also are marked as translational toxicological efforts from molecular mechanisms to global public health.

ABBREVIATIONS

A. flavus	= *Aspergillus flavus*
A. parasiticus	= *Aspergillus parasiticus*
AFB_1	= aflatoxin B_1
AFM_1	= Aflatoxin M_1
FAO	= Food and Agriculture Organization
FDA	= United States Food and Drug Administration
GEMS	= Global Environment Monitoring System
HBsAg	= Hepatitis B virus surface antigen
HBV	= hepatitis B virus
HCC	= hepatocellular carcinoma
HCV	= Hepatitis C virus
IARC	= International Agency for Research on Cancer
JECFA	= Joint Food and Agriculture Organization / World Health Organization Expert Committee on Food Additives
OR	= Odds ratio
PAR	= Population attributable risk
RR	= Relative risk
WHO	= World Health Organization

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