PEANUT BUTTER CONSUMPTION AND HEPATOCELLULAR CARCINOMA IN SUDAN

Ragaa El Hadi Omer

Promotor:	Prof.dr.ir. F.J. Kok Wageningen Universiteit
Co-promotor:	Dr.ir. P. van 't Veer Wageningen Universiteit
Samenstelling Promotiecommissie:	Prof.dr. J.H. Koeman Wageningen Universiteit
	Prof.dr.ir. F.E. van Leeuwen Vrije Universiteit Amsterdam
	Prof.dr. C.E. West Katholieke Universiteit Nijmegen Wageningen Universiteit
	Prof.dr. J. Jansen Katholieke Universiteit Nijmegen

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To the spirit of
my lovely father

Abstract

PEANUT BUTTER CONSUMPTION AND HEPATOCELLULAR CARCINOMA IN SUDAN

Ph.D. thesis by Ragaa El Hadi Omer, Division of Human Nutrition and Epidemiology, Wageningen University, Wageningen, The Netherlands.
12th March 2001.

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world with 80% of cases occurring in developing countries in sub-Saharan regions in Africa, South-East Asia and China. The cancer is highly fatal and survival is generally less than 1 year from diagnosis. Clinical records suggest that the incidence of this cancer is high and increasing in Sudan. Major risk factors of HCC have been identified as dietary exposure to aflatoxins, chronic infection with hepatitis B virus (HBV) and other hepatitis viruses and the intake of alcoholic beverages. Climatic conditions, agricultural practices and the dietary patterns in Sudan, as well as in many of the sub-Saharan African countries, are contributing to the contamination of food with aflatoxins and possibly to the incidence of HCC.

The aim of this study was to investigate the role of aflatoxins from peanut butter in the etiology of HCC in Sudan, and to substantiate this by studying polymorphisms of potential relevant genes for aflatoxin metabolism. The ultimate goal of this study was to find clues for prevention and control of HCC in Sudan, by investigating the relative importance of aflatoxins (peanut butter intake) and hepatitis infections. Accordingly, implications for agricultural and public health policy can be substantiated.

First, an explorative study was conducted among 24 cases and 34 controls. This study confirmed that high amounts of aflatoxins (20 times above the guidelines of the World Health Organization) are present in Sudanese peanut butter and suggested that humid storage conditions might contribute to the risk of HCC. Furthermore, procedures for the main study were tested and adapted.

In the main study 150 cases with HCC and 200 controls, matched for sex, were enrolled from West and Central Sudan. In this study, an about four-fold increased risk of HCC was observed among subjects with a high daily consumption of peanut butter. This association was considerably stronger for subjects having genetic polymorphisms of glutathione S-transferase M1 (GSTM1). For the polymorphisms in glutathione S-transferase T1 (GSTT1) and microsomal epoxide hydrolase (EPHX), no association was observed. This might indicate that GSTM1 is a more important rate-limiting factor in the metabolism of aflatoxins. In addition, we observed a positive association between the intake of peanut butter stored in humid storage conditions although less clear than it was in the explorative study. Regarding hepatitis infections, a fifteen-fold increased risk was found for those who had experienced HBV infections.

The results of our study point at both exposure to aflatoxins through peanut butter intake and hepatitis B infection as important risk factors of HCC in Sudan. Although hepatitis B infection was a much stronger risk factor as compared to aflatoxins/peanut butter, the latter was almost equally important to public health because of the high prevalence of aflatoxin contamination. About fifty percent of all cases of HCC might be attributable to contamination of peanut butter.

In order to reduce HCC in Sudan, pre and post harvest measures in agricultural policy need to be reinforced. At the community level, specific attention should be given to selling and processing of peanuts and their products at local markets. In addition, immunization of infants and high-risk groups against HBV infections has to be considered as a short-term strategy. Finally, future research would benefit from improved hospital and population registries.

Propositions

1

Reducing the aflatoxin content of peanuts to permissible levels might halve the number of cases of hepatocellular carcinoma among the Sudanese population (*this thesis).*

2

Consumption of peanut butter contaminated with aflatoxins is an important cause of hepatocellular carcinoma in Sudan, especially among subjects with the GSTM1 null genotype, *i.e.* those who are unable to detoxify the aflatoxins properly (*this thesis*).

3

Because of the biological interaction with aflatoxins, subjects with acquired susceptibility (e.g., hepatitis B infection) and subjects with genetic susceptibility may benefit most from aflatoxin reducing strategies.

4

Genetic susceptibility will often be an important source of individual variation in risk within populations, but less so between populations.

5

Epidemiological research in developing countries is crucial to implement sensible public health measures adapted to the local circumstances.

6

There is nothing like returning to a place that remains unchanged to find the ways in which you yourself have altered (*Nelson Mandela, A long walk to freedom*).

7

"Say: If the ocean were ink (wherewith to write out) the words of my Lord, sooner would the ocean be exhausted than would be the words of my Lord, even if we added another ocean like it, for its aid." (*Soera 18, ayia 109, Holy Koran*).

Propositions belonging to the thesis
"Peanut butter consumption and hepatocellular carcinoma in Sudan"

Ragaa El Hadi Omer
Wageningen, 12 March 2001

Contents

1

Introduction

Aflatoxins

Historical background

The possibility that contamination of dietary staples by aflatoxins is one of the etiologic factors for hepatotoxic disease, has been suggested by many authors soon after the outbreak of Turkey "X" disease and it was put forward that a fungal metabolite might be involved. Aflatoxins were detected for the first time in Brazilian groundnuts, which caused the death of about 100,000 turkeys in Britain during the year 1960[1,2].

However a number of less well-described incidents in humans and different animal species had preceded this outbreak. In the mid-1940s, a diet for laboratory animals that contained peanuts[3] was associated with a non-infectious disease in guinea pigs and rats; this disease was characterized by edema, ascites, and hepatic injury and ultimately by liver cancer in rats[4,5]. During the period 1945-1953, an outbreak of a non-infectious, hepatotoxic disease in swine, cattle and dogs was encountered in the southeastern United States; the swine and cattle disease was associated with moldy feeds. In all documented outbreaks in dogs, the commercially prepared feed contained peanut meal as a source of dietary protein. Later studies using diets from the same source produced similar symptoms and lesions in dogs; the peanut meal in these latter studies was found to be contaminated with aflatoxins[6].

In humans there have been numerous outbreaks that tended to be endemic in certain areas throughout years. In spite of limited clinical aflatoxicosis in humans, there is ample evidence for substantial exposure of populations in many areas of the world[7]. For example, in the Ukraine, the use of over-wintered wheat for bread making resulted in an epidemic of a fatal disease known at that time as alimentary toxic aleukia[8]. The event was attributed to certain fungal toxins ingested with food, but the toxin and its dose could not be specified. In children in Thailand a disease was identified with symptoms of Reye's syndrome[9,10] characterized by vomiting, and fat infiltration in the liver, kidney and heart. As the symptoms of Reye's syndrome are approximately similar to those observed with acute aflatoxicosis in monkeys[11] aflatoxin poisoning was proposed as another possible explanation for the Thailand's children disease.

Thus aflatoxin-related diseases have been described in animals and humans in the decades before the dramatic outbreak of Turkey "X" disease in 1960. However this outbreak marks a turning point that demonstrated the seriousness of the problem, led to the recognition of the carcinogenicity of aflatoxins, and ultimately led to the acknowledgement that aflatoxin is both an economic and public health problem in many areas of the world.

Aflatoxin contamination of foods and occurrence

Aflatoxins are a group of highly toxic metabolites produced by fungi, *Aspergillus flavus* and the closely related species *Aspergillus parasiticus*[12]. Not all isolates of the two fungi produce aflatoxins, but there are indications that many species are capable of toxin production. *A. flavus* produces only aflatoxin B_1 and occasionally B_2, whereas *A. parasiticus* produces other aflatoxins as well (see page 4).

Aspergillus flavus and *Aspergillus parasiticus* can infect peanuts and produce aflatoxins at pre and post harvest stages. In addition, cereal grains have been proven to be good substrates for aflatoxin production in laboratory studies. Aflatoxins can be found in peanut kernels, in unrefined peanut oil, in peanut cake and in peanut butter processed from aflatoxin-contaminated peanuts[12]. Generally, the preferred carbon sources for aflatoxin production are glucose, sucrose or fructose. Amino acids such as glycine and glutamic acid were also found to be essential for aflatoxin production[13].

Among environmental factors, temperature and moisture content showed a stimulatory effect on production of aflatoxins. The optimal growth conditions of *A. flavus* for aflatoxin production are found to be at temperatures ranging between 28-30°C and moisture content level 15-35%[14]. Therefore, it is likely that aflatoxin contamination of foods is ubiquitous in areas of the world with hot, humid climates, including sub-Saharan Africa, South-East Asia and China. In these countries, human exposure to aflatoxins results from contamination of dietary staple foods. For instance in India high aflatoxin production was observed in food samples stored at a temperature of 31°C and 81.0% relative humidity[15]; in this study, a hazardous level of aflatoxin production was reached after 5 months of storage. The risk of contamination with aflatoxins can be increased due to drought stress to peanut pods during the pre harvest stage[16]; physical damage of peanut kernels can also lead to higher vulnerability to mould growth especially during storage periods.

Contamination with aflatoxins can cause economic damage to farmers and merchants, national losses through export reduction, cost of returning shipments of rejected crops and cost of control at pre and post harvest stages. Thus, the frequent occurrence of these toxins in agricultural commodities has a potential negative impact on the economies of the affected regions, especially in developing countries where harvest and post harvest techniques are not adequate to prevent mould growth. It has been estimated that around 25% of yearly world's crops are contaminated by mycotoxins[17]. Therefore, measures have been put forward by a joint FAO/WHO expert committee on food additives in 1987[18]. More recently, the FAO/WHO[19] committee (1999) supported the evidence that aflatoxins should be treated as carcinogenic food contaminants, the intake of which should be reduced as much as reasonably can be achieved. In addition, they referred to the previous measures.

In Sudan peanuts and its products are of great economic importance, and are important to agricultural export. However, fungal contamination in Sudanese groundnuts from West Sudan was reported to be 64 to 90%[20]. Sudanese groundnuts imported into the USA in the year 1981 were found to contain high levels of aflatoxins, i.e. more than 50% of the samples contained over 26 μg/kg[21]. It has been determined that improper handling and storage of peanuts in Sudan lead to multiplication of aflatoxin producing fungi and hence production of high levels of aflatoxins[22].

Apart from economic losses, contamination with aflatoxins also constitutes a nutrition and health problem. It has been suggested that consumption of aflatoxin contaminated foods may be related to the occurrence of kwashiorkor[22b], aflatoxicosis and liver cancer. In Sudan poor quality peanut kernels tend to be mixed with the good ones to be used for local consumption. Therefore, the health effects of aflatoxins may be most serious at local level communities, where peanuts are used as an important dietary component.

Chemical and physical structure of aflatoxins

Four aflatoxins commonly occur in contaminated food and feeds: Aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) Fig (1). AFB_1 is usually found in the highest concentration followed by AFG_1, AFB_2 and AFG_2. A total of 17 aflatoxins have been isolated, but the term refers generally to the four metabolites of this group.

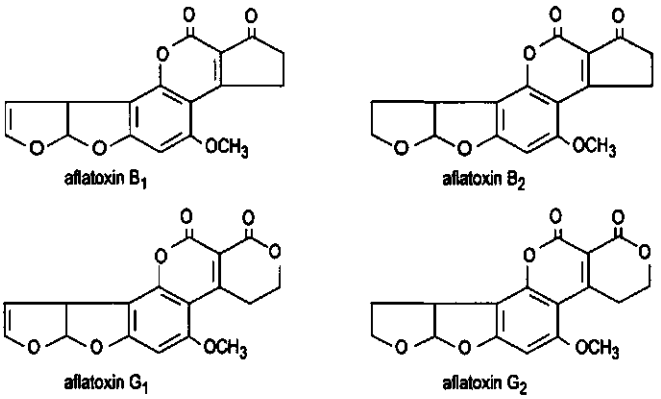

Figure 1. Aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*

The four compounds are distinguished on the color of their fluorescence under long-wave ultraviolet illumination (B = blue, G = green), with the subscripts relating to their relative chromatographic mobility. Aflatoxins are slightly soluble in water (10-30 μg/ml), insoluble in non-polar solvents, and freely soluble in moderately polar organic solvents (e.g., chloroform and methanol)[23]. The lactone ring is susceptible to alkaline hydrolysis, aflatoxins are also degraded by ammonia or sodium hypochlorite. The melting points of aflatoxins are very high ranging between 237-289 °C, so aflatoxins can not readily be degraded under normal home cooking conditions such as boiling and frying (approx. 150 °C)[24].

Many investigators have proposed that all the other aflatoxins can be derived from aflatoxin B_1[25-27]. However, the biosynthetic relationship between the AFB group and the AFG group is controversial.

Biotransformation

The liver is the principal target organ for aflatoxin metabolism. Acute structural and functional liver damage has been observed in field outbreaks and has been reproduced experimentally in most laboratory animal species.

Following transport across the liver cell plasma membrane, the AFB_1 molecule is activated by microsomal (tubular endoplasmic reticulum (ER)-associated) mixed-function mono-oxygenases, requiring presence of catalytic enzymes (cytochrome P450s) to form the highly

reactive AFB_1-8,9-epoxide[28]. AFB_1 exo-8,9-epoxide is the only known genotoxic metabolite of AFB_1 that can be formed by the P450s. In humans, P450 3A4 appears to be the dominant P450 involved in the formation of the exo-epoxide at all AFB_1 concentrations[29-31]. This epoxide may become hydrated to its dihydrodiol followed by sequential oxidation to dialdehyde and condensation with amino groups of lysine, forming Schiff bases[32]. AFB_1-8,9-epoxide may also conjugate to glutathione (GSH) mediated by glutathione S-transferase (GST)[33,34]. This AFB_1-glutathione conjugate can be excreted primarily through the bile[35]. It has been described that polymorphisms in the $GSTM_1$ and epoxide-hydrolase (EPHX) genes may lead to less efficient detoxification of the AFB_1-exo-8,9-epoxide, resulting in higher levels of this highly reactive compound. Since the glutathion S-transferase polymorphism is widely distributed in the human population, the role of this particular enzyme in liver cancer could be of great importance.
Although AFB_1-exo-8,9-epoxide is a very unstable molecule, it results in nuclear damage[7] since it reacts efficiently with high concentrations of DNA to form the guanyl N^7 adduct [36] (Figure 2).

AFB_1 can also be oxidized by P450 1A2 to deactivated products such as aflatoxin M_1 (AFM_1), aflatoxin Q_1 (AFQ_1) and both AFB_1 exo-8,9-epoxide and AFB_1 endo-8,9-epoxide.

Toxicity of aflatoxins

Aflatoxins are reported to be toxic to a wide range of organisms, from bacteria to vertebrates and primates[37]. Wide differences in toxicity have been detected among the various chemical members of the aflatoxin family. The relative potency of the four aflatoxin types as assessed in ducklings is, $AFB_1 > AFG_1 > AFB_2 > AFG_2$ with LD50 values of 0.36, 0.78, 1.70, and 3.44 mg/kg, respectively[38]. However, data on clinical aflatoxicosis in humans is limited although evidence exists for aflatoxicoses of human populations in many areas of the world. Evidence of acute aflatoxin toxicity has been reported from Taiwan and Uganda[9,10] ; it is characterized by vomiting, abdominal pain, pulmonary oedema, and fatty infiltration and necrosis of the liver, but the intake level was not reported. An outbreak of aflatoxin poisoning as a result of the consumption of a heavily moldy corn (6-16 mg aflatoxin/kg), was also described in Western India[10,39]. The hydroxylated metabolite of aflatoxin B_1, especially AFM_1 was detected in urine of Filipinos consuming peanut butter contaminated with ~0.5 mg AFB_1/kg[40]. Acute hepatic encephalopathy, due to severe aflatoxicosis occurred among Malaysians (mainly children) in the year 1988[41]. Epidemiological investigations determined that attack rates were significantly

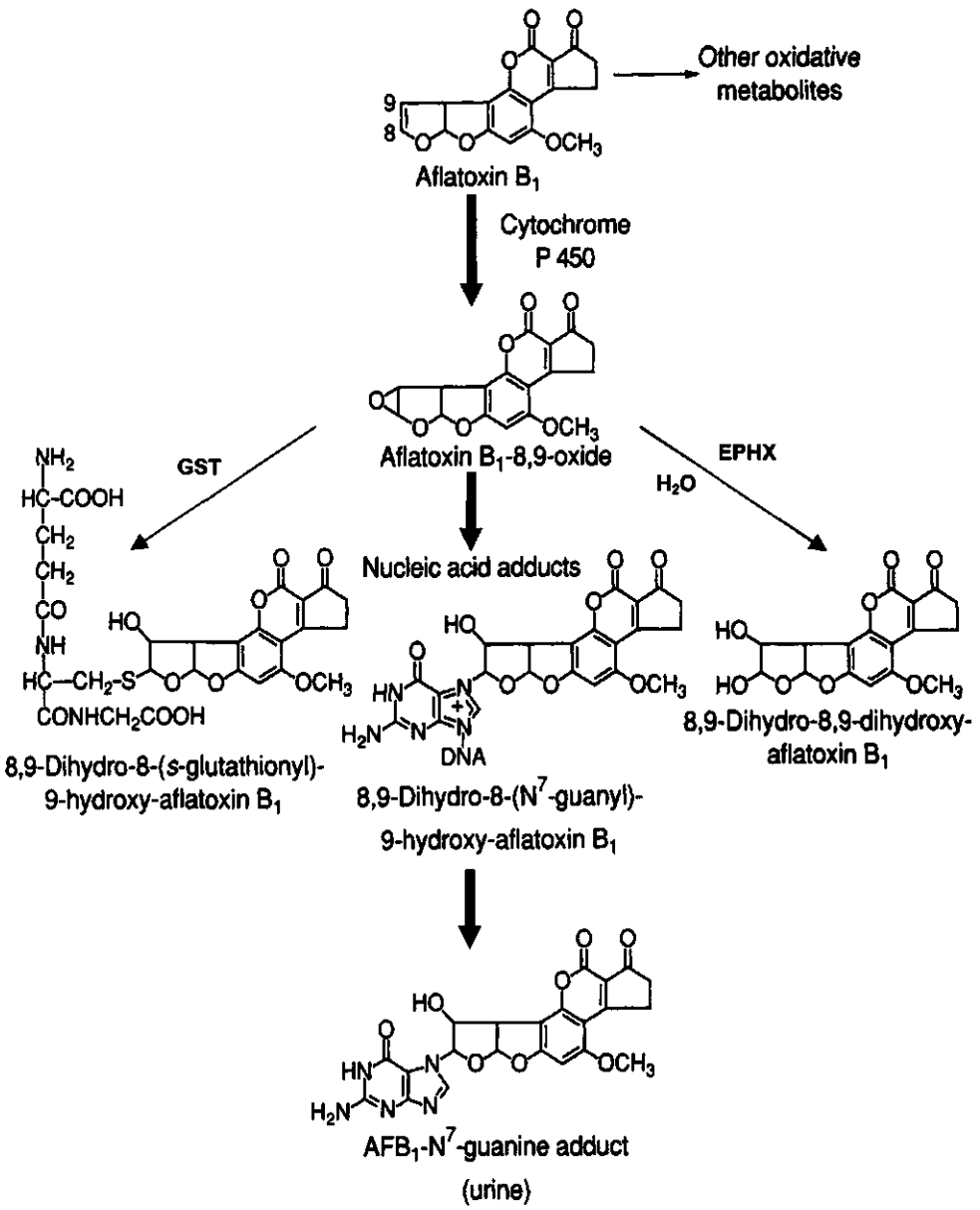

Figure 2. Metabolic pathway for activation and subsequent detoxification of aflatoxin B_1
GST, glutathionine-S-transferase enyzmes; EPHX, epoxide hydrolase

higher among children who had eaten Chinese noodles one day before they died than those who did not. High levels of aflatoxins and their metabolites were found in blood and other postmortem tissues of 10 children out of 13 deaths.

Carcinogenicity of aflatoxin

The aflatoxins are among the most potent mutagenic and carcinogenic substances known[42-45]. AFB_1 is the most important of this family and is a so-called category one human carcinogen[21]. The liver is the principal target organ in all species studied, probably due to the fact that the formation of AFB_1-DNA adducts is greater in the liver than in other organs; which can be explained by the enzymes expressed in the liver. The dose of AFB_1 required for a certain level of DNA-adduction in general correlates with species susceptibility[46]. Aflatoxin-epoxide can bind to the N-7 guanine codon 249 of the p53 tumor suppressor gene in human liver cells to cause a G → T transversion mutation at this site; this provides further evidence for a putative role of aflatoxin exposure in p53 mutagenesis[47]. Formation of DNA adducts of AFB_1-epoxide is well characterized e.g., it has been observed that many aflatoxin-initiated hepatic tumours in trout carry cells with point mutations in the Ki-ras proto-oncogene[48]. The dominant species for all aflatoxins was a codon 12 GGA → GTA transversion. So the susceptibility of AFB_1 towards G → T transversions is compatible to its mutagenic properties[43].

Descriptive Epidemiology of Hepatocellular carcinoma

World wide occurrence

Liver cancer or hepatocellular carcinoma (HCC) has been recognized for many years as one of the most common malignancies. It is the sixth most common cancer in the world, with an estimated world wide incidence of about 540,000 in the year 1996, accounting for 5.2% of all new cancers[49]. HCC incidence varies largely throughout the world; it is considered to be rare in the United States and western countries, ranking 25th among cancers and occurring at a rate of four to five per 100,000 population in North America, United States and Australia[50,51]. In the developing world rates of HCC are much higher (about 6-9 times), e.g. 30 per 100,000 males in Chad and 36 per 100,000 males in China[52]. HCC in developing countries accounts for more than 80% of the global cases, while 55% of the world new cases are accounted for by China alone[53]. The mortality of HCC is practically 100%, which is about 7.5% of all cancer deaths[49]; survival rates are very low, estimated to be 6% in five years in the USA; and it might be even

lower in developing countries. The incidence of HCC increases with age, affects men more often than women, (sex ratio of 3:1) and it usually occurs in the 5th and 6th decades of life[54].

Occurrence of hepatocellular carcinoma in Sudan

In Sudan as well as many other countries of the developing world, no national or regional wide cancer registers have been established. Data on cancer risk in Sudan is very limited. Figures from National Health Laboratory records reported liver cancer being 1.7% of total cancer incidence in the period 1970-74 and 3.8% in the period 1979-84. This data suggest that HCC might be indeed relevant to Sudan. In addition, a higher incidence was reported from West Sudan than from Central Sudan[55]. The supposed higher risk in West Sudan might be real, since the ethnic groups in West Sudan are comparable to populations in Chad (neighboring country), where HCC incidence is reported to be high (see page 8). Thus, despite the lack of well-established cancer registries, it is likely that HCC is a relevant public health problem to Sudan, as it is for other sub-Saharan African countries.

Etiology of hepatocellular carcinoma

The principal etiological factors for hepatocellular carcinoma have been identified as exposure to aflatoxins, chronic infection with viral hepatitis, and other factors such as alcohol consumption, possibly tobacco smoking[56] and oral contraceptives use.

Exposure to aflatoxins

Ingestion of aflatoxin contaminated foods has been studied as a risk factor of liver cancer by a number of ecological and analytical epidemiological studies in different parts of the world. For example, the incidence of liver cancer in eight regions in Taiwan was positively associated with HCC mortality rates[57]; a perfect linear relationship was observed between dietary aflatoxin exposure and HCC mortality rates in four communities in southern China[58]. Table 1 shows the association between aflatoxins and HCC as observed in cohort studies, nested case-control studies and retrospective case-control studies. A total of four cohort studies have been reported and they all observed statistically significant positive associations between HCC risk and markers of aflatoxin exposure. Two studies from the same cohort[59,60] used urinary aflatoxin as a marker of exposure; they reported a positive association for several urinary metabolites of aflatoxins. Four

Table 1. Aflatoxin exposure and the risk of hepatocellular carcinoma (HCC): Cohort, nested case-control and case-control studies.

Author, year, place	Exposure assessment	Study size No. of subjects	Main results	Remarks
Cohort studies				
Olsen et al, 1988[62], Denmark	Aflatoxins contaminated dust	Industrial cohort of livestock-feed workers (men from 241 companies)	Exposure for ≥10 years to 179 ng AFB_1 per day (in dust) vs non exposed (other industries): 7 cases observed, 2.8 expected, RR=2.5 (1.08-4.86)	Hepatitis B virus and alcoholism did not appear to be risk factors in this study. Lungs did not seem to be a target organ for the carcinogenic effect of aflatoxins.
Ross et al, 1992[59], China	Urinary AFB_1-N7-Gua adducts, AFM_1, AFP_1 and AFB1	18,244 men with 35,299 person-years of follow up 22 cases	RR=3.8 (1.2-12.2) for detectable urinary aflatoxin metabolites vs below detectable levels. AFB_1-N7 Gua : RR=4.9 (1.5-16.3) AFM_1:RR=3.0 (1.0-9.3) AFP_1: RR=6.2 (1.8-12.2). AFB_1: RR=2.3 (0.9-5.9)	The follow-up time (<2 years) was rather short. Urinary aflatoxin may not accurately reflect the long-term exposure, due to day-to-day variability. Results were adjusted for HBV, education and cigarette smoking. Interaction was observed between HBsAg positivity and detectable urinary aflatoxin metabolites RR=60 (6.4-562).
Qian et al, 1994[60], China	Urinary AFB_1-N7-Gua adducts, AFM_1, AFP_1 and AFB_1	18,244 men 70,000 person-years of follow up 55 cases	AFB_1-N7 Gua : RR=9.1 (2.9-29.2) AFM_1:RR=16.1 (3.6-72.5) AFP_1:RR=11.0 (2.4-50.9) AFB_1: RR=5.7 (1.3-26.0)	Results adjusted for HBsAg and smoking. Among the various AFB-contaminated foods, peanuts contributed 55% to the total dietary exposure of aflatoxins.
Sun et al, 1999[63], China	Urinary adducts AFM_1	145 male HBV carriers men, 10 years of follow up 22 cases	RR=3.3 (1.2-8.7) for detectable urinary AFM_1 (>3.6 ng/L) vs below detectable level.	Four men with detectable AFM_1 and HCC all had a mutation in codon 249 of the P53 gene in cancer tissues. Family history of HCC increased the risk 5.6 fold. The risk due to HCV infection was RR= 5.8 (2.0-17).
Nested case-control studies				
Chen et al, 1996[65], Taiwan	AFB_1-albumin adducts	32 cases and 73 controls nested in cohort of HBV carriers n=4,841	Comparing undetectable, low and high AFB1-adduct RR=1.0, 0.7, 1.4 (1.0-16.9) at GSTM1 non-null genotype and RR=1.0, 4.1, 12.4 (1.7-92.7) at GSTM1 null genotype.	The interaction between the level of AFB_1-albumin adduct and the polymorphisms of GSTM1 & T1was borderline statistically significant (0.05< P <0.1).
Chen et al, 1996[64], Taiwan.	AFB_1-albumin adducts	20 cases and 86 controls nested in cohort (n=6,487)	OR=5.5 (1.2-24.5) for the presence vs absence of AFB_1-albumin adduct and HCC.	For other HCC risk factors, OR=129.4 (25.4-659) for HBsAg and OR=1.8 (0.2-13.3) for HCV positive cases. Result for $AFB_1$was adjusted for other risk factors of HCC.

Wang et al, 1996[66], Taiwan	AFB_1-albumin adducts and urinary aflatoxin	56 cases and 220 controls nested in cohort n=12,024 males and 13,594 females.	After adjustment for HBV, the OR for high urinary aflatoxin level vs low=3.8 (1.1-12.8), and for detectable vs non-detectable AFB_1-albumin adducts OR=1.6 (0.4-5.5). When analysis were limited to HBV carriers, the OR=112 (13.8-905) for highest vs lower urinary aflatoxins, and OR=70.0 (11.8-415.4) for detectable vs non-detectable AFB_1-albumin adducts.	HBsAg positive subjects with high aflatoxin intake at a higher risk than subjects with high aflatoxin exposure only or HBsAg only.
Yu et al, 1997[67], Taiwan	AFB_1-albumin adducts and urinary aflatoxin	43 cases and 86 controls nested in a cohort of HBV carriers n=7,342 men	Comparing high vs low urinary AFM_1 level OR=6.0 (1.2-29.0). A very high risk was found among those having both markers (AFB_1-albumin adducts and urinary AFB_1-N7-guanine adducts), OR=10.0 (1.6-60.9).	No elevated risk observed for those who were positive for either AFB_1-albumin adducts or urinary AFB_1-N7-guanine adducts marker alone. There was a synergistic interaction between the GSTM1 null genotype and AFB_1 exposure in HCC risk.
Case-control studies				
Lam et al, 1982[68], Hong Kong	Dietary sources of aflatoxin Food frequency questionnaire	107 cases 107 controls	Consumption frequency of ≥ once/week vs less Peanuts OR=1.5 other grains OR=2.2 corn OR=1.0 beans OR=0.9 mung beans OR=1.0	Dietary sources of aflatoxin intake didn't appear as strong risk factor in this study. This may be due to misclassification of aflatoxin exposure. Results were adjusted for HBsAg status.
Bulatao etal, 1982[69], Philippine	Dietary aflatoxin	90 cases and 90 controls	Heavy aflatoxin exposure (≥ 7μg) vs low exposure (0-3μg) in peanuts and its products RR=10.0 (P< 0.01) Heavy (≥ 7μg) vs low (0-3μg) overall mean aflatoxin load in the diet RR=17.0 (P<0.05).	Combining aflatoxin load and alcohol intake yielded a synergistic and statistically significant effect, heavy vs low aflatoxin among heavy alcohol drinkers RR=35.0 (P< 0.05) and among light alcohol drinkers RR=17.0 (P< 0.05). Hepatitis infections were not considered.
Srivatanakul et al, 1991[70], Thailand	AFB_1-albumin adduct Dietary sources of aflatoxin	65 cases and 65 controls	Presence vs absence of adduct OR=1.0 (0.4-2.7). Peanuts > than twice per month vs less OR= 0.6 (0.1-0.6) Powdered peanut > than once per month vs less OR= 0.3 (0.1-0.6).	No increased risk was found from recent aflatoxin intake as estimated by consumption of possible contaminated foods or by presence of AFB_1-albumin adducts in serum. This might be explained by the fact that cases might have reduced their food intake. HBV infection was the major risk factor RR=15.2.
Zang et al, 1998[71], China	Dietary sources of aflatoxin	152 cases and 115 controls	Peanuts and peanut oil consumption OR=13.8 (3.7-51.5). Corn consumption OR=19.4 (3.7-103)	For HBV infection, OR=44.6 (12.5-158.5). A dose-response relationship for alcohol intake was found, heavy drinkers experienced 3-4 folds risk.

nested case-control studies were reported from Taiwan; all of them reported OR/RRs exceeding 1.0 and most of them were statistically significant. Four case-control studies from four different countries addressed dietary sources of aflatoxins; two of these showed clear positive associations while the two others failed to observe an association.

In conclusion, the four cohort studies and the four nested case-control studies reported RRs ranging from about 3 to 16 for high versus low levels of biomarkers of exposure to aflatoxins. The four case-control studies, addressing dietary intake, reported ORs between 1.0 and 35 for high versus low level of intake.

Virtually all studies in Table 1 were conducted in Asian countries. Since climatological conditions in these countries are similar to sub-Saharan tropical countries like Sudan, contamination of food with aflatoxins is likely to be relevant to these African countries as well. So far, this is only supported by ecological studies from Kenya[61].

Viral hepatitis

Infections with hepatitis viruses are considered to be major risk factors of liver diseases including hepatocellular carcinoma. There are different hepatic virus types, but they are not all considered to be carcinogenic, e.g. hepatitis A and E.

Chronic infection with hepatitis B virus can act as both initiator and promotor of liver cancer. It results in deletion and alteration of the host DNA genome; a point mutation of the P53 gene is also observed particularly in areas where there is accompanying exposure of aflatoxins in food, suggesting a joint role of HBV infection and aflatoxin exposure in causing HCC[72]. Chronic HBV results in widespread cell death and hence cell proliferation; this can lead to cirrhosis, which can provide an environment for cell with specific genetic damage to have a growth advantage. Hepatitis C virus is an RNA virus, and is therefore active via a mechanism other than integration into the host DNA.

The etiological role of chronic hepatitis B infection in the development of HCC has been established extensively, numerous analytical epidemiological studies from around the world[73] indicate the importance of HBV in the etiology of HCC and also from a meta-analysis study done by Donato *et al.*[74].The importance of hepatitis C virus (HCV) in the etiology of HCC was

initially recognized by studies in Japan[75,76]. Further studies have demonstrated the strength of this association[77-79], but others did not[80, 81]. Other viruses, such as hepatitis G virus (HGV) were not found to be a major risk factor for HCC in an HBV endemic area in Thailand: the risk of HGV was found to be ten times lower than that of HBV[82]. The etiological role of the new human DNA virus named TT virus (TTV) on HCC was not supported, according to a case-control study undertaken in Italy, 1999[83]. However, as the number of studies on these other viruses is still limited, different results in future studies cannot be excluded yet.

Other risk factors

Alcohol consumption is considered to be a risk factor for liver cancer. In areas where the prevalence of aflatoxins and HBV is very low, it is clear that alcohol consumption is associated with increased risk of the development of HCC, as shown in alcoholic drinkers from northern Europe and North America[84,85]; a case-control study from China has reported similar findings[71] (Table 1). However, two cohort studies from China[59] (Table 1) and Japan[86] reported a non-significant positive association between alcohol consumption and HCC; the same conclusion was also reached by a case-control study from Japan that investigated heavy drinking vs non drinking[87]. Synergistic effects of alcohol consumption with aflatoxin intake and with hepatitis infections have been shown in case-control studies from Philippine[69] (Table 1) and Italy[78] respectively.

Tobacco use may be a contributing factor in areas where liver cancer is not virally induced since the increased HCC risk from tobacco use is apparent among HBsAg-negative subjects[88]. A moderate excess risk of HCC among cigarette smokers has been suggested by Ross, 1992[59].

Exogenous steroids, i.e. the contraceptive hormones may also be relevant in the etiology of HCC[89]. This is not likely being relevant among the poor socio-economic groups in the developing world.

Toxins from blue-green algae have also been considered as an etiological factor of HCC. Some studies have suggested that people who drink bond-ditch water contaminated with blue-green algae experience higher HCC mortality rates than people who did drink deep-well water[90].

The role of various nutritional factors including protein, minerals and vitamins in liver cancer is extensively reviewed[53].

The evidence for many dietary factors such as cereal (grains), vegetables and fruits, meat and dairy products in the association with liver cancer is limited and most of the study results are inconsistent. In ecological studies, protein has been suggested to protect against liver cancer. However, in areas where protein intake is low, the prevalence of chronic hepatitis B infection and exposure to aflatoxins tend to be high. However, no evidence is available from individual-based case-control and cohort studies, either addressing protein or other macronutrients. Human and animal evidence concerning the effect of minerals such as iron and selenium on liver cancer is not considered sufficient yet to prove this association. However, several studies have suggested that iron overload might be involved in increasing the risk of liver cancer, and deficient intake of selenium has been found to increase hepatocarcinogenesis in laboratory animals. Epidemiological and animal studies have also shown that low serum retinol concentration is associated with liver cancer, and dietary vitamin A has been suggested to reduce the risk of hepatocellular carcinoma.

Regarding the mechanism involved, it has been demonstrated that vitamins A and C can inhibit the formation of AFB_1-DNA adducts in woodchuck hepatocytes[91]. Along similar lines oltipraz, a chemopreventive agent, is considered to decrease the metabolism of aflatoxin to its carcinogenic form and to increase the detoxification pathway[92].

Aims and outline of the thesis

Aflatoxin contamination of food and hepatitis B viral infection are involved in the etiology of hepatocellular carcinoma (HCC) in many developing countries. As appears from previous sections, climatic conditions in Sudan are likely to contribute to the contamination of food with aflatoxins, possibly contributing to the risk of HCC. So far only ecological studies have been reported from African countries, while evidence from individual-based epidemiological studies is lacking. The studies described in this thesis are designed to identify the most important etiological factors relevant to the incidence of HCC in Sudan, one of sub-Saharan tropical regions in Africa experiencing a high risk of HCC.

Objective. The primary aim of this study is to investigate the role of peanut butter contaminated with aflatoxins in the etiology of HCC, in order to find clues for prevention and control among the Sudanese population. Following an explorative study, a case-control study (main study) was conducted in two regions in Sudan, supposed to be different in exposure to aflatoxins as well as HCC risk.

Explorative study. To investigate the possibilities and limitations of conducting a case-control study in Sudan, we first conducted an explorative study in the two regions. The aims of this study were to compare HCC risk factors in a supposed high risk area compared to a low risk area, to pretest the questionnaire and the procedure for recruitment of cases and controls. Furthermore, it was meant to explore the possibilities to include collection of biological markers in the main study. The results of this study are incorporated in Chapter 2.

Main study. The aims of the main study are to address questions of direct relevance to Sudan i.e. to establish the role of aflatoxins in the etiology of HCC. This is evaluated at the level of life style and environmental factors such as food consumption and storage conditions of peanuts. In order to achieve a better understanding of the mode of action of aflatoxins, and to strengthen the scientific rationale we also addressed polymorphisms in susceptibility genes relevant to aflatoxin metabolism. This is guided by the following research questions:

1. Is the usual intake of peanut butter associated with increased risk of HCC in West and Central Sudan? Are subjects who lack the GSTM1 gene at particularly high risk? (Chapter 3).

2. Are other genetic polymorphisms involved in the metabolism of HCC relevant risk factors and/or modifiers of HCC risk? (Chapter 4).

Subsequently, the alleged role of aflatoxins needs to be addressed relative to other risk factors of HCC. This was guided by the following research questions:

3. What is the role of hepatitis B and C viral infections in the etiology of HCC in Sudan (Chapter 5).
4. What is the relevance of joint exposure to aflatoxins and hepatitis B virus infections to the prevention and control of HCC in Sudan? (Chapter 6).

Scope. The ultimate aim of this research project is to contribute to a feasible strategy to reduce HCC in Sudan. However, the validity of the estimated public health effects of aflatoxins depend on the internal validity of the study, the underlying biological mechanisms, and the generalizability of results. These issues are incorporated in the general discussion (Chapter 7).

References

The references to this introduction are provided at the end of Chapter 7.

2

Aflatoxin and liver cancer in Sudan

R.E. Omer, M.I. Bakker, P. van't Veer, R.L.A.P. Hoogenboom, T.H.G. Polman, G.M. Alink, M.O. Idris, A.M.Y. Kadaru, F.J. Kok.
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Abstract

This study investigated whether aflatoxin contamination of peanut products may contribute to the incidence of hepatocellular carcinoma (HCC) in Sudan. Thirty-seven peanut butter and peanut samples were collected from local markets. Aflatoxin concentrations were significantly higher in West Sudan [87.4 ± 197.3 (SD) μg/kg], a high-risk area, compared with Central Sudan (8.5 ± 6.8 μg/kg), a low risk area. In West Sudan, humid local storage conditions of peanut products were related to high aflatoxin concentrations. In a small case-control study of HCC patients (n=24) and controls (n=34), an odds ratio of 7.5 (95% confidence interval = 1.4-40.2) was observed for humid versus dry local storage conditions. Development of an index of individual HCC exposure was less successful, probably due to year-to-year variability in aflatoxins in food. These preliminary findings justify further research into the role of aflatoxins and hepatitis in HCC incidence in Sudan.

Introduction

Aflatoxin contamination of groundnuts is a serious problem in many parts of the world. It is considered to be an important cause of hepatocellular carcinoma (HCC), one of the most common cancers in developing tropical countries[1]. Studies in Mozambique, Swaziland, China and Korea[2-5] have reported high frequency of liver cancer, compared with western countries[6]. Food mycotoxins like aflatoxin, malnutrition, hepatitis B virus infection, hepatitis C virus infection, alcohol intake and various parasitic infections are frequently found to coexist with HCC in many areas of the developing world, and there is evidence that these factors interact in the etiology of HCC[7,8].

The groundnut is an important crop in Sudan and one of the major export products. Few systematic surveys to study aflatoxin contamination of Sudanese groundnut have been conducted. Habish and Abdalla[9] reported a wide range of aflatoxins levels in groundnut samples from West Sudan. Ahmed[10] reported on a consignment of the Sudanese groundnut, which was rejected as export because of aflatoxin contamination. Omer[11] showed that low quality kernels, because of groundnut damage by insects and moulds, are seriously contaminated with aflatoxin. These groundnuts of poorer quality containing the higher levels of aflatoxins, are used locally as an inexpensive cheap source of protein especially among the poor.

Population-based incidence data are not available, but figures from the Sudan Cancer Registry[12] show a doubling of the proportion of HCC among all cancers over the period 1970-1974 to 1979-1984 (from 1.7% to 3.8%); although this increase might partially be due to initial underdiagnosis because of a low percentage of biopsies or autopsies, these figures suggest an increasing incidence. Clinical experience and Khartoum hospital records, raised the suspicion that incidence of HCC is substantially higher in the West than in the Central Sudan. Field and laboratory studies have attributed the higher incidence of HCC in West Sudan to hepatitis B virus infection[13]. No study was performed to investigate the role of aflatoxin on HCC in Sudan.

To assess whether aflatoxin contamination in peanut products contributes to the HCC incidence in Sudan, we compared peanut consumption, aflatoxin levels and types of storage in an alleged high-incidence area (West Sudan) and a low-incidence are (Central Sudan). Furthermore, we

explored the feasibility of conducting a case-control study in these regions to evaluate the role of aflatoxins in the etiology in HCC in Sudan.

Methods

Study period and study area

A survey was conducted over a period of seven months (May-Nov. 1995), in West and Central Sudan, representing regions of higher and lower incidence of liver cancer. Sudan is situated in North East Africa between latitude 4 and 23 degrees north and longitudes 22 and 38 degrees east, within the African tropical zone.West Sudan (North Kordofan State) is an area of 229,694 km², and a population size of 1,500,000. The groundnut is one of the major cultivated crops in this region, and its cultivation is critically dependent on rain as the only source of water. Central Sudan (Gazira State) is an area of 151,639 km², and a population size of 2-3,000,000. Here, the groundnut is one of the main products as well; however, cultivation depends mainly on irrigation systems for water supply.

Peanut butter samples

From West and Central Sudan, three provinces were chosen randomly; in each province a district and in each district one health service were chosen randomly. Peanut butter samples were collected from a local market of each village where the health services were located, each market serving approximately 1500 subjects. Twenty-three peanut butter samples and four groundnut samples were collected from the West Sudan after seven months of local storage. Ten peanut butter samples were collected from Central Sudan after five months of local storage. Sample size (0.5-1 kg), sampling date, and type of storage were recorded. The samples were then transferred to Khartoum, where they were kept in a refrigerator (5°C).

Storage was classified as mud building, dry grass house and brick house or a combination of these. A mud building is constructed of raw mud, the floor is not covered by any means of infrastructure, and it has a thatched roof. A dry grass house has walls and a roof made of straw supported by rafter and studs; the floor is not concrete. A brick house is constructed of mud bricks stacked together by means of a mixture made of mortar plasticizer and sand, the floor is concrete, and the roof is covered with metal sheets.

Aflatoxin Analysis

The samples were analyzed at the Department for Food Safety and Health at RIKILT-DLO by high-performance liquid chromatography using an extraction procedure based on AOAC-method No. 993.17[14]. As a principle of this method, aflatoxin B_1 (AFB_1) is extracted from samples with 85:15 (vol/vol) methanol/water. The filtrate is diluted 1:1 with 10% NaCl solution and defatted with hexane. AFB_1 is partitioned into chloroform, which is then removed by evaporation. Extracts are purified using 0.5g silica gel columns (Baker) and quantified by high-performance liquid chromatography. The samples were injected onto an LC18DB column (250 x 4.6 mm I.D; Supelco, Zwijndrecht, The Netherlands), and eluted with 1 N 400:510:40:50 (vol/vol/vol/vol) methanol/water/acetonitrile/(formic acid/NaOH-buffer), pH 3.6. Aflatoxins were detected by ultraviolet absorption at 365 nm. Except for three of the groundnut samples, the analyses were done in two independent samples, originating from the same portion as bought at the market place. The standard error of these duplicate analyses was 31%. Because these samples were not homogenized before analysis, this value represents within-sample heterogeneity rather than laboratory error. The mean values of the duplicates were used in further statistical analyses. Recoveries of AFB_1 in samples spiked at 16 and 250 ppb were 108 and 74%, respectively. We report data for Aflatoxin B_1, the most toxic and the most commonly occurring aflatoxin[15]; however, Aflatoxin B_2 was observed as well, in particular in the highly contaminated samples, comprising 20-40% of the AFB_1 levels.

Case and Control Recruitment

Twenty-four patients (20-75 years of age) were obtained by using the admission lists of four out of six Khartoum (capital) hospitals during the survey period. This resulted in 15 patients from West Sudan and 9 patients from Central Sudan. Because HCC cannot be treated in the health centers in West and Central Sudan, patients from West and Central Sudan who are seriously ill tend to receive their treatments at Khartoum hospitals, where better diagnosis and treatment are possible. Diagnosis of HCC was based on a series of tests with increasing certainty, i.e. liver function test (enzymes and protein), ultrasound investigations and liver biopsy. In this study 14 (58%) cases had been diagnosed by biopsy.

Controls were recruited from the health services as mentioned in Peanut Butter Samples. At each health service, five to seven controls (24-72 yr of age), who had no liver, stomach, or intestinal complaints, were randomly chosen from the admission lists. The controls were matched for sex

and age. This resulted in a total of 16 subjects from West and 18 from Central Sudan. The response rate was 100% since all invited cases and controls agreed to participate in the study.

Exposure Assessment

A questionnaire was developed to evaluate the degree of exposure among the two groups in a standardized manner. Questionnaires were completed during an oral interview. The questionnaire contained 36 items and focused on food habits and especially on peanut butter and other forms of groundnut consumption in terms of frequency, main composition of meals, quantity and storage conditions. To estimate aflatoxin intake of the subjects, an aflatoxin index was constructed, containing the following factors: frequency and quantity (portion size) of the peanut butter consumption, and aflatoxin content of peanut butter according to storage conditions and region. The oral questionnaire also inquired about potential confounders of the association of interest, i.e., hepatitis infections, alcohol consumption, and bilharzia.

Data Analysis

Because the AFB_1 concentrations of the samples were not normally distributed we tested for statistical significance between levels of AFB_1 between region and between storage conditions using a Wilcoxon rank-sum test. In further analyses the distribution was normalized by using the $^{10}\log AFB_1$ concentrations, and Student's t-test and regression analyses were used.

Crude and Mantel-Haenszel adjusted odds ratios (ORs) were computed for factors representing the exposure to aflatoxin and other risk factors of HCC. Adjustment was carried out for age but not for gender, because of the few women in the dataset.

Results

Table 1 shows the prevalence of life style and risk factors for HCC in the alleged high-low risk areas (West and Central Sudan, respectively). West Sudan is socioeconomically less well developed: the increased odds ratio's show that people are less educated and more frequently farmer and laborers. Differences also relate to food pattern: in West Sudan, peanut butter is eaten in larger quantities and groundnuts are eaten more frequently; roasted peanut butter is typical for Central Sudan. Furthermore, storage conditions differ between West and Central Sudan: in Central Sudan, a mud building, which is considered to have the highest humidity, is used most often (56%), while in West Sudan, only 13% use mud buildings. In contrast, 47% of the cases

Table 1. Prevalence of Life style and Risk factors for Hepatocellular Carcinoma in West an Central Sudan, Based on the Control group of the Case-Control study

Variables	West Sudan[a] (high risk area)	Central Sudan[a] (low risk area)	Prevalence OR[b]	χ^2	p-value
Demographic factors					
Age					
50 – 75yr	7 (44)	15 (83)	3.9 (0.8-19.0)	3.0	0.08
20 – 49yr	9 (57)	3 (17)	1.0		
Gender					
Male	13 (81)	17 (94)	0.3 (0.02-2.7)	1.4	0.23
Female	3 (19)	1 (6)	1.0		
Socioeconomic factors					
Literacy					
Illiterate	10 (63)	4 (22)	5.8 (1.3-26.2)	5.7	0.02
Literate	6 (37)	14 (78)	1.0		
Occupation					
Farmer/laborer	11 (69)	9 (50)	2.2 (0.5-9.0)	1.2	0.3
Merchant/soldier	5 (31)	9 (50)	1.0		
Peanut butter consumption					
Frequency					
≥3 times/week	9 (56)	8 (44)	1.6 (0.4-6.2)	0.5	0.5
<3 times/week	7 (44)	10 (56)	1.0		
Quantity					
Full meal	14 (88)	8 (44)	8.8 (1.5-50.3)	6.9	0.01
Part meal or less	2 (12)	10 (56)	1.0		
Preparation					
Roasted	6 (37)	17 (94)	0.04 (0.004-0.3)	12.6	0.000
Unroasted	10 (63)	1 (6)	1.0		
Type of storage[c]					
Mud building	2 (12)	10 (56)	0.2 (0.03-1.3)	3.0	0.08
Dry grass house	8 (50)	1 (6)	8.0 (0.8-85.3)	3.5	0.06
Brick House	6 (38)	6 (33)	1.0		
Groundnut consumption					
Frequency					
≥1 ounce/day	11 (69)	4 (22)	7.7 (1.7-35.7)	7.4	0.006
<1 ounce/day	5 (31)	14 (78)	1.0		
Risk factors liver disease					
Hepatitis infection					
Yes	13 (81)	8 (44)	5.4 (1.1-25.8)	4.9	0.03
No	3 (19)	10 (56)	1.0		
Bilharzia infection					
Yes	4 (25)	8 (44)	0.4 (0.1-1.8)	1.4	0.2
No	12 (75)	10 (56)	1.0		
Alcohol consumption					
≥10 bottles/month	4 (24)	4 (22)	1.0 (0.2-5.7)	0.0	1.0
<10 bottles/month	5 (31)	7 (39)	0.7 (0.2-3.4)	0.2	0.7
None	7 (44)	7 (39)	1.0		
Years of alcohol consumption					
≥10 yr	6 (37)	6 (33)	1.2 (0.3-4.9)	0.1	0.8
<10 yr	10 (63)	12 (67)	1.0		

[a] Value in Parentheses is percentage. [b] Value in Parentheses is 95% confidence interval. OR, odds ratio.
[c] One control in Central Sudan combined mud building and dry grass house

from West Sudan use mud buildings. Hepatitis infections were reported with high frequency (21 of 34 controls, 62%), far more frequent in West Sudan (prevalence 81% as compared with 44% in Central Sudan).

The AFB_1 concentrations are between <1 μg/kg (detection limit is 1 μg/kg) and 781 μg/kg. Sixty-three percent of the samples from West Sudan were above the permissible level of 10 μg/kg, and 50% of the samples from Central Sudan exceeded this level (Figure 1). Mean AFB_1 concentration differ significantly between West (87.4 ± 197.3 (SD)) and Central (8.54 ± 6.75 μg/kg) Sudan ($p = 0.04$, Wilcoxon rank-sum test). Aflatoxin concentrations in groundnuts and peanut butter were similar. The highest concentrations were found in samples from mud buildings in West Sudan (Table 2). Mean AFB_1 concentration of samples from mud buildings and dry grass houses within West Sudan differ significantly ($p = 0.005$, Wilcoxon rank-sum test); in Central Sudan no differences were found between the types of storage. Multiple regression of $^{10}\log AFB_1$ concentrations showed independent associations of region ($p=0.031$) and storage conditions ($p=0.010$). Although adding an interaction term between region and storage conditions did not significantly improve the fit of the model ($p=0.23$), the mean and observed values by region and storage conditions (Figure 1, Table 2) suggest that storage conditions are relevant to aflatoxin concentrations in West Sudan only.

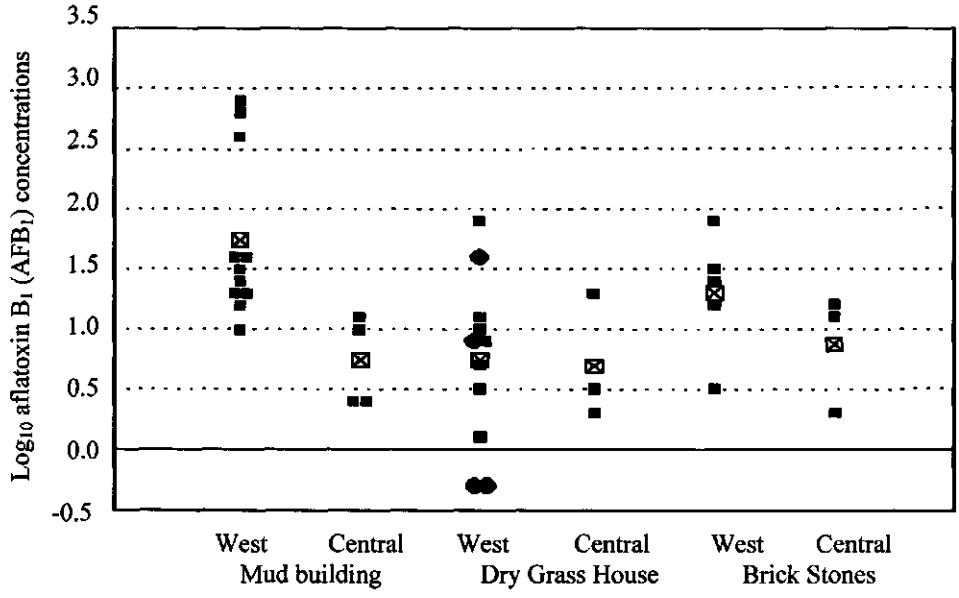

Figure 1. Log_{10} aflatoxin B_1 (AFB_1) concentrations according to region and storage.
***Filled squares*, peanut butter; *filled circles*, groundnut; *crossed open squares*, mean value.**

Table 2. Mean aflatoxin B_1 concentrations in peanut products by region and type of storage[a,b]

Storage	West Sudan	Central Sudan
Mud Building	55.2 (21.6-141.1)[*]	5.5 (2.2-13.4)[‡]
Dry Grass House	5.5 (2.1-14.1)	4.9 (1.3-19.6)
Brick House	19.8 (6.8-57.6)[†]	7.4 (2.0-27.0)[§]
Overall	17.8 (8.9-35.4)[□]	5.8 (3.2-10.6)

[a]: Values are means expressed in µg/kg with 95% confidence interval in parentheses. Concentrations were transformed from analyses on a $\log_{10}$ scale.
[b]: statistical significance is as follows: *: p = 0.003; †: p = 0.13; not different from dry grass house in Central Sudan; ‡: p = 0.69; §, p = 0.90; □: border line significantly different from overall in Central Sudan (p = 0.07).

Table 3 shows risk factors of HCC among cases and controls. The use of mud buildings for storage was strongly and significantly associated with HCC, which remained after adjustment for age and hepatitis infection or for education as an indicator of socioeconomic conditions. In addition, Table 3 shows the association of the aflatoxin index and HCC. This index was weakly associated with HCC (OR = 1.2), and was slightly strengthened within the subjects infected with hepatitis virus (OR = 1.5, 95% CI = 0.9-2.5). No relation was found between HCC and hepatitis infection, but it became weakly positive when adjusted for age. Low socioeconomic status, as indicated by illiteracy and job, was positively associated with HCC, but this association was weakened when adjusted for age, and was also related to region (not adjusted for). Other factors did not affect the association between the aflatoxin index and HCC in this small dataset.

Table 3. Association of Hepatocellular Carcinoma With Factors Representing Exposure to Aflatoxin, Risk Factors for Liver Disease, Socioeconomic and Demographic factors

Variables	*n*		OR[a]		
	Cases	Controls	Crude	age adjusted MH-OR	age + hepatitis adjusted MH-OR
Exposure to aflatoxin					
Peanut butter frequency	12	17	1.0 (0.4-2.8)	1.2 (0.4-3.6)	1.1 (0.4-3.4)
≥3 times/week	12	17	1.0	1.0	1.0
<3 times/week					
Peanut butter quantity					
Full meal	16	22	1.1 (0.4-3.6)	1.3 (0.4-4.5)	1.3 (0.4-4.6)
Part meal or less	8	12	1.0	1.0	1.0
Type of storage					
Mud building[b]	15	12	7.5 (1.4-40.2)	6.1 (1.0-38.8)	5.1 (1.0-25.2)
Dry Grass House	5	9	3.3 (0.5-21.3)	1.7 (0.3-8.9)	3.1 (0.4-23.4)
Brick House	2	12	1.0	1.0	1.0
Aflatoxin index[c]					
High (index≥20)	14	17	1.4 (0.5-4.0)	1.4 (0.4-4.2)	1.2 (0.4-3.9)
Low (index<20)	10	17	1.0	1.0	1.0
Risk factors for liver disease					
Hepatitis infection					
Yes	15	21	1.0 (0.4-3.0)	1.4 (0.4-4.6)	N.A.
No	9	13	1.0	1.0	
Bilharzia infection					
Yes	7	12	0.8 (0.2-2.3)	0.8 (0.3-2.8)	1.0 (0.3-3.5)
No	17	22	1.0	1.0	1.0
Alcohol consumption					
≥10 yr	11	12	1.6 (0.5-4.5)	1.1 (0.3-3.4)	0.9 (0.3-3.1)
<10 yr	13	22	1.0	1.0	1.0
Socio-economic factors					
Region					
West	15	16	1.9 (0.6-5.4)	1.5 (0.5-4.6)	1.3 (0.4-3.9)
Central	9	18	1.0	1.0	1.0
Literacy					
Illiterate	18	14	4.3 (1.4-13.5)	2.6 (0.7-9.3)	2.3 (0.7-8.3)
Literate	6	20	1.0	1.0	1.0
Occupation					
Farmer/laborer	21	20	4.9 (1.2-19.7)	3.7 (0.9-14.7)	3.9 (0.9-16.6)
Merchant/soldier	3	14	1.0	1.0	1.0

[a] Values in parentheses are 95% confidence intervals. MH, Mantel-Haenzel; NA, not applicable.

[b] Two additional cases and 1 control reported a use of a mud building with other storage condition; depending on the classification, this might slightly weaken or strengthen the association.

[c] Aflatoxin index = frequency (times/month) * quantity (full meal = 2/part meal = 1) * aflatoxin according to storage condition based on Table 2; index ranges from 0 to 1.042, with high and low index defined by median among controls (index = 20); on average, a low index corresponds to 10 and a high value to 175.

Discussion

We found the highest peanut butter consumption and highest aflatoxin concentrations in West Sudan, the region with the alleged highest liver cancer incidence. In this region peanut products stored in mud buildings were related to the highest aflatoxin concentrations and mud buildings were also associated with HCC in the case-control analysis. Although confirmation in a larger study is required, these findings support the idea that peanut butter consumption and storage conditions are important determinants of exposure to aflatoxins in Sudan. The study also shows that it is feasible to conduct a case-control study in these regions in Sudan.

For *Aspergillus flavus* and the more prevalent *Aspergillus parasiticus*[16] the optimal conditions to get maximum aflatoxin production are temperatures between 25 and 28°C, moisture content of 25% and a relative humidity up to 99%. Especially when combined with pre-harvest drought stress, these post-harvest wet-storage conditions result in high aflatoxin contamination[17]. The temperatures are quite similar in Central and West Sudan, but in Central Sudan higher precipitation[18] and better irrigation and drainage systems help to avoid drought stress in the peanuts, whereas pre-harvest drought stress is much more common in West Sudan. In line with this, we observed the highest aflatoxin concentrations in mud building in West Sudan; however, the small samples size and two months shorter post-harvest storage time in Central Sudan may provide an alternative explanation of this observation.

In the case-control study we observed an association between storage in mud buildings and HCC, but the use of the aflatoxin index did not reveal a clear association. This apparent discrepancy might originate from lack of reliability in the aflatoxin index as a marker of long term individual exposure, relevant to the risk of HCC. Despite great expansion of groundnut production in Sudan during the past decades, food habits and storage conditions of the local population tend to be stable over long periods of time, and aflatoxin contamination in West Sudan has been reported already in 1971[9]. In line with this, we observed higher consumption of peanut products and higher levels of aflatoxins in West Sudan. In calculating the aflatoxin index for cases and controls, however, several types of error are multiplied, i.e. mismeasurement of individual dietary habits by the food frequency, weather-related year-to-year variability in contamination levels, and the small sample size (of peanut products). Furthermore, as compared with the population size of West Sudan, subjects from Central Sudan were underrepresented among the

controls; because they eat fewer peanut products, the association between the aflatoxin index and HCC will be further attenuated. Individual dietary assessment and aflatoxin analysis in different food items has been used successfully in a case-control study in the Philippines, where a strong significant association between primary liver cancer and overall mean aflatoxin load in the diet was found (relative risk = 17.0, significant at 0.05 level)[19]. In that study, however, aflatoxin exposure mainly originated from two food products with high concentrations: 1) cassava and 2) peanut butter. In Central and West Sudan, however, peanut butter is the main aflatoxin containing staple food, and regional food patterns, weather and storage conditions might be the main determinants of exposure to aflatoxins.

Hepatitis infection can be a confounder in the relation between aflatoxins and HCC. Indeed, we observed the highest frequency of self-reported hepatitis infections in West Sudan, the region with high consumption of peanut products and the alleged high incidence of HCC. The high prevalence of self reported hepatitis (21 of 34 controls), however, suggests low specificity for this question, resulting in misclassification. For this reason, hepatitis did not appear as a risk factor in the case-control study and adjustment for confounding was insufficient. Furthermore, the association between aflatoxin and HCC might be modified by hepatitis. Ross and co-workers[4] observed a strong interaction between hepatitis B surface antigen positivity and biomarkers of aflatoxin exposure in risk of liver cancer. In future studies in Sudan, the role of hepatitis infections need be addressed using blood tests.

In conclusion, the small size of our study does not permit firm conclusions. However, the results support the initial idea that contamination of peanut products with aflatoxin might indeed contribute to HCC in Sudan. If these results are confirmed in a larger study, improving methods of local storage of peanut products may contribute to lowering liver cancer in Sudan.

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References

1. World Health Organization. *Prevention of liver cancer.* (*WHO Tech. Rep. Ser.* 691) Geneva. World Health Organisation. Switzerland. 1983.
2. Van Rensburg SJ, Cook-Mozaffari P, van Schalkwyk DJ, van der Watt JJ, Vincent TJ, Purchase IF. Hepatocellular carcinoma and dietary aflatoxin in Mozambique and Traskei. *Br. J. Cancer.* 1985; **51:** 713-26.
3. Peers F, Bosch X, Kaldor J, Lindsell A, Pluijmen M. Aflatoxin exposure, hepatitis B virus infection and liver cancer in Swaziland. *Int J Cancer.* 1987; **39:** 545-53.
4. Ross RK, Yuan JM, Yu MC, Wogan CN, Qian GS, *et al.* Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet.* 1992; **339:** 943-46.
5. Shin HR, Lee CU, Park HJ, Seol SY, Chung JM, *et al.* Hepatitis B and C Virus, Clonorchis sinensis for the Risk of Liver Cancer: A Case-Control Study in Pusan, Korea. *Int. J. Epidemiol.* 1996; **25:** 933-40.
6. Doll R, Payne PM, Waterhouse JAH. *The cancer incidence in five continents.* UICC publication. Berlin Spinger-Verlag, 1966.
7. Newberne PM. Chemical carcinogenesis: mycotoxins and other chemicals to which humans are exposed. *Seminars in liver disease.* 1984; **4:** 122-35.
8. Stuver SO, Trichopoulos D. Liver Cancer. *Cancer Surveys.* 1994; **19/20:** 99-124.
9. Habish HA, Abdalla MA. The incidence of aflatoxin in Sudanese groundnut. *Trop. Sci.* 1971; **13:** 279-87.
10. Ahmed AI. Studies on Aspergillus flavus link in relation with aflatoxin production in groundnut in the Sudan. M. Sc. Thesis, University of Khartoum, 1981.
11. Omer RE. Study on the effect of field and storage environments on aflatoxin contamination of Rahad Agriculture Scheme Groundnut. M. Sc. Thesis, University of Khartoum, 1999.
12. National Health Laboratory. Sudan Cancer Registry Records, Ministry of Health. 1970-1984.
13. Mohamadien A. Biochemical tumour markers in HCC and other liver disease. M.D. Thesis, University of Khartoum, 1993.
14. Parker DL, Mary WT, Stanly N, Michael S, Richard FN. Solvent-efficient Thin Layer Chromatographic Method for the determination of aflatoxin B1, B2, G1 and G2 in corn and peanut products: Collaborative study. *Journal of AOAC International.* 1994; **77:** 638-46.
15. Steyn PS. Mycotoxins, general view, chemistry and structure. *Toxicology Letters.* 1995; **82/83:** 843-51.
16. Gourama H, Bullerman LB. *Aspergillus flavus* and *Aspergillus* parasiticus: aflatoxigenic Fungi of concern in Foods and Feeds: A Review. *J. Food Protection.* 1995; **58:** 1395-404.
17. Wilson M, Payne GA. Factors affecting *Aspergillus flavus* group infection and aflatoxin contamination of crops In: *The toxicology of aflatoxins, human health, veterinary, and agricultural significance*, Eaton DL, Groopman JD (eds.). Academic Press, Inc., San Diego. 1994; 309-25.
18. Lebedev AN. The climate of Africa. Part 1, Air temperature, precipitation. Jerusalem, Israel Program for Scientific Translations Ltd, 1970.
19. Bulatao JJ, Almero EM, Castro MC, Jardeleza MTR, Salamat LA. A case-control dietary study of primary liver cancer risk from aflatoxin exposure. *Int. J. Epidemiol.* 1982; **11:** 112-19.

3

Peanut butter intake, GSTM1 genotype and hepatocellular carcinoma: A case-control study in Sudan

R.E. Omer, L.P.B. Verhoef, P. van't Veer, M.O. Idris, A.M.Y. Kadaru, E. Kampman, J.E. Bunschoten, F.J. Kok.
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Abstract

Hepatocellular carcinoma (HCC) is one of the major cancers in the world. In Sudan, the incidence is thought to be high and increasing.

Objective. This study aims to assess the association between peanut butter intake, as a source of aflatoxins, and the genetic polymorphism of glutathione-S-transferase M1 (GSTM1) in the etiology of HCC.

Method. A case-control study was conducted among 150 patients and 205 controls from two regions in Sudan. Food habits with special reference to peanut butter consumption as well as peanuts storage systems have been investigated, as well as confounders such as hepatitis, drinking and smoking habits, and demographic characteristics. GSTM1 genotype was assessed in DNA extracted from blood samples (110 cases, 189 controls).

Results. A positive association was observed for highest vs lowest quartile of peanut butter intake, humid storage system and HCC, with OR's (95% CI) being 3.0 (1.6-5.5) and 1.6 (1.1-2.5) respectively. The positive association with peanut butter intake was essentially limited to subjects with GSTM1 null genotype with OR for highest vs lowest quartile 16.7 (2.7-105).

Conclusion. Peanut butter consumption has been identified as a strong risk factor of HCC in a region with endemic aflatoxin contamination in Sudan and was essentially limited to subjects with the GSTM1 null genotype.

Key Words: Peanut butter, genotype, liver carcinoma, Sudan

Introduction

Aflatoxins are food-borne toxins, produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*[1-3], and are regarded as potent toxins, mutagens, teratogens and carcinogens[4]. The most carcinogenic member of this group is aflatoxin B_1 (AFB_1)[5], which is also the most commonly found. Aflatoxins have been implicated in the etiology of hepatocellular carcinoma (HCC), which is one of the major cancers in the world and a public health concern in many developing countries. Several epidemiological studies in areas of high aflatoxin exposure particularly in Africa, Southern China and Taiwan[6-8] have further substantiated this idea. The geographical variation of HCC also suggests the involvement of environmental factors, such as exposure to aflatoxins, interaction with hepatitis B (HBV) and hepatitis C viruses (HCV), drinking of contaminated water, and alcohol consumption.

In Sudan, oil seeds are of great importance in the traditional and irrigated agriculture practices. Peanut is the most important of the oilseeds, both for export and for local consumption. Peanut butter, one of the main peanut products, is considered to be a popular meal in different parts of the country especially among the poor socio economic groups. Improper agricultural practices, storage and processing lead to multiplication of aflatoxin producing fungi and hence production of high levels of mycotoxins. Peanuts kernels (used for peanut butter processing) are frequently damaged by insects, rodents and moulds and are found to be seriously contaminated with aflatoxins[9]. A wide range of aflatoxin levels has been reported in peanut samples from West Sudan[10]. In an explorative study conducted in West and Central Sudan, peanut butter samples from the western region scored the highest aflatoxin levels, three times as high as in Central Sudan, coinciding with the higher incidence of liver cancer in this region. The same study suggested that storage of peanuts inside humid types of stores contributes to higher aflatoxin levels in peanut butter in West Sudan (five fold the WHO permissible level)[11].

The μ-class enzyme glutathione-S-transferase M1 (GSTM1) plays an important role in the defense of the body against reactive compounds. Detoxification of AFB_1 metabolites in hepatocytes by conjugation to glutathione is one of the activities of this phase II enzyme[12]. The GSTM1 gene has been shown to be polymorphic[13]: individuals may lack GST μ enzyme activity due to homozygous deletion. Lack of the enzyme activity leaves more of the mutagenic metabolite to bind to DNA[14]. Individuals homozygous for the null allele may therefore be at

increased cancer risk when exposed to potential carcinogenic compounds. Former studies suggest that the risk of cancer conferred to individuals homozygous for GSTM1 gene deletion appears to be small, though interactions of GSTM1 with exposure factors and risk for lung cancer have been described[15]. A small case-control study among hepatitis infected subjects in Taiwan suggested that the GSTM1 genotype might be a modifier of the association between aflatoxin exposure and HCC[16].

In order to assess the potential involvement of dietary aflatoxin exposure in the etiology of HCC in Sudan, we compared peanut butter consumption for cases and controls in West and Central Sudan, as a high and low incidence area, respectively. GSTM1 genotypes of cases and controls were compared to determine the potential modification of the association between aflatoxins and HCC by genetic susceptibility.

Methods

Geographical background

A case-control study was conducted during a period of two years (Sept. 1996-Sept. 1998), in West and Central Sudan representing regions of higher and lower incidence of liver cancer respectively. West Sudan (North Kordofan State) is an area of 230,000 km^2, and a population size of 1,500,000. Central Sudan (Gazira State) is an area of 152,000 km^2, and a population size of 2,500,000. All cases and controls resided in these regions. Since adequate diagnosis and treatment of liver cancer in not available in the regional hospitals, suspected cases are usually referred to one of the hospitals in Khartoum, the capital of the country, situated 650 Km from West Sudan and 500 Km from Central Sudan.

Case recruitment

A number of 150 patients between 21-70 years were enrolled from the two study regions, in a period of two successive years. Cases were recruited from 5 out of 6 hospitals in Khartoum (Tropical Medicine Hospital, Oumdurman Hospital, Khartoum Hospital, Soba Hospital and Ibn Sena Hospital). The admission lists from the departments of Internal Medicine in these hospitals were screened weekly to identify newly admitted HCC cases; then the responsible doctor and patients were contacted. Blood samples were obtained from 115 of these patients; missing blood samples were attributable to study logistics only. For all included subjects diagnosis of HCC was verified clinically, by liver function test and by ultrasound. Due to

severity of the disease, liver biopsy examinations and histological confirmations were obtained from 95% of the cases. All cases identified by these procedures agreed to participate in the study.

Control recruitment

Controls were community-based subjects, recruited from the same catchment areas as the cases; the number of controls was chosen proportional to the population size of the two areas. Therefore, four localities from West Sudan and six localities from Central Sudan were chosen randomly by means of a map of each region. In each locality there are 10-15 sugar shops, each serving a population size of 2,000-2,500 households. The sugar shops hold a complete registry of households with only names and sex (i.e. they were not identified by age); registration by the shops is required for all inhabitants in the locality, irrespective of their income. From each locality, one sugar shop was chosen randomly and twenty households were taken randomly from the shop list. Since liver cancer is more prevalent among men, this selection was made in a ratio 1:3 women to men. A total of 205 controls aged 25-70 years were enrolled, from 199 blood samples were obtained (80 from West and 119 from Central). All chosen controls agreed to cooperate in the study.

Diet and life style factors

Based on the experience obtained during the explorative study[11], we designed a questionnaire to assess peanut butter intake in a standardized manner. All participating subjects were interviewed personally, at hospitals for cases and at home for controls. All interviews were done by one person (R.O.) using the standardized questionnaire. Almost all patients were able to provide answers to the questionnaire except for a few who required the help of a family member who is always present in the hospital to assist the patient (so-called "co-patient"). The questionnaire inquired on the frequency and quantity of peanut butter on a daily or weekly basis. Frequency was inquired both "in season" and "off season", the former corresponding to the period of the year with active agricultural practices following the start of the rainy season, i.e. June – September (3 months), the latter is during the dry season, i.e. October-May (9 months). Quantity was assessed in two ways, first in terms of meal pattern, i.e. it was asked whether peanut butter is eaten as a part of the meal or as a full meal; second, the amount of peanut butter consumed per meal was inquired and divided by the number of subjects usually sharing the meal. To calculate average monthly intake, a weighted average of the frequency

consumption "in" and "off" season was used (see Table 2, note Υ). In addition to peanut butter consumption, we also assessed the mode of storage of peanuts because this may be relevant to the aflatoxin content of the peanut butter bought at the local markets. Storage was classified as "Mud Building", "Dry Grass House", and "Brick Stones House". A Mud Building is constructed of raw mud, the floor is not covered by any means of infrastructures, and it has a thatched roof. A Dry Grass House has walls and a roof made of straw supported by rafter and studs, the floor is not a concrete one. A Brick Stones House is constructed of mud bricks stacked together by means of a mixture made of mortar plasticizer and sand, the floor is concrete, and the roof is covered with metal sheets. As shown in our explorative study, the Mud building storage type is associated with highest aflatoxin levels, especially in West Sudan where agricultural practices are very poor[11]. The questionnaire also identified the potential confounders and other risk factors of HCC, e.g. hepatitis infections, smoking and drinking habits.

Blood samples

Ten ml of blood was obtained from cases and controls using the venoject system, with tubes containing a gel layer for separation of blood serum and cells (i.e. no coagulant material was used).

From each patient, the obtained blood sample was centrifuged immediately after venapuncture at (2000 round per minute) for 10 minutes; the separated blood cells were then kept at minus 20°C. Immediately after venapuncture, blood samples from controls were put in isolated foam boxes (at 4°C); on the same day, the samples were transported to the regional hospital laboratory where the serum and the cells were separated as mentioned above. These vials were kept at minus 20°C until they were transferred in isolated foam boxes at less than 0°C to Khartoum National Health Laboratory where they were stored at minus 20°C. The blood samples from both cases and controls were transported to Wageningen, the Netherlands in dry ice, for further analysis.

Laboratory analysis

Blood samples of 110 cases and 189 controls were available for GSTM1 genotyping. Loss of 5 cases and 10 controls was caused by reasons unrelated to study design. DNA was extracted from white blood cells present in a 200 µl blood residue using the QIAamp blood kit (Qiagen

Inc. Valencia, California, USA). The total yield of DNA varied between 12 and 70 µg. The presence or absence of GSTM1 and GSTT1 was determined as proposed by Arand *et al.*[17], but instead of amplification of the albumin gene, ß-Globin was coamplified as an internal positive control[18]. Water controls were coamplified as external negative controls. GSTT1 results were not used for analysis in this study. PCR reactions were carried out in 25 µl volume containing approximately 100 ng DNA, 10 pmol of each GSTM1 primer, 10 pmol of each GSTT1 primer, 5 pmol of ß-globin gene primers, 7.5 µmol deoxynucleoside triphosphates, 0.25 units of DNA TAQ polymerase (SphaeroQ) and 2.5 µl of Super TAQ buffer (100 mM Tris-HCl pH 9, 15mM $MgCl_2$, 500 mM KCl, 0.1% gelatin, 1% Triton X-100) (HT Biotechnology Ltd. Cambridge, UK). After 4 minutes at 94°C for primary denaturation, the samples were processed through 36 temperature cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C. The last elongation step was extended to 5 min. All reactions were performed in a thermal cycler (PTC-100, MJ Research, Inc. Watertown, Massachusetts, USA). The amplified products were visualized under UV light after DNA electrophoresis on an ethidium bromide-stained 2% agarose gel. The fragments of GSTM1, ß-globin and GSTT1 were 215, 350 and 480 bp in size respectively.

Hepatitis B and hepatitis C virus infection, was determined in blood serum, and was based on Hepatitis B surface antigen (HBsAg) and HCV antibodies, as determined by Hepanostika HBsAg and ORTHO HCV 3.0 ELISA test System with Enhanced SAVe test kits (Organon Teknika, Boxtel, The Netherlands). The tests were performed according to the manufactures instructions.

Data analysis

The usual frequency and amount of peanut butter consumption, mode of storage and genotype were compared between cases and controls followed by calculation of crude odds ratio's, X^2 testing and determination of confidence intervals. Subsequently, analyses were stratified for age, region, gender, hepatitis infections, education and job type as potential confounders and/or modifiers. A variable was considered an effect modifier if the X^2 of the interaction term was significant at $p<0.05$. A variable was considered a confounder if it altered the OR in the trend test by 10% or more. The test for trend was conducted by scoring the subsequent categories of intake from 1 to 4 and entering this score as a continuous variable in a logistic regression model. Finally, multivariate analysis was used to establish the association between usual

consumption of peanut butter and HCC, accounting simultaneously for the potential confounders, and in strata for potential modifiers. The final model is adjusted for age, region, hepatitis infections, storage type and stratified for genotype. All analyses were performed by using SAS 6.12 (SAS Institute Inc., Cary, NC, USA). All odds ratios are presented with 95% confidence intervals (95% CI). The final ORs for test for trend can be interpreted as the relative risk for developing HCC at high amount of peanut butter consumption compared to one class lower amount of peanut butter consumption in West and Central Sudan. OR to the power 3 gives relative risk of the highest vs lowest quartile of peanut butter consumption.

Results

Table 1 provides main characteristics of cases and controls. Regarding variables that are related to the study design and conduct, the cases appeared to be older because the intended frequency matching on age could not be realized during the fieldwork. A higher percentage of cases were obtained from West Sudan, while the distribution among controls represents the population size of the two areas, as intended. HCC was three times more frequent among males and matching on gender was successful.

Concerning the potential confounders, indicators of poor socio-economic status, i.e. illiteracy and being farmer or laborer were more prevalent among cases ($P<0.001$). As expected, infection with hepatitis B or C was much more prevalent among cases, with OR=12.9 (95% CI 6.9-24.2). For drinking and smoking habits, prevalence among cases and controls was similar ($P> 0.05$).

Table 1. Main characteristics of cases and controls.

Potential risk factor	Cases (n=150) No.	(%)	Controls (n=205) No.	(%)
Design related variables				
Age				
<40	15	(10)	81	(40)
40-59	53	(35)	99	(48)
60+	82	(55)	25	(12)
Region				
West	93	(62)	80	(40)
Central	57	(38)	119	(60)
Gender				
Male	113	(75)	153	(75)
Female	37	(25)	52	(25)
Potential confouders				
Education				
Illiteracy	107	(71)	72	(35)
Literacy	43	(29)	133	(65)
Job type				
Farmer + Laborer	82	(55)	74	(36)
Others	68	(45)	131	(64)
Drinking history				
Yes	57	(38)	63	(31)
No	93	(62)	142	(69)
Smoking history				
Yes	55	(37)	78	(38)
No	95	(63)	127	(62)
Hepatitis B or C				
Yes	61	(53)	16	(8)
No	54	(47)	183	(92)

Table 2 shows the reported food habits, storage methods and genotype. The frequency of peanut butter consumption is found to be substantially higher for cases both in-season and off-season; meal serving size indicated either by meal pattern or portion size was also higher among cases. For all indicators of peanut butter consumption, the ORs exceeded 2.0 and were statistically significant. The above variables, combined to give the average consumption, show odds ratios increasing from 1.0 to 3.0 with test of trend being statistically significant (OR=1.5, 95% CI=1.2-1.8). Other dietary factors, susceptible to contamination by mycotoxins (sorghum, millet and corn), were not materially associated to HCC; the age and region adjusted odds ratios for their associations with HCC were 0.8 (0.4-1.7), 1.6 (0.8-2.9) and 1.5 (0.9-2.5) for sorghum, millet and corn consumption respectively. Regarding storage, humid storage was found to be more prevalent among cases than Brick Stones and Dry Grass House (dry) storage types, with OR = 1.6 (95% CI=1.1-2.5). The GSTM1 null genotype, which indicates aflatoxin detoxification inability, was not associated with risk of HCC (OR=0.9, CI=0.6-1.5).

Table 3 compares potential risk factors of HCC and peanut butter consumption among the control group. Age categories were approximately similar represented in both high and low peanut butter consumption levels. Although socio-economic status shows a slight association with peanut butter consumption, other dietary sources susceptible to be contaminated with aflatoxins or other mycotoxins such as sorghum, millet and corn were similarly present among high and low peanut butter consumption. Other variables such as region, gender, smoking and drinking habits showed almost no differences among the groups. Hepatitis infection was slightly more frequent among those with high consumption of peanut butter. The GSTM1 null genotype had similar frequency (± 40%) in subjects with high and low intake of peanut butter.

Table 2. Factors relevant to the exposure to aflatoxins among cases and controls.

Potential risk factor	Cases (n=150)* No.	(%)	Controls (n=205)* No.	(%)	Odds Ratio (95% CI)
Peanut butter consumption					
Frequency consumption in season					
< 3 times /week	76	(52)	152	(75)	1.0
>= 3 times /week	70	(48)	52	(25)	2.7 (1.7-4.2)
Frequency consumption off season					
< 3 times /week	96	(66)	162	(79)	1.0
>= 3 times /week	50	(34)	42	(21)	2.0 (1.2-3.3)
Meal pattern in season					
Part of a meal	49	(34)	117	(57)	1.0
Full meal	97	(66)	87	(43)	2.6 (1.7-4.1)
Meal pattern off season					
Part of a meal	78	(53)	141	(69)	1.0
Full meal	68	(47)	63	(31)	2.0 (1.3-3.0)
Portion size [quantity /person]					
Low portion size (< 25g)	50	(34)	106	(52)	1.0
High portion size (>25g)	96	(66)	98	(48)	2.1 (1.3-3.2)
Average consumption (g/month)$^{\Upsilon}$					
< 70	22	(15)	53	(26)	1.0
71-150	25	(17)	48	(24)	1.3 (0.6-2.5)
151-300	36	(25)	52	(25)	1.7 (0.9-3.2)
> 300	63	(43)	51	(25)	3.0 (1.6-5.5)
Main staple food consumption					
Sorghum					
No	26	(17)	25	(12)	1.0
Yes	124	(83)	180	(88)	0.7 (0.4-1.2)
Millet					
No	100	(67)	164	(80)	1.0
Yes	50	(33)	41	(20)	2.0 (1.1-3.2)
Corn					
No	100	(67)	137	(67)	1.0
Yes	50	(33)	68	(33)	1.0 (0.6-1.6)
Storage type					
Dry type[2]	51	(34)	94	(46)	1.0
Humid type[1]	99	(66)	111	(54)	1.6 (1.1-2.5)
Genotype					
GSTM1$^{\bullet}$					
Present	65	(59)	115	(61)	1.0
Null	45	(41)	74	(39)	0.9 (0.6-1.5)

* = Peanut butter consumption data from 4 cases and 1 control were missing.

Υ= Calculated as: [3*freq "in season" + 9*freq "off season"] * [portion size] * $^{1}/_{12}$ (see methods section)

[1] = Mud building storage system [2] = Brick stones + dry grass house storage systems.

$^{\bullet}$= 110 cases and 189 controls.

Table 3. Potential risk factors of hepatocellular carcinoma stratified by peanut butter intake.

Potential risk factor	Low average peanut butter consumption (n=101)		High average peanut butter consumption (n=103)	
	No.	(%)[#]	No.	(%)[#]
Age				
<40	45	(44)	36	(35)
40-59	44	(44)	54	(52)
60+	12	(12)	13	(13)
Region				
West	44	(44)	38	(37)
Central	57	(56)	65	(63)
Gender				
Male	76	(75)	76	(74)
Female	25	(25)	27	(26)
Education				
Illiteracy	31	(31)	40	(39)
Literacy	70	(69)	63	(61)
Job type				
Farmer + Laborer	32	(32)	41	(40)
Others	69	(68)	62	(60)
Main staple food				
Sorghum				
Yes	86	(85)	93	(90)
No	15	(15)	10	(10)
Millet				
Yes	18	(18)	23	(22)
No	83	(82)	80	(78)
Corn				
Yes	34	(34)	33	(30)
No	67	(66)	70	(70)
Drinking history				
Yes	29	(29)	34	(33)
No	72	(71)	69	(66)
Smoking history				
Yes	41	(41)	37	(36)
No	60	(59)	66	(64)
Hepatitis B or C infection[*]				
Yes	6	(6)	10	(10)
No	92	(94)	90	(90)
Genotype[**]				
Null	39	(42)	35	(37)
Present	55	(58)	59	(63)

[#] For each potential risk factor stratum frequencies for high and low peanut butter consumption is shown.
[*] n=199 [**]n=188

Table 4 shows ORs in quartiles of peanut butter consumption with test for trend and X^2 for interaction. As compared to the crude analysis (Table 2), it appears that adjustment for age tends to increase the OR slightly; since matching for age was intended, we adjusted for this variable throughout further analysis. Moreover, the OR for the highest vs lowest level of exposure was 3.0 in crude analysis (Table 2) and increased only slightly (to 3.3) upon adjustment for age; furthermore, this OR approximately equals the OR for trend to the power of three (e.g., $1.50^3 = 3.4$). When the other potential confounders were added the results for all categories of intake remained essentially similar, as did the test for trend. When the potential confounders were added jointly (combinations of three) results were similar (data not shown). The result for the test for trend was similar increased after adjustment for GSTM1 genotype. Tests for interaction identified region, storage type and genotype as potential modifiers in the association of peanut butter consumption with risk of HCC.

Hepatitis infections, a supposed modifier of the aflatoxin-HCC association, did not show a significant interaction. After adjustment for age, OR and (95% CI) for the test for trend among hepatitis positive subjects was 1.23 (0.68-2.24), while it was 1.54 (1.13-2.11) among hepatitis negative subjects.

Table 4. Odds ratios and test of trend for peanut butter consumption and hepatocellular carcinoma, adjusted for possible confounders.

Peanut butter Intake (g/month)	Odds Ratios (95% Confidence Interval)				Test for trend[1]	Effect modification	
Adjusted for	< 70	71-150	151-300	> 300	(95% CI)	X^2	(p-value)
Age	1.0	1.3 (0.6-2.9)	1.9 (0.9-3.9)	3.3 (1.7-6.5)	1.50 (1.20-1.86)	-	
Age + region	1.0	1.5 (0.7-3.2)	2.0 (1.0-4.2)	3.1 (1.6-6.3)	1.46 (1.18-1.82)	11.87	(0.0001)
Age + sex	1.0	1.3 (0.6-2.9)	1.9 (0.9-3.9)	3.3 (1.7-6.6)	1.50 (1.21-1.86)	0.57	(0.4509)
Age + education	1.0	1.4 (0.6-3.2)	2.0 (1.0-4.3)	3.2 (1.6-6.4)	1.47 (1.18-1.83)	0.61	(0.4365)
Age + job type	1.0	1.3 (0.6-2.7)	1.8 (0.9-4.0)	3.0 (1.5-6.1)	1.47 (1.18-1.82)	0.00	(0.9912)
Age + storage	1.0	1.3 (0.6-2.9)	1.8 (0.9-3.7)	3.5 (1.8-7.1)	1.53 (1.23-1.91)	3.92	(0.0478)
Age + hepatitis[2]	1.0	1.6 (0.6-4.4)	1.9 (0.7-5.1)	3.3 (1.4-8.1)	1.47 (1.12-1.93)	0.56	(0.4545)
Age + GSTM1[3]	1.0	1.8 (0.7-4.5)	2.0 (0.8-5.0)	4.0 (1.8-9.2)	1.56 (1.21-2.00)	4.44	(0.0350)

[1] = Categories scored 1 to 4 respectively; the OR represents the 'average' OR comparing two adjacent categories of intake. OR to the power 3 approximately equals the OR for the highest vs lowest quartile of exposure.

[2] = The analyses were limited to a subset of 115 cases and 199 controls for whom blood samples were available and tested for hepatitis B and C.

[3] = The analysis were limited to a subset of 110 cases and 189 controls for whom blood samples were available for determination of genotype.

Because of the modifiers identified, Table 5 shows results of simple stratified analysis with adjustment for age only. It appeared that the association between peanut butter consumption and HCC was essentially confined to West Sudan, dry storage type and the GSTM1 null genotype. When all potential confounders and the three interactions (see Table 4) were included in a multivariate model, the OR for trend was 2.06 (0.95-4.47) and 1.73 (0.76-3.92) in West and Central Sudan, with the interaction being negligible (OR=1.19, 95% CI: 0.61-2.34). We therefore eliminated this interaction term in further models.

However, the interaction with storage type remained (OR for interaction=0.51, 0.27-0.98), with OR=1.73 (0.76-3.92) for dry storage and OR=0.88 (0.42-1.85) for humid storage. As these associations are both compatible with the null-hypothesis and in opposite direction, we decided to simplify the final model by eliminating this interaction term. Again, elimination of this interaction term did not materially alter the interaction term for GSTM1 and peanut butter intake, i.e. the strength of the association in the two strata for genotype remains the same. Moreover, This GSTM1-peanut butter interaction remained in strata for region, and was not affected when other dietary factors (sorghum, millet and corn) were included in the model.

Table 5: Odds Ratios and test of trend for peanut butter consumption and hepatocellular carcinoma, in strata for possible effect modifiers.

Peanut butter consumption	Age-adjusted odds ratios (95% confidence intervals)				Test for trend[2]
	< 70	71-150	151-300	> 300	(95% CI)
Simple stratified					
Region					
West	1.0	3.0 (1.0-9.3)	4.2 (1.4-12.1)	8.7 (3.4-22.5)	1.98 (1.47-2.66)
Central	1.0	0.6 (0.2-1.8)	0.8 (0.3-2.2)	0.7 (0.2-2.1)	0.93 (0.66-1.32)
Storage					
Humid	1.0	1.4 (0.5-3.7)	1.4 (0.6-3.5)	2.2 (0.9-5.6)	1.28 (0.96-1.71)
Dry	1.0	1.4 (0.3-5.4)	2.9 (0.8-11.0)	6.8 (2.1-21.6)	1.98 (1.38-2.85)
GSTM1					
Null	1.0	4.0 (0.6-27.2)	6.0 (1.0-36.3)	18.9(3.1-116.6)	2.44 (1.48-4.03)
Present	1.0	1.3 (0.5-3.7)	1.3 (0.4-4.0)	2.1 (0.8-5.4)	1.27 (0.95-1.71)
Multi-variate					
GSTM1[1]					
Null	1.0	2.8 (0.4-19.5)	4.1 (0.7-25.5)	16.7 (2.7-104.8)	2.47 (1.44-4.25)
Present	1.0	1.2 (0.3-4.7)	1.1 (0.3-4.3)	1.0 (0.3-3.3)	0.98 (0.67-1.44)

[1]= X^2 for interaction=7.00 (P=0.0081); model includes: age, region, storage type, hepatitis.
[2]= See Table 4, note 1.

The final model in Table 5 shows ORs in strata for GST, controlled for confounding effects. In this model, storage type, genotype, region and hepatitis infection were added simultaneously, and as before region ceased to be a modifier (P for 'interaction'>0.60); hepatitis was still found to be a confounder, but addition of education and job type did not change the results of the test for trend. GSTM1 null genotype remained a modifier throughout all analyses. In this final model, the association of peanut butter consumption and HCC is significantly higher in subjects with GSTM1 null genotype, compared to subjects with GSTM1 present. Risk by humid storage type remains approximately unchanged, with OR=1.5 (95% CI 0.7-2.9).

Discussion

A positive association has been observed in this study for highest vs lowest quartile of peanut butter intake, humid storage system and HCC, with ORs (95% CI) being 3.0 (1.6-5.5) and 1.6 (1.1-2.5), respectively. The positive association with peanut butter intake is essentially limited to subjects with the GSTM1 null genotype with OR (95% CI) being 16.7 (2.7-105) for the highest vs lowest quartile.

Cases were enrolled, using the registration lists of five out of six hospitals in Khartoum. In these five hospitals almost all cases with HCC from the two study regions are diagnosed and treated. Moreover, Ibn Sena hospital (one of the five hospitals) is a National Center for Gastrointestinal and Liver diseases so a large proportion of suspected HCC patients is referred to this hospital. The only non-participating hospital serves a catchment area that is of minor relevance to patients from West and Central Sudan. Therefore, we expect that our weekly screening of the hospital admission lists will only have missed few patients, i.e. those who might have been deceased before they could reach the hospital and those who had been missed during the periods of control recruitment (a total of four weeks during the two-year study period); in Sudan, this can not be verified by health administration systems, however.

Controls were chosen on a random basis, in a manner that it is independent of exposure to dietary habits, with the number of controls from West and Central Sudan proportional to the population size in these regions. For those regions several sugar shops were chosen randomly (see: methods part). As social, cultural and demographic characteristics are homogeneous within the two regions, the random chosen sugar shops are thought to be representative for food habits and aflatoxin exposure in the regions at large. In theory, referral patterns of cases

and controls might be associated with aflatoxin exposure, e.g. mediated by socioeconomic factors such as education, job or sex. However, these factors were not materially associated with peanut butter consumption (Table 3) and statistical adjustment for these factors did not alter the results (Table 4).

In order to assess the usual intake of peanut butter, frequency and portion size were assessed over a time frame of at least one year. For all indicators of peanut butter consumption, a positive association with HCC was observed; moreover, the consumption frequency in and off-season were positively correlated, as was the quantity expressed by either portion size or by the meal pattern suggesting a stable food pattern over the year. In the Sudanese population, use of peanut butter is a common and traditional practice and there is no knowledge or concern on aflatoxin among the population. Given these stable food habits, the design and standardized format of the questionnaire and the fact that all subjects were questioned by only one person, systematic errors in assessment of peanut butter intake are not considered a serious problem to our investigation. Therefore, information bias is not a likely explanation for the positive association between peanut butter consumption and HCC; moreover, the observed interaction with GSTM1 genotype cannot be affected by information bias.

The assessment of storage conditions, however, might have been a less reliable indicator of exposure to aflatoxins. The kernels used for local production of peanut butter may be the poor ones that are not considered suitable for export. Moreover, the consumers are not always aware of the storage conditions and they may differ even when the peanut butter is bought from the same local market. As reported earlier, the western region is considered to have a higher incidence of HCC[11,19]; peanuts from this region were reported to contain high levels and a wide range of aflatoxins[10]. We considered that this might be caused by the use of mud storage type in this region, as in our small explorative case-control study a strong association was observed for humid vs dry type of storage with OR (95%CI) of 7.5 (1.4-40.2)[11]. However, this positive association is clearly much weaker in the present study (OR=1.6). Therefore, the results for storage condition need to be interpreted with some caution.

Age, region, education level and job types were evaluated as potential confounders of the association between peanut butter and HCC. The potential bias was accounted for by stratified analysis (Mantel Haenszel method), followed by logistic regression that accounted for several

confounders simultaneously. In fact only age appeared to be a mild confounder that required adjustment in further analyses.

The presence of three potential modifiers of the association between peanut butter intake and HCC complicated the analysis and interpretation. Moreover, in order to reduce chance findings in this relatively small data set, stratification by two factors simultaneously was not considered appropriate, and we have to interpret the findings using independent scientific information. In this section we will motivate our interpretation by first discussing the role of GST (our primary hypothesis), followed by region and storage conditions.

In this study, high consumption of peanut butter gives a 17-fold increased risk on HCC, which was limited to the subject with the GSTM1 null genotype. However, the prevalence of the GSTM1 null genotype was similar in cases and controls (about 40%), with OR=0.9 (95%CI 0.6-1.5), indicating that genotype alone is not a risk factor. Since the GSTM1 gene codes for a carcinogen-detoxification enzyme, increased risk may be present only in individuals with exposure to aflatoxins[18]. Thus, the effect modification by GSTM1 is in line with aflatoxins being the actual risk factor for HCC in this population. In other studies, subjects with the GSTM1 null genotype also tended to be at greater risk for HCC when exposed to aflatoxins[16]. Moreover, the interaction between peanut butter consumption and GSTM1 remained statistically significant in all multivariate analyses we conducted.

The results of Table 4 suggest that region is also an effect modifier of the association between peanut butter intake and HCC, since the positive association was confined to the western region. In theory, modification by region might be due to differences in ethnicity existing in the two regions, since West and Central Sudan are populated by subjects from African and Arabic origin, respectively. Racial differences in GSTM1 genotype have been described: Frequencies of homozygous deletions vary between 20% in Africans from Nigeria and 50% in Malay from Singapore[15]. Although differences might be present for other relevant genetic properties, the frequency of GSTM1 null genotype was equal in both regions. Moreover, while the modification by genotype remained, the modification by region ceased to exist in multivariate and stratified analysis.

Finally, a positive association was observed between humid storage conditions and HCC (Table 2). However, the interaction between storage type and peanut butter intake observed in Table 4 is difficult to interpret. Apart from a possible lack of reliability in its assessment, this may be due to its limited relevance to aflatoxin exposure and statistical reasons.

First, aflatoxin level in peanuts varies about 3 fold by storage type[11], whereas intake of peanut butter varies 20-fold. In line with this, peanut butter consumption was much more stronger associated with risk of HCC than was storage type, when both variables were included in the model. Second, we were surprised that peanut butter processed from humid storage conditions showed a weaker association with HCC (OR=1.28) than peanut butter obtained from dry storage conditions (OR=1.98, Table 5). When both the interaction with GSTM1 and region were taken into account in the analysis, the estimates for humid and dry storage conditions came close to 1 and lost statistical significance. By doing this, results for other variables (GSTM1, the interaction term, age and hepatitis) remained essentially similar.

Results from some other studies have suggested synergism between aflatoxin exposure and chronic HBV infection in the causation of HCC[26,27,28]. In our study, the OR for high vs low peanut butter consumption was 1.23 (0.74-2.03) and 1.46 (1.10-1.95) for hepatitis positive vs negative subjects. Thus, hepatitis and peanut butter consumption seem to be statistically independent risk factors, affecting risk of HCC in a multiplicative fashion. Of course, as the hepatitis positive subjects have a tenfold increased risk of HCC, the attributable risk of peanut butter consumption among these subjects will far exceed the attributable risk among the hepatitis-negative subjects. Regarding the population attributable risk due to aflatoxin contaminated peanut butter; it appears from our data that almost 50% of all HCC could be avoided when peanut butter consumption would be limited to the lowest quartile, or when aflatoxin intake could be reduced correspondingly. Among subjects with GSTM1 null genotype this would be even 80-90%.

The observed positive relationship between exposure to aflatoxin and cancer[20] has modified the believe that "aflatoxin ingestion may increase the risk of liver cancer"[3], to a conclusion that aflatoxin is a definite human carcinogen[20]. In Swaziland in regions of little variation of HBV infection, HCC incidence was found to be coinciding with daily intake levels of aflatoxin[6]. In Mozambique and Transkei, the mean daily aflatoxin intake, in particular AFB_1, was found to

be significantly related to the incidence of HCC[21]. On the other hand Campbell *et al.*[22], failed to find a positive association between aflatoxin exposure and HCC mortality rate in a cross-sectional study undertaken in China. Biomarkers of aflatoxin intake have been used to assess the postulated association with HCC more clearly. Urinary aflatoxins were measured in eight districts in Kenya, showing a reasonable correlation between HCC and AFB_1[23]. Hatch *et al.*[24] have also reported a correlation between aflatoxin urinary metabolites and HCC incidence in regions in Taiwan. In nested case-control studies in Taiwan, AFB_1-albumin adducts were found to correlate with HCC risk[16,25]. In a cohort study in an area with high HCC in China, the estimated AFB_1 intake was positively correlated to HCC mortality rates[7]. Thus, ecological and analytical epidemiologic studies support a positive association between aflatoxin and HCC[26-28].

In conclusion, in a region with endemic aflatoxin contamination of peanuts we identified peanut butter consumption to be a strong risk factor of HCC, essentially limited to subjects with the GSTM1 null genotype, i.e. those who are considered unable to detoxify the aflatoxins properly. Deletion of the GSTM1 gene is common in Sudan, as 40% of the study population appears to have the null genotype. This implies that the proportion of cancer attributable to the combination of aflatoxin exposure and this genetic polymorphism may be large in the general population in Sudan.

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References

1. Lilly VG, Bareett HL. *Physiology of the Fungi.* McGraw Hill. New York, 1969.
2. Goldblatt LA. *Aflatoxin.* Academic Press. New York, 1969.
3. World Health Organisation Task Group on Environmental Health Criteria for Mycotoxins. *Mycotoxins.* Environmental Health Criteria. Geneva, Switzerland. 1979; **11:** 1-127.
4. Ueno Y, Ueno I. Toxicity and biochemistry of mycotoxins. In: Uraguchi K, Yamazaki M. (eds.). *Toxicology, Biochemistry and Pathology of Mycotoxins.* Kodansha Press, Tokyo. 1978; 107-88.
5. International Agency for Research on Cancer (IARC). Monographs on the Evaluation of Carcinogenic risks to humans, *some naturally occurring substances. Food items and constituents, heterocyclic aromatic amines and mycotoxins.* Lyon. 1993; 245-395.
6. Peers FG, Bosch X, Kaldor J, Linsell A, Pluijmen M. Aflatoxin exposure, hepatitis B virus infection and liver cancer in Swaziland. *Int. J. Cancer.* 1987; **39:** 545-53.
7. Yeh FS, Yu MC, Mo CC, Luo S, Tong MJ, Henderson BE. Hepatitis B virus, aflatoxin and hepatocellular carcinoma in Southern Guangxi, China. *Cancer Res.* 1989; **49:** 2506-09.
8. Lunn RM, Zhang YJ, Wang LY, *et al.* P53 Mutations, Chronic Hepatitis B virus Infection, and Aflatoxin Exposure in Hepatocellular Carcinoma in Taiwan. *Cancer Res.* 1997; **57:** 3471-77.
9. Omer RE. Study on the effect of field and storage environments on aflatoxin contamination of Rahad Agricultural Scheme-Groundnut. M. Sc. Thesis, University of Khartoum. 1992.
10. Habish HA, Abdulla MA. The incidence of aflatoxin in Sudanese groundnuts. *Trop. Sci..* 1971; **13:** 279-87.
11. Omer RE, Bakker MI, van't Veer P, *et al.* Aflatoxin and liver cancer in Sudan. *Nutrition and Cancer.* 1998; **32:** 174-80.
12. McGlynn KA, Rosvold EA, Lustbader ED, *et al.* Susceptibility to hepatocellular carcinoma is associated with genetic variation in the enzymatic detoxification of Aflatoxin B1. *Proc. Natl. Acad. Sci.* 1995; **92:** 2384-87.
13. Bell DA, Thompson CL, Miller CR, *et al.* Genetic monitoring of human polymorphic cancer susceptibility genes by polymerase chain reaction: application to glutathione S-transferase μ. *Environmental Health Perspectives.* 1992; **98:** 113-17.
14. Guengerich FP, Johnson WW, Ueng Y, Yamazaki H, Shimada T. Involvement of cytochrome P450, glutathione S-transferase and epoxide hydrolase in the metabolism of aflatoxin B1 and relevance to risk of human liver cancer. *Environmental health perspectives.* 1996; **104 S3:** 557-62.
15. Rebbeck TR. Molecular epidemiology of the human glutathione S- transferase genotypes GSTM1 and GSTT1 in cancer susceptibility. *Cancer Epidemiology, Biomarkers and prevention.* 1997; **6:** 733-43.
16. Chen CJ, Yu MW, Liaw YF, *et al.* Chronic hepatitis B carriers with Null genotypes of glutathione S-Transferase M1 and T1 Polmorphisms who are exposed to aflatoxin are at increased risk of hepatocellular carcinoma. *Am. .J Hum. Genet.* 1996; **59:** 128-34.
17. Arand M, Muhlbauer R, Hengstler J, *et al.* A multiplex polymerase chain reaction protocol for the simultaneous analysis of the glutathione S-transferase GSTM1 and GSTT1 polymorpisms. *Analytical Biochemistry.* 1996; **236:** 184-86.

18. Bell DA, Taylor JA, Paulson DF, *et al.* Genetic risk and carcinogen exposure: a common inherited defect of the carcinogen-metabolism gene glutathione S-transferase M1 (GSTM1) that increases susceptibility to bladder cancer. *J. Nat. Cancer. Institute.* 1993; **85:** 1159-63.

19. Zaki M. Aetiological factors in HCC. M.D. Thesis, Faculty of Medicine, University of Khartoum. 1991.

20. International Agency for Research on Cancer (IARC). Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, *Aflatoxins*. Lyon, 1987; **S7:** 83-87.

21. Van Rensburg SJ, Cook-Mozaffari P, Van Schalkwyk DJ, Van der Watt JJ, Vincent TJ, Purchase IF. Hepatocellular carcinoma in Mozambique and Transkei. *Br. J. Cancer.* 1985; **51:** 713-26.

22. Campbell TC, Chen J, Liu C, Li J, Parpia B. Non association of aflatoxin with primary liver cancer in a cross-sectional ecological survey in the People's Republic of China. *Cancer Res.* 1990; **50:** 6882-93.

23. Atrup H, Seremet T, Wakhisi J, Wasunna A. Aflatoxin exposure measured by urinary extraction of aflatoxin B1-guanine adduct and hepatitis B virus infection in areas with different liver cancer incidence in Kenya. *Cancer Res.* 1987; **47:** 3430-33.

24. Hatch MC, Chen CJ, Leven B, *et al.* Urinary aflatoxin levels hepatitis B virus infection and hepatocellular carcinoma in Taiwan. *Int. J. Cancer.* 1993; **54:** 931-34.

25. Wang LY, Hach MC, Chen CJ, *et al.* Aflatoxin exposure and the risk of hepatocellular carcinoma. in Taiwan. *Int. J. Cancer.* 1996; **67:** 620-25.

26. Yu MW, Lien JP, Chiu YH, *et al.* Effect of aflatoxin metabolism and DNA adduct formation on hepatocellular carcinoma among chronic hepatitis B carriers in Taiwan, *J. Hepatol.* 1997; **27:** 320-30.

27. Ross RK, Yuan JM, Yu MC, *et al.* Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet.* 1992; **339:** 943-46.

28. Qian GS, Ross RK, Yu MC, *et al.* A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in shanghai, People's Republic of China. *Cancer Epidemiology Biomarkers and Prev.* 1994; **3:** 3-10.

4

Role of genetic polymorphism of glutathione-S-transferase T1 and microsomal epoxide hydrolase in aflatoxin-associated hepatocellular carcinoma

E.W. Tiemersma, R.E. Omer, J.E. Bunschoten, P. van't Veer, F.J. Kok, M.O. Idris, A.M.Y. Kadaru, S.S. Fedail, E. Kampman.
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Abstract

Exposure to aflatoxins is a risk factor for hepatocellular carcinoma (HCC). Aflatoxins occur in peanut butter and are metabolized by genetically polymorphic enzymes such as glutathione-S-tranferases, encoded by GSTM1 and GSTT1, and microsomal epoxide hydrolase encoded by EPHX. The rate at which aflatoxins become activated or detoxified may depend on polymorphisms in the encoding genes. GSTM1 homozygous was indeed found to modify the association between peanut butter consumption and HCC. In this paper, we investigate possible roles of GSTT1 and EPHX polymorphisms in this relationship.

In this study, we analysed data of incident 112 cases and 194 controls. All participants were interviewed using a standardized questionnaire inquiring about social and demographic factors, peanut butter consumption, and other known HCC risk factors.

Univariate analysis showed that GSTT1 polymorphism was not associated with HCC, whereas EPHX 113HH&139HH genotype increased risk of HCC (OR 3.10, 95% CI 1.18-8.12). Adjustment for age and region of origin slightly attenuated this association (OR 2.56, 95 CI 0.83-7.95). Interestingly, unlike GSTM1, both GSTT1 and EPHX polymorphism did not modify the association between peanut butter consumption and HCC.

In conclusion, these epidemiological findings do not suggest important roles of GSTT1 and EPHX in aflatoxin metabolism, although EPHX polymorphism is possibly related to increased risk of HCC. Further studies are needed to investigate mechanisms by which the EPHX polymorphism potentially modifies cancer risk.

Introduction

Hepatocellular carcinoma (HCC) is one of the major cancer types in developing countries, where most important risk factors are chronic hepatitis virus infection and exposure to aflatoxins[1]. Aflatoxins are produced by *Aspergillus* fungi which mainly occur in poorly stored maize and peanuts. In Sudan, aflatoxin exposure most likely occurs via consumption of peanut butter which is a popular food[2]. As we reported previously[3], peanut butter consumption is indeed related to increased risk of HCC in Sudan. Genetic differences in aflatoxin metabolism may explain the observed differences between prevalence rates of HCC[4] in populations with similar aflatoxin exposures and hepatitis infection rates.

The most potent mutagenic and carcinogenic of the aflatoxins is AFB_1. Figure 1 depicts the metabolism of AFB_1, which is mainly metabolized by cytochrome P450 3A4 into the genotoxic metabolite AFB_1-8,9-exo-epoxide. This metabolite can bind to DNA causing G to T transversions[5] that may ultimately lead to cancer. Detoxification prevents formation of DNA adducts; the metabolite may be conjugated to glutathione by glutathione-S-transferases or may be hydrolyzed. Hydrolysis occurs spontaneously or is catalyzed by microsomal epoxide hydrolase (mEH)[6,7].

Two genetically polymorphic glutathione-S-transferases play a role in AFB_1 detoxification: GST-μ, encoded by the GSTM1 gene, and GST-θ, encoded by GSTT1. Homozygous deletion of part of these genes (null genotype) results in enzyme deficiency and might therefore lead to hampered detoxification. Several studies, among which our own Sudanese case-control study, showed that of populations exposed to aflatoxins only subjects carrying the GSTM1 null genotype are at increased risk of HCC[3,8,9]. After GST-μ, GST-θ showed highest efficiency for conjugation of glutathione to AFB_1-8,9-exo-epoxide[10-12] (Figure 1). Homozygous deletion of the GSTT1 gene was recorded in 24 to 38% of people from African origin[13]. One study showed a positive association between GSTT1 null genotype and aflatoxin-albumin adduct level among chronic HBV antigen carriers[8], but this was not confirmed by another study[9]. Microsomal epoxide hydrolase might play a role in AFB_1-8,9-exo-epoxide hydrolysis[14]. The encoding EPHX gene has two polymorphic sites occurring with allele frequencies of about 30%[15,16]. In exon 3, the 113Y allele encodes tyrosine incorporation at position 113 of the resulting protein whereas 113H codes for histidine. The allelic variants in exon 4, 139H and

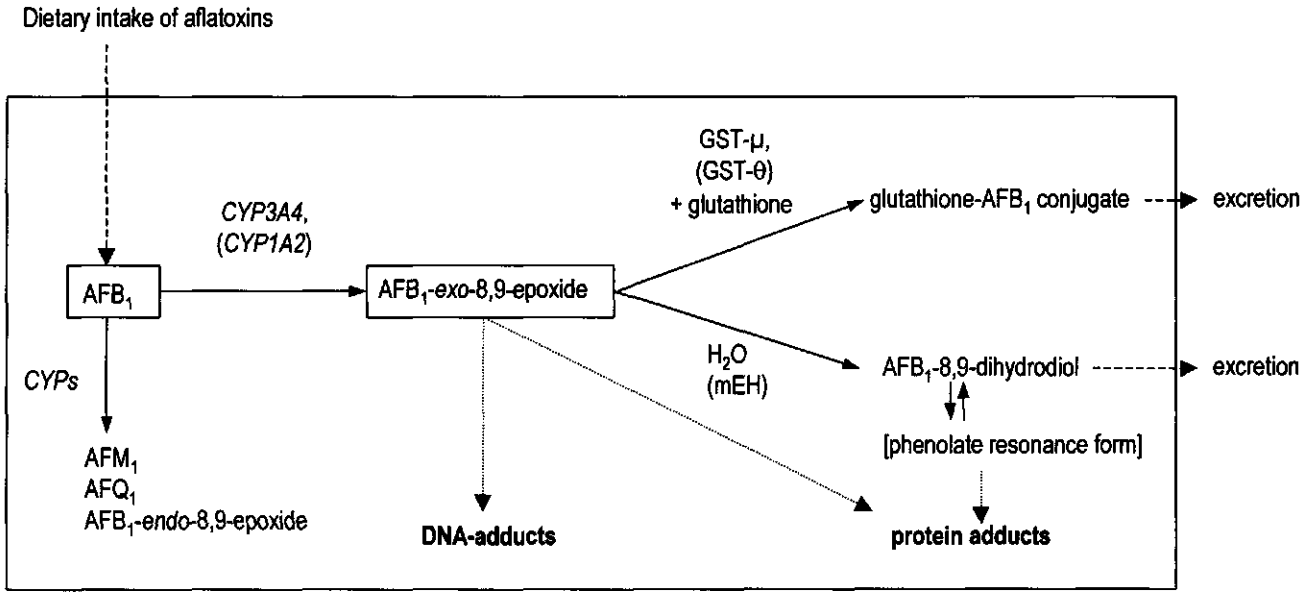

Figure 1: Proposed metabolism of aflatoxin B_1(AFB_1) in hepatocytes. Adapted from Guengerich *et al*[7] and Eaton *et al*[6].

139R, result in proteins with histidine and arginine at position 139, respectively[15]. Both the 113H and the 139H allele are related to relatively decreased enzyme expression or stability[15,17,18] and may therefore be associated with increased risk of HCC. Indeed, EPHX 113HH genotype increased risk of HCC in a small case-control study[19], but was not associated to aflatoxin-albumin adduct levels in another study[9].

GSTT1 and EPHX polymorphisms may especially be important among subjects carrying the GSTM1 null genotype. Few studies addressed the role of GSTT1 and EPHX polymorphism in HCC etiology and only one[9] simultaneously studied GSTM1 polymorphism. Moreover, there is some discrepancy between biochemical and epidemiological studies concerning possible roles for GST-μ, GST-θ, and mEH in AFB_1 detoxification and ultimately in aflatoxin-related HCC etiology. Therefore, we evaluate the possible roles of GSTT1 and EPHX polymorphism and their interaction with aflatoxin exposure (as estimated by peanut butter consumption) in HCC in a Sudanese case-control study, in all subjects and in the subgroup of GSTM1 null genotype carriers.

Methods

Population

A case-control study investigating risk factors for HCC was conducted in Sudan between September 1996 and September 1998. The design and conduct of this study were described in detail by Omer *et al.*[3]. In short, subjects residing in West and Central Sudan were eligible. HCC cases were diagnosed clinically by liver function test and ultrasound in five of the six hospitals in the capital Khartoum situated between West and Central Sudan. Additional diagnosis by liver biopsy and histological examination was available for 95% of cases. About 5% of HCC patients died before they could be contacted to participate in the study. All 150 contacted HCC patients provided oral informed consent. Community-based controls were selected randomly in a 1:3 woman-to-men ratio, since HCC is more prevalent among men, and in proportion to the respective population sizes of the two regions. Recruitment was done through sugar shops which hold a complete registration of inhabitants of their serving area. In four randomly chosen localities in West Sudan and six localities in Central Sudan, one out of the 10 to 15 sugar shops was selected randomly, and 20 households were selected from the sugar shop's registration list. In each household one control was recruited. All 205 invited subjects agreed to participate.

Data collection

Based on the experience obtained during the explorative study[20], a questionnaire was designed to assess peanut butter intake in a standardized manner. This questionnaire was administered orally by one of the authors (R.E.O.). All participants were interviewed personally, although some cases needed help of an accompanying family member because of serious illness. Cases were interviewed in the hospital and controls were interviewed at home. Frequency of peanut butter consumption was assessed on a daily or weekly basis and was inquired both 'in season' and 'off season', the former corresponding to the period of the year with active agricultural practices, usually June - September. To assess quantity of consumption we asked whether peanut butter is eaten as a part of the meal or as a full meal and we inquired the amount of peanut butter eaten per meal and the number of persons such a meal is shared with.

Monthly frequency of peanut butter consumption was calculated as a weighted average of the frequencies of peanut butter consumption 'in season' and 'off season'. The quantity of peanut butter consumed per month was subsequently calculated by multiplication of the average frequency of peanut butter consumption by the amount of peanut butter consumed per meal per person. The questionnaire also identified other HCC risk factors, such as hepatitis infection, smoking, and alcohol consumption.

For determination of genotypes and chronic hepatitis infection, blood samples were collected in 10-ml Venoject tubes. Blood samples of cases were drawn at the hospital, centrifuged immediately and subsequently stored at minus 20°C. Controls were sampled at home and blood samples were first transferred in coolers at 4°C to regional hospital laboratories where they were centrifuged and then transferred to Khartoum National Health Laboratory. Here, all samples were kept at minus 20°C until transport to Wageningen, the Netherlands, for further analysis.

Laboratory analysis

Blood samples of 115 cases (77% of cases) and 199 controls (97% of controls) were available for genotyping. DNA was isolated from 200 µl whole blood using the QIAamp blood kit (Qiagen Inc., USA), stored at 4°C, and directly used as a template in PCR analyses. A multiplex PCR was done to determine presence or absence of the GSTM1 and GSTT1 genes simultaneously according to Arand and co-workers[21]. As a positive PCR control, however, we

used primers derived from ß-globin[22] instead of histidine. Primers derived from GSTM1, GSTT1, and ß-globin were 5'-GAA CTC CCT GAA AAG CTA AAG C-3' and 5'-GTT GGG CTC AAA TAT ACG GTG G-3', 5'-TTC CTT ACT GGT CCT CAC ATC TC-3' and 5'-TCA CCG GAT CAT GGC CAG CA-3', and 5'-CAA CTT CAT CCA CGT TCA CC-3' and 5'-GAA GAG CCA AGG ACA GGT AC-3', respectively. After DNA electrophoresis on an ethidium bromide stained agarose gel, the amplified products were visualized under UV light. The GSTM1 fragment was 215 bp, the GSTT1 fragment was 480 bp, and the ß-globin fragment was 350 bp in size.

Genetic polymorphism in EPHX exon 3 (113Y and 113H alleles) and exon 4 (139H and 139R alleles) were determined by RFLP analysis after amplification of the exons. For amplification of exon 3 we used primers described by Smith and Harrison[23] (5'-GAT CGA TAA GTT CCG TTT CAC C-3' and 5'-ATC TTA GTC TTG AAG TGA GGA T-3'). A mismatch in the reverse primer incorporated an EcoRV restriction site in the amplicon of the 113Y allele resulting in digestion products of 23 and 140 bp. The 113H allele remained undigested. Amplification of exon 4 was done using primers described by Hassett *et al.*[15] (5'-GGG GTA CCA GAG CCT GAC CGT-3' and 5'-AAC ACC GGG CCC ACC CTT GGC-3'), followed by restriction analysis with RsaI. The 139H allele was digested into two fragments (295 and 62 bp) and the 139R allele was digested into fragments of 174, 121 and 62 bp in size. The amplified products were visualized under UV light after DNA electrophoresis on an ethidium bromide stained agarose gel.

Determination of chronic hepatitis B and C virus infection was done using Hepanostika HBsAg and ORTHO HCV 3.0 ELISA test systems with enhanced SAVe test kits, according to the instructions of the manufacturer.

Data analysis

Analyses were restricted to subjects who completed the questionnaire and of whom genotyping results were available for at least one polymorphism, i.e. 112 cases and 194 controls. The GSTM1 and GSTT1 null genotypes were a priori considered as the high-risk genotypes. For EPHX, we first conducted the analyses separately for both polymorphic sites. Thus, when analyzing the exon 3 polymorphism, we did not consider the exon 4 polymorphism and vice versa. Based on the available literature on the association between EPHX genotype and

phenotype, we considered 113HH and 139HH genotypes and the combination of these to be the EPHX high-risk genotypes[24]. Based on the median peanut butter consumption in controls, we classified all subjects as low or high peanut butter consumers. Analyses were done using the SAS statistical software package (release 6.12). After univariate analyses we adjusted for age (in tertiles, according to age distribution in the control group). Multiple variables (e.g., education level, job type, region of origin and hepatitis infection) were considered to be included in the model if numbers in the various cells were sufficiently large (i.e. more than 5 after one-way stratification). Variables remained in the model if their inclusion caused a change of 10% or more in the β-estimates. Although adjustment for age did not change the β-estimates this variable was forced into the model since cases were significantly older than controls and age is known to be related both to several HCC risk factors as to the disease itself.

Results

Table 1 shows the characteristics of the study population. Cases were older than controls, less educated, and more often resided in West Sudan. They also consumed significantly more peanut butter, both in amount as in frequency per month. Besides, more cases were chronic carriers of the hepatitis B or C virus. Frequencies of the GSTM1 and GSTT1 null genotypes were similar for cases and controls. There were no statistically significant differences in frequency of EPHX variants in exons 3 and 4. However, cases more often had the EPHX 113HH genotype in combination with the EPHX 139HH genotype than controls (Table 1). Allelic variants of the studied polymorphic genes occurred at similar frequencies among controls from West and Central Sudan. Maximal differences were observed for EPHX exon 3: 113HH and 113YH genotypes occurred in 10% and 30% of West Sudanese controls and in 6% and 23% of Central Sudanese controls, respectively. Adjustment for age did not change these results importantly.

In Table 2, ORs of HCC are shown for genetic polymorphism of GSTM1, GSTT1 and EPHX. Univariate ORs indicate that only the combination of the EPHX 113HH and 139HH increased risk of HCC. Adjustment for age and region in a multivariate model did not change the results on GST polymorphisms, although the association between the EPHX 113HH&139HH genotype and HCC lost statistical significance. Even though inclusion of a variable for hepatitis infection changed ORs importantly (by increasing ORs and 95%CIs for the GSTM1 null and EPHX 139HH and 139HR genotypes to statistically significant values), it was not

included in the multivariate model because this substantially inflated the standard errors of the β-estimates. This was probably due to limited data: only 16 controls showed evidence of hepatitis infection, of whom one presented the EPHX 113HH&139HH genotype and 5 carried the GSTM1 null genotype.

Table 1. General characteristics of the study population

	Cases (n=112)	Controls (n=194)
Demographic variables		
Mean age (SD)	57.0 (12.2)	44.9 (10.9) [c]
Gender, % males (se) [a]	76.8 (4.0)	75.3 (3.1)
Region, % from West Sudan (se)	63.4 (4.6)	41.2 (3.5) [c]
Education, % illiterate (se)	69.6 (4.3)	34.5 (3.4) [c]
Peanut butter consumption, mean (SD)		
Average consumption (kg/month)	0.68 (1.1)	0.29 (0.6) [c]
Average frequency (times/month)	11.3 (11.8)	7.9 (9.1) [c]
Other risk factors, frequency (se)		
Hepatitis B or C infections [b]	52.3 (4.7)	8.3 (2.0) [c]
Positive history of alcohol consumption	39.3 (4.6)	30.9 (3.3)
Positive history of smoking	40.2 (4.6)	39.2 (3.5)
Genotype, frequency (se)		
GSTM1 null [b]	42.7 (4.7)	38.8 (3.6)
GSTT1 null [b]	35.8 (4.6)	37.8 (3.5)
EPHX exon 3 [b]		
113YY	61.8 (4.6)	66.3 (3.4)
113YH	25.5 (4.2)	25.9 (3.2)
113HH	12.7 (3.2)	7.8 (1.9)
EPHX exon 4 [b]		
139HH	57.3 (4.7)	55.4 (3.7)
139HR	40.0 (4.7)	37.5 (3.6)
139RR	2.7 (1.5)	7.1 (1.9)
EPHX exon 3 & 4 combined, 113HH&139HH	10.9 (3.0)	3.8 (1.4) [c]

[a] standard errors were calculated as follows: $se=\sqrt{(p(1-p)/n)}*100$

[b] information on hepatitis infections was available of 109 cases and 193 controls, information on GSTM1 and GSTT1 genetic polymorphism of 109 cases and 188 controls, on EPHX exon 3 of 110 cases and 193 controls, and on EPHX exon 4 of 110 cases and 184 controls.

[c] significantly different from cases ($P < 0.05$)

Table 2. Genotypes of GSTM1, GSTT1, and EPHX and risk of HCC

	ORs (95% CI)		
	Univariate	Adjusted for age	Multivariate [a]
GSTM1			
Present	1.00 (Referent)	1.00 (Referent)	1.00 (Referent)
Null	1.18 (0.73-1.90)	1.26 (0.73-2.16)	1.26 (0.73-2.19)
GSTT1			
Present	1.00 (Referent)	1.00 (Referent)	1.00 (Referent)
Null	0.92 (0.56-1.50)	0.94 (0.54-1.64)	0.94 (0.54-1.65)
EPHX exon 3			
113YY	1.00 (Referent)	1.00 (Referent)	1.00 (Referent)
113YH	1.05 (0.61-1.82)	0.94 (0.51-1.73)	0.93 (0.50-1.71)
113HH	1.76 (0.80-3.85)	1.68 (0.69-4.09)	1.51 (0.61-3.75)
EPHX exon 4			
139RR	1.00 (Referent)	1.00 (Referent)	1.00 (Referent)
139HR	2.75 (0.75-10.25)	3.67 (0.89-15.06)	3.63 (0.88-15.02)
139HH	2.68 (0.73-9.76)	3.19 (0.80-12.79)	3.22 (0.80-13.01)
EPHX exon 3 & 4 combined			
All other combinations	1.00 (Referent)	1.00 (Referent)	1.00 (Referent)
113HH&139HH	3.10 (1.18-8.12)	2.97 (0.99-8.85)	2.56 (0.83-7.95)

[a] multivariate model adjusted for age (tertiles) and region of origin.

To investigate if genetic polymorphism of GSTT1 or EPHX modified the association between peanut butter consumption and HCC, we stratified for peanut butter consumption. Results are shown in Table 3. We previously showed that peanut butter consumption was a risk factor for HCC especially in GSTM1 null genotype carriers[3]. Interestingly, we observed no effect modification by GSTT1 or EPHX genotype (ORs for the interaction terms were 1.3 and 1.0, respectively). These results did not change importantly after adjustment for age and region.

In GSTM1 null genotype carriers, presence of the GSTT1 null genotype or the EPHX 113HH&139HH genotype did not increase risk of HCC; ORs and 95%CIs were 0.7, 0.3-1.9 and 2.0, 0.4-11.2, respectively. Strikingly, only those with GSTM1 non-null genotypes in combination with the EPHX 113HH&139HH genotype were at increased risk of HCC (OR 5.7, 95% CI 1.2-28.2). Similarly, subjects with GSTT1 non-null genotypes and the EPHX 113HH&139HH genotype had increased risk of HCC (OR 22.2, 95% CI 2.4-205.8). Since there were no cases and only three controls carrying a combination of all three high-risk genotypes, i.e., GSTM1 null, GSTT1 null, and EPHX 113HH&139HH, we could not evaluate if presence of this combination specifically increased risk of HCC.

Table 3. Genotypes of GSTM1, GSTT1 and EPHX as risk factors for HCC, stratified for peanut butter consumption[a]

Genotype	Peanut butter consumption[b]		Peanut butter consumption[c]	
	low	high	low	high
All genotypes	1.00 (Referent)	2.62 (1.50-4.57)	1.00 (Referent)	2.50 (1.43-4.38)
GSTM1				
Present	1.00 (Referent)	1.57 (0.77-3.17)	1.00 (Referent)	1.43 (0.70-2.93)
Null	0.55 (0.21-1.47)	2.75 (1.30-5.78)	0.55 (0.21-1.47)	2.60 (1.22-5.54)
GSTT1				
Present	1.00 (Referent)	2.71 (1.30-5.63)	1.00 (Referent)	2.71 (1.30-5.63)
Null	1.25 (0.51-3.11)	2.28 (0.99-5.23)	1.26 (0.51-3.11)	2. 28 (0.99-5.23)
EPHX exon 3				
113YY	1.00 (Referent)	2.47 (1.24-4.94)	1.00 (Referent)	2.26 (1.12-4.56)
113YH	0.74 (0.25-2.23)	2.34 (1.02-5.38)	0.66 (0.22-2.01)	2.28 (0.99-5.25)
113HH	1.47 (0.35-6.20)	4.54 (1.33-15.49)	1.35 (0.31-5.78)	3.75 (1.07-13.12)
EPHX exon 4				
139RR	1.00 (Referent)	1.90 (0.12-31.48)	1.00 (Referent)	1.70 (0.10-28.24)
139HR	2.48 (0.24-25.30)	7.85 (0.83-74.67)	2.37 (0.23-24.07)	7.18 (0.76-67.98)
139HH	2.88 (0.30-27.44)	6.54 (0.69-61.37)	2.82 (0.30-26.80)	6.02 (0.64-56.22)
EPHX exons 3 & 4 combined				
All other combinations	1.00 (Referent)	2.57 (1.43-4.62)	1.00 (Referent)	2.43 (1.34-4.39)
113HH&139HH	3.02 (0.51-18.01)	7.68 (1.65-35.69)	2.60 (0.41-16.69)	6.31 (1.32-30.05)

[a] low consumption defined as consumption of ≤135 g of peanut butter per month, high consumption defined as consumption of > 135 g of peanut butter per month, based on the median consumption among controls.
[b] adjusted for age (tertiles).
[c] adjusted for age (tertiles) and region of origin.

Discussion

GSTT1 null genotype alone or in combination with peanut butter consumption was not a risk factor for HCC in this Sudanese case-control study. EPHX polymorphism might play a modest role in HCC. However, no interaction with peanut butter consumption was observed, indicating that the mEH enzyme may not be important in aflatoxin detoxification.

As in all case-control studies, several types of bias might have occurred in this study. Selection bias probably did not occur, since almost all suspected HCC cases are referred to one of the participating hospitals. Therefore, we estimate that only few cases from the two selected regions were missed. Only cases being able to travel to Khartoum were included in this study and of these, we analyzed results of those providing blood and for whom genotyping results were available. These might be the relatively mildly diseased ones, since few cases might have

died before reaching the hospital and blood sampling failed for severely ill cases. It is unlikely that this introduced selection bias, since HCC is fatal in almost all cases and survival time is very short[1]. Moreover, we do not expect GST and EPHX genotypes to influence HCC survival. We think the control population correctly reflects the Sudanese population in the two regions with respect to habitual peanut butter consumption and exposure to other HCC risk factors, since both regions are inhabited by homogeneous populations with culturally determined, relatively constant food habits. Also, the frequency of the GSTM1 null genotype in our control group was in between frequencies reported for Europeans and sub-Saharan Africans[13,16,25] and the frequency of the GSTT1 null genotype was equal to prevalences found in other African studies[9,13]. EPHX genotype distributions varied between West and Central Sudan, although not with statistical significance. EPHX polymorphism is known to vary greatly across populations[9] and a high genetic variation in EPHX genotype frequencies between populations living at close distance from each other was also found by Masimirembwa and colleagues[16], who determined EPHX genotype in Venda and Zimbabwean blood donors.

Controls were frequency matched to cases for sex. No age matching was done, since information on age was not available from sugar shop registries. Because cases were older than controls we adjusted for age in all analyses, although this did not change the results importantly. Controls were enrolled from the same two regions as the cases, proportional to the population size of each region. These regions were chosen to investigate if differences in HCC prevalence may be explained by differences in aflatoxin exposure. Since Central Sudan has more inhabitants than West Sudan whereas HCC is more prevalent in West Sudan, controls were more likely to be from Central Sudan and cases were more likely to be West-Sudanese. Apart from HCC prevalence and peanut butter consumption, other life style factors (but not hepatitis prevalence) and genetic composition differed between the regions, since the West Sudanese population is African whereas Central Sudan is mainly Arabic. Thus, region of origin was an important factor in our analyses, both as a co-factor and as a potential effect modifier. Effect modification was not observed, but region indeed contributed to the model as a co-factor: adding this variable to the model weakened the associations between EPHX genotypes and HCC. This could be the result of reduction of the difference in EPHX genotype frequencies between the two regions.

Information bias is not a major consideration in this study, since the population has no knowledge on aflatoxin contamination and its potential hazards. Moreover, we used a standardized questionnaire and all interviews were conducted by the same person.
Like GSTM1, the GSTT1 null genotype itself was not associated with HCC in this study. In contrast to GSTM1, GSTT1 did not modify the relation between peanut butter consumption and HCC. This may suggest that GSTT1 polymorphism is not important in aflatoxin metabolism or in HCC etiology. Possibly, conjugation of glutathione to AFB_1-8,9-exo-epoxide is efficiently catalyzed by GST-μ and in case of GST-μ deficiency GST-θ does not take over the function of GST-μ, but the metabolite is detoxified via hydrolysis instead. Our results correspond with those of a study in Gambia including 328 healthy subjects, showing that GSTT1 polymorphism was not related to aflatoxin-albumin adduct levels[9]. Chen and colleagues, however, reported that HBV positive, GSTT1 null genotype carriers, had increased albumin adduct levels and were at increased risk of HCC in a small study on 32 cases and 73 controls[8].

We found that the combination of EPHX 113HH and 139HH genotypes increased risk of HCC. In a study in China including 52 cases and 116 controls, McGlynn and colleagues found that carriers of the 113HH genotype were at increased risk of HCC[19]. Wild and colleagues, however, reported that this genotype was not associated with aflatoxin-albumin adducts[9]. Both research groups did not study the EPHX exon 4 polymorphism. We found no indications for the EPHX polymorphism to modify the relationship between peanut butter consumption and HCC. Biochemical studies showed that mEH-catalyzed hydrolysis is probably not a rate-limiting step in aflatoxin detoxification, since hydrolysis of the AFB_1-8,9-exo-epoxide can occur spontaneously at a rate comparable to that of mEH-catalyzed hydrolysis[14]. The role of mEH in detoxification of polycyclic aromatic hydrocarbons might be more important[26]. This could explain the increased risk of liver[19], ovarian[27], and colon cancer[28] associated with several allelic variants of EPHX. However, in vitro studies did not reveal a clear genotype-phenotype correlation for the polymorphic sites of EPHX[15,18,29,30]; the studied polymorphic sites might be linked to other genetically polymorphic sites, e.g., in non-coding regions, modifying regulation of gene transcription[31].

Wild and coworkers found that presence of GSTM1 and GSTT1 null genotypes in combination with the EPHX 113HH genotype was related to elevated aflatoxin-albumin adduct levels,

although not statistically significant[9]. We could not evaluate the effect of such a combination, since none of the cases and only three controls in our study had this combination of genotypes. However, the combinations of GSTM1 or GSTT1 non-null genotypes with the EPHX 113HH&139HH genotype increased risk of HCC, which is not in line with the proposed metabolism (see Figure 1). These unexpected findings might be due to chance because of low cell counts, but they might also indicate that mEH is involved in other metabolic pathways rather than AFB_1 detoxification.

We have estimated that about 50% of HCC cases in Sudan might be attributed to hepatitis B virus infection. Hepatitis infection has been reported to modify the relation between genetic polymorphism and HCC[19,32]. However, we could not study such effect modification in our study population, since only the number of controls with evidence of hepatitis B or C virus infection was too small.

Although being the largest study on aflatoxin-associated HCC and genetic polymorphism to date, the population was still small to study relatively weak gene-environment interactions. Especially our results on EPHX should be interpreted with care, because several variants occurred at low frequency and after stratification some cells had less than five observations.
In conclusion, our results do not indicate that GSTT1 plays a role in HCC. EPHX polymorphism might be related to HCC, although the encoded enzyme may not be important in AFB_1 detoxification in which GST-μ plays a major role. This seems to be in line with in vitro studies[11]. Since EPHX polymorphism appears to be associated with cancer risk, the specific effect of this polymorphism on gene expression and enzyme function, and the role of the mEH enzyme in various metabolic pathways need to be addressed in future studies.

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References

1. Thomas London W, McGlynn KA. Liver cancer. *In:* Cancer Epidemiology and Prevention. Schottenfeld D, Fraumeni J.F. (eds.). New York: Oxford University Press. 1996; 772-93.
2. Habish HA, Abdulla, M.H. The incidence of aflatoxin in Sudanese groundnuts. *Trop. Sci.* 1971; ***13*: 279-87.**
3. Omer RE, Verhoef L, van't Veer P, *et al.* Peanut butter intake, GSTM1 genotype and hepatocellular carcinoma: a case-control study in Sudan. Cancer Causes Control (in press).
4. IARC. Cancer incidence in five continents. Parkin DM, Whelan SL, Ferlay J, Raymond L, Young J. Lyon, France, *IARC Sci. Publ.* 1997; **7**.
5. Wang JS, Groopman JD. DNA damage by mycotoxins. *Mutat. Res.* 1999; **424:**167-81.
6. Eaton DL, Ramsdell HS, Neal GE. Biotransformation of aflatoxins. In: The Toxicology of Aflatoxin. Human Health, Veterinary, and Agricultural Significance. Eaton DL. Groopman JD. (eds.). San Diego: Academic Press Inc. 1994; 45-71.
7. Guengerich FP, Johnson WW, Ueng YF, Yamazaki H, Shimada T. Involvement of cytochrome P450, glutathione S-transferase, and epoxide hydrolase in the metabolism of aflatoxin B1 and relevance to risk of human liver cancer. *Environ. Health Perspect.* 1996; **104** ***S3*: 557-62.**
8. Chen CJ, Yu MW, Liaw YF, *et al.* Chronic hepatitis B carriers with null genotypes of glutathione S-transferase M1 and T1 polymorphisms who are exposed to aflatoxin are at increased risk of hepatocellular carcinoma. *Am. J. Hum. Genet.* 1996; **59:** 128-34.
9. Wild CP, Yin F, Turner PC, *et al.* Environmental and genetic determinants of aflatoxin-albumin adducts in the Gambia. *Int. J. Cancer.* 2000; **86:** 1-7.
10. Liu YH, Taylor J, Linko P, Lucier GW, Thompson CL. Glutathione S-transferase mu in human lymphocyte and liver: role in modulating formation of carcinogen-derived DNA adducts. Carcinogenesis. 1991; **12:** 2269-75.
11. Guengerich FP, Johnson WW, Shimada T, Ueng YF, Yamazaki H, Langouet S. Activation and detoxication of aflatoxin B1. *Mutat. Res.* 1998; **402:** 121-28.
12. Johnson WW, Ueng YF, Mannervik B, *et al.* Conjugation of highly reactive aflatoxin B1-8,9-exo-epoxide catalyzed by rat and human glutathione transferases: estimation of kinetic parameters. *Biochemistry.* 1997; **36:** 3056-60.
13. Rebbeck TR. Molecular epidemiology of the human glutathione S-transferase genotypes GSTM1 and GSTT1 in cancer susceptibility. *Cancer Epidemiol. Biomark and Prev.* 1997; **6:** 733-743.
14. Johnson WW, Yamazaki H, Shimada T, Ueng YF, Guengerich FP. Aflatoxin B1 8,9-epoxide hydrolysis in the presence of rat and human epoxide hydrolase. *Chem. Res. Toxicol.*. 1997; **10:** 672-76.
15. Hassett C, Aicher L, Sidhu JS, Omiecinski CJ. Human microsomal epoxide5hydrolase: genetic polymorphism and functional expression in vitro of amino acid variants. *Hum. Mol. Genet.* 1994; **3:** 421-28.
16. Masimirembwa CM, Dandara C, Sommers DK, Snyman JR, Hasler JA. Genetic polymorphism of cytochrome P4501A1, microsomal epoxide hydrolase, and glutathione S-transferases M1 and T1 in Zimbabweans and Venda of southern Africa. *Pharmacogenetics.* 1998; **8**: 83-85.

17. Hassett C, Lin J, Carty CL, Laurenzana EM, Omiecinski CJ. Human hepatic microsomal epoxide hydrolase: comparative analysis of polymorphic expression. *Arch. Biochem. Biophys.* 1997; **337:** 275-83.
18. Laurenzana EM, Hassett C, Omiecinski CJ. Post-transcriptional regulation of human microsomal epoxide hydrolase. *Pharmacogenetics.* 1998; **8:** 157-67.
19. McGlynn KA, Rosvold EA, Lustbader ED, *et al.* Susceptibility to hepatocellular carcinoma is associated with genetic variation in the enzymatic detoxification of aflatoxin B1. *Proc. Natl. Acad. Sci.* U.S.A. 1995; **92:** 2384-87.
20. Omer RE, Bakker MI, van't Veer P, *et al.* Aflatoxin and liver cancer in Sudan. *Nutr. and Cancer.* 1998; **32:** 174-80.
21. Arand M, Muhlbauer R, Hengstler J, *et al.* A multiplex polymerase chain reaction protocol for the simultaneous analysis of the glutathione S-transferase GSTM1 and GSTT1 polymorphisms. *Anal. Biochem.* 1996; **236**: 184-86.
22. Bell DA, Taylor JA, Paulson DF, Robertson CN, Mohler JL, Lucier GW. Genetic risk and carcinogen exposure: a common inherited defect of the carcinogen-metabolism gene glutathione S-transferase M1 (GSTM1) that increases susceptibility to bladder cancer. *J. Natl. Cancer Inst.* 1993; **85:** 1159-64.
23. Smith CA, Harrison DJ. Association between polymorphism in gene for microsomal epoxide hydrolase and susceptibility to emphysema. *Lancet.* 1997; **350:** 630-33.
24. Pastorelli R, Guanci M, Cerri A, *et al.* Impact of inherited polymorphisms in glutathione S-transferase M1, microsomal epoxide hydrolase, cytochrome p450 enzymes on DNA, and blood protein adducts of benzo(a)pyrene-diolepoxide. *Cancer Epidemiol. Biomark. Prev.* 1998; **7:** 703-9.
25. Zhao L, Alldersea J, Fryer A, *et al.* Polymorphism at the glutathione S-transferase GSTM1 locus: a study of the frequencies of the GSTM1 A, B, A/B and null phenotypes in Nigerians. *Clin. Chim. Acta.* 1994; **225:** 85-88.
26. Shou M, Gonzalez FJ, Gelboin HV. Stereoselective epoxidation and hydration at the K-region of polycyclic aromatic hydrocarbons by cDNA-expressed cytochromes P450 1A1, 1A2, and epoxide hydrolase. *Biochemistry.* 1996; **35:** 15807-13.
27. Lancaster JM, Brownlee HA, Bell DA, *et al.* Microsomal epoxide hydrolase polymorphism as a risk factor for ovarian cancer. *Mol.Carcinog.* 1996; **17:** 160-62.
28. Harrison DJ, Hubbard AL, MacMillan J, Wyllie AH, Smith CD. Microsomal epoxide hydrolase gene polymorphism and susceptibility to colon cancer. *Br. J. Cancer.* 1999; **79:** 168-71.
29. Omiecinski CJ, Hassett C, Hosagrahara V. Epoxide hydrolase-polymorphism and role in toxicology. *Toxicology Letters.* 2000; **112-113:** 365-70.
30. Hassett C, Robinson KB, Beck NB, Omiecinski CJ. The human microsomal epoxide hydrolase gene (EPHX1): complete nucleotide sequence and structural characterization. *Genomics.* 1994; **23:** 433-42.
31. Raaka S, Hassett C, Omiencinski CJ. Human microsomal epoxide hydrolase: 5'-flanking region genetic polymorphisms. *Carcinogenesis.* 1998; **19:** 387-93.
32. London WT, Evans AA, Buetow K, *et al.* Molecular and genetic epidemiology of hepatocellular carcinoma: studies in China and Senegal. *Princess. Takamatsu. Symp.* 1995; **25:** 51-60.

5

The role of hepatitis B and hepatitis C viral infections in the incidence of hepatocellular carcinoma in Sudan

R.E. Omer, P. van't Veer, A.M.Y. Kadaru, E. Kampman, I.M. El Khidir, S.S. Fedail, F.J. Kok.
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Abstract

Background: In Sudan, the incidence of hepatocellular carcinoma (HCC) is high and increasing. Hepatitis B virus (HBV) and hepatitis C virus (HCV) are important risk factors of HCC. This study aims to assess the role of HBV and HCV viral infections in the incidence of HCC in two regions of Sudan.

Methods. A case-control study was conducted among 150 HCC patients and 205 controls from two regions in Sudan. Their demographic characteristics as well as food habits and chronic conditions have been investigated. In this study, 115 cases and 199 controls were tested for HBsAg and HCV antibodies.

Results. Strong positive associations were found between HBV, HCV, and HCC with odds ratios (OR) 9.8 (95% CI 5.1-18.9) and 8.3 (95% CI 2.3-29.9) respectively. After adjustment for age, by logistic regression, the ORs for HBV and HCV were 16.1 (95% CI 7.4-34.9) and 4.5 (95% CI 1.1-18.6) respectively. Further adjustment for region, education level and job type did not appreciably affect the results.

Conclusion. Given prevalence of HBV and HCV of 7.0% and 1.5% among controls, about 57% of all HCC cases can be attributed to these viral infections. Hepatitis infections seem to be important risk factors for HCC in Sudan.

Key words: Hepatitis B virus, hepatitis C virus, HCC, Sudan.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most frequent tumor in the world. Although it is relatively rare in Western Europe, North America and Australia, it is considered to be one of the most common malignancies in sub-Saharan Africa, South East Asia and China[1]. In spite of the fact that the tumor was under diagnosed because of lack of autopsies and biopsies, HCC has shown an increasing incidence in the past two decades, in The Sudan[2]. Daoud *et al.*[3], reported 35 cases of HCC (2.7% of all malignancies) seen in Khartoum hospitals in the year 1968; more recently, medical workers at Khartoum hospitals have reported much higher numbers, especially from West Sudan[4].

It is well-established world wide that HCC is strongly associated with chronic infection with hepatitis B virus[5]. HBV infections have also been reported in Sudanese patients with HCC[6]. In some Asian countries, such as Taiwan, Hong Kong, main land China, and countries within sub-Saharan Africa, HBV prevalence is high and considered to be a major cause of HCC, either acting on its own or in combination with aflatoxins[7-11].

The association between non-A non-B transfusion hepatitis and HCC was investigated soon after the hepatitis C virus (HCV) genome had been cloned and specific assays for circulating anti-HCV were developed[12]. Several case-control studies suggest that HCV may have a role in the etiology of HCC, in most of these a significant association between anti-HCV positive cases and HCC was found[13-20]. In Japan the rising incidence of HCC has been linked to the increasing prevalence of HCV infection, even in regions with moderate HBV[21]. The importance of HCV as an etiologic factor of chronic liver diseases including HCC was also observed in Egypt[22]. On the other hand, in Senegal, where HBV remains the main viral cause of HCC, the epidemiological association between HCV and HCC was not proven[23].

In order to assess the potential contribution of HBV and HCV viral infections to the incidence of HCC in Sudan, we compared hepatitis B surface antigen (HBsAg) and anti-HCV for HCC cases and controls collected from two areas in Sudan.

Methods

A case-control study was conducted during a period of two years (Sept. 1996-Sept. 1998), in West and Central Sudan representing regions of higher and lower incidence of liver cancer respectively. West Sudan (North Kordofan State) is an area of 230,000 km², and a population size of 1,500,000. Central Sudan (Gazira State) is an area of 152,000 km², and a population size of 2,500,000. All cases and controls were recruited from these regions; subjects consent was obtained after they had been informed.

Case recruitment

A total of 150 HCC cases between 21-70 years were recruited from the two study regions, in a period of two successive years; from 115 of these blood samples were obtained and tested for HBsAg and HCV antibodies. Cases were recruited from 5 out of 6 Khartoum (capital) hospitals (Tropical Medicine Hospital, Oumdurman Hospital, Khartoum Hospital, Soba Hospital and Ibn Sena Hospital). In these hospitals liver cancers are diagnosed, following referral from the regional hospitals. The admission lists from the departments of Internal Medicine in the above mentioned hospitals were weekly screened to identify new admitted cases and the responsible doctor and patients were contacted. For all 115 cases, the diagnosis of HCC was verified clinically, by liver function tests and by histopathological examination of a liver biopsy.

Control recruitment

Controls were community-based subjects, recruited from the same catchment areas as the cases; the number of controls was chosen proportional to the population size of the two areas. Therefore, four localities were chosen from West Sudan and six localities in Central Sudan. From each locality one sugar shop was chosen randomly; these sugar shops hold a complete registry of all inhabitants in the locality and registration by the shops is required for obtaining subsidized sugars, a product that is highly appreciated in regions of Sudan. Twenty households were taken randomly from the shop list. Since liver cancer is more prevalent among men, the households selection was made in a ratio 1:3 women to men. A total of 199 controls were enrolled (80 from West and 119 from Central) aged 25-70 years. All chosen subjects agreed to cooperate in the study.

Blood samples

Blood samples from cases were centrifuged immediately at about 1200g for 10 min, and the sera were then kept at -20°C for HBV and HCV testing. Blood samples from controls were kept immediately after venapuncture in isolated foam boxes containing cooling materials at 4°C, to be transported from the locality to the regional hospital laboratory where it was separated using the same technique as mentioned above. The separated blood sera were then kept at -20°C until they were transferred in isolated foam boxes at less than 0°C to Khartoum National Health Laboratory where they were kept at -20°C.

Hepatitis analysis

The samples were analysed at the National Health Laboratory - Virology Department. Hepatitis B virus surface antigen (HBsAg) was determined by Hepanostika HBsAg. In short, 100 µL of (undiluted) sample and control were pipetted into assigned wells. Each well was then soaked and washed four times with phosphate buffer after it had been incubated at 37 ± 2 °C for one hour. 100 µL of TMB substrate was then pipetted into each well. The strips were then incubated at 18 - 25 °C for 30 min. 100 µL of sulfuric acid (1mol/L) was added to each well to stop the reaction. The absorbency of the solution in each well was read at 450 ± 5 nm. 25% of the positive samples (selected randomly) were also positive when confirmed by the Hepanostica HBsAg Uni-form microelisa system.

Detection of hepatitis C virus (HCV) was done by ORTHO HCV 3.0 ELISA Test System with Enhanced SAVe. For this assay a diluted test specimen was incubated in a test well at 37 °C for 30 min. Then it was washed five times to remove unbound serum proteins. 200 µL of murine monoclonal antibody conjugated to horseradish peroxidase was added to the micro well and incubated for 30 min. at 37°C. This was followed by subsequent washing for five times to remove the unbound conjugate. The specimen was then incubated for 15 min. after the addition of an enzyme detection system composed of o-phenylene-diamine (OPD) and hydrogen peroxide. 50 µL of 4N sulfuric acid was then added to stop the reaction. The colour intensity was measured with a micro-well reader (photometer) using 492 nm filter. Positive samples were then confirmed by CHIRON* RIBA* HCV 3.0 SIA.

Background data

The orally administered questionnaire was designed to identify the potential confounders and other risk factors of HCC. The data for cases and controls were collected by the same investigator (RO) and entered in the database.

Data analysis

Prevalence of hepatitis B and C were compared between cases and controls, followed by calculation of crude odds ratio's, X^2 testing and determination of confidence intervals. Subsequently, analyses were stratified by the potential confounders age, region, gender, education and job type. Finally, logistic regression analysis was used SAS 6.12 (SAS Institute Inc., Cary, NC, USA) to establish the association between hepatitis B or C and HCC accounting for potential confounders such as age, region, education and job type. Variables were considered as potential confounders, when their inclusion in the model altered the OR for HBV or HCV by 10% or more.

Results

Table 1 shows the main characteristics of HCC cases and controls from the two study regions West and Central Sudan. The prevalence of hepatitis B and C infections are higher among cases than controls; the prevalence of either hepatitis B or C is also higher (cases 53%, controls 8%). Cases are older than controls, more often of the western residence, less educated and more frequently farmers and laborers. No difference was observed between cases and controls for a history of alcohol consumption.

To identify potential confounders, Table 2 shows the association between hepatitis and age, region, gender, education and job type. Hepatitis B virus prevalence is higher among people less than 40 years old, and among the western population, less educated people, and farmers and laborers. Alcohol history was not related to HBV. The prevalence of HCV among controls was much lower than prevalence of HBV. Although the few data do not permit conclusions there is no reason to suspect that the prevalence of HCV over strata of potential confounders is different than the distribution of HBV.

Table 1. Main characteristics of cases and controls.

Potential risk factor	Cases (n=115) No.	(%)	Controls (n=199) No.	(%)
Hepatitis infections				
Hepatitis B				
Yes	49	(43)	14	(7)
No	66	(57)	185	(93)
Hepatitis C				
Yes	13	(11)	3	(2)
No	102	(89)	196	(98)
Hepatitis B or C				
Yes	61	(53)	16	(8)
No	54	(47)	183	(92)
Design related variables				
Age				
<40	11	(10)	80	(40)
40-59	38	(33)	96	(48)
60+	66	(57)	23	(12)
Region				
West	74	(64)	80	(40)
Central	41	(36)	119	(60)
Gender				
Male	88	(77)	150	(75)
Female	27	(23)	49	(25)
Potential confouders				
Education				
Illiteracy	80	(70)	68	(34)
Literacy	35	(30)	131	(66)
Job type				
Farmer + Labour	66	(57)	70	(35)
Others	49	(43)	129	(65)
Alcohol history				
Yes	45	(39)	61	(31)
No	70	(61)	138	(69)

Table 2. Potential risk factors for liver cancer and HBV, HCV infection.

Potential risk factor	HBV (n=199) Positive	(%)	HCV (n=199) Positive	(%)
Overall	14/199	(7)	3/199	(1.5)
Age				
<40	7	(9)	1	(1)
40-59	7	(7)	2	(2)
60+	0	(0)	0	(0)
Region				
West	7	(9)	1	(1)
Central	7	(6)	2	(2)
Gender				
Male	10	(7)	2	(1)
Female	4	(8)	1	(2)
Education				
Illiteracy	8	(12)	2	(3)
Literacy	6	(5)	1	(1)
Job type				
Farmer + Laborer	9	(13)	2	(3)
Others	5	(4)	1	(1)
Alcohol history				
Yes	5	(8)	0	(0)
No	9	(7)	3	(100)

Strong associations of HBV and HCV with the incidence of HCC are observed (Table 3). Age appears a clear negative confounder for HBV; hence the odds ratio rises from 9.8 (crude) to 16.1 (adjusted), while it acts as a positive confounder for HCV and HCC. Region, education and job type are no clear confounders for HBV; region and education appeared to be negative confounders for HCV, although confidence intervals are very wide. After adjustment for age, the variables region, education and job type were no substantial counfounders anymore. Thus, the age adjusted ORs of 16.1 for HBV and 4.5 for HCV provide reasonable estimates of the association between hepatitis infections and HCC in this population.

Table 3. Crude and adjusted Odds Ratios for hepatitis B and hepatitis C virus infection and liver cancer.

	HBV OR (95% CI)	HCV OR (95% CI)
Crude OR	9.8 (5.1-18.9)	8.3 (2.3-29.9)
Adjusted For		
Age	16.1(7.4-34.9)	4.5 (1.1-18.6)
Region	9.0 (4.6-17.6)	10.1 (2.7-37.4)
Education	8.7 (4.4-17.4)	13.1 (3.4-50.2)
Job Type	8.5 (4.4-16.7)	7.6 (2.1-27.8)
Adjusted For		
Age + Region	15.0 (6.9-32.7)	5.5 (1.3-23.9)
Age + Education	14.5 (6.7-31.6)	6.0 (1.4-24.8)
Age + Job Type	14.8 (6.8-32.3)	4.5 (1.0-19.3)

Discussion

In this study we found that HBV and HCV virus infections are strongly associated with HCC in Sudan. The data suggested that HBV infection have a stronger association with HCC than HCV infection. The prevalence of HBV was also found to be higher (7.0%) than the prevalence of HCV (1.5%) among this study population.

Regarding selection of cases in our study, the use of registration lists of five hospitals in Khartoum for case recruitment, enabled all cases from the two regions to be included in the study, except for those who might have been deceased before they could be included in the study; furthermore few were missed during the two periods of control recruitment accounting for a total of four weeks during the two-year study period.

Moreover, one of the five hospitals is a National Center for Gastrointestinal and Liver diseases, so that HCC patients from all over the country are sent to that hospital for further diagnoses and better treatment. From five of six hospitals in Khartoum150 cases were obtained during the two study years. This number clearly exceeds the number of 42 liver and gall bladder cancer cases reported from Khartoum hospitals by (Daoud *et al.*) during the period 1957-1965.

Selection of controls was done at random basis, with number of controls from West and Central Sudan proportional to the population size, and these regions are the catchment areas

that provided cases in the study. From those regions several sugar shops (see: methods part) were chosen randomly. As social, cultural and demographic characteristics are homogeneous within the two regions, the random chosen sugar shops are thought to yield a representative sample of the subjects living in the region at large.

Age, place of origin, education level and job type were evaluated as possible confounders of the association between HBV or HCV and HCC. The potential bias was excluded by Mantel Haenszel analysis, followed by logistic regression that accounted for several confounders simultaneously. In fact, age appeared to be the only relevant confounder; alcohol drinking and gender were not considered as confounders since they were not related to HCC in the data.

Regarding hepatitis infections, a study conducted in the year 1989 in Sudan showed that HBsAg and HBcAb (200X) were detected more often in HCC patients than in controls[6]. The summary odds ratios for HBsAg positivity and anti-HCV/HCV RNA positivity from 32 published case-control studies included in a meta-analysis by Donato *et al.*[24], were found to be 13.7 and 11.5 respectively. In areas where HBV infection was low to intermediate endemic and HCV infection is predominant among HCC cases, the summary odds ratios for HBsAg and anti-HCV/HCV RNA positivity were 8.5 and 16.8 respectively. In the same study, in a total of 14 studies from sub-Saharan and southern African countries where HBV is endemic, summary odds ratios of 16.7 for HBsAg and 6.2 for anti-HCV/HCV RNA were observed. Our results for HBsAg and anti-HCV positivity coincide with the findings of Donato *et al*, for sub-Saharan countries with high HBV prevalence. Despite to the ethnical and cultural heterogeneity of the different regions of Sudan, this suggests that our results may also apply to a large geographical area including most other regions of Sudan. Given the observed prevalence of hepatitis and the ORs in this study, HBV might account for 52%, and HCV would account of 5% for HCC in Sudan.

In addition, the study by Donato *et al.* suggested synergism between the 2 viruses i.e. the increase in HCC risk when both HBV and HCV infections were present is higher as compared with each infection alone. However, since in our study HBV and HCV infections coincided in only one case and one control, we were not able to study this synergism especially because of the very low prevalence of HCV (1.5%). The average age of HCC cases in areas where HBV is endemic is lower than among cases from areas where HCC is predominantly due to HCV

infection (Donato *et al.*). Similarly in our study, the mean age of HCC patients whom are positive for HBsAg was 55 ± 10 while it was 65 ± 5 for cases positive for anti-HCV. This different age distribution, along with the lower OR for HCV might be related to different ages at hepatitis infection or differences in pathogenesis. Thus studies are needed to determine the age of infection and mode of transmission of hepatitis viruses in the Sudanese population.

Apart from the role of HBV and HCV in the etiology of HCC, clues have also been obtained from studies in other African and Asian countries with similar demographic, social and cultural characteristics. In most of these countries, HBV is believed to act in combination with other environmental and dietary exposures such as aflatoxins. In these areas where food and feed stuffs are highly contaminated with aflatoxins, the interaction between aflatoxins exposure and chronic HBV infection has been suspected to play an important role in the incidence of HCC. For instance, it is thought that HBV increases HCC risk by either integrating in the DNA or causing proliferation of cells with a mutated p53 gene, which is seen in areas where there is excessive exposure to aflatoxin in foods. Therefore, the strong association we observed with HBV may partially result from synergism with aflatoxin exposure, which is common in some regions in Sudan, including West Sudan[25].

In conclusion, hepatitis infections, especially HBV, are important risk factors for HCC in Sudan. They may account for about 57% of HCC, which may partially be due to a combination with exposure to environmental carcinogens such as aflatoxins.

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References

1. Muir CS. Cancer incidence in five continents. *IARC Sci. Publ.* Lyon 1992; **120:** 25-30.
2. Zaki M. Aetiological factors in HCC. MD thesis, Faculty of Medicine, University of Khartoum. 1991.
3. Daoud EH, El-Hassan AM, Zak F, Zakova N. Cancer in Africa (Isted): Cliford P, Linsell CA, Timms GL, (eds.). East Africa Publishing House, Nairobi 1968.
4. Mohamadien A. Biochemical Tumour Markers in HCC and Other Liver Disease. MD thesis, Faculty of Medicine, University of Khartoum. 1993.
5. IARC, Hepatitis viruses. IARC monographs on the evaluation of carcinogenic risks of humans, IARC, Lyon 1994; **59.**
6. Itoshima T, Fedail SS, Souliman I, Ali AK, Higashi T, Tsjit T. Hepatitis B virus markers in patients with shistosomiasis, liver cirrhosis and hepatocellular carcinoma in Khartoum, Sudan. *Acta. Med. Okayama.* 1989; **43:** 241-44.
7. Bukh J, Miller RH, Kew MC, Purcell RH. Hepatitis C virus RNA in Southern Africa Blacks with hepatocellular carcinoma. *Proc. Natl. Acad. Sci.* 1993; **90:**1848-51.
8. Coursget P, Leboulleux D, Lecann P, *et al.* Hepatitis C virus in cirrhosis and Primary hepatocellular carcinoma in Senigal. *Trans. R.. Soc. Trop. Med. Hyg.* 1992; **86:** 552-53.
9. Jeng JE, Tsai JF. Hepatitis C virus antibody in hepatocellular carcinoma in Taiwan. *Med. Virol.* 1991; **34:** 74-77.
10. Leung NW, Tam JS, Lai Jy. Does hepatitis C virus infection contribute to hepatocellular carcinoma in Hong Kong? *Cancer.* 1992; **70:** 40-44.
11. Wild CP, Shrestha SM, Anwar WA, Montesano R. Field studies of aflatoxin exposure, Metabolism and induction of genetic alteration of HBV infection and hepatocellular carcinoma in Gambia and Thailand. *Toxicolog. Letters.* 1992; **64/65:** 455-61.
12. Ming WY, Chien JC. Hepatitis B and C virus in the development of hepatocellular carcinoma. Critical reviews in *Oncology/Hematology.* 1994; 7: 71-91.
13. Kew MC, Houghton M, Choo QL, Kuo G. Hepatitis C virus antibodies in Southern Africa blacks with hepatocellular carcinoma. *Lancet.* 1990; **335:** 873-74.
14. Yu MW, You SL, Shang AS, Lu SN, Liaw YF, Chen CJ. Association between hepatitis C virus antibodies and hepatocellular carcinoma in Taiwan. *Cancer Res.* 1991; **51:** 5621-25.
15. Chuang WL, Chang WY, Lu SN, *et al.* The role of hepatitis B and C viruses in hepatocellular carcinoma in a hepatitis B endemic area. *Cancer.* 1992; **69:** 2052-54.
16. Tanaka K, Hirohata T, Koga S, *et al.* Hepatitis C and hepatitis B in the etiology of hepatocellular carcinoma in The Japanese population. *Cancer Res.* 1991; **5:** 2842-47.
17. Kaklamani E, Trichopoulos D, Tzonou A, *et al.* Hepatitis B and C viruses and their interaction in the origin of hepatocellular carcinoma. *JAMA.* 1991; **265:** 1974-76.
18. Zavitanos X, Hatzakis A, Kaklamani E, *et al.* Association between hepatitis C virus and hepatocellular carcinoma using assays based on structural and non structural hepatitis C virus peptides. *Cancer Res.* 1992; **52:** 5364-67.

19. Yu MC, Yuan JM, Ross RK, Govindarajan S. Presence of antibodies to the hepatitis B surface antigen is associated with an excess risk for hepatocellular carcinoma among non-Asian in Los Angeles county, California. *Hepatology.* 1997; **25:** 226-28.
20. Di Bisceglie AM, Order SE, Klein JL, *et al.* The role of chronic viral hepatitis in hepatocellular carcinoma in The United States. *Am. J. Gasterenterol.* 1991; **86:** 335-38.
21. Kiyosawa K, Furuta S. Clinical aspects and epidemiology of hepatitis B and C viruses in hepatocellular carcinoma in Japan. *Cancer Chemother. Pharmacol.* 1992; **31:** 5150-56.
22. Waked IA, Saleh SM, Moustafa MS, Raouf AA, Thomas DL, Strickland GT. High prevalence of hepatitis C in Egyptian patients with chronic liver disease. *Gut.* 1995; **37:** 105-7.
23. Ka MM, Herve P, Leguenno B, *et al.* Low prevalence of anti-hepatitis C virus antibodies in hepatocellular carcinoma in Senegal. *Ann. Gastroenterol. Hepatol.* 1996; **31:** 329-32.
24. Donato F, Boffetta P, Puoti M. A meta-analysis of epidemiological studies on the combined effect of hepatitis B and C virus infections in causing hepatocellular carcinoma. *Int. J. Cancer.* 1998; **75:** 347-54.
25. Omer RE, Bakker MI, van't Veer, P, *et al.* Aflatoxin and liver cancer in Sudan. *Nutrition and Cancer.* 1998; **32:** 174-80.

6

Public health relevance of peanut butter intake and hepatitis B virus infection with respect to hepatocellular carcinoma: a case-control study in Sudan

R.E. Omer, A. Kuijsten, A. M.Y. Kadaru, F.J. Kok, M.O. Idris, I.M. El Khidir, P. van 't Veer.

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Abstract

Objective. This study aims to assess the effect of exposure to aflatoxins (through peanut butter intake) and hepatitis B virus (HBV) infection, either alone or in combination in the etiology of hepatocellular carcinoma (HCC) in Sudan.

Method. A case-control study was conducted among 150 patients and 205 controls from Sudan. Peanut butter consumption and HBV have been investigated, as well as drinking and smoking habits, and demographic characteristics. HBV infection was assessed from blood samples (115 cases and 199 controls).

Results. A clear dose-response relation was observed between increasing peanut butter consumption and HCC. In virus negative subjects, age-adjusted OR for the highest vs lowest quartile of aflatoxin exposure was 5.1, with test for trend being statistically significant; the OR for HBV in the lowest category of peanut butter was 32. The joint exposure to aflatoxin intake and HBV infection in increasing HCC risk appeared to be more than the sum and less than the product of the ORs for each factor alone. Based on these data, about 80% of the HCC cases are attributable to peanut butter consumption and HBV infection in Sudan. Depending on assumptions in the data analysis, 39-60% of all cases can be attributed to aflatoxin exposure and 49-52% to HBV infection; of these figures, 7-34% reflects a shared responsibility of the two factors.

Conclusion. Reduction of aflatoxin exposure may be a useful long-term strategy in HCC prevention in Sudan, while hepatitis B virus vaccination of children and high risk groups in endemic areas might be a feasible short-term strategy.

Key Words: Peanut butter intake, Hepatitis B virus, Public health, HCC, Sudan.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most frequent tumor in the world. Aflatoxins, hepatitis B virus (HBV) and C virus (HCV) infections are major risk factors for this cancer in many areas of the developing world. There are currently 300-400 million chronic HBV carriers worldwide who continue to be at risk of HCC, and many of these are in parts of the world with high exposure to aflatoxin contaminated foods[1].

Epidemiological studies[2-6] suggest a multiplicative increase in HCC risk in individuals chronically infected with HBV and exposed to dietary aflatoxins. This is supported by experimental studies in HBV transgenic mice and woodchucks, which suggest a biological interaction between these two risk factors in HCC induction[7,8]. This was not the case in ducks, however, where liver injury associated with chronic HBV infection is less marked than in the former two models[9].

In public health policy aiming to reduce cancer risk among exposed populations, it is not only important to understand the biological background of disease, but also to quantify the relative contribution of hepatitis infections and aflatoxin to HCC.

The aim of the present analysis is to quantify the potential impact of public health measures in Sudan, where both HBV infections and dietary intake of aflatoxins are common. The results obtained from this study may help to develop a feasible strategy for prevention of HCC optimal to Sudan.

Methods

Study population

The design of the case-control study has been described in detail previously[10]. Briefly, a case-control was conducted during a period of two years (Sept. 1996- Sept. 1998), in West and Central Sudan representing regions of higher and lower incidence of liver cancer respectively. West Sudan (North Kordofan State) is an area of 230,000 km^2, and a population size of 1,500,000. Central Sudan (Gazira State) is an area of 152,000 km^2, and a population size of 2,500,000. All cases and controls were recruited from these regions; subjects consent was obtained after they had been informed.

Recruitment

A total of 150 HCC cases and 205 controls between 21-70 years were recruited from the two study regions, in a period of two successive years. Cases were recruited from 5 out of 6 Khartoum (capital) hospitals. In these hospitals liver cancers are diagnosed, following referral from the regional hospitals. The admission lists from the departments of Internal Medicine in these five hospitals were weekly screened to identify new admitted cases and the responsible doctor and patients were contacted. For all cases, the diagnosis of HCC was verified clinically, by liver function tests and by ultrasound, due to the severity of the disease histopathological examination of a liver biopsy could be verified for 95% of the participated cases.

Controls were recruited from the same catchment areas as the cases; the number of controls was chosen proportional to the population size of the two areas. Therefore, four localities were chosen from West Sudan and six localities in Central Sudan. From each locality one sugar shop was chosen randomly; these sugar shops hold a complete registry of all inhabitants in the locality and registration by the shops is required for obtaining subsidized sugars, a product that is highly appreciated in these regions of Sudan. Twenty households were taken randomly from the shop list. Since liver cancer is more prevalent among men, selection was made in a ratio 1:3 women to men. All subjects agreed to cooperate in the study.

Diet and life style factors

Based on the experience obtained during the explorative study[11], we designed a questionnaire to assess peanut butter intake in a standardized manner. All participating subjects were interviewed personally, at hospitals for cases and at home for controls. All interviews were done by one-person (R.O.) using the standardized questionnaire. The questionnaire inquired on the frequency and quantity of peanut butter on a daily or weekly basis. Frequency was inquired both "in season" and "off season", the former corresponding to the period of the year with active agricultural practices following the start of the rainy season, i.e. June-September (3 months), the latter is during the dry season, i.e. October-May (9 months). Quantity was assessed in two ways, first in terms of meal pattern, i.e. it was asked whether peanut butter is eaten as part of the meal or as full meal; second, the amount of peanut butter consumed per meal was inquired and divided by the number of subjects usually sharing the meal. To calculate average monthly intake, a weighted average of the frequency consumption "in" and "off" season was used. In addition to peanut butter consumption, we also assessed the mode of storage of peanuts because

this may be relevant to the aflatoxin content of the peanut butter bought at the local markets. The questionnaire also identified the potential confounders and other risk factors of HCC, e.g. smoking and drinking habits.

Blood samples

Blood samples from cases were centrifuged immediately at rpm 2*1000 U/min (2000 round per minute units) in 10 minutes, and the sera were then kept at minus 20°C for HBV testing. Blood samples from controls were kept immediately after venapuncture in a foam fridge containing cooling materials at 4°C, to be transported from the locality to the regional hospital laboratory where it was separated using the same technique as mentioned above. The separated blood sera were then kept at minus 20°C until they were transferred in a foam fridge at less than 0°C to Khartoum National Heath Laboratory where they were kept at minus 20°C.

Hepatitis analysis

The samples were analyzed at the National Health Laboratory – Virology Department. HBV surface antigen (HBsAg) was determined by Hepanostika HBsAg. In short, 100 µl of (undiluted) sample and control were pipetted into assigned wells. Each well was then soaked and washed four times with phosphate buffer after it had been incubated at 37 ± 2°C for one hour. 100µl of TNB substrate was then pipetted into each well. The strips were then incubated at 18-25°C for 30 minutes. 100µl of sulfuric acid (1 mol/l) was added to each well to stop the reaction. The absorbency of the solution in each well was read at 450 ± 5 nm. Twenty five percent of the positive samples (selected randomly) were also positive when confirmed by the Hepanostica HBsAg Uni-form microelisa system.

Data-analysis

We analyzed data of 114 cases and 198 controls. Blood samples were obtained and tested for HBsAg antibodies from 115 cases and 199 controls (80 from West and 119 from Center); missing blood samples were not related to the study design. Food consumption data from one case and one control were missing.

To examine the interaction of dietary aflatoxin exposure (peanut butter consumption) and HBV infection, the joint distribution of these factors was compared between cases and controls, followed by calculation of crude odds ratio's (ORs) and confidence intervals (CIs).

Subsequently, logistic regression analysis was used (SAS statistical package) to adjust the association between peanut butter consumption, HBV infection and HCC for potential confounders such as sex, age, region, education, occupation, cigarette smoking and alcohol consumption (one at a time). Only age appeared to be relevant as a confounder.

The ORs describing the joint effect of peanut butter consumption and HBV infection on HCC were obtained in four different ways, aiming to obtain the best estimate of the separate and joint effect under different assumptions.

First, we described the data without any assumption on additivity or multiplicativity of effects. This was done by fitting a model, with indicator variables for each of the 7 combinations of peanut butter consumption and HBV infections (versus the reference group). Second, we assumed multiplicativity of effects and obtained ORs from logistic regression models with only main effects for peanut butter consumption and HBV infection (i.e. without a statistical product term or interaction). Third, we obtained ORs assuming additivity of the two factors by inspecting the results from the first model (Figure 1) and using the ORs for peanut butter consumption among (the relatively large group of) virus negative subjects. Finally, we combined our results with other studies to obtain an overall summary estimate with respect to the OR for peanut butter consumption, HBV, and their joint effect. To quantify these results, we used inverse variance weighted pooled estimates of the ln-transformed ORs from three studies published before[2-4]. In order to allow pooling with these other studies, we dichotomized our data for peanut butter consumption by joining the three highest categories.

Calculation of the etiological fraction

The aim of this study is to quantify the relative contribution of HBV infections and aflatoxin exposure to HCC, i.e., the (population) attributable risk (percent), also denoted as the attributable or etiological fraction (EF). The EF represents the proportion of all new cases in a given period that are attributable to the risk factors of interest. For a combination of two risk factors with levels i=1 to 4 (categories of low to high peanut butter consumption), and j=1,2 (without, with HBV infection), the EF is calculated as:

$$EF_{++} = (ID_{++} - ID_{11})/ ID_{++} = 1 - (ID_{11} / \Sigma_{ij} F_{ij} * ID_{ij}) = 1 - (1/ \Sigma_{ij} F_{ij} * IDR_{ij})$$

where ID_{++} is the overall incidence density in the candidate population with exposure to both factors, ID_{11} is the rate in the reference group, $IDR_{11}=1.0$ and the summation is over i and j[12] .

Thus, the EF is a function of two types of information: (1) the frequency of the risk factors in the candidate population (F_{ij}), and (2) the strength of the association between the risk factor and the disease (IDR_{ij}). It is chiefly the first quantity that makes the EF specific to a particular population, while the second is estimated by the OR_{ij} obtained from our case-control study.

Subsequently, we used the ORs derived under the four different assumptions (see data-analysis) to calculate the EF. For peanut butter consumption, we calculated

$$EF_{pb} = 1 - (\Sigma_j F_{+j} * IDR_{1j}) / (\Sigma_{ij} F_{ij} * IDR_{ij})$$

and for HBV infection we calculated

$$EF_{hbv} = 1 - (\Sigma_i F_{i+} * IDR_{i1}) / (\Sigma_{ij} F_{ij} * IDR_{ij})$$

In these formula F_{i+} and F_{+j} represent the marginal exposure distribution for peanut butter consumption and HBV respectively; IDR_{i1} and IDR_{1j} denote the IDRs for peanut butter among virus negative subjects and of HBV among the reference category for peanut butter intake, as estimated under the before-mentioned four assumptions, respectively.

As argued by Rothman[13] public health effects must be evaluated on an additive scale. The part of the EF shared by both factors, denoted by EF* is a measure of super additivity or public health interaction by both factors and can be calculated as $EF^* = EF_{pb} + EF_{hbv} - EF_{++}$. This formula can be derived by determining IDR^*_{ij} assuming additivity of the observed separate effects, i.e. $IDR^*_{ij} = 1 + (IDR_{i1}-1) + (IDR_{1j}-1)$ and calculation of $EF^* = (ID_{++} - ID^*_{++}) / ID_{++} = 1 - \Sigma_{ij} F_{ij} * IDR_{ij} * / \Sigma_{ij} F_{ij} * IDR_{ij}$, which reduces algebraically to the easier formula of EF* given before.

Results

In this analysis, 114 cases and 198 controls were included. As shown previously, cases appeared to be older because the intended frequency matching on age could not be realized during fieldwork. A higher percentage of cases were obtained from West Sudan, while distribution among controls represents the population size of the two areas, as intended. HCC was three times more frequent among males. Illiteracy and being farmer or laborer were more prevalent among cases. For drinking and smoking habits, prevalence among cases and controls was similar. Age, sex, region, education level and job type were evaluated as potential confounders of the association between peanut butter and HCC. The potential bias was accounted for by stratified analysis, followed by logistic regression that accounted for several confounders

simultaneously. When the potential confounders were added to the models the results for all categories of exposure remained essentially similar (data not shown). Only age appeared to be a mild confounder and was adjusted for in all analyses.

The combined effects of peanut butter consumption and HBsAg carrier status on the development of HCC are shown in Table 1. A clear dose-response relation between increasing peanut butter consumption and HCC was observed. In virus negative subjects, the OR for the highest vs lowest level of exposure was 4.18 in crude analysis and increased only slightly to 5.06 upon adjustment for age, with the test for trend being statistically significant. Furthermore, the ORs for virus positive subjects were much higher than for virus negative subjects: The OR in the highest category of peanut butter consumption was 41.5, while it was 32.2 for the reference category. Thus, the OR for peanut butter consumption among virus positive subjects was 41.5/32.2 = 1.3 and the test for trend for peanut butter consumption among this subgroup was not statistically significant. Partially due to the small number of HBV positive subjects, the X^2 for statistical interaction between hepatitis virus infection and peanut butter consumption was not significant either (p=0.17).

Table 1. Combined effects of peanut butter intake and HBsAg antibody status on the development of hepatocellular carcinoma.

Peanut butter intake	Virus negative				Virus positive			
(g/m)	cases	controls	OR*	(95% Cis)	cases	controls	OR*	(95% CIs)
<70	8	49	1.00		6	2	32.16	(4.02-257.45)
71-150	11	45	1.89	(0.60-5.95)	10	2	46.46	(6.82-316.73)
151-300	16	46	2.67	(0.91-7.87)	10	3	35.00	(5.87-208.59)
>300	30	44	5.06	(1.84-13.91)	23	7	41.52	(11.15-154.54)

*OR adjusted for age.
Test for trend; X^2 =10.94 (p<0.01) among virus negative subjects, X^2 =0.02 (p=0.88) among virus positive subjects; X^2 for interaction between peanut butter consumption and HBV = 1.89 (p=0.17).

Figure 1 shows the best estimates of ORs based on the former estimates (thick lines), as well as assuming a multiplicative effect between peanut butter consumption and hepatitis B infection (thin lines). The ORs of the multiplicative model fit within the confidence intervals of the previous model, suggesting that both models adequately describe the data. Moreover, the figure also suggests that an additive model for peanut butter consumption and HBV infection might describe the data equally well. The figure suggest that an additive effect might be estimated by about 35 units on the OR scale. Moreover, the size of our study lacks the statistical power required to discriminate between these different models.

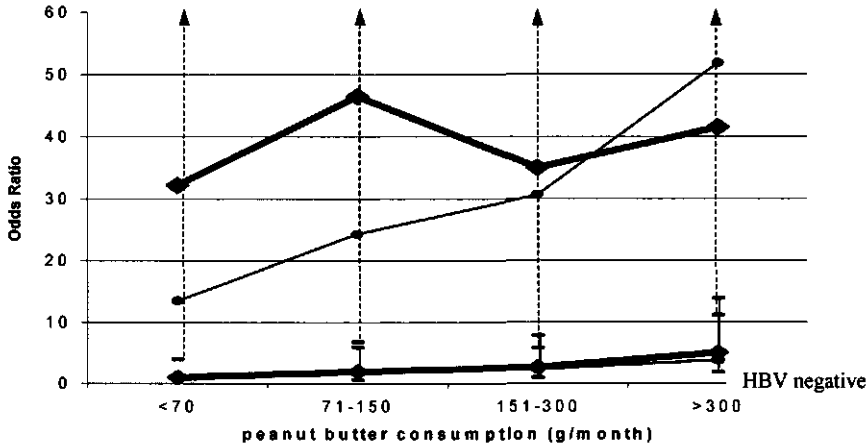

Figure 1. Association between peanut butter consumption, hepatitis B virus infection and HCC, fitted by different statistical models (see legend).

Legend to figure 1. Subjects with peanut butter consumption <70g/month and without HBV infection served as the reference category. Thick lines connect the estimates fitted with 7 dummies for all combinations of peanut butter consumption and 95% confidence intervals for these estimates are indicated as vertical lines (HBV-negative: continuous lines; HBV-positive: dashed lines). Thin lines connect estimates of the OR fitted using the assumption of multiplicativity of ORs for peanut butter consumption and HBV infection. All models were adjusted for age.

In order to obtain a better estimate for the joint effect of peanut butter consumption and HBV infection, and to evaluate the relation between aflatoxin exposure, HBV infection, and HCC quantitatively, we pooled ORs and 95% CI's of our study with three previous individual-based studies[2-4] (Table 2). Results for peanut butter consumption from our study (OR=3.3, Table 2) are obtained by the joining the three highest categories of consumption with the reference category (as shown in Table 1); in the other studies detectable vs non detectable levels were compared for urinary levels of aflatoxin-metabolites and adducts. The pooled OR for HBsAg-negative subjects with high aflatoxin exposure was 2.78 (95% CI= 1.5-5.0). The pooled ORs for HBsAg-positive subjects with low and high aflatoxin exposure were respectively 9.55 (95% CI=4.5-20.4), and 55.2 (95% CI=26.1-117). Thus, even when combined with our results, the OR for the joint exposure (55.2) exceeded multiplicativity (9.6*2.8=26.9) of the separate associations by about a factor 2.

Table 2. Overview of four studies addressing the separate and joint effect of aflatoxins and HBV.

References	Aflatoxin exp.	Odds Ratios (95% CI) Hepatitis B virus (HBV) Negative	Positive	Prevalence of HBV	ORs adjusted for
Wang[4] (1996)					
38 cases	low	1.0	22.8 (3.6-143.4)		Alcohol drinking
136 controls	high	1.7(0.3-10.8)	111.9 (13.8-905.0)	15%	Cigarette smoking
Qian[3] (1994)					
50 cases	Low	1.0	7.3 (2.2-24.4)		
267 controls	High	3.4 (1.1-10.0)	59.4 (16.6-212.0)	14%	Cigarette smoking
Ross[2] (1992)					
22 cases	Low	1.0	4.8 (1.2-19.7)		
140 controls	High	1.9 (0.5-7.5)	60.1 (6.4-561.8)	11%	
Omer (this paper)					
114 cases	Low	1.0	32.0 (4.0-255.8)		
198 controls	High	3.3 (1.3-8.3)	40.7 (12.7-130.9)	7%	Age
Pooled results					
224 cases	Low	1.0	9.6 (4.5-20.4.8)		
741 controls	High	2.8 (1.5-5.0)	55.2 (26.1-117)		

Figure 2 shows the proportion of cases among the population that can be attributed to peanut butter consumption and hepatitis i.e. the etiological fraction. This was calculated based on estimates obtained from the four previously mentioned statistical models. First, additivity was assumed with independence of the effect of the two risk factors i.e. no biological interaction between peanut butter consumption and HBV infection. Under this assumption, the population attributable risk for peanut butter and hepatitis were 27% and 52% respectively; results were only slightly different when a smaller (30) or larger (40) OR for hepatitis was used. Second, the logistic model with seven dummy variables was used (as in Table 1). For this model, the population attributable risk for peanut butter consumption was 39% and for HBV was 49%; for the joint exposure to HBV and peanut butter consumption, the model showed an excess to additivity of 7%, suggesting some synergism between these factors. The multiplicative model (Figure 2, column 3, see also Figure 1) resulted in a shared responsibility for the two risk factors of about 34%; in this case, the population attributable risk of aflatoxins (60%) exceeds that of HBV (52%). When ORs from the four pooled studies (including ours) were used, the shared responsibility of both factors was even larger (48%) and the population attributable risk of aflatoxins exceeded 70%. All four models showed that around 80% of the HCC cases in Sudan are attributable to these two risk factors.

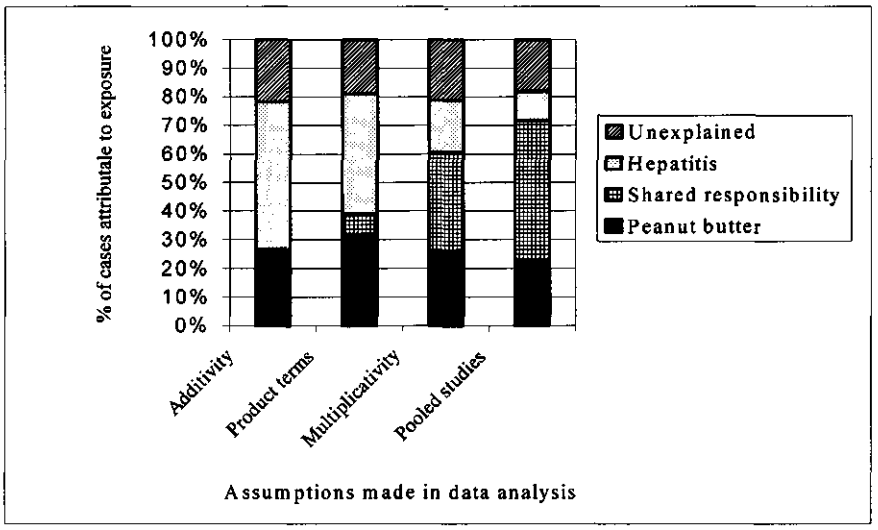

Figure 2. Percent of cases attributable to peanut butter consumption, hepatitis and their joint effect (shared responsibility) according to different assumptions in data analysis.

Discussion

In the present study, we investigated the combined effects of HBV infection and aflatoxin exposure on the development of HCC. A clear dose-response relation between increasing peanut butter consumption and liver cancer was observed. Moreover, HBV infection was strongly associated with HCC. The risk for joint exposure to HBV and aflatoxin consumption appeared to be more than additive but less than multiplicative for each factor alone. Based on these data, 32% all cases can be attributed to aflatoxin exposure alone, 42% to HBV infections alone and 7% to a shared responsibility of the two factors, i.e. exceeding additivity of both effects.

There is no obvious source of bias to explain our results. Regarding selection and information bias we would like to refer to a previous paper[10]. Briefly, we weekly screened the hospital admission lists of all 5 relevant hospitals in Khartoum, enabling us to include all cases from the two regions in the study; we only missed the few that were admitted during the periods of control recruitment (a total of four weeks during the two years study-period) and those that might have been deceased before they could reach the hospital. Although we expect that we included most cases, we might have missed the most severely ill subjects who might have been more likely to be exposed, i.e. the strength of the association may have been underestimated.

Selection of controls was done at random basis, from the same catchment areas that provided cases; the total number of controls from each region was proportional to the population size in each region. In order to assess the usual intake of peanut butter, frequency and portion size were assessed over a time frame of at least one year. In the Sudanese population, use of peanut butter is a common practice and there is no knowledge or concern on aflatoxin among the population. Given these food habits, the design and standardized format of the questionnaire and the fact that all subjects were questioned by only one person, systematic errors in assessment of peanut butter intake are not considered a serious problem to our investigation. Therefore, information bias is not a likely explanation for the positive association between peanut butter consumption and HCC.

Regarding the pooled analysis, several issues need to be addressed. First, we must be careful in interpreting the quantitative outcomes of the pooled ORs, because the previous studies used aflatoxin guanine-adducts and/or urinary aflatoxin metabolites as markers of aflatoxin. In contrast, we used peanut butter intake as a measure of aflatoxin exposure. Aflatoxin is detectable in various foods, but the level of contamination is usually low. Peanuts, peanut-containing foods, and soy sauce (not relevant to Sudan) are important exceptions.

Urinary aflatoxin metabolites including aflatoxin guanine-adducts may reflect only recent dietary exposure to aflatoxins (24 hours), and they show considerable day-to-day variation in their excretion. Dietary aflatoxin exposure reflects a much longer period of exposure e.g. in our study one year before the disease became clinically manifest (for cases); furthermore, eating pattern through different seasons was also accounted for in our dietary questionnaire. Secondly, it should be noted that each of the four studies has limited statistical power, which justifies pooling of the results to arrive at more stable estimates. Although our results (Table 1, Figure 1) suggest that the joint effect of both exposures is between additivity and multiplicativity of the separate effects, the pooling results clearly suggests super-multiplicativity. Therefore, the results from Sudan appear to be compatible with the usual assumption of multiplicativity.

Regarding HBV alone as a risk factor of HCC, results from the pooling with the three other studies, resulted in an OR of 9.6 (95% CI 4.5 – 20.4). This is in line with the results from a meta-analysis study by Donato *et al.*,[14] which included 32 published case-control studies from different parts of the world (OR for HBV = 13.7). The pooled odds ratio of 14 of these studies

conducted in sub-Saharan, Southern African and Asian regions where HBV is endemic was 16.7 (95% CI 14.5-19.3). The strong relative risk of HBV seems to overrule the risk of aflatoxin exposure. However, regarding aflatoxin exposure, among HBV carriers in China it was observed that the mortality rate from liver cancer was 10 times higher in villages where the individual dietary exposure to aflatoxins was high compared to individuals from villages with lower aflatoxin exposure[5]. A case-control study conducted in the Philippines Bulatao *et al.,*[15] observed a risk ratio (RR) of 17.0 in individuals exposed to very high levels of dietary aflatoxins (>7μg per day), and a RR of 13.9 in those exposed to moderate levels (4 to 6 μg per day) when compared to those who were exposed to relatively low levels (0 to 4 μg per day). Since the latter study did not measure HBV serology of the subjects and the Philippines are a HBV endemic region, it is uncertain how much of this significant association could also be attributed to HBV carrier status. Thus, we argue that the risk of HCC seems to depend on the proportion exposed to both factors.

In Sudan, HBV and aflatoxin exposure accounts for about 80% of HCC either alone, or in combination. The results shown in Figure 2 (column 2), provide the best description of our dataset obtained from modeling with product terms. However, a multiplicative model (column 3) is also compatible with our data and corresponds better to the pooled data. Thus, the multiplicative model might give a valid estimate for Sudan, which brings the population attributable risk for aflatoxins in Sudan to 60% exceeding that for hepatitis (52%). As for primary prevention strategies, the purpose is to limit the incidence of liver cancer by controlling exposure to risk factors or by increasing individual resistance to them (e.g. by vaccination). The proportion in a population that could be prevented depends on the RRs and prevalence of the exposure (either HBV or aflatoxins). Reduction of aflatoxins from Sudanese food may serve as along-term strategy for Sudan, but a short-term strategy by HBV immunization of children as well as high risk groups in endemic areas is also feasible.

In summary, peanut butter consumption and hepatitis infections, are important risk factors of HCC. In Sudan, reduction of aflatoxin contamination of foods seems to be slightly more important for the prevention of HCC than HBV immunization.

References

1. Wild CP, Hall AJ. Hepatitis B virus and liver cancer unanswered questions. *Cancer Surveys: Infections and Human Cancer,* Imperial Cancer Research Fund. 1999; **33:** 35-54.
2. Ross RK, Yuan JM, Yu MC, *et al.* Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet.* 1992; **339:** 943-46.
3. Qian GS, Ross RK, Yu MC, *et al.* A follow-up study of urinary markers of flatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. *Cancer Epidemiol. Biomark and Prev.* 1994; **3:** 3-10.
4. Wang LY, Hatch M, Chen CJ, *et al.* Aflatoxin exposure and risk of hepatocellular carcinoma in Taiwan. *Int. J. Cancer.* 1996; **67:** 620-25.
5. Yeh FS, London WT. Risk factors for hepatocellular carcinoma in Guangxi, People's Republic of China. *Natl. Cancer Inst. Monogr.* 1985; **69:** 47-48.
6. Peers F, Bosch X, Kaldor J, Lindsell A, Pluijmen M. Aflatoxin exposure, hepatitis B virus infection and liver cancer in Swaziland. *Int. J. Cancer.* 1987; **39:** 545-53.
7. Sell S, Hunt JM, Dunsford HA, Chisari FV. Synergy between hepatitis B virus expression and chemical hepatocacinogens in transgenic mice. *Cancer Res.* 1991; **51:** 1278-85.
8. Bannasch P, Khoshkhou NI, Hacker HJ, *et al.* Synergistic hepatocarcinogenic effect of hepadnaviral infection and dietary aflatoxin B_1 in woodchucks. *Cancer Res.* 1995; **55:** 3318-30.
9. Cova L, Wild CP, Mehrotra R, *et al.* Contribution of aflatoxin B1 and hepatitis B virus infection in the induction of liver tumours in ducks. *Cancer Res.* 1990; **50:** 2156-63.
10. Omer RE, Verhoef L, van't Veer P, *et al.* Peanut butter intake, GSTM1 genotype and hepatocellular carcinoma: a case-control study in Sudan. *Cancer Causes and Control.* (in press).
11. Omer RE, Bakker MI, van't Veer P, *et al.* Aflatoxin and Liver Cancer in Sudan. *Nutr. Cancer.* 1998; **32:**174-180.
12. Kleinbaum DG, Kupper LL, Morgenstern H. Epidemiologic Research, Principles and Quantitative Methods. Life time Learning Publications. London. 1982.
13. Rothman KJ, Sander G. Modern Epidemiology. 2nd edition. Lippincott-Raven publishers. 1998; 47-64, 329-342.
14. Donato F, Boffetta P, Puoti M. A meta-analysis of epidemiological studies on the combined effect of hepatitis B and C virus infections in causing hepatocellular carcinoma. *Int. J. Cancer.* 1998; **75:** 347-54.
15. Bulatao-Jayme J, Almero EM, Castro MCA, Jardeleza MTR, Salamat LA. A case-control dietary study of primary liver cancer risk from aflatoxin exposure. *Int. J. Epidemiology.* 1982; **11:**112-119.

7

General discussion

Introduction

The studies reported in this thesis were designed to quantify the impact of aflatoxins on the etiology of hepatocellular carcinoma (HCC) particularly in Sudan. The ultimate objective was to find clues for prevention and control of HCC among the Sudanese population. This objective was pursued by conducting a case-control study in two regions in Sudan (West and Central), in order to maximize heterogeneity in exposure to aflatoxins and HCC risk. Apart from these issues of direct relevance to the Sudanese population, we also addressed issues of general scientific interest that help to substantiate our findings e.g. genetic factors and albumin adducts.

First, an explorative study was carried out (Chapter 2), aiming to address contamination of peanuts with aflatoxins and to investigate the feasibility of conducting a case-control study. In this study we also compared the population from both regions, we tested the questionnaire to be used in the main study, and we identified the possibilities to include biological markers in the main study.

The main case-control study included ca 150 cases and 200 controls. In this study we evaluated the risk of peanut butter consumption, storage conditions and GSTM1 (Chapter 3). In addition the possible role of polymorphisms in GSTT1 and EPXH has been investigated in detail (Chapter 4). Subsequently, hepatitis B and C infections have been studied as important risk factors of HCC (Chapter 5). In order to evaluate potential public health measures for HCC prevention in Sudan, the combined effect of hepatitis B virus and intake of aflatoxins was studied in more detail (Chapter 6).

In this chapter, the main findings of our studies are summarized (Table 1). Methodological issues that may interfere with the interpretation of the results are discussed (page 101-110). Subsequently, our results are compared to those from related studies (page 110-113). Relevant public health measures and directions for future research will be presented at the end of this chapter (page 113-116).

Table 1. Main findings of the studies described in this thesis.

Relevant subject	Main findings
Contamination of peanuts with aflatoxins *(Chapter 2)*	Mean contamination level is 20 times the WHO permissible level (10 µg/kg).
Peanut butter consumption and HCC *(Chapter 3)*	Strong positive association especially among subjects deficient in aflatoxin detoxification, (GSTM1 null genotype).
Genetic susceptibility *(Chapter 3,4)*	Whereas GSTM1 has a major role in aflatoxins detoxification, GSTT1 plays no role in HCC. For EPHX polymorphisms, no interaction with peanut butter consumption was observed.
Hepatitis infections and HCC *(Chapter5)*	Hepatitis infections are major risk factors, showing strong associations with HCC.
Public health *(Chapter 6)*	Around 80% of HCC cases are attributable to exposure to aflatoxins (peanut butter intake) and hepatitis B infection. Ca 50% is due to aflatoxins whereas similar percentage could be attributed to hepatitis B virus infection.

Methodological issues

Epidemiological studies are susceptible to several forms of bias. Issues related to validity and precision of the studies presented in this thesis will be discussed in this chapter. Due to poor infrastructures in a developing country like Sudan, special consideration will be paid to the conduct of these studies under such circumstances. Internal validity will be discussed in terms of selection bias (recruitment of subjects, page 101), information bias (assessment of exposure and use of biomarkers, page 104 and 105, respectively) and confounding and effect modification (in the comparison between regions, page 106). Conclusions of the above mentioned will be presented on page 109.

Recruitment of cases

In a case-control study the proper ascertainment of cases and controls is of crucial importance. Apart from objective criteria for the diagnosis of the disease and eligibility criteria for cases

and controls (extensively described in Chapter 3), this also requires unbiased recruitment procedures.

With respect to recruitment procedures, cases might be identified through regional health registers, through records of large ambulatory-care practices, or through admission to hospitals. In this study cases were recruited from the admission lists of five out of six hospitals in Khartoum for the main study (four in the explorative study, chapter 2). The use of these lists enabled us to include cases from the two study regions irrespective of their exposure history. Since HCC is a rare and serious disease, new suspected incident cases are usually referred from the regional to the hospitals in Khartoum for adequate diagnosis and treatment. Moreover, one of the hospitals, which served as a main source for cases in both the explorative and the main study, is a national centre for gastro-intestinal diseases in Sudan; HCC cases diagnosed in other hospitals in Khartoum are referred to this centre for treatment. This leaves the one (two) non-cooperating hospital(s) even less important; furthermore, the latter hospital(s) is of minor importance to case-recruitment since it obtains the majority of its cases from northern Sudan i.e. it does not represent the catchment area of cases originating from West and Central Sudan.

During the conduct of this study, it appeared that absence of proper hospital registries in Sudan, made it rather difficult to verify completeness of case recruitment, and qualitative arguments have to be used indeed. One might expect that some cases were missed especially from the larger western region, which is situated at a slightly further distance from Khartoum. Therefore, the difference in risk between the two regions (and the association with intake of peanut butter) might be underestimated. Nevertheless, as we anticipated, we do see a higher risk of HCC in the western region.

Therefore, improvement of registries in the study regions as well as in other regions in Sudan seems to be of crucial importance as it could provide reliable incidence figures for comparing the regions in this study and it could offer better possibilities for monitoring and evaluation of public health in Sudan. Furthermore, such registries might help to compare the health issues in Sudan to other countries. For instance, due to the lack of proper cancer registries, currently no data on cancer incidence are available from Sudan in the WHO-IARC series "cancer incidence in five continents", 1997[93].

In conclusion, we are confident that the cases that have been missed have not seriously affected our results. However, in order to substantiate such a statement quantitatively, hospitals and health registers in Sudan need to be improved.

Recruitment of controls

The studies focused especially on two regions in Sudan (West and Central), where peanut consumption is considered to be habitual and that were suspected to be different in exposure to aflatoxins as well as the incidence of HCC. This approach was intended to increase the heterogeneity of exposure to aflatoxins. Controls were obtained randomly by the use of infrastructure available in the area e.g. health centers (explorative study) or sugar shops (main study).

In the explorative study, the controls from both regions were recruited from subjects attending three health centers chosen randomly by means of a multistage sampling procedure. The same statistical procedure was adopted in the main study for the random selection of sugar shops from West and Central Sudan (four and six sugar shops, respectively). Registration by sugar shops is required for all inhabitants in the region irrespective to their income status. Controls were then randomly recruited out of the sugar shop lists. In Sudan, this was the only available infrastructure that can provide a frame of potentially eligible controls in the target population; the population enrolled via the health centers can be biased as compared to the target population under consideration, because of their illness and complaints. The sugar shop lists provide advantages over the health centers lists as they could give an unbiased procedure for control recruitment.

Unfortunately the sugar shop lists do not provide inhabitant's ages. Therefore, age matching could not be properly attained for the control groups in this study, because it would require too much extra work and time. However, because of time constraints in the conduct of our study, we had to make a choice between proper age matching of controls and blood collection from controls. We considered the latter to be more important to properly address hepatitis infections, genetic susceptibility and albumin adducts.

From the former, the necessity of population registries establishment arises. Such registries could make age matching more easy and feasible for studies like ours. In addition, they may

provide good sources for descriptive data relevant to other studies, as well as producing statistics on the occurrence of cancer in Sudan to be compared to other countries.

In conclusion, although recruitment of controls by means of sugar shop lists did not allow matching by age, we consider the controls in this study representative of the target population when age is accounted for by statistical methods. Yet, the presence of population registries would enable age matching and improve the statistical efficiency of similar studies. When combined with cancer registries, such registries could provide a proper estimate of the incidence figures of different tumors in Sudan.

Assessment of exposure to aflatoxins

In retrospective studies, dietary questionnaires should be designed to investigate the exposure of cases and controls with similar validity, with the reference period coinciding with the etiological relevant time frame. In the present study, intake of aflatoxins in the decade(s) before disease occurrence is considered relevant to the etiology of HCC. The intake of aflatoxins depends on both the food pattern and the aflatoxin content of foods. In Sudan, these two determinants of aflatoxin intake have different characteristics, relevant to exposure assessment.

Food pattern. In this respect the questionnaire investigates the food habits especially peanut butter consumption in terms of frequency, daily quantity and number of consumers (resulting in average consumption). In order to account for seasonal differences in eating pattern, intake was assessed over a whole year by using questions on intake during harvest period "inseason" and "off season". Except for this seasonal variation, food patterns tend to be constant for long periods of time because of limited food sources and possible variations. Other main components of meals, which are suspected to be possible sources of aflatoxins (i.e. sorghum, millet or corn), were also investigated. Therefore, given these stable foods habits and the design of the questionnaire, recall bias is not considered a serious problem. As dietary habits of cases tend to change when symptoms of liver cancer arise, the reference period for this food pattern was one year before symptoms occurred. Misclassification of exposure might occur when a population is in a transition stage e.g. due to changes in the economic situation. Thus, as a result of the recent economic decline in Sudan, the central region might be in a transition stage regarding food habits i.e. people tend to shift to a cheaper source of protein (peanut butter).

Aflatoxin content of foods. To assess the extent of aflatoxin contamination, duration and type of storage were investigated as indicators of this contamination. Samples of peanut butter were collected randomly at different time intervals after harvest and from different storage types in the two study regions. Subsequently the level of aflatoxins was determined in these samples. However, we suspected that aflatoxin content of peanut butter might be highly heterogeneous within the same storage type, and from year-to-year; this is probably due to the yearly variation of environmental factors such as temperature, humidity and precipitation. Therefore, aflatoxins content of samples is not considered reliable enough to calculate the individual exposure to aflatoxins over a long time period.

Genetic polymorphisms. Considerable differences regarding the metabolism of aflatoxins determine the susceptibility to liver cancer. The carcinogenicity of aflatoxins partially results from the incapability of producing aflatoxin-detoxification enzymes e.g. glutathione S-transferase (GSTM1). Genetic polymorphisms in such enzymes result in higher exposure of the target organ (target DNA molecules) to carcinogenic aflatoxin metabolites.

Assessment of exposure using biological markers

Hepatitis infections. The high prevalence of self-reported hepatitis infections observed in the explorative study as well as the main study was due to the high prevalence of non-carcinogenic types of hepatitis. Thus the prevalence of anti-hepatitis A virus in adult Sudanese subjects was found to be 96%[94]. However, only hepatitis B and C viruses are relevant to liver cancer. In the explorative study self-reported hepatitis was not associated to HCC, which was attributed to the low specificity of self-reported hepatitis, leading to misclassification results of the biologically relevant forms of hepatitis. For this reason, the laboratory checking for this important risk factor was considered a necessity in the main study, even more important than improved statistical efficiency by matching for age (see page 103). In the main study both the questionnaire data and blood samples were available. Indeed, sensitivity and specificity of self-reported hepatitis indicate that questionnaires are not suitable to assess hepatitis infections (Table 2).

Table 2. Lack of association between self-reported hepatitis infection and serological laboratory data

Lab results	Self-reported hepatitis			Test characteristics
	Yes	No	Total	
HBV				
Positive	31	32	63	Sensitivity = 50%
Negative	119	132	251	
Total	150	164	314	Specificity = 53%
HCV				
Positive	6	10	16	Sensitivity = 43%
Negative	144	154	298	
Total	150	164	314	Specificity = 52%

Albumin adducts. Beyond the intake of aflatoxins, its metabolites can be measured in humans and animals as markers of exposure. In an extension to this study, aflatoxin albumin adducts will be measured in blood serum as an indicator of recent exposure (integrating over about three months). However, cases might have changed their eating habits. In our study more than 80% of cases stopped eating peanut butter at the time they became diseased. Therefore, aflatoxin albumin adducts are intended to be determined among the controls only and will be used to compare the exposure of aflatoxins in the two regions and its association with peanut butter intake as well as with genotype.

In conclusion. Human biomarkers are considered to provide useful information for epidemiological research. Thus, the contribution of hepatitis infections in HCC can be validated properly by the use of serological laboratory analysis for hepatitis markers.

Comparisons between regions.

The previous findings by Zaki, 1991[55] and the clinical observations of a relatively high number of HCC cases from West Sudan as compared to Central Sudan contributed to the rationale of the present study. The population of the two regions together was taken as the study population, with the number of controls proportional to the population size of each region. Thus, the range of exposure to aflatoxins was considered to be large, which might result in observing a stronger association (provided that confounders did not differ markedly between the regions). However, region appeared to be a modifier of the association between peanut butter consumption and HCC in crude data analysis, although this disappeared largely in

further analysis. Therefore, we systematically discuss the regional differences and the possible background of the effect modification by region (if any) in this paragraph.

In order to explain the risk difference between the high and lower HCC incidence region and also to provide information directly relevant to exposure to aflatoxins, comparisons were made at the group level between the two regions. Our findings clearly show that the western region has the higher incidence of HCC as compared to the central region (i.e. OR=1.9). Central Sudan has a good infrastructure and higher socio-economic status so that people can possibly reach Khartoum hospitals more easily when they are diseased. This way, cases that would have been missed during the study period would probably be living in West Sudan. Thus, the OR of 1.9 is not likely to overestimate the risk differences between the regions. Nevertheless, we will evaluate whether this risk difference can be explained by confounding by other risk factors that differ between the regions (i.e. life style, food patterns and hepatitis infections), or by effect modification (i.e. stability of food habits, aflatoxin content of food and genetic susceptibility).

Potential confounders underlying the region effect

Life style. Alcohol consumption and cigarette smoking were found to differ between the two regions. They were not materially associated with HCC, however. Thus, when they were included in a multivariate regression model to control for their confounding effect, the regional risk difference remained the same.

Food patterns. Other food patterns differed between the two regions. When these foodstuffs are suspected to be contaminated with aflatoxins or other mycotoxins (e.g. millet consumption), this may play a role in the risk difference between the regions. However, when included in a multivariate logistic model the difference in the risk between the two regions remained of similar strength (OR=1.8).

Hepatitis infections. The association between region and HCC may be confounded by hepatitis infections. However, the prevalence of hepatitis infections is 10% and 8% in West and Central Sudan respectively. When multivariate logistic regression was conducted the risk difference between the two regions remained of similar strength.

Potential modifiers underlying the region effect

Aflatoxins content. Peanut butter samples were collected from different storage types in the two regions. As mentioned before, these levels fluctuated highly during the explorative and the main study periods (Table 3). Results from the explorative study are in line with previous findings from West Sudan[20]. Incidentally the main study period 1997-1998 has shown very high aflatoxins levels in peanuts from Central Sudan (reports from Quality Control Dept. Sudan). In addition, the long-term aflatoxins exposure in West Sudan is more likely to explain the higher HCC incidence in West over Central Sudan.

Stability of food habits. Given the fact that most subjects in West Sudan are farmers and labourers, consumption of peanut butter in West Sudan is more stable over longer periods. In contrast, Central Sudan has recently become a transition society i.e. as a result of economic decline in the latest period (3-4 years) people at different educational levels and occupations are shifting to cheap sources of protein like peanut butter. Therefore, the food frequency questionnaire over a one year reference period may not reflect long-term exposure to aflatoxins in the central region, but it does in the western region. The resulting misclassification of the long-term exposure may lead to attenuation of the association, especially in Central Sudan.

Genetic factors. Genetic factors not included in this study may, in theory, explain the risk difference between the two regions. GSTM1, a main modifier of the association between peanut butter intake and HCC was found to be similar in the two regions. Genetic factors that are different among Sudanese from Arab origin and those from African origin may partially explain the difference in risk between the two regions, indeed the EPHX polymorphism was slightly different between the regions, but we lack sufficient power to reach a firm conclusion.

Table 3. Mean value of AFB_1 concentration over 1-year period in samples of different storage types in West and Central Sudan.

Region	Storage type	Main study (1997-1998)			Explorative study (1995)		
		#samples	µg/kg*	(log_{10})	#samples	µg/kg*	(log_{10})
West Sudan	Dry storage	10	20.6	(1.32)	16	8.7	(0.94)
	Humid storage	11	15.1	(1.18)	11	55.2	(1.74)
Central Sudan	Dry storage	4	27.5	(1.44)	6	6.0	(0.78)
	Humid storage	5	13.0	(1.11)	4	5.5	(0.74)

* = Concentrations were retransformed from mean value on a log_{10} scale.

Thus, life style, food patterns and hepatitis infections can not explain the high HCC risk in West Sudan as compared to Central Sudan. The strong association between peanut butter consumption and HCC in West Sudan might in theory be due to a high aflatoxin content and higher prevalence of EPHX polymorphisms in West Sudan and/or less stable food habits in Central Sudan. Although the effect modification disappeared in multivariate analysis, it is difficult to say whether this is truly and completely absent or whether its presence reflects limitations of the study size for such types of analysis.

Conclusions

Selection of cases. Cases enrolled in this study were community-based subjects, from the same catchment area of the controls. In addition, cases were clinically and pathologically confirmed as HCC patients (95% of participating cases were histologically confirmed). Our weekly screening of the admission lists of the five hospitals in Khartoum will enable almost all cases from the two regions independent to their history of exposure to aflatoxins to be included in the study. Those who might have been missed during the conduct of the study were not considered to affect the internal validity so much, but the completeness could not be verified by health administration systems in Sudan, unfortunately.

Selection of controls. The random-based selection of controls enabled each control from the target population irrespective to his or her exposure history to have an equal chance to participate in the study. However, the ideal balance of age distribution in cases and controls could not be properly attained; nevertheless, age did not appear to be strongly related to peanut butter consumption and therefore, it did turn out not to be a strong confounder.

Assessment of exposure to aflatoxins. The use of peanut butter is considered to be common practice in the Sudanese population, and there is no knowledge or concern on aflatoxins among the population. Given these stable food habits, the design and standardized format of the questionnaire and the fact that only one person questioned all participating subjects, information bias is not likely to explain the positive association between peanut butter consumption and HCC. Moreover, the observed interaction with GSTM1 genotype cannot be affected by information bias.

Assessment of hepatitis infection. Hepatitis infections appeared to be an independent risk factor of HCC in this study. Questionnaire information is less reliable for hepatitis infections, due to the high prevalence of the non-carcinogenic types of hepatitis among Sudanese.

Comparison between regions. Differences in life style, food patterns, and case ascertainment can not explain the difference in HCC risk between the two regions. Aflatoxin content of peanut butter is higher in West Sudan than in Central Sudan, although this was clearer in the explorative study than in the main study. Peanut butter consumption is a habitual practice in West Sudan whereas it considered as a recent habit in Central Sudan (transition society), which may lead to misclassification of exposure especially in Central Sudan.

In conclusion. The findings reported from the studies in this thesis (Table 1, page 101) are not likely to be seriously affected by issues related to the internal validity (i.e. selection bias, information bias and confounding). However, to ensure the completeness of subject recruitment, additional data from hospitals and population registries would be welcomed.

Comparison with other studies

Results from this case-control study provide evidence for an etiological role of aflatoxin intake in hepatocellular carcinoma in Sudan. In this section, we will discuss the discrepancies of our findings with the retrospective and prospective epidemiological studies mentioned in the introduction (Chapter 1). Our findings on dietary exposure to aflatoxins will be compared to other epidemiological studies, which used dietary sources of aflatoxins or/and markers of exposure. The findings for genetic susceptibility and hepatitis infections will be compared to other relevant studies presented previously. The potential interaction of hepatitis B virus with aflatoxins has been compared to relevant literature in chapter 6; this chapter contains a discussion on potential public health implications of our findings for Sudan.

Retrospective studies using dietary sources and biomarkers of aflatoxins exposure

The positive association between the intake of aflatoxins or peanut butter and HCC (chapter 3) is in line with two large retrospective studies (Bulatao *et al.*, 1982 and Zang *et al.*, 1998)[69,71], whereas two other studies may have been too small to detect the association (e.g. Srivatanakul *et al.*,1991)[70]. Furthermore, the latest two studies (Srivatanakul *et al.*, 1991 and Lam *et al.*,

1982)[68,70] addressed exposure of cases in the hospital, whereas usual exposure before occurrence of symptoms was addressed in the present study. Thus, the apparent inconsistency between our findings and these two studies might be due to altered diet in the cases because of illness. Indeed in our own study ca 80% of cases reported that they had reduced their intake of peanut butter recently, whereas 2% of controls reported so. For the same reason comparison of biomarkers of aflatoxins between cases and controls in case-control studies is of limited value (e.g. Srivatanakul *et al.*, 1991)[70]. In the study by Lam *et al.*,[68] the low range in the frequency of consumption of presumed aflatoxin-contaminated food might have led to misclassification of exposure. Moreover, in the latter study aflatoxin contamination of food was based on a market survey that had been performed 10 years before the study.

Prospective studies using biomarkers of aflatoxins exposure

Our finding for dietary intake of peanuts (or aflatoxins) (Chapter 3) are consistent with the cohort and the nested case-control studies mentioned in the introduction. In most of these studies markers of aflatoxins (albumin and/or urinary adducts) were examined as recent markers of exposure to aflatoxins. Since the samples were obtained from cases before the disease became clinically manifest, the comparison of markers between cases and controls in prospective studies is unlikely to be affected by altered dietary habits. However, the comparison of dietary exposure of aflatoxins in our study allows to address a dose-response relationship, whereas in most of the previously mentioned studies the comparison of albumin and urinary adducts had to be made for a dichotomy of detectable vs. non-detectable levels.

Genetic factors

Humans differ with respect to the metabolism of AFB_1-exo-8,9-epoxide conjugation to glutathione S-transferase (GST). Our results on genotypes clearly show that the risk of HCC from peanut butter consumption is mainly restricted to people who have the GSTM1 null genotype (Chapter 3). Chen *et al.*, 1996[65] reported similar findings; they observed that high AFB_1-adduct levels were restricted to subjects having the GSTM1 null genotype. Our finding is in line also with others (e.g. McGlynn *et al.*, 1995)[95] who postulated that the high risk of developing AFB_1-adducts may be mainly among individuals with the GSTM1 null genotype. Such an interaction between GSTM1 and albumin adducts was also shown in a study including 328 healthy Gambian subjects[96].

The relation between GST activity and HCC is also clear from interspecies differences; animal species found to be resistant to aflatoxin carcinogenesis such as mouse, have three to five times more GST activity than susceptible species, such as rats. Humans have even lower GST activity than rats or mice and are supposed to experience higher risk of HCC[21]. Thus, our observations on the interaction between peanut butter consumption and GSTM1 null genotype are also in line with these biochemical interspecies differences.

Apart from GST-μ, other enzymes such as GST-θ and microsomal epoxide hydrolase (mEH) might be involved in detoxification of aflatoxins in hepatocytes, although less prominent[97]. However, we did not observe an association between the GSTT1 null genotype and HCC (Chapter 4), whereas in a relatively small study (32 cases and 73 controls)[65] a positive association between the GSTT1 null genotype and HCC was observed. Our findings are in line with those in the Gambia study by Wild *et al.*[96], who did not observe an association between GSTT1 and AFB_1-adducts levels. As also described in Chapter 4, our results indicated that with respect to EPHX polymorphism, carriage of the combination of EPHX 113HH and 139HH genotypes is associated to a moderately increased risk of HCC. It has been found by McGlynn *et al.*,[95] that EPHX 113HH genotype carriers had a higher risk of HCC than those without this genotype. These results however, could not be confirmed in the Gambian study[96] investigating the association between this genotype and high levels of AFB_1-adducts, and similarly our results did not indicate presence of an association between the EPHX 113HH genotype and HCC. No interaction between EPHX genotype and peanut butter consumption was observed in this study; this is consistent with biochemical studies which showed that mEH-catalyzed hydrolysis may not be a limiting factor in aflatoxin detoxification since hydrolysis of aflatoxin 8,9-epoxide can occur spontaneously at equally high efficiency[98].

In conclusion, the observation of an interaction between peanut butter consumption and GSTM1 genotype is consistent with other epidemiological studies that used either AFB_1-albumin adducts levels or HCC as endpoints. This adds further credibility to the interpretation that the peanut butter consumption is indeed a causal factor of HCC in Sudan.

Hepatitis virus infections

In this study hepatitis B and hepatitis C viral infections were found to be strongly associated with HCC (Chapter, 5). Our findings for this association are consistent with the findings shown

by Donato and colleagues[74] from a meta-analysis of 32 published case-control studies from different parts of the word. Thus, our findings were closely related to the summary odds ratios of 14 of these studies from sub-Saharan and Southern Africa regions where hepatitis B infection is highly endemic. In our study, the prevalence of HBV among healthy subjects is found to be higher than the prevalence of HCV, and was similar to what has been reported from an Ethiopian population[99] (country neighbouring to Sudan). However, HBV prevalence from our study was not in line with a small Sudanese study[94] in which a HBV prevalence of 24% was assessed from 21 young blood donors. The anti-HCV prevalence in our study population (1.5%) is quite similar of what has been published from Saudi Arabia (1.5%)[100]. The prevalence of both HBV and HCV among HCC cases from our study (50%) are similar to the findings from a Sudanese study by Moudawi *et al.* (59%)[101].

Implications for prevention and future research

Implications for agricultural policy and public health

The results of our study have pointed at both aflatoxins and hepatitis as important factors in the etiology of HCC in Sudan (Chapter 6). Therefore, both agricultural and public health measures can be considered in order to reduce this risk.

Measures in *agricultural policy* could be proposed to reduce exposure to aflatoxins, including action at the level of agricultural practices and at the level of human behavior. Regarding agricultural practices, the following measures are considered relevant. They are known for along time already and our results suggest that they need to be implemented in Sudan as well:

- Agricultural measures should aim to reduce contamination with aflatoxins. In first instance this should aim at minimizing mould growth. To do this, several pre-harvest control measures have been identified (FAO/WHO, 1987)[17], and have been reinforced (FAO/WHO 1999)[18]. These include selection of high resistance varieties especially at rain fed areas, prevention of physical damage to crop by insects, and appropriate crop rotation.
- Similarly harvest precautions should be taken e.g. harvesting of the crop at the proper stage of maturity, proper handling to avoid physical damage and removal of contaminated field soils. Subsequently, contamination during sun drying can be avoided by introduction of mechanical drying and subsequent packing of the crop in clean fumigated sacks.

- Storage conditions are of major importance therefore; it should be promoted by development of dry storage facilities, both at local production sites as well as in big cities.
- Other important measures such as regular quality control at local selling points (markets) and before processing of peanuts. An aflatoxin-detoxification plant, present in Port Sudan city, should be re-established. This plant is built to chemically destruct aflatoxin B_1 in contaminated feed stuffs so as to prevent aflatoxins entering the human food chain. This would probably serve both national health issues as well as economic benefits (export).

In addition to harvesting and storage precautions, the key persons to put agricultural advices into practice need to be taken into account. From this point of view, the following issues are considered relevant:

- Farmers and laborers are key persons controlling the agricultural production. Improvement of the health situation of populations in high-risk areas should focus on these key persons. Ultimately, reduction of aflatoxin contamination will lead to better agricultural production, an improved trade position and economic development.
- Screening of kernels by excluding the damaged or discolored peanuts manually or mechanically or by electronic methods, prior to processing and sales is an important way of minimizing exposure to aflatoxins. Since people in small communities benefit from selling these small-sized and damaged kernels for peanut butter production, this is probably one of the most critical points in reducing exposure to aflatoxins at the individual level.

Based on our results (Chapter 6), the following *public health priorities* for Sudan can be put forward:

- Measures could be taken to reduce the incidence of HBV and HCV infection, such as immunization for HBV of infants in high incidence areas and of high-risk groups in low incidence areas.
- Screening of donor blood should not only address HBV but should be extended to HCV antibodies as well. This might prevent HCV prevalence to reach the levels that are seen in other neighbouring countries such as Libya[102] and Egypt[103].
- Hospital registries should be improved and population-based cancer registries should be established. This can provide full information on the disease burden and geographical patterns of cancer in Sudan. Furthermore, this might help to focus research on aetiology and prevention of diseases of large public health importance.

In the preceeding part various agricultural and public health measures have been indicated to reduce HCC in Sudan. The results of our study suggest that both measures are of similar importance regarding HCC prevention in Sudan.

Unless much cheaper vaccines become available, the costs required for hepatitis vaccination will put a great burden on the governmental and national economics. The immunization for HBV of infants in high incidence areas and of high risk groups in both high and low incidence areas, seems to be of high importance as a short-term prevention strategy for HCC. However, our data suggest that elimination of HBV alone would not be sufficient to eradicate HCC in Sudan.
On the other hand, implementing agricultural policy with proper attention to key persons is put forward to reduce the contamination of peanuts with aflatoxin. Reduced aflatoxin exposure might lead to improved health and higher productivity of farmers and labourers. Ultimately this may lead to a better trade-position and hence the development of the national income as well.

In conclusion we do recommended that the measures in agricultural policy provide the best strategy for a long-term and lasting contribution to the health of the Sudanese people. For the short-term, however, immunization of HBV of infants and high-risk groups in areas where hepatitis is endemic, has to be considered as well.

Future research

Based on our results and insights obtained during this project, several types of research can be considered. These include research directly related to aflatoxins, to their health effects, or concerning prevention of hepatitis. In addition, questions of more general scientific interest could be considered.

First, the occurrence of aflatoxins in other cereals (grain) products and the occurrence of other mycotoxins such as fumonisins, ochratoxin etc. and their relevance to human health in Sudan need a closer look. In addition, the use of bond-ditch water and the possibility of its contamination by blue green algae toxins may require investigation.

The rationale for reduction of aflatoxins in food in highly exposed regions in Sudan might be strengthened by additional research on the role of aflatoxins in other diseases, nutritional status, child growth and morbidity. Thus, aflatoxins are incriminated in neonatal jaundice and

are supposed to cause prenatal death and reduced birth weight. Aflatoxin M_1 was found to occur in up to 40% of breast milk samples from tropical Africa, and this might contribute to kwashiorkor among breast-fed children[22b]. Furthermore, a role of aflatoxins has been proposed in the pathogenesis of malaria. Aflatoxins may contribute to morbidity among young children, and loss of economic productivity by the working force.

In order to substantiate the public health measures to reduce hepatitis transmission among Sudanese people, research questions relevant to hepatitis infections need to be addressed. These include the mode of transmission of hepatitis B, e.g. by scars and other tattoo practices among children particularly in regions where hepatitis is endemic. The relative importance of such cultural-based transmission should be compared to prevention mediated by hepatitis immunisation programs.

Research questions of general scientific interest are also worthwhile to be addressed in future research including a comparison of aflatoxin albumin adducts in different regions in Sudan, or evidence of codon 249 mutations in the p53 gene as a finger print of exposure to aflatoxins among Sudanese HCC patients. Others topics might include intervention with oltipraz, a chemopreventive agent that can lower the biological effect of aflatoxins by decreasing its metabolism to the ultimate carcinogenic form and increasing the detoxification pathway.

Despite of the research questions of direct relevance to Sudan and those of general scientific interest, the research issues should not be used as an argument to postpone the agricultural and public health measures mentioned in the previous section (page 113).

References

1. Carnaghan RBA, Sargeant K. The toxicity of certain groundnut meals V. Poultry. *Vet. Rec.*1961; **73:** 726-27.
2. Blount WP. Turkey "X" disease. *J. Brit. Turkey Fed.* 1961; **9:** 52-54.
3. Bruce HM, Parkes AS. Peanut meal diets for laboratory animals. *J. Hyg. Camb.* 1947; **45:** 70-72.
4. Paget GE. Exudative hepatitis in guinea pigs. *J. Pathol. Bacteriol.* 1954; **67:** 393-96.
5. Schoental R. Liver changes and primary liver tumors in rats given toxic guinea pig diet (MRC Diet 18). *Br. J. Cancer.* 1961; **15:** 812-15.
6. Newberne PM, Russo R, Wogan GN. Acute toxicity of aflatoxin B1 in the dog. *Pathol. Vet.* 1966; **3:** 331-40.
7. Busby WF Jr, Wogan GN. Aflatoxin. In: *Chemical Carcinogenesis.* Searle CE (ed.), American Chemical Society, Washington, D. C. 1984; 945-1136.
8. Cited by Mclean M, Michael FD. Cellular interactions and metabolism of aflatoxin: An update. *Pharmac. Ther.* 1995; **65:** 163-92.
9. Shank RC. Epidemiology of aflatoxin carcinogensis. In: "Environmental Cancer" Kraybill HF, Mehlman MA, (eds.), John Wiley and Sons, New York. 1977; 291-318.
10. Shank RC, Environmental toxicosis in humans. In: Mycotoxins and N-Nitroso-Compounds: Environmental Risks. Shank RC (ed.). CRC press, Boca Raton, Florida. 1981; **1:** 107-40.
11. Bourgeois CH. Acute aflatoxin B1 toxicity in the macaque and its similarities to Reye's syndrome. *Lab. Invest.* 1971; **24:** 206-16.
12. Mehan VK. Analytical methods for detection and estimation of aflatoxins. Paper presented at the Regional Plant Protection Group Meeting, Harare, Zimbabwe, February 15-21, 1987.
13. Jarvis B. Factors affecting the production of mycotoxins. *J. Appl. Bact.* 1971; **34:** 199-213.
14. Brackett RE. Strategies for dealing with aflatoxins in peanuts. In: Trends in Food Product Development. Yam TC, Tan C (eds.). Singapore institute of food science and technology. Singapore, 1989; 83-91.
15. Mukherjee K, Lakshminarasimham AV. Aflatoxin contamination of sorghum seeds during storage under controlled conditions. *Int. J. Microbiol. Virol. Parasitol. Infect. Dis.* 1995; **282:** 237-43.
16. Sanders TH, Cole RJ, Blankenship PD, Dorner JW. Aflatoxin contamination of peanuts from plants drought stressed in pods or root zones. *Peanut Sci.* 1993; **20:** 5-8.
17. Mannon J, Johnson E. Fungi down on the farm. *New Sci.* 1985; **105:** 12-16.
18. World Health Organization Technical Report Series No. 759. Evaluation of certain food additives and contaminants. Thirty-first report on the joint FAO/WHO expert committee on food additives, World Health Organization, Geneva. 1987; 33-37.
19. World Health Organization Technical Report Series No. 884. Evaluation of certain food additives and contaminants. Forty-nineth report on the joint FAO/WHO expert committee on food additives, World Health Organization, Geneva. 1999; 69-88.
20. Habish HA, Abdulla MA. The incidence of aflatoxin in Sudanese groundnut. *Trop. Sci.* 1971; **13:** 279-87.
21. International Agency for Research on Cancer (IARC). Monographs On The Evaluation of Carcinogenic Risks To Humans, *Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins.* 1993; **56:** 244-395.

22. Omer RE. Study on the effect of field and storage environments on aflatoxin contamination of Rahad Agricultural Scheme-Groundnut. M. Sc. Thesis, University of Khartoum, 1992.

22b. Hendrickse RG, Coulter JB, Lampluch SM, *et al.* Aflatoxins and kwashiorkor. Epidemiology and clinical studies in Sudanese children and findings in autopsy liver samples from Nigeria and South Africa. *Bull. Soc. Pathol. Exotique et de ses Filiales.* 1983; **76:** 559-66.

23. Cole RJ, Cox RH. Handbook of Toxic Fungal Metabolites, New York Academic Press, 1981; 1-66.

24. Kamimura H. Removal of mycotoxins during food processing. In: Mycotoxins and Phycotoxins. Natori S, Hashimoto K, Ueno Y (eds.). Elsevier Science Publishers. Amsterdam, 1989; 169-76.

25. Hesseltine CW, Sorenson WG, Smith M. Taxonomic studies of the aflatoxin producing strains in the *Aspergillus flavus* group. *Mycologia.* 1970; **62:** 123-32.

26. Maggon KK, Gupta SK, Venkitasubramanian TA. Biosynthesis of Aflatoxin. *Bacteriol. Rev.* 1977; **41:** 822-55.

27. Maggon KK, Gupta SK, Venkitasubramanian TA. Metabolism of aflatoxin B1 and G1 by *Aspergillus parasiticus. Experientia.* 1973; **29:** 1210-11.

28. Swenson DH, Miller EC, Miller JA. Aflatoxin B_1-2,3-oxide: evidence for its formation in rat liver in vivo and by human liver microsomes in vitro. *Biochem. Biophsy. Res. Commun.* 1974; **60:** 1036-43.

29. Shimada T, Guengerich FP. Evidence for cytochrome $P450_{NF}$, the nifedipine oxidase, being the principle enzyme involved in the bioactivation of aflatoxins in human liver. *Proc. Natl. Acad. Sci.* 1989; **86:** 462-65.

30. Raney KD, Shimada T, Kim DH, Groopman JD, Harris TM, Guengerich FP. Oxidation of aflatoxins and sterigmatocystin by human liver microsomes: significance of aflatoxin Q1 as a detoxification product of aflatoxin B1. *Chem. Res. Toxicol.* 1992; **5:** 202-10.

31. Ueng YF, Shimada T, Yamazaki H, Guengerich FP. Oxidation of aflatoxin B1 by bacterial recombinant human cytochrome P450 enzymes. *Chem. Res. Toxicol.* 1995; **8:** 218-25.

32. Sabbioni G, Skipper P, Buchi G, Tannenbaum SR. Isolation and characterization of the major serum albumin adduct formed by aflatoxin B1 in vivo in rats. *Carcinogenesis.* 1987; **8:** 819-24.

33. Degan GH, Neuman HG. The Major metabolite of aflatoxin B1 in the rat is a glutathione conjugate. *Chem. Biol. Interact.* 1978; **22:** 239-55.

34. Degan GH, Neuman HG. Differences in aflatoxin B1-susceptibility of rat and mouse are correlated with the capability in vitro to inactivate aflatoxin B1-epoxode. *Carcinogenesis.* 1981; **2:** 299-306.

35. Hsieh DPH. Mode of action of mycotoxins. In: Mycotoxins in Food, Krogh P (ed.). Academic Press, Cambridge. 1987; 149-76.

36. Johnson WW, Guengerich FP. Reaction of aflatoxin B1 exo-8,9-epoxide with DNA: Kinetic analysis of covalent binding and DNA-induced hydrolysis, *Proc. Natl. Acad. Sci. U.S.A.* 1997; **94:** 6121-25.

37. Hill RA, Blankenship PP, Cole RJ, Sanders TH. Effects of soil moisture and temperature on preharvest invation of peanuts by *Aspergillus flvus*, and subsequent aflatoxin development. *Appl. Env. Microbiol.* 1983; **45:** 628-33.

38. Carnaghan RBA, Hartley RD, O'Kelly J. Toxicity and fluorescence properties of the aflatoxins. *Nature.* 1963; **200:** 1101-02.

39. Van Rensburg SJ. Role of epidemiology in the elucidation of mycotoxin in health risks. In: Mycotoxins in Human and Animal Health, Rodricks, JV, Hesseltine CW, Mehlman, MA. (eds.), Pathotox, Forest Park South, Illinois. 1977; 699-711.
40. Campell TC, Caedo JP, BulataoJayme J, Salamat L, Engel RW. Aflatoxin M1 in human urine. *Nature.* 1970; **227:** 403-4.
41. Lye MS, Ghazali AA, Mohan J, Alwin N, Nair RC. An Outbreak of acute hepatic encephalopathy due to severe aflatoxicosis in Malaysia. *Am. J. Trop. Med. Hyg.* 1995; **53:** 68-72.
42. McCann J, Choi E, Yamasaki E, Ames BN. Detection of carcinogens in the Salmonella microsome test: Assay of 300 chemicals. *Proc. Natl. Acad. Sci.* 1975; **72:** 5135-39.
43. Foster PL, Eisenstadt E, Miller JH. Base substitution mutations induced by metabolically activated aflatoxin B1 Proc. *Natl. Acad. Sci. U. S. A.* 1983; **80:** 2695- 98.
44. el-Zawahri M, Moubasher A, Morad M, el-Kady I. Mutagenic effect of aflatoxin B1. *Ann. Nutr. Aliment.* 1977; **31:** 859-66
45. Levy DD, Groopman JD, Lim SE, Seidman MM, Kraemer KH. Sequence specificity of aflatoxin B1-induced mutations in a plasmid replicated in xeroderma pigmentosum and DNA repair proficient human fibroblasts. *Cancer Res.* 1992; **52:** 5668-73.
46. Cole KE, Jones TW, Lipsky MM, Tump BF, Hsu IC. In vitro binding of aflatoxin B1 and 2-acetylaminofluorene to rat, mouse and human hepatocyte DNA: The relationship of DNA binding to carcinogenicity. *Carcinogenesis.* 1988; **9:** 711-16.
47. Aguilar F, Hussain SP, Cerutti P. Aflatoxin B1 induce the transversion of G > T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.* 1993; **90:** 8586-90.
48. Chang YJ, Mathews C, Mangold K, Marien K, Hendricks J, Bailey G. Analysis of ras gene mutations in rainbow trout liver tumors initiated by aflatoxin B1. *Mol. Carcinogen.* 1991; **4:**112-19.
49. The World Health Report 1997. Conquering suffering enriching humanity. Report of the Director-General. *World Health Organization.* Geneva. 1997.
50. Beasley R. Hepatitis B virus: the major etiology of hepatocellular carcinoma. *Cancer.* 1988; **61:** 1942-56.
51. Di Biscegli A. Hepatocellular carcinoma: molecular biology of its growth and relationship to hepatitis B virus infection. *Med. Clin. North .Am.* 1989; **73:** 985-95.
52. IARC website www-dep.iarc.fr/cgi-bin/exe/globocan/rate.exe, 18th Oct. 1999.
53. World Cancer Research Fund Food. *Nutrition and the prevention of cancer: a global perspective.* American Institute for Cancer Research. Washington DC. 1997.
54. Bruix J, Calvet X, Bru C, Rodes J. Clinical pattern and national history of hepatocellular carcinoma. *Eur. Clin. Digest. Dis.* 1992; **1:** 97-102.
55. Zaki M. Aetiological factors in HCC. Thesis. Dept. of Medicine, Faculty of Medicine University of Khartoum, 1991.
56. Munoz N, Bosch FX. Epidemiology of hepatocellular carcinoma. In: Neoplasms of the liver. Okuda K, Ishaki KG (eds.). Springer: Tokyo. 1987; 3-19.
57. Hatch MC, Chen CJ, Levin B, *et al.* Urinary aflatoxin levels hepatitis B virus infection and hepatocellular carcinoma in Taiwan. *Int. J. Cancer.* 1993; **54:** 931-34.

58. Yeh FS, Yu MC, Mo CC, Luo S, Tong MJ, Henderson BE. Hepatitis B virus, aflatoxin and hepatocellular carcinoma in Southern Guangxi, China. *Cancer Res.* 1989; **49:** 2506-09.

59. Ross RK, Yuan JM, Yu MC, *et al.* Urinary aflatoxin biomarkers and risk of epatocellular carcinoma. *Lancet.* 1992; **339:** 943-46.

60. Qian GS, Ross RK, Yu MC, *et al.* A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in shanghai, people's Republic of China. *Cancer Epidemiol., Biomarkers & Prev.* 1994; **3:** 3-10.

61. Autrup H, Sermet T, Wakhisi J, Wasunna A. aflatoxin exposure measured by urinary exposure of aflatoxin B1-guanine adduct and hepatitis B virus in areas with different liver cancer incidence in Kenya. *Cancer Res.* 1987; **47:** 3430-3.

62. Olsen JH, Dragsted L, Autrup H. Cancer risk and occupational exposure to aflatoxins in Denmark. *Br. J. Cancer.* 1988; **58:** 392-6.

63. Sun Z, Lu P, Gail MH, *et al.* Increased risk of hepatocellular carcinoma in male hepatitis B surface antigen carriers with chronic hepatitis who have detectable urinary aflatoxin metabolite M1. *Hepatology.* 1999; **30:** 379-83.

64. Chen CJ, Wang LY, Lu SN, *et al.* Elevated aflatoxin exposure and increased risk of hepatocellular carcinoma. *Hepatology.* 1996; **24:** 38-42.

65. Chen CJ, Yu MW, Liaw YF, *et al.* Chronic hepatitis B carriers with Null genotypes of glutathione S-Transferase M1 and T1 Polmorphisms who are exposed to aflatoxin are at increased risk of hepatocellular carcinoma. *Am. J. Hum. Genet.* 1996; **59:** 128-34.

66. Wang LY, Hach MC, Chen CJ, *et al.* Aflatoxin exposure and the risk of hepatocellular carcinoma in Taiwan. *Int. J. Cancer.* 1996; **67:** 620-25.

67. Yu MW, Lien JP, Chiu YH, Santella RM, Liaw YF, Chen CJ. Effect of aflatoxin metabolism and DNA adduct formation on hepatocellular carcinoma among chronic hepatitis B carriers in Taiwan. *J. Hepatol.* 1997; **27:** 320-30.

68. Lam KC, Tong MJ. Analytical epidemiology of primary liver carcinoma in the pacific Basin. *Natl. Cancer Inst. Monogr.* 1982; **62:** 123-7.

69. Bulatao JJ, Almero EM, Castro MC, Jardeleza MT, Salamat LA. A case-control dietary study of primary liver cancer risk from aflatoxin exposure. *Int. J. Epidemiol.* 1982; **11:** 112-19.

70. Srivatanakul P, Parkin DM, Khlat, M, *et al.* Liver cancer in Thailand. II. A case- control study of hepatocellular carcinoma. *Int. J. Cancer.* 1991; **48:** 329-32.

71. Zhang JY, Wang X, Han SG, Zhuang H. A case-control study of risk factors for hepatocellular carcinoma in Henan, China. *American J. of Trop. Med. Hyg.* 1998; **59:** 947-51.

72. Ruddon RW. Cancer Biology, Third Edition, Chapter 6, Viral carcinogenesis. Oxford University Press. 1995; 264-76.

73. IARC. Hepatitis viruses. IARC monographs on the evaluation of carcinogenic risks of humans, IARC, Lyon. 1994; **59:** 45-242.

74. Donato F, Boffetta P, Puoti M. A meta-analysis of epidemiological studies on the combined effect of hepatitis B and C virus infections in causing hepatocellular carcinoma. *Int. J. Cancer.* 1998; **75:** 347-354.

75. Sakamoto M, Hirohashi S, Tsuda H, *et al.* Increasing incidence of hepatocellular carcinoma possibly associated with non-A, non-B hepatitis in Japan, disclosed by hepatitis B virus DNA analysis of surgically reselected cases. *Cancer Res.* 1988; **48:** 7294-7.
76. Okuda K, Fujimoto I, Hanai A, Urano Y. Changing incidence of hepatocellular carcinoma in Japan. *Cancer Res.* 1987; **47:** 4967-72.
77. Kiyosawa K, Furuta S. Clinical aspects and epidemiology of hepatitis B and C viruses in hepatocellular carcinoma in Japan. *Cancer Chemother. Pharmacol.* 1992; **31:** 5150-56.
78. Tagger A, Donato F, Ribero ML, *et al.* Case-control study on Hepatitis C virus (HCV) as a risk factor for hepatocellular carcinoma: the role of HCV genotype and the synergism with hepatitis B virus and alcohol. Bresica HCC study. *Int. J. Cancer.* 1999; **81:** 695-9.
79. Yates SC, Hafez M, Beld M, *et al.* Hepatocellular carcinoma in Egyptians with and without a history of hepatitis C virus (HCV) infection but not with (HCV) RNA level. *Am. J. Trop. Med. Hyg.* 1999; **60:** 714-20.
80. Reid AE, Koziel MJ, Aiza, I, *et al.* Hepatitis C virus Genotypes and viremia and hepatocellular carcinoma in the United States. *Am. J. Gastroenterol.* 1999; **94:** 1619-26.
81. Ka MM, Herve P, Leguenno B, *et al.* Low prevalence of anti-hepatitis C virus antibodies in hepatocellular carcinoma in Senegal. *Ann. Gastroenterol. Hepatol.* 1996; **31:** 329-32.
82. Tangkijvanich P, Hirsch P, Theamboonlers A, Nuchprayoon I, Poovorawan Y. Association of hepatitis virus with hepatocellular carcinoma in Thailand. *J. Gastroenterol.* 1999; **34:** 227-33.
83. Tagger A, Donato F, Ribero ML, *et al.* A case-control study on a novel DNA virus (TT virus) infection and hepatocelluar carcinoma. The Brescia HCC study. *Hepatology.* 1999; **30:** 294-9.
84. Adami HO, Hsing AW, McLaughlin JK, *et al.* Alcohilism and liver cirrhosis in the etiology of primary liver cancer. *Int. J. Cancer.* 1992; **51:** 898-902.
85. Cited by Giorgio S. Review article. Primary liver cancer is of multifactorial origin: Importance of hepatitis B virus infection and dietary aflatoxin. *J. Gastroenterol. Hepatol.* 1995; **10:** 604-8.
86. Tsukuma H, Hiyama T, Tanaka S, *et al.* Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *New Engl. J. Med.* 1993; **328:** 1797-801.
87. Tanaka K, Hirohata T, Takeshita S, *et al.* Hepatitis B virus cigarette smoking and alcohol consumption in the development of hepatocellular carcinoma: A case-control study in Fukuoka, Japan. *Int. J. Cancer.* 1992; **51:** 509-14.
88. Trichopoulos D, Day NE, Kaklamani E, *et al.* Hepatitis B virus, tobacco smoking and ethanol consumption in the etiology of hepatocellular carcinoma. *Int. J. Cancer.* 1987; **39:** 45-49.
89. World Health Organization, International Agency for Research on Cancer. On The Evaluation Of Carcinogenic Risks To Humans, *Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs.* 1987; **42(S7):** 297-308.
90. Yu SZ. Primary prevention of hepatocellular carcinoma. *J. Gastroenterol. Hepatol.* 1995; **10:** 674-82.
91. Yu MW, Zhang YJ, Blaner WS, Santella RM. Influence of vitamins A, C and E and β-carotene on aflatoxin B1 binding to DNA in woodchuck hepatocytes. *Cancer.* 1994 ; **73:** 596-604.

92. Davidson NE, Egner PA, Kensler TW. Transcriptional control of glutathione S- transferase gene expression by the chemopreventive agent 5-(2-pyrazinyl)-4-methyle-1,2-dithiole-3-thione (oltipraz) in rat liver. *Cancer Res.* 1990; **50:** 2251-55.

93. Parkin DM, Whelan SL, Ferlay J, Raymond L, Young J. *Cancer incidence in five continents.* International Agency for Research on Cancer. IARC Sci. Publ., 1997.

94. Itoshima T, Fedail SS, Souliman I, Ali AK, Higashi T, Tsjit T. Hepatitis B virus markers in patients with shistosomiasis, liver cirrhosis and hepatocellular carcinoma in Khartoum, Sudan. *Acta Med. Okayama.* 1989; **43:** 241-44.

95. McGlynn KA, Rosvold EA, Lustbader ED, *et al.* Susceptibility to hepatocellular carcinoma is associated with genetic variation in the enzymatic detoxification of aflatoxin B1. *Proc. Natl. Acad. Sci.* 1995; **92:** 2384-87.

96. Wild CP, Yin F, Turner PC, *et al.* Environmental and genetic determinants of aflatoxin-albumin adducts in the Gambia. *Int. J. Cancer.* 2000; **86:**1-7.

97. Guengerich FP, Johnson WW, Shimada T, Ueng YF, Yamazaki H and Langouet S. Activation and detoxication of aflatoxin B1. *Mut. Res.* 1998; **402:** 121-28.

98. Johnson WW, Yamazaki H, Shimada T, Ueng YF, Guengerich FP. Aflatoxin B1 8,9-epoxide hydrolysis in the presence of rat and human epoxide hydrolase. *Chem. Res. Toxicol.* 1997; **10:** 672-76.

99. Kefene H, Rapicetta M, Rossi GB, *et al.* Ethiopian national hepatitis study. *J. Med. Virol.* 1988; **24:** 75-84.

100. al-Karawi MA, Shariq S, el-Shekh-Mohamed AR, Saeed AA, Ahmed AM. Hepatitis C Virus infection in chronic liver disease and hepatocellular carcinoma in Saudi Arabia. *J. Gastroenterol. Hepatol.* 1992; **7:** 237-9.

101. Moudawi SB. Prevalence of Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) in Sudanese Patients with Hepatocellular Carcinoma. Thesis. Dept. of Medicine, Faculty of Medicine University of Khartoum, 1998.

102. Saleh MG, Pereira MMB, Tibbs CJ, *et al.* High prevalence of hepatitis C virus in the normal Libyan population. *Trans. R. Soc. Trop. Med. Hyg.* 1994; **88:** 292-294.

103. Darwish MA, Issa SA, Aziz AM, Darwish NM, Soliman AH. Hepatitis C and B viruses, and their association with hepatocellular carcinoma in Egypt. *J.Egypt. Public Health Assoc.* 1993; **68:** 1-9.

Summary

Liver cancer or hepatocellular carcinoma (HCC) has been recognized for many years as one of the most common malignancies in sub-Saharan countries in Africa, South-East Asia and China. Mortality is practically 100% and an estimated number of 250,000 subjects die annually, most of them middle-aged males. The death rate is still rising even in low-incidence areas. In Sudan, clinical records suggested that liver cancer is high and increasing. Figures from the National Health Laboratory reflected an increase in the incidence over the period 1970-74 from 1.7% to 3.8% (of all reported cancers) in the period 1979-84.

Aflatoxin contamination of food, hepatitis B viral infection, malnutrition, and various parasitic infestations are frequently found to coexist with HCC in many developing countries. Climatic conditions, agricultural practices and the dietary pattern in Sudan, as well as in many of the sub-Saharan African regions, are contributing to the contamination of food with aflatoxins and possibly to the hazard of HCC.

This study was designed to investigate the etiological factors relevant to the incidence of hepatocellular carcinoma among the population in two regions in West and Central Sudan, in order to find leads for prevention and control. The two regions were suspected to differ in exposure to aflatoxins and the risk of HCC. To our knowledge, this is the first individual-based epidemiological study so far to investigate the contribution of aflatoxin exposure on HCC among an African population, since only ecological studies from Africa have been published.

In order to assess the feasibility of conducting a large case-control study, we first conducted a small explorative study (Chapter 2) among 24 confirmed HCC patients and 34 healthy subjects, from the two study regions in Sudan. West Sudan, the region with the alleged highest liver cancer incidence, showed the highest peanut butter consumption and highest aflatoxin concentration (87.4 ± 197.3) compared to Central Sudan (8.5 ± 6.8). In this region, peanut butter stored in mud buildings was related to highest levels of aflatoxin concentration; these mud buildings were also found to be associated with HCC (OR 7.5, 95% CI 1.4-40.2). Despite of this, the use of a calculated index of individual aflatoxin intake did not reveal a clear association with HCC, probably due to lack of reliability of this index as a marker of long-term

individual exposure. These results supported the idea that contamination of peanut butter with aflatoxin might contribute to HCC in Sudan.

In the explorative study, hepatitis infections were considered as a potential confounder in the association of aflatoxin and HCC. However, since hepatitis infections were determined by questionnaire, misclassification resulted in an unacceptable dilution of the association with HCC and adjustment for potential confounding was not possible. This pointed at the need to use serological blood tests to assess hepatitis infections in the main study.

In the main study the role of aflatoxins from peanut butter in the etiology of HCC in Sudan was investigated (Chapter 3). The scientific evidence was further substantiated by addressing the role of genetic polymorphisms involved in aflatoxin-mediated HCC (Chapter 3 and 4, respectively). The role of hepatitis infections as important risk factors of HCC is addressed in Chapter 5. The potential relevance of simultaneous exposure to both peanut butter (aflatoxins) and hepatitis B infection for the prevention and control of HCC in Sudan is addressed in Chapter 6.

The main study included ca 150 cases and 200 controls enrolled from the two study regions in Sudan (West and Center). It accounted for the limited size and other methodological limitations of the explorative study. HCC cases from the two regions were recruited using of admission lists of the five most important hospitals in Khartoum. Controls from each region were recruited by means of a multistage sampling procedure using lists of inhabitants present at so-called "sugar shops" in each chosen locality. The population of the two regions together was taken as the study population, with the number of controls proportional to the population size of each region. Thus, the range of exposure to aflatoxins was considered to be large, which may contribute to observing a strong association. All participants were investigated using a standardized questionnaire inquiring about social and demographic factors, food habits with special reference to peanut butter consumption and peanuts storage systems. In addition, confounders such as hepatitis have been investigated as well as drinking and smoking habits.

The association between peanut butter intake, as a dietary source of exposure to aflatoxins and the GSTM1 genotype in the etiology of HCC has been studied (Chapter 3). A positive association was observed for the highest vs lowest category of peanut butter consumption with

the OR (95% CI) being 3.0 (1.6-5.5). The positive association with peanut butter consumption was limited to subjects having the GSTM1 null genotype with the OR for highest vs lowest quartile being 16.7 (2.7-105). However, the association between mud building storage types and HCC was less clear in the main study (OR 1.6,1.1-2.5) than in the explorative study (Chapter 2).

The role of the genetic polymorphisms of GSTT1 and EPHX in aflatoxin-related HCC was studied in Chapter 4. Like GSTM1, GSTT1 showed no association with HCC. The combinations of EPHX 113HH/139HH genotype carriers showed a moderate increased risk of HCC with OR 3.10 (1.2- 8.1), but in further analysis this association lost its significance. Dissimilar to GSTM1, neither GSTT1 nor EPHX polymorphism modified the association between peanut butter consumption and HCC. This might indicate that the latter two genes are not important limiting factors in the metabolism of aflatoxins.

In Chapter 5, the role of hepatitis B virus and hepatitis C virus infections in the etiology of HCC in the two study regions is described. The prevalence of HBV and HCV among healthy subjects was found to be 7.0% and 1.5% respectively. Hepatitis infections appeared to be an important risk factor of HCC since a strong positive association was found between HBV, HCV and HCC with odds ratios being 9.8 (5.1-18.9) and 8.3 (2.3-29.9) respectively. In further logistic regression analysis, age-adjustment altered these ORs for HBV and HCV to 16.1 (7.4-34.9) and 4.5 (1.1-18.6) respectively.

The synergistic effect between hepatitis B virus infection and peanut butter consumption in the etiology of HCC is illustrated in Chapter 6. The joint effect of aflatoxin exposure (contamination of peanut butter) and hepatitis B infection, was found to be more than additive but might also be compatible with a multiplicative effect. Together they may account for 80% of all HCC cases in Sudan, about half of this being attributable to aflatoxins.

The main findings of the studies presented in this thesis and the methodological issues that might interfere with the interpretation of the results are discussed in Chapter 7. These findings were subsequently compared to other related studies in the same field. Potential implications for agriculture and public health measures are discussed including reduction of exposure to aflatoxins, which can be achieved by addressing measures required for elimination of fungal

contamination of crops at both agricultural policy and community level. In addition, health measures have to be considered such as immunization for HBV of infants in high incidence areas and of high-risk groups in both high and low incidence areas. Finally, directions for future research of direct relevance to Sudan and of more general scientific interest are considered.

بسم الله الرحمن الرحيم

ملخص الرسالـة

عرف سرطان الكبد الخلوى HEPATOCELLULAR CARCINOMA منذ عديد من السنوات على أنه من أكثر الأمراض الخبيثه شيوعا في الأقطار الأفريقية ، منطقة جنوب شرق أسيا والصين . وتعادل نسبة الوفيات 100% من المرض ، ويقدر عددهم ب (250,000)سنويا معظمهم من الرجال في منتصف العمر ولاتزال هذه النسبة في إرتفاع حتي في الأقطار التي لا يحدث فيها المرض كثيرا. وفي السودان توضح السجلات الطبية أن نسبة الإصابة بسرطان الكبد الخلوى عالية وفي تزايد مستمر . وتعكس الأرقام من المعمل الصحي القومي زياده في هذا المرض في الفترة (1970-1974 من 1,7% الي 3,8 في الفترة (1984- 1979) عن كل أمراض السرطان المسجلة . ظهر أن التلوث بالافلاتوكسين (AFLATOXIN) التهاب الكبد الوبائى (الفئه ب) سؤ التغذية والإصابات الطفيلية المختلفة ذات إرتباط وثيق بسرطان الكبد الخلوى في معظم الحالات في كثير من الأقطار النامية . وتسهم الاحوال المناخية والممارسات الزراعية ونوعية الغذاء في السودان كما في كثير من مناطق أفريقيا جنوب الصحراء في تلوث الطعام بالافلاتوكسين (AFLATOXIN) ولربما في مخاطر سرطان الكبد الخلوى (H C C).

صممت هذه الدراسة لتقصي العوامل المسببة لسرطان الكبد الخلوى (H C C) وسط السكان في منطقتي غرب ووسط السودان لأجل إيجاد سبل لمنع هذا المرض والسيطره عليه . وتعتبر هاتين المنطقتين معرضتين بشكل مختلف للافلاتوكسين ومخاطر سرطان الكبد . وفي مبلغ علمنا فإن هذه أول دراسة تبني علي معلومات الأفراد (INDIVIDUAL-BASED STUDY) والتي تتقصى علاقة التعرض للافلاتوكسين بسرطان الكبد الخلوى (H C C) وسط السكان الافارقة ، نسبة لأن كل الدراسات التي نشرت من أفريقيا والتي تخص سرطان الكبد الخلوى (H C C) هي دراسات بيئية فقط . (ECOLOGICAL STUDIES)

ومن أجل تقييم إمكانية تطبيق دراسة كبرى مبنية علي معلومات المرض والأصحاء -CASE (CONTROL STUDY) أجرينا أولا دراسة إستكشافية صغيرة (الباب الثاني) وسط عينة (24) من المرضى الذين تأكد أنهم يعانون من سرطان الكبد الخلوى ، و (34) من الأشخاص الأصحاء من كلا منطقتي الدراسة في السودان . وقد أظهرت منطقة غرب السودان ذات النسبة العليا من الإصابة بسرطان الكبد الخلوى الإستهلاك العالي لزبدة الفول السودانى (الدكوة) كما أظهرت أيضا تركيزا عاليا للافلاتوكسين (78,4± 197,3) بالمقارنة مع (6,8±8,5) بوسط السودان . وفي هذه المنطقة (غرب السودان) أبان تخزين الفول السودانى في مخازن مبنية من الطين بأن له علاقة بالتركيز العالي للافلاتوكسين في هذه المنطقة . كما إتضح أن التخزين في هذه المباني (مخازن الطين) يرتبط بصورة كبيرة بسرطان الكبد الخلوى (HCC) (OR = 7.5,95 CI 1.4-402) . وبالرغم من ذلك فإن إستخدام نسبة محسوبة من تعاطى الافلاتوكسين فرديا (INDEX) لم توضح أى إرتباط

وأضح بسرطان الكبد ربما بسبب إنعدام المصداقية في هذه النسبة المحسوبة (INDEX) كمؤشر للتعرض الفردى على المدى الطويل . وهذه النتائج تؤيد فكرة أن تلوث زبدة الفول (الدكوة) بالافلاتوكسين قد تسهم في تسبب سرطان الكبد الخلوى (HCC) فى السودان .

وفي هذه الدراسة الإستكشافية فقد أعتبر التهاب الكبد الوبائى كعامل متداخل (CONFOUNDER) للعلاقة بين الافلاتوكسين وسرطان الكبد الخلوى (H CC) . وبما أن الإستيضاح عن التهاب الكبد الوبائى قد حدد بواسطة الإستبيان فقط فإن الخطأ في المعلومات الإستبيانية أدى الي تخفيف غير متوقع للعلاقة بين إلتهاب الكبد الوبائى بسرطان الكبد ، وبذا فإن تصحيح هذا الخطأ بالأساليب الإحصائية لم يكن ممكناً . وقد إتضح من ذلك الحوجة الي إستخدام إختبارات الدم لكشف الإصابة بإلتهاب الكبد الوبائى للمشاركين في الدراسة الرئيسية .

في الدراسة الرئيسية تم إستقصاء دور الافلاتوكسين في زبدة الفول (الدكوة) في التسبب بسرطان الكبد الخلوى في السودان ، (الباب الثالث) . وقد تم تأييد الشواهد العلمية بدرجة أكبر بتقصى دور الإختزال الجينى (GENETIC POLYMORPHISM) المتعلقة بسرطان الكبد الخلوى (HCC) كوسيط له (الباب الثالث والرابع علي التوالى) . كذلك تم توضيح دور التهاب الكبد في الباب الخامس . ومن أجل إيجاد السبل لمنع سرطان الكبد الخلوى ومكافحته فقد تم بحث مخاطر التعرض للافلاتوكسين (زبدة الفول) والإصابة بإلتهاب الكبد الوبائى (الفئه ب)، في نفس الوقت (الباب السادس) .

وقد شملت الدراسة الرئيسية علي 150 حالة سرطان كبد خلوى (HCC) و 200 حالات أصحاء من منطقتى الدراسة في السودان (الغرب والوسط) ، مع الوضع في الإعتبار الحجم المحدود والمحدوديات المنهجية الأخرى للدراسة الإستكشافية . وقد تم التحصل علي حالات سرطان الكبد من المنطقتين بإستخدام كشف الدخول الي خمسة من أهم المستشفيات في الخرطوم . أما مجموعة الأصحاء فقد تم إختيارها من كل منطقة بإستخدام طريقة العينة متعددة المراحل وذلك بإستخدام كشوفات السكر في كل محلية مختارة . وقد تم أخذ سكان المنطقتين معاً كمجتمع للدراسة ، مع تحديد عدد أفراد مجموعة الأصحاء بنسبة حجم السكان في كل منطقة .

لذا فإن مدى التعرض للافلاتوكسين قد أعتبر كبيراً مما قد يسهم في ملاحظة أرتباط قوى .

وقد تم إستقصاء كل المشاركين بإستخدام الإستبانة المعيارية التى تبحث في العوامل الديمغرافية والإجتماعية ، عادات الأكل بإشارة خاصة لإستهلاك زبدة الفول (الدكوة) ونظم تخزينها بالإضافة الي ذلك فقد تم الإستقصاء عن إلتهاب الكبد الوبائى ، وكذلك عن عادات شرب الكحول والتدخين كأسباب أخرى وقد تعمل معاً مع الافلاتوكسين (CONFOUNDER) للإصابة بسرطان الكبد الخلوى .

وقد تمت دراسة الإرتباط بين تناول زبدة الفول (الدكوة) كمصدر للعرضة بالافلاتوكسين والإختزال الجينى لـ (GSTM1genotype) في التسبب بسرطان الكبد (HCC) (الباب الثالث) وقد لوحظ وجود إرتباط إيجابى بين أعلى وأدنى نسبة الخطورة بين المصابين مقارنة بغير المصابين (الأصحاء) تصل الي ((OR, 95%CI=3.0 (1.6-5.5) . كما وضحت الدراسة بأن الإرتباط الإيجابى

لإستهلاك زيدة الفول (الدكوة) محدوداً بإفراد ذوى الجين المختزل (GSTM1null genotype) لذلك فإنها تفقد المقدرة الطبيعية علي تكسير الافلاتوكسين داخل الجسم ، بمعدل إرتفاع نسبة الخطورة لأعلي وأدنى معيار (OR,95% CI = 16.7(2.7-105 . وعلي أى حال فإن الإرتباط بين التخزين في المبانى الطينية وسرطان الكبد (HCC) أقل وضوحا في الدراسة الرئيسية (OR,1.6 (1.1-2.5 مما كان عليه في الدراسة الإستكشافية (الباب الثاني) .

كذلك تمت دراسة دور الإختزال الجينى (GENETIC POLYMORPHISM) لجينات الـ (GSTT1) و (EPHX) المتعلقة بالافلاتوكسين فى سرطان الكبد الخلوى (HCC) (الباب الرابع). ومثل الـ (GSTM1) أوضح (GSTT1) أنه لا يرتبط بسرطان الكبد الخلوى . إن الجمع بين حالات الجينات (EPHX 113HH/139HH) إرتبط بمخاطر طفيفه لزيادة سرطان الكبد الخلوى (HCC) بمعدل إرتفاع نسبة الخطورة (1.2-8.1) OR 3.10 لكن بعد مزيد من التحليلات الإحصائية فقد فقد هذا الإرتباط أهميه . وخلافاً لإختزال الـ (GSTM1) فإن إختزال جينات الـ (GSTT1) والـ (EPHX) لم توضح إرتباطاً بين إستهلاك زيدة الفول (الدكوة) وسرطان الكبد الخلوى (HCC) . وقد يشير ذلك الى أن الجينين الاخيرين ليس ذوى أهمية كعاملين يحدان من التمثيل الغذائى للأفلاتوكسين .

الباب الخامس من هذه الدراسه تعرض لدور التهابات الكبد الوبائيه (الفئه ب) و (الفئه ج) في التسبب بسرطان الكبد الخلوى (HCC) في منطقتى الدراسة . وقد وجد أن إنتشار فيروس التهاب الكبد الوبائى (ب) و (ج) وسط مجتمع الدراسة هو (7.5%) و (1.3%) على التوالى . كما إتضح أن التهابات الكبد الوبائيان الفئتين (ب) و (ج) هما عاملان خطوره هامان فى حدوث سرطان الكبد الخلوى بالسودان لوجود إرتباط إيجابى كبير بينهما وحدوث سرطان الكبد الخلوى (HCC) بمعدل إرتفاع نسبة الخطورة (ORs) للفيروس (ب) (5.1-18.9) OR, 9.8 و الفيروس (ج) (2.3-29.9) OR, 8.3 ، وبمزيد من التحليلات الإحصائية بضبط عامل العمر بين المرضى والأصحاء فقد تعدلت هذه العلاقة (ORs) لكل من الفيروسين ب و ج الى (7.4-34.9) 16.1 و (1.1-18.6) 4.5 علي التوالى .

وضح الباب السادس الأثر المتعاضد بين التهاب الكبد الوبائى الفئه (ب) والتعرض للأفلاتوكسين (الدكوه) في التسبب بسرطان الكبد (HCC) . فقد أبان الأثر المشترك للتعرض للعاملين مجتمعين { التهاب الكبد (الفئه ب) والتعرض للأفلاتوكسين } ، أكثر من حاصل إضافة أثر كل عامل منفردا كما أنه قد يكون متوافقا أيضا مع حاصل ضرب الأثرين . ومعا فإن 80% من حالات سرطان الكبد الخلوى (HCC) كانت نتيجة لتعرض للأفلاتوكسين (دكوه الفول) والتهاب الكبد الفئه ب ، بنسب متقاربه لكل المسببين أى 50% لكل منهما .

أن النتائج النهائية للدراسات المقدمه فى هذه الرسالة والمسائل المنهجية التى قد تتداخل مع تفسير النتائج المقدمة تمت مناقشتها في الباب السابع . كما تمت مقارنة هذه النتائج فى نفس الباب بالدراسات الأخرى المتعلقه بهذا الموضوع فى نفس المجال . فى هذا الباب أيضا تمت مناقشة إمكانية تطبيق الحلول التى سوف تترتب على الزراعه والصحه العامه وإجراءتهما مما يشمل تخفيض

التعرض للأفلاتوكسين الشيء الذى يمكن تحقيقه بتطبيق الأجراءات المطلوبه لمنع تلوث المحاصيل الزراعيه بما فى ذلك الفول السودانى بفطر الأسبيرجلس(ASPERGILLUS FLAVUS) والفطريات الأخرى التى تفرز الأفلاتوكسين ، وذلك على مستوى السياسات الزراعيه وجهود المجتمع ، بالإضافه الى ذلك ، يجب إعطاء الإعتبار للإجراءات الصحية ، مثل تطعيم الأطفال ضد إلتهاب الكبد الوبائى الفئه (ب) فى المناطق ذات الإنتشار العالى لهذا الفيروس كما يجب تطعيم المجموعات التى تكون عرضه للإصابه فى مناطق الإنتشار العالى والمنخفض على حد السواء . كما تشير الأبحاث المستقبليه فى نهاية هذا الباب الى إرتباط هذه الأبحاث بحوجة السودان كما وأنها ذات إهتمامات علمية كبيرة .

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About the author

Ragaa El Hadi Omer was born on 26 November 1961 in Sudan. She completed her secondary education in Sudan. In 1985 she obtained her B.Sc. from Zagazige University Egypt, and worked for four years at the Quality Control Department, Ministry of Commerce, Sudan. In 1992, she received her MSc. degree in Environmental Studies from the University of Khartoum. This was based on investigations into the role of different modes of storage in the occurrence of aflatoxin contamination in Sudan. Early 1993, she joined a training course in post harvest technology of food crops at Silsoe College, UK. The year 1994, she attended a training program in quality assurance and marketing in the International Agricultural Center (IAC), Wageningen, the Netherlands. In the same year she joined the newly established Sudanese Standards and Meteorology Organization, Sudan, and she received the approval for her PhD. in Wageningen University, the Netherlands.

She started her PhD. at the Division of Human Nutrition and Epidemiology in December 1995, after she finished the data collection for the explorative study, during the preceding seven months in Sudan. During the first phase of the PhD. program (Dec 1995 - July 1996) the main study was designed and data of the explorative study were analyzed. The field work and data collection of the main study was conducted during the period August 1996 – August 1998. During this period she followed an Intensive Course in Epidemiology and Medical Statistics, London School of Hygiene & Tropical Medicine; tuition fees and bursary were granted by Elementa Company, Khartoum-Switzerland and Agri-Products Company, Khartoum-London. The last phase of the Ph.D. started June 1999. During this phase she attended a course on Modern Statistical Methods organized by the Netherlands Institute for Health Science (NIHES) at Erasmus Medical Center, Rotterdam, with tuition fees paid by the Graduate School VLAG (Advanced Studies in Food Technology, Agrobiotechnology, Nutrition and Health Science).

Upon completion of her PhD. she will resume her duties in the Sudanese Standards and Meteorology Organization. A plan for future collaboration with the Department of Public Health, Ministry of Health, Sudan will be established.

List of abbreviations

AFB, AFM, etc	**Aflatoxin B, aflatoxin M, etc.**
CI	**Confidence interval, usually 95% CI**
DNA	**Deoxyribo nucleic acid, molecular carrier of genetic information**
EF	**Etiological fraction, proportion of cases attributable to an etiological factor**
EPHX	**DNA sequence coding for microsomal epoxide hydrolase (mEH)**
HBV	**Hepatitis B virus**
HBcAb	**Hepatitis B core antibody**
HBsAg	**Hepatitis B surface antigen**
HCV	**Hepatitis C virus**
HCC	**Hepatocellular carcinoma, primary liver cancer**
ID	**Incidence density, rate of disease occurrence**
IDR	**Incidence density ratio, comparing ID in exposed versus unexposed, this be estimated in a case- control study by the OR**
GSTM1	**DNA sequence coding for glutathione-S-transferase 1 (GST-mu)**
GSTT1	**DNA sequence coding for glutathione-T-transferase 1 (GST-theta)**
MH-OR	**MH-OR is the OR adjusted for potential confounders using the Mantel-Haenszel procedure**
OR	**Odds ratio, estimate of disease risk in exposed versus unexposed**
RNA	**Ribo nucleic acid, transcript from DNA to be translated to amino acid sequence of proteins**
RR	**Relative risk, disease risk in exposed versus unexposed**
SE	**Standard error, sampling error of the estimate of a mean (SE = SD/√n), proportion (SE = √[p(1-p)/n] or other for measures of association (e.g. difference of means or the OR)**
SD	**Standard deviation**