KUOPION YLIOPISTON JULKAISUJA D. LÄÄKETIEDE 408
KUOPIO UNIVERSITY PUBLICATIONS D. MEDICAL SCIENCES 408

NEKTARIA POLYCHRONAKI

Biomarkers of Aflatoxin Exposure and a Dietary Intervention

Studies in Infants and Children from Egypt and Guinea and Young Adults from China

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium L21, Snellmania building, University of Kuopio, on Saturday 9th June 2007, at 12 noon

School of Public Health and Clinical Nutrition
Department of Clinical Nutrition, Food and Health Research Centre
University of Kuopio

KUOPION YLIOPISTO

KUOPIO 2007

Distributor: Kuopio University Library
P.O. Box 1627
FI-70211 KUOPIO
FINLAND
Tel. +358 17 163 430
Fax +358 17 163 410
www.uku.fi/kirjasto/julkaisutoiminta/julkmyyn.html

Series Editors: Professor Esko Alhava, M.D., Ph.D.
Institute of Clinical Medicine, Department of Surgery

Professor Raimo Sulkava, M.D., Ph.D.
School of Public Health and Clinical Nutrition

Professor Markku Tammi, M.D., Ph.D.
Institute of Biomedicine, Department of Anatomy

Author´s address: Food and Health Research Center
Department of Clinical Nutrition
University of Kuopio
P.O. Box 1627
FI-70211 KUOPIO
FINLAND
Tel. +358 17 163 615
Fax +358 17 162 792

Supervisors: Professor Hannu Mykkänen, Ph.D.
School of Public Health and Clinical Nutrition
University of Kuopio

Adjunct Professor Hani El-Nezami, Ph.D.
Food and Health Research Center
University of Kuopio

Professor Seppo Salminen, Ph.D.
Functional Food Forum
University of Turku

Reviewers: Professor Anthony Kafatos, M.D., Ph.D.
Department of Social Medicine
University of Crete, Greece

Adjunct Professor Liisa Valsta, D.Sc.
Department of Health Promotion and
Chronic Disease Prevention
National Institute of Public Health, Finland

Opponent: Associate Professor Christopher Loffredo, Ph.D.
Department of Epidemiology and Preventive Medicine
Georgetown University, Washington DC, USA

ISBN 978-951-27-0668-6
ISBN 978-951-27-0745-4 (PDF)
ISSN 1235-0303

Kopijyvä
Kuopio 2007
Finland

Polychronaki, Nektaria. Biomarkers of aflatoxin exposure and a dietary intervention: studies in infants and children from Egypt and Guinea and young adults from China.
Kuopio University Publications D. Medical Sciences 408. 2007. 114 p.
ISBN 978-951-27-0668-6
ISBN 978-951-27-0745-4 (PDF)
ISSN 1235-0303

ABSTRACT

Aflatoxins are fungal metabolites which are toxic, carcinogenic, and mutagenic, and have been proposed as altering immunocompetence and growth in humans. Aflatoxins, together with hepatitis B and C, have been recognized as the major risk factors for the development of hepatocellular carcinoma (HCC) in parts of Africa and Southeast China. Children are a highly susceptible population to environmental carcinogens and a number of studies indicate that susceptibility to aflatoxin is greatest in the young. Aflatoxin exposure has been associated with growth faltering and immune suppression in young children. Early life exposures could be a contributing factor towards the early onset of HCC.

The undertaken research aimed 1) to assess using specific biomarkers the exposure patterns and levels of aflatoxin exposure among nursing Egyptian mothers and their children, 2) to compare the exposure levels of the Egyptian children with children from Guinea, a country with a known high aflatoxin exposure, and 3) to assess the effectiveness of probiotic supplementation in the reduction of the biologically effective dose of aflatoxin exposure in a highly exposed region of Southern China.

In Egypt, 388 lactating mothers were screened for aflatoxins in their breast milk, and 50 of them with high aflatoxin M_1 (AFM_1) levels were recruited for a year of follow up with monthly collections of breast milk samples. The aflatoxin exposure of the 50 Egyptian children was additionally compared to the exposure of 50 Guinean children by monitoring urinary biomarkers of aflatoxin exposure. In China, 90 young adults were selected out of a group of 300, to participate in a randomized double blind placebo controlled intervention trial testing the effectiveness of a probiotic supplementation in reducing aflatoxin exposure.

Aflatoxin was found in the breast milk supplied by 138 out of 388 nursing Egyptian mothers [median 13.5 pg/ml, IQR (10.27-21.43)]. Non-working status [OR= 2.87, 95% CI (1.2-6.8)], obesity [OR= 3.01, 95% CI (1.43-6.33)], high corn oil consumption [OR= 2.21, 95% CI (1.3-3.7)], number of children (>1) [OR= 1.99, 95% CI (1.09-3.64)], and early lactation stage (<1 month) [OR=3.57, 95% CI (1.15-1.09)], contributed to the occurrence of AFM_1 in breast milk. In the follow up cohort, the most dominant factor affecting the presence of AFM_1 in breast milk was the seasonal effect. AFM_1 was observed most frequently during summer months (May-September) with the highest rates observed in June [OR 63, 95% CI (7.6, 522)]. The duration of lactation [OR= 1.08, 95% CI (1.02, 1.13)] also affected AFM_1 presence, while peanut consumption [OR= 1.69, 95% CI (0.9, 2.9)] hinted at an effect. Exposure of the Guinean children to aflatoxin was more prevalent and at higher levels as compared to the Egyptian children (86% vs. 38%, p= 0.000). Probiotic supplementation in young Chinese adults clearly reduced the biologically effective dose of AFB_1 as indicated by the urinary excretion of AFB_1-N^7-guanine adduct (probiotic group 0.24 ng/ml, placebo group 0.49 ng/ml, p= 0.005).

These data would suggest that aflatoxin exposure does occur in children and young adults residing in highly exposed regions and measures should be initiated to reduce this exposure. The identification and understanding of factors determining the presence of toxicants in human milk is important and may provide a knowledge driven basis for controlling the transfer of chemicals to infants. Probiotic supplementation could represent affordable and feasible means to reduce the bioavailability of aflatoxin and consequently the health risks associated with aflatoxin exposure in aflatoxin endemic areas.

National Library of Medicine Classification: QU 120, QW 630.5.M9, WS 125
Medical Subject Headings: Aflatoxins/adverse effects; Aflatoxins/urine; Biological Markers/analysis; Breast Feeding; Child; Infant; China/epidemiology; Egypt/epidemiology; Guinea/epidemiology; Maternal Exposure; Milk, Human/chemistry; Milk, Human/toxicity; Mothers; Mycotoxins/adverse effects; Probiotics

“After all, science is essentially international..”

-Marie Curie

Acknowledgements

This work was carried out in the Department of Clinical Nutrition and in the Food and Health Research Centre at the University of Kuopio, and in the Molecular Epidemiology Unit at the University of Leeds. My sincere thanks to the personnel of both facilities, especially to the people below:

To Hannu Mykkänen, my principal supervisor, for his sincere support and essential guidance during all these years. For having a direct way to deal with our problems, for being above and ahead of the situations, for always having time to advise and discuss our problems.

To Hani El-Nezami, my supervisor, for believing in me and providing me with the opportunity to do these studies, work in research and travel the world. Thank you Hani for your supervision, for being a constant positive support and motivation during all the years in both happy and difficult times while in Kuopio or in Leeds.

To Seppo Salminen, my third supervisor, for his constructive remarks in reviewing my thesis, and to Risto Juvonen for his support during the first years of my PhD.

To Chris Wild and Paul Turner, for giving me the opportunity to study in the Molecular Epidemiology Unit, and gain from their experience and knowledge. Thank you for your support and supervision during my time there and afterwards. My sincere gratitude to Chris for his support, and for opening a door for me to start a post doctoral career and find a way back home.

To Anthony Kafatos and Liisa Valsta for kindly accepting to review this thesis, for providing constructive and valuable comments, and for their support and encouragement during the whole process.

To all people that worked with me in and out of the lab, or shared with me in any way the PhD experiences: Silvia, Ursi, Quoc, Huilian, Otto, Yunyun, Ann, Michael, Robert, Seppo, Kay, Susan, Maarit, Margaret, Jo, Joe, John, Heather, Mike, Lizzy, my dear Ellie, Marcelo, Julia, and many more.

To my dear foreign-Finnish friends Quoc, Petteri, Anne, Ferdinand, Iain, Jakub, Otto, Jonas, Antonio, Kaisu, Jani, Masha, Tatjana, for their company and for making those days special and memorable. Especially to Silvia and Ursi, my dear friends, that walked all these years with me, taught me to enjoy snowy forests and lakes, filled my life with experiences and shared hard and happy times both in research and personal life.

To my dear friends Maria, Darin, Vana, Christos, Elina, Stefanos, Leena, Carme, Marc, Laya. Petros, Katja, Sirkka, Maria and Andrei, Radovan and Paivi, Olivia and Aurora, for being my beloved Greek family abroad, and to all my Ilomantsi friends for bringing me to Finland.

To my family in Greece for their love and constant support, and last but most importantly, to God, for everything.

For financial support of this work, I would like to thank the University of Kuopio, the Finnish Graduate School on Applied Bioscience: Bioengineering, Food and Nutrition, Environment, The Finnish Society of Nutrition Research and the Marie Curie Fellowship European program.

Kuopio, June 2007

Nektaria Polychronaki

ABBREVIATIONS

AFB_1	aflatoxin B_1
AFB_2	aflatoxin B_2
AFG_1	aflatoxin G_1
AFG_2	aflatoxin G_2
AFM_1	aflatoxin M_1
AFM_2	aflatoxin M_2
AFP_1	aflatoxin P_1
AFQ_1	aflatoxin Q_1
AF-albumin	aflatoxin B_1 albumin
AFB_1-N^7-guanine	aflatoxin B_1 N^7 guanine
BMI	body mass index
CI	confidence intervals
FFQ	food frequency questionnaire
HAZ	height to age z score
HBsAg	hepatitic B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HPLC	high performance liquid chromatography
IQR	intra quartile range
WAZ	weight to age z score
WHZ	weight to height z score

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their Roman numerals I-IV:

I: Polychronaki N, Turner PC, Mykkänen H, Gong Y, Amra H, Abdel-Wahhab M, El-Nezami H. 2006. Determinants of aflatoxin M_1 in breast milk in a selected group of Egyptian mothers.
Food Additives and Contaminants, 23: 700-708.

II: Polychronaki N, West RM, Turner PC, Amra H, Abdel-Wahhab M, Mykkänen H, El-Nezami H. 2007. A longitudinal assessment of aflatoxin M_1 excretion in breast milk of selected Egyptian mothers.
Food and Chemical Toxicology, 45: 1210-1215.

III: Polychronaki N, Wild CP, Mykkänen H, Amra H, Abdel-Wahhab M, Sylla A, Diallo M, El-Nezami H, Turner PC. 2006. Urinary biomarkers of aflatoxin exposure in young children from Egypt and Guinea.
Submitted to Food and Chemical Toxicology.

IV: Mykkänen H, Zhu H, Salminen E, Juvonen RO, Ling W, Ma J, Polychronaki N, Kemiläinen H, Mykkänen O, Salminen S, El-Nezami H. 2005. Fecal and urinary excretion of aflatoxin B_1 metabolites (AFQ_1, AFM_1 and AFB_1-N^7-guanine) in young Chinese males.
International Journal of Cancer, 115: 879-884.

V: El-Nezami HS, Polychronaki N, Ma J, Zhu H, Ling W, Salminen EK, Juvonen RO, Salminen SJ, Poussa T, Mykkänen HM. 2006. Probiotic supplementation reduces a biomarker for increased risk of liver cancer in young men from Southern China.
American Journal of Clinical Nutrition, 83: 1199 -203.

In addition, some unpublished results are presented.

CONTENTS

1. INTRODUCTION

Aflatoxins are a family of highly toxic and carcinogenic fungal metabolites produced by several species of *Aspergillus* which frequently contaminate dietary staples (groundnuts, maize) all over the world, though mostly in the developing countries mainly due to the environmental conditions. Aflatoxins, together with hepatitis B (HBV) and C viruses (HCV), have been recognized as the major risk factors for the development of hepatocellular carcinoma (HCC) in the high risk incidence regions for the disease. Often the regions of high HBV infection rates are the same as those with the highest aflatoxin exposure. Individual exposure as assessed by measurements of aflatoxin specific biomarkers such as urinary metabolites or albumin adducts, have been repeatedly reported in West Africa and Southeast China**.** In comparison, individual aflatoxin exposure in Egypt is known to occur at moderate levels. However, for genotoxic carcinogenic substances such as aflatoxins, it is impossible to define a threshold value below which there is no risk for human health.

Children are exceptionally vulnerable and highly susceptible to environmental hazards and there is a strong link between growth and health of the fetus and infant and disease risk in later life. Aflatoxins are potent carcinogens and in addition, childhood aflatoxin exposure has been associated with growth faltering and reduced salivary IgA levels. In West Africa, elevated biomarker levels in children have been repeatedly reported, with children exhibiting higher biomarker levels when infected with HBV. Maternal aflatoxin exposure during the pregnancy and the lactation period leads to in utero and early infancy exposures through breast milk contamination. Early pre- and postnatal exposure to aflatoxins via mother's milk or weaning foods could be one of the factors contributing to growth faltering and/or the early onset of liver cancer in adulthood.

Prevention of HCC among high risk populations can be mainly achieved by immunization against HBV infection and by reductions of aflatoxin exposure. Reductions in aflatoxin exposure can be achieved by several approaches. Where the source of contamination is clearly defined, such as in Guinea, West Africa, post harvest changes to the way that risk foods are dried and stored have proven to be effective. Where dietary sources are less well defined such as in Egypt, reduction in uptake of aflatoxins may be achieved by dietary modulation, eg. with chlorophyllin, or probiotics.

In the present work, we aimed to investigate the patterns and levels of maternal and child aflatoxin exposure in a group of mothers-children in Egypt, and to compare the exposure levels of the Egyptian children with children from Guinea, a country with a known high aflatoxin exposure. Another goal was to assess the effectiveness of probiotic supplementation in the reduction of the biologically effective dose of aflatoxin exposure in a highly exposed region of Southern China. The ultimate goal of the present work was to create the basis for an intervention to be targeted at reducing maternal and child aflatoxin exposure in Egypt.

2. REVIEW OF THE LITERATURE

2.1. Aflatoxins and natural occurrence

Primary aflatoxins

Aflatoxins are the most widely studied group of mycotoxins. They are a family of highly toxic and carcinogenic fungal metabolites produced by *Aspergillus* moulds and particularly by the species of *Aspergillus flavus*, *Aspergillus paraciticus* and the rare *Aspergillus nomius*. *A. flavus* produces aflatoxins B_1 (AFB_1) and B_2 (AFB_2), whereas *A. parasiticus* produces also aflatoxins G_1 (AFG_1) and G_2 (AFG_2) (IARC 1993). Figure 1 shows the chemical structures of the four major naturally occurring aflatoxins produced by the *Aspergillus* species.

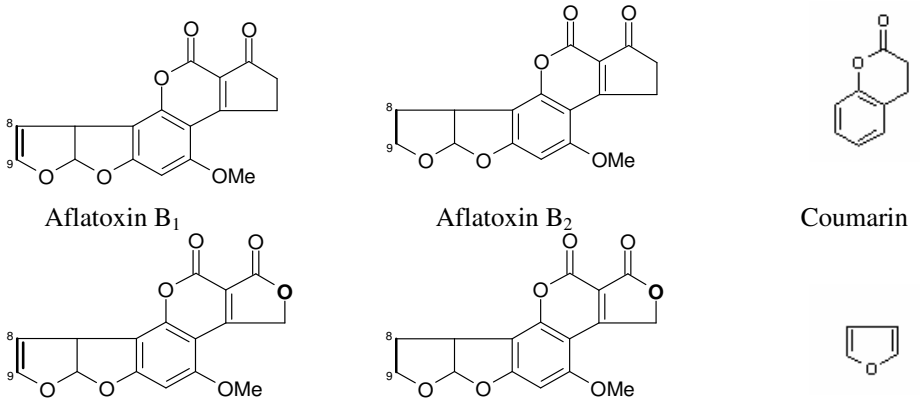


Figure 1: The chemical structures of the major naturally occurring aflatoxins.

Aflatoxins were first identified in 1961 in animal feed contaminated by *Aspergillus* fungi (Sargeant *et al.* 1961). The name 'aflatoxin' was introduced by Nesbitt et al. (1962) from the abbreviation of '*A. flavus* toxin'. Aflatoxins are strongly fluorescent under ultraviolet (UV) light and the four major aflatoxins are named accordingly to the color of the emitted light; B for blue and G for green. The subscripts refer to the chromatographic mobility of the toxins during thin-layer chromatography (Sargeant *et al.* 1963, Bennett and Klich 2003).

Chemically, aflatoxins are difuranocoumarins composed from two furans and a coumarin ring (Figure 1). The unsaturated bond in the terminal furan ring carbons 8 and 9 (atoms are numbered according to instructions given by the International Union of Pure and Applied Chemists, IUPAC) is the site at which its bio-activation forms a highly reactive epoxide structure. AFB_1 is the most potent of the aflatoxins and consequently the most widely studied.

Aflatoxins in milk

Shortly after the discovery of aflatoxins, food safety concerns about the presence of aflatoxin residues in milk (Allcroft and Carnaghan 1963) prompted numerous studies on the conversion of AFB_1 in dairy cattle feed to aflatoxin metabolites in milk. This lead to the identification of aflatoxin M (De Longh *et al.* 1964), a name derived to indicate its original isolation from milk. The structure of aflatoxin M was revealed by Holzapfel and coworkers (1966), who found that aflatoxin M consisted of two components designated as aflatoxin M_1 (AFM_1) and M_2 (AFM_2), and which were identified as the 4-hydroxyderivatives of AFB_1 and AFB_2, respectively. Later, another hydroxyderivative of AFB_1 was detected in milk (Lafont *et al.* 1986) AFM_4, but the knowledge about AFM_4 is very limited. Considerable attention has been paid to AFM_1. A Detailed discussion of AFM_1 can be found in chapter 2.2, under the sections of biotransformation and excretion of aflatoxins.

Natural occurrence of primary aflatoxins

Aspergillus fungi are common contaminants in the agricultural envrionment. Fungal growth and subsequently dietary aflatoxin contamination are ubiquitous in areas of the world with hot and humid weather conditions as in several African (El-Nezami *et al.* 2000b, Wild and Hall 2000, Wild and Turner 2002) and Asian countries (Groopman *et al.* 1992a, Qian *et al.* 1994). In Africa, *A. flavus* and *A. paraciticus* are widely distributed whereas in south-east Asia, *A. flavus* is present to the virtual exclusion of all other species (IARC 1993). The fungal infection of the crops can occur pre-harvest in the field or post-harvest during transportation or storage. Pre-harvest infections are mostly associated with drought stress, whereas post-harvest contamination occurs mostly during the storage of the crops under conditions that favor mould growth

(Bennett and Klich 2003). During storage, usually the most important variables are the moisture content of the substrate and the relative humidity of the surroundings (Wilson and Payne 1994).

Human dietary exposure to aflatoxins at levels of nanograms to micrograms per day occurs mainly through consumption of a wide variety of contaminated crops like maize, groundnuts, cottonseed, soybeans, sorghum, rice and wheat (IARC 1993). The high level contamination of groundnuts and maize are of particular concern since these crops comprise the dietary staples of many populations in the developing countries (Table 1). In aflatoxin contaminated samples, AFB_1 is the compound most frequently present followed by AFG_1. AFB_2 and AFG_2 are typically present at much lower quantities (IARC 1993).

The aflatoxin limits for human foods vary from 4 to 30 µg aflatoxin /kg of food, with the limit depending on the country (Williams *et al.* 2004). The maximum limits set by the European Commission for cereals, nuts, dried fruits and processed products intended for human consumption are 2 µg/kg for AFB_1 and 4 µg/kg for total aflatoxins (AFB_1+AFB_2+AFG_1+AFG_2) (EC 2001). For the health protection of the infants and young children, a vulnerable population group, the European Commission states that it is appropriate to establish the lowest maximum level that is achievable through a strict selection of the raw materials used for the manufacturing of the infant milk formulas and baby foods. The maximum permissible limits of AFB_1 in baby foods and processed cereal based foods intended for infants and young children is set at 0.1 µg/kg, and for AFM_1 in infant foods, milk formulas and follow up formulas at 0.025 µg/kg (EC 2004). The European Commission has fixed 0.05 µg/kg (EC 2001) as the limit for AFM_1 in milk and 0.05 mg/kg (EC 1999) as the limit for AFB_1 in feeds. The action level for AFM_1 in milk and dairy products in the United States is tenfold higher (0.50 µg/kg) than the current level in the EC. In Egypt, there is a little known about levels of AFM_1 in the milk of lactating animals. The presence of aflatoxins in feed however has been reported (Abdelhamid 1990), with mean AFB_1 levels claimed to be 58± 29 µg/kg (range 4-577), i.e. above the limits set by the European Commission.

Table 1: Occurrence of primary aflatoxins in staple foods in developing countries

Country	Foods	Aflatoxins	% of detection (n)	Mean level or range, µg/kg	Reference
Egypt	corn	AFB_1, AFB_2	23 (40)	NS*	(El-Shanawany *et al.* 2005)
Egypt	corn and corn products	AFB_1 AFG_1	2.5 (57)	>35000 >16000	(El-Sayed *et al.* 2003)
Egypt	peanut and seeds spices herbs and plants dried vegetables cereal grains	AFB_1	82 40 29 20 36	24 25 49 20 36	(Selim *et al.* 1996)
Egypt	spices	AFB_1, AFB_2 AFG_1, AFG_2	13 (120)	8-35	(El-Kady *et al.* 1995)
Egypt	corn, rice, wheat, cotton seed, peanuts	AFB_1, AFB_2 AFG_1, AFG_2	44 (95)	5-400	(Abdelhamid 1990)
Egypt	corn, lentils, beans, peanuts, cottonseed	AFB_1, AFB_2 AFG_1, AFG_2	33 (42)	3-12	(Girgis *et al.* 1977)
China	corn groundnuts	AFB_1	70 24	36.5 (max 1098) 80.2 (max 437)	(Wang and Liu 2006)
China	corn peanut oil rice	AFB_1	76 (30) 66 (30) 23 (30)	0.4-128 0.1-52 0.3-2	(Wang *et al.* 2001)
China	maize	AFB_1, AFB_2, AFG_1	85	9-2496	(Li *et al.* 2001)
Guinea	groundnuts corn rice	AFB1	61 (46) 22 (9) 0 (66)	1-112 NS* NS*	(Turner *et al.* 2002)
Gambia	peanuts	AFB_1, AFB_2, AFG_1,AFG_2	87 (47)	1-100	(Wild *et al.* 1992)
Kenya	corn	AFB_1	54 (480)	0-58000	(Muture and Ogana 2005)
Benin and Togo	corn, groundnuts	AFB_1	11.6 (502) 4.6 (175)	0-≥20	(Egal *et al.* 2005)
Benin	corn	AFB_1	100 (3)**	15.2	(Fandohan *et al.* 2005)

* not specified, **three replicates of 5 kg each

2.2. Toxicokinetics of aflatoxins

Four processes are involved in toxicokinetics: absorption, distribution, biotransformation, and elimination (Hsieh and Wong 1994). These are inter-related processes as illustrated in the following figure (Figure 2). AFB_1 is both the most frequently occurring and the most potent of the naturally occurring aflatoxins and thus will be the focus of this section. Other aflatoxins will be discussed only when relevant. Excretion of AFB_1 metabolites from the human body will be discussed here, but further information on aflatoxin metabolites in human biological fluids will be presented in chapter 2.3.2 Biomarkers of aflatoxin exposure.

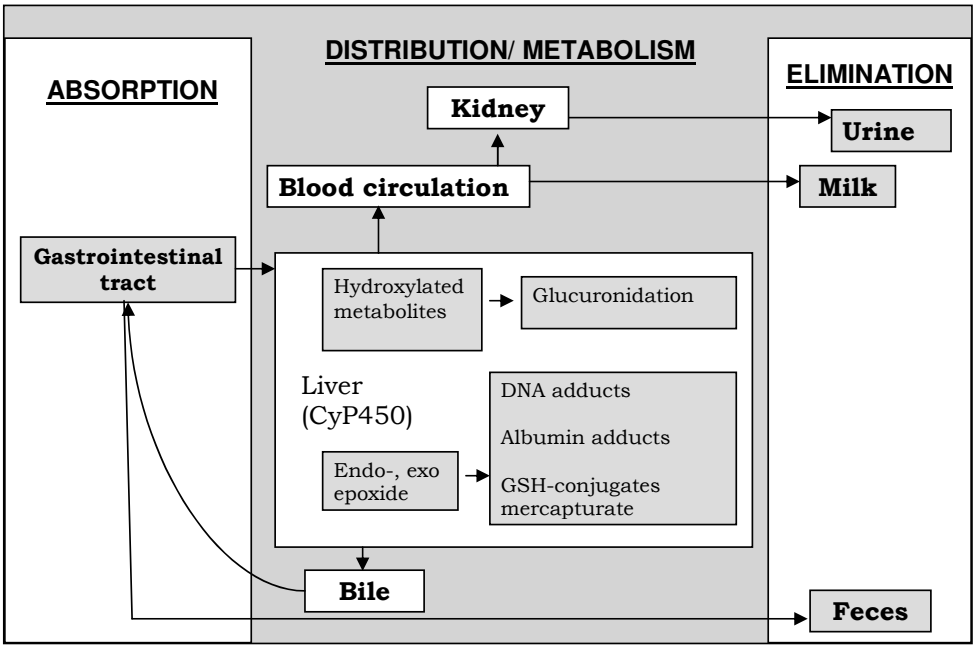


Figure 2: Toxicokinetics of aflatoxins

2.2.1. Absorption

Absorption is the process by which toxicants gain entrance into the body but the extant of absorption varies greatly depending on the route of exposure. The absorbed dose (internal dose) is only a fraction of the exposure via skin, oral or respiratory tract routes (external dose). For substances injected directly into the body, the external dose is the

same as the internal dose. The oral intake is the main route of exposure to aflatoxins. AFB_1 is a relatively low molecular weight, lipophilic molecule, indicating that there is likely to be efficient absorption after ingestion. Animal studies have shown that under normal conditions 50% of the orally administered dose of AFB_1 is quickly absorbed from the duodenal region of the small intestine (Coulombe and Sharma 1985, Kumagai 1989) and then reaches the liver via the hepatic portal blood supply (Wilson *et al.* 1985). Aflatoxins have to pass through the gastrointestinal mucosa, crossing several membranes before entering the systemic circulation. They are absorbed by passive diffusion and the lipophilicity of the aflatoxin determines the rate of absorption. For AFB_1 the rate of absorption is considerably higher than for AFG_1, which is a less lipophilic analogue (Kumagai 1989).

Enterohepatic circulation

Absorption of aflatoxins from the gastrointestinal tract can also occur via of the enterohepatic circulation (Hsieh and Wong 1994). This is a process of excretion of aflatoxin metabolites from the liver via the bile into the intestinal tract and reabsorption and return to the liver by the portal circulation. Since aflatoxin metabolites are excreted in the bile as water soluble glucuronides, they are not likely to be reabsorbed as such. However, enzymes present in the intestinal microbiota are capable of hydrolyzing some glucuronide conjugates, and thereby release the less polar compounds that may then be reabsorbed.

2.2.2. Distribution

Distribution is the process during which an absorbed chemical is transferred from its site of absorption to other areas of the body. Following absorption, AFB_1 is concentrated in the liver not only after oral exposures but also after *intravenous* and *intraperitoneal* dosing. This is due to the high permeability of the hepatocyte membrane for AFB_1, and its active metabolism and subsequent covalent binding with hepatic macromolecules (Busby and Wogan 1984)**.** The kidneys also concentrate AFB_1 but to a much lesser extent (Hayes *et al.* 1977, Hsieh and Wong 1994). The major route for distribution of an absorbed xenobiotic, e.g. aflatoxins, is via the blood circulation and this is greatly affected by binding of the compound to plasma proteins (Hsieh and Wong

1994). Within the circulating blood, the non-bound (free) portion is in equilibrium with the bound portion. However, only the free substance is available to pass through the capillary membranes. Given the high efficiency of the liver to extract free AFB_1 from the blood, binding of AFB_1 to serum albumin already at the site of intestinal absorption can be considered as a major detoxification mechanism, preventing aflatoxin from evoking potential cell interactions (Hsieh and Wong 1994).

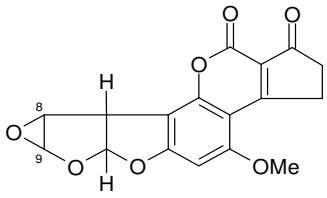
2.2.3. Biotransformation

Biotransformation or metabolism is the process by which the body transforms the substances into new chemicals (metabolites) i.e. a process by which the parent compound is eliminated from the exposed biological system. The aflatoxins, like other xenobiotics undergo phase I (activation) and phase II (detoxification) biotransformation. Although the primary site of aflatoxin metabolism is the liver (and to a lesser extent in the kidney), the gastrointestinal tract also participates in this function (Eaton *et al.* 1994).

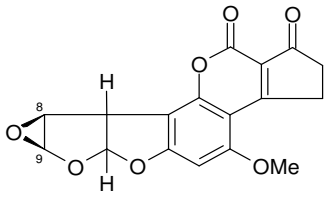
Phase I metabolism (AFB_1 activation, macromolecule binding and formation of hydroxylated metabolites)

The cytochrome P450 (CYP450) mixed-function monooxygenase system is the most important element in Phase I reactions of xenobiotics modifying them by adding a functional group. CYP450 enzymes are a family of haemoproteins that catalyze the metabolism of a large number of xenobiotics, including aflatoxins (Guengerich 1999). Although predominantly localized in the endoplasmic reticulum of liver cells, CYP450's are additionally present in the lung, bladder, uterus, kidney, and small intestine (Krishna and Klotz 1994). Of the CYP450 enzymes, CYP1A2 and CYP3A4 are the main enzymes responsible for the metabolism of the absorbed aflatoxins (Essigmann *et al.* 1982, Forrester *et al.* 1990, Omiecinski *et al.* 1999). CYP3A4 is one of the major CYP enzymes in the liver (Guengerich *et al.* 1998) and it is the dominant P450 involved in the activation of AFB_1 at all AFB_1 concentrations.

Oxidative activation of the 8,9 double bond in the terminal furan moiety of AFB_1 by CYP450 1A2 and 3A4, forms the 8,9-AFB_1 epoxide (Johnson and Guengerich 1997). The epoxide has two stereomeric forms, *exo* and *endo*-epoxide (Figure 3).



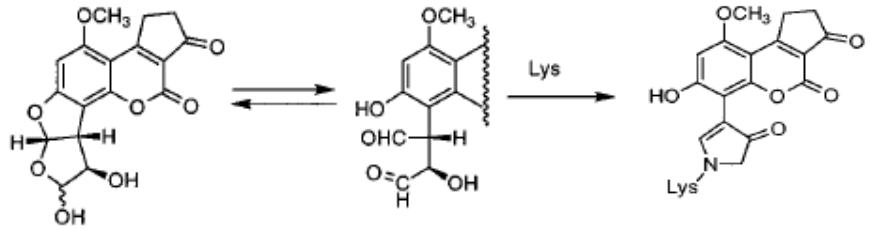
Aflatoxin B_1-8,9-*exo*-epoxide



Aflatoxin B_1-8,9-*endo*-epoxide

Figure 3: The *exo* and *endo*-epoxides of aflatoxin B_1-8,9-epoxide.

AFB_1 epoxides are highly unstable in water ($t_{1/2}$ of <1 sec, 23° C and neutral pH) and rapidly and non-enzymatically hydrolyse to the more stable AFB_1-8,9 dihydrodiol (Guengerich *et al.* 1998) AFB_1-8,9 dihydrodiol, can undergo ring opening and this results in the formation of a resonating dialdehyde phenolate ion, which is capable of forming adducts with protein amino groups, particularly lysine (Figure 4) (Sabbioni *et al.* 1987). The dihydrodiol/dialdehyde equilibrium is complex and both forms are present at physiological pH (Guengerich *et al.* 2002).



AFB_1 dihydrodiol AFB_1 dialdehyde

Figure 4: Reaction of AFB_1 dialdehyde with lysine to form the aflatoxin B_1-lysine adduct (Guengerich *et al.* 2002).

AFB_1 *exo*-epoxide, is the only known genotoxic product of AFB_1 (Wang and Groopman 1999). When added to DNA or guanosine containing double-stranded oligodeoxynucleotides, it readily adducts the N^7 position of guanine by covalent binding

to C^8 of AFB_1 epoxide (Johnson and Guengerich 1997). This reaction yields the predominant AFB_1–DNA adduct identified as trans-8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB_1 (AFB_1-N^7-guanine) (Figure 5) (Essigmann *et al.* 1977).

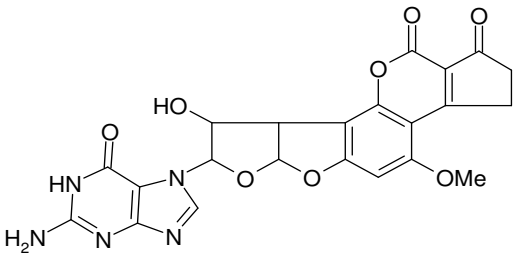


Figure 5: Aflatoxin B_1- N^7-guanine

AFB_1-N^7-guanine comprises over 95% of all AFB_1-DNA adducts (Iyer *et al.* 1994). The presence of adducts creates a positive charge in the imidazole ring of guanine, which results in one of the two alternatives: 1) the majority of the AFB_1-N^7-Gua adducts are removed from DNA rapidly as they are relatively unstable within the DNA structure and are excreted exclusively into urine, having a half life of 7.5 hours (Wang and Groopman 1999), 2) a chemical rearrangement occurs resulting in the imidazole ring opening to form an AFB_1-formamidopyrimidine structure (AFB_1-FAPY) (Lin *et al.* 1977) (Figure 6).

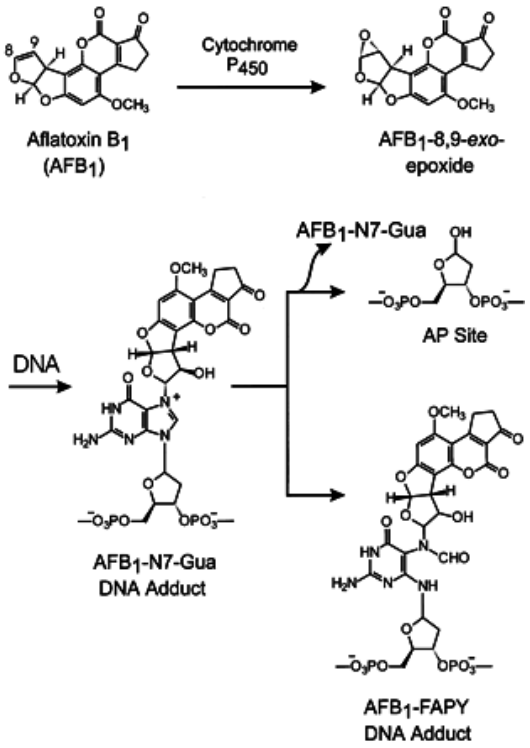


Figure 6: Pathway of metabolic AFB_1 activation leading to AFB_1-N^7-guanine adduct formation. This adduct has a destabilized glycosidic bond and either depurinates to form an apurinic site in the DNA or undergoes opening of its imidazole ring, giving rise to the chemically and biologically stable formamidopyrimidine adduct (AFB_1-FAPY) (Smela *et al.* 2001).

CYP 3A4 and 1A2 also oxidize AFB_1 to various other derivatives than epoxides, the major ones being the hydroxylated metabolites of AFM_1, aflatoxin Q_1 (AFQ_1) and the demethylation metabolite of aflatoxin P_1 (AFP_1) (Figure 7).

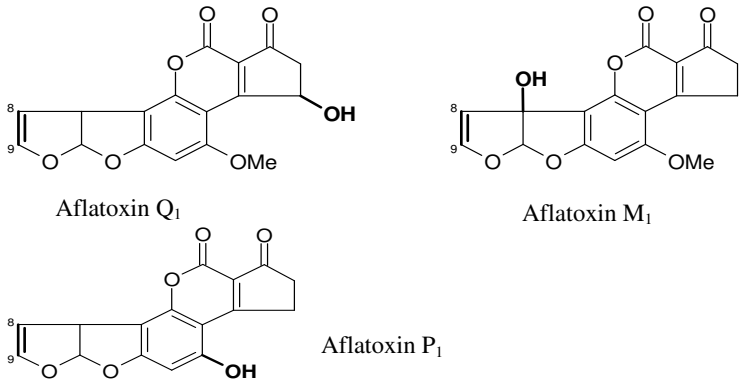


Figure 7: The structure of aflatoxin M_1, Q_1 and P_1; hydroxylated metabolites of aflatoxin B_1.

AFM_1 appears to be formed preferentially by CYP 1A2 and AFQ_1 mainly by CYP 3A4 (Raney *et al.* 1992). AFM_1 is the major aflatoxin metabolite in humans (Groopman *et al.* 1985). AFM_1 can be further activated to form an AFM_1-8,9-epoxide that binds to DNA and is excreted into urine in the form of AFM_1-N^7-guanine (Egner *et al.* 2003). AFQ_1 and AFP_1 are not appreciably oxidized by human liver microsomes and are not very genotoxic (Raney *et al.* 1992). Generally, the hydroxylated AFB_1 metabolites (AFM_1, AFQ_1, AFP_1), are poorer substrates for epoxidation and have reduced genotoxicity compared to AFB_1. Thus they are generally considered as detoxification products. However, in the case of AFM_1, the reported high cytotoxicity of the compound (Neal *et al.* 1998) highlights the caution that needs to be exercised in designating the formation of AFM_1 as detoxification product.

The formation of one further metabolite, aflatoxicol, a reduced aflatoxin species, has also been shown and it is reported to have an equal potency to AFB_1 as a carcinogen (Coulombe *et al.* 1982).

Phase II conjugation reactions (detoxification)

Phase II reactions consist of enzymatic reactions that conjugate the modified xenobiotic with another substance e.g. with glucuronic acid or glutathione. The conjugated products are larger molecules than the substrate and are generally polar in nature (water-soluble) and thus, they can be readily excreted from the body.

In the case of AFB_1, detoxification is accomplished by enzymatic conjugation of the hydroxylated metabolites with glucuronic acid to form water soluble glucuronide esters that are excreted in urine or bile. Additionally, AFB_1 in the epoxide form can be conjugated to glutathione (GSH) and subsequently be excreted in the bile (Busby and Wogan 1984).

The conjugation of the epoxide to GSH is catalyzed by the cytosolic glutathione-S-transferases (GST) (Neal *et al.* 1987). These transferases play a significant role in the detoxification of AFB_1 epoxide and in the species susceptibility to the toxic effects of AFB_1. The most striking species difference has been observed between the mouse and the rat. Although the mouse has very high microsomal epoxidation activity, it is very resistant to AFB_1-induced tumors in comparison to the rat, which is a sensitive species. The conjugation of GSH to the AFB_1 epoxide has been shown to be higher in the mouse than in the rat, and the very high level of GST activity towards AFB_1 epoxide in the mouse appears to be the basis of the resistance of this species (Eaton *et al.* 1994).

Glucuronidation of the hydroxylated AFB_1 metabolites by UDP-glucuronyl transferases is another reaction for the elimination of the metabolites from the body. However, AFM_1, since it is a tertiary alcohol (the C-OH carbon is bonded to 3 carbons), is not a good substrate for glucuronidation (Busby and Wogan 1984). Glucuronic acid conjugates are primarily eliminated via the bile, thus the enterohepatic circulation could prolong the effective half-life of AFB_1. Figure 8 summarizes the known pathways involved in AFB_1 metabolism.

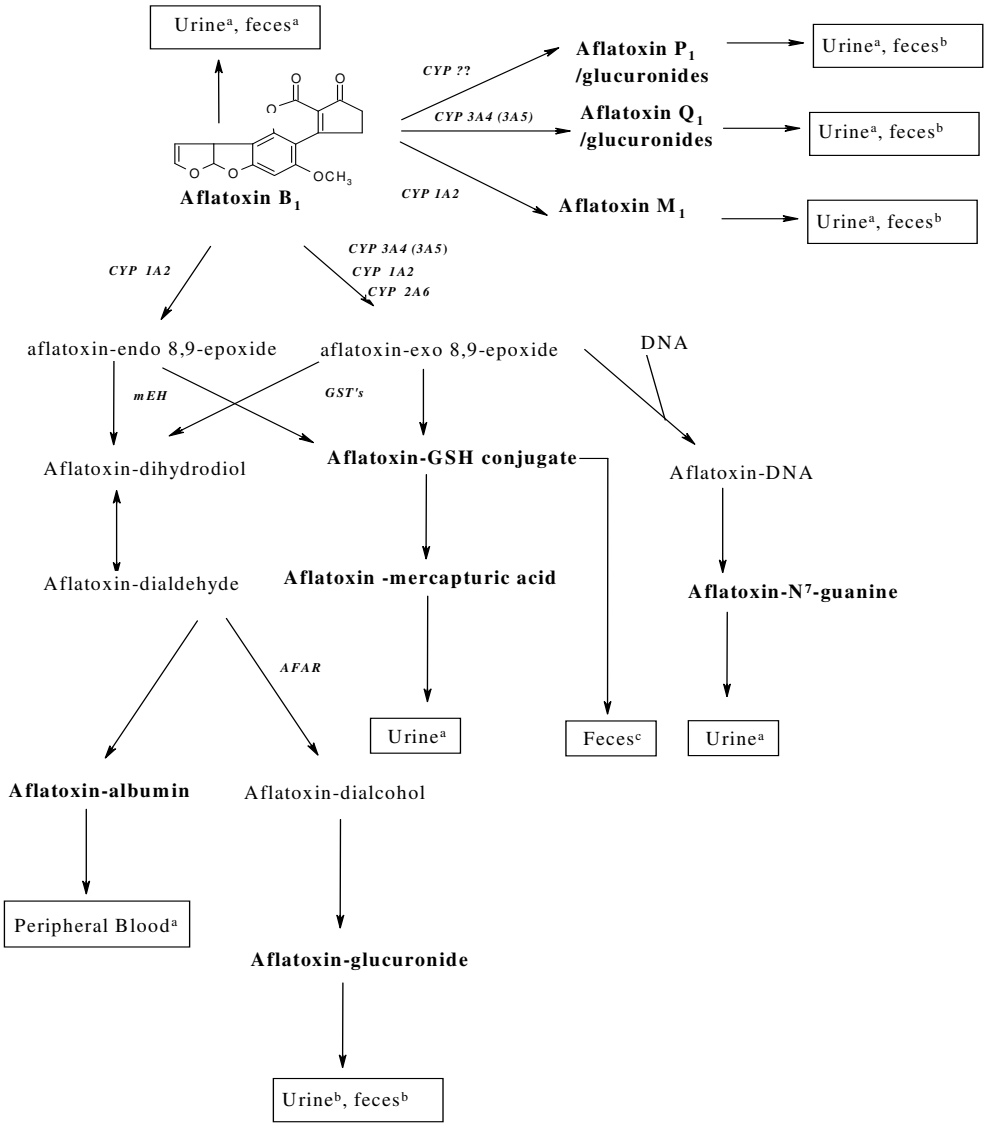


Figure 8. AFB_1 elimination in humans: a schematic diagram for the known pathways of AFB_1 metabolism in humans and possible routes of excretion of different AFB_1 metabolites (modified from Eaton et al. (2001) and Wild and Turner (2002)).

[a]Extensive human and experimental evidence on excretion of this metabolite; [b]Scant or no evidence available; [c]Only experimental evidence available (no human data).
GST = glutathione S-transferases; mEH = microsomal epoxide hydrolase; AFAR = aflatoxin B_1-aldehyde reductase.

2.2.4. Excretion

AFB_1 and its metabolites are excreted primarily through the fecal and urinary routes (Figure 8). In the case of lactating mothers, aflatoxin metabolites are also excreted in breast milk after consumption of aflatoxin contaminated food.

Fecal excretion

Aflatoxins are excreted in the feces in two ways, excretion of the unabsorbed aflatoxin from the lumen of the gastrointestinal tract and biliary excretion to the intestine. The biliary route is the major route of excretion of the absorbed aflatoxins and their metabolites. AFB_1-GSH, which is a stable, non toxic, polar product, together with glucuronidated aflatoxin metabolites, is excreted from the liver via the bile into the gastrointestinal tract. Subsequently, these metabolites will either be excreted by the fecal route or metabolized by enzymes in the intestinal microbiota capable of hydrolyzing some glucuronide conjugates which then release the aflatoxins that may then be reabsorbed by the gastrointestinal tract (enterohepatic circulation) (Hsieh and Wong 1994).

Urinary excretion

The urinary excretion is an important route of excretion of absorbed aflatoxins and their metabolites. AFM_1, AFQ_1, AFP_1 (Groopman *et al.* 1985, Egner *et al.* 2003) and AFB_1-N^7-guanine (Essigmann *et al.* 1982, Groopman *et al.* 1992a) are excreted through the urinary route. The AFB_1-GSH metabolite undergoes sequential metabolism in the liver and kidneys to form mercapturic acid conjugates (aflatoxin-N-acetylcysteine) also excreted in the urine (Scholl *et al.* 1997). The glucuronidated aflatoxin metabolites can be excreted both by biliary and urinary routes, the former being the primary route of excretion (Hsieh and Wong 1994).

In many animal species (rats, sheep, pigs, cows), AFM_1 is the main unconjugated AFB_1 metabolite in the urine and accounts for 2-9% of the dose (IARC 1993). AFM_1 is also the major urinary aflatoxin metabolite in human subjects exposed to dietary AFB_1 (Zhu *et al.* 1987). Only small amounts, between 1.2 and 2.2% of dietary AFB_1, have been shown to be present in the urine as AFM_1 (Zhu *et al.* 1987), and 0.2% of the AFB_1 was calculated to be excreted in urine as AFB_1-N^7-guanine (Groopman *et al.* 1992c).

Excretion in milk

The presence of aflatoxins in breast milk provides the main source of aflatoxin for newborn infants. Milk synthesis in the mammary gland cells is under hormonal control, promoted by the increasing serum prolactin levels occurring shortly after delivery. Milk production is regulated and adjusted by the infant's demands and therefore normally increases gradually during the lactation period. Furthermore, milk production is influenced by a variety of factors including age, stress, nutritional and health status of the mother (Jensen and Slorach 1991).

The composition of the breast milk varies strongly with time of lactation. Milk volume and concentrations of fat, lactose and water-soluble vitamins increase, while those of proteins, fat-soluble vitamins, phospholipids and cholesterol decrease from the first postpartum week's breast milk (colostrum) to the mature milk (Lawrence 1999).

Some of the components in the human milk are formed through synthesis in the breast gland itself, e.g. most proteins, lactose and triglycerides. Other compounds are transferred from the blood, e.g. vitamins, fatty acids and minerals. In the latter case the concentration may be higher than that in the blood, and the components are then transported by an active process through the epithelial cells to the milk duct lumen (Jensen and Slorach 1991).

The excretion of a chemical into breast milk and the amount of the chemical that passes into the milk is affected by many factors that can be divided into chemical and physiological/maternal characteristics. The chemical characteristics refer to those aspects of the compound that affect its ability to be taken up in milk, such as lipid solubility, degree of ionization, molecular weight, and ability to bind to maternal blood and/or milk components (Clewell and Gearhart 2002). The physiological/maternal characteristics can be separated into external (dietary and environmental exposure) and internal factors (maternal age and body weight, parity, length of lactation, fat mobilization and fat concentration in milk) (Harris *et al.* 2001, Clewell and Gearhart 2002). The most critical factor determining the chemical dose delivered to the infant is obviously the extent and pattern of maternal exposure (Clewell and Gearhart 2002).

AFM_1 is the primary aflatoxin metabolite in both animals and human milk, comprising 95 % of the total amount of aflatoxins excreted in milk. It has been estimated that 0.09–

0.43% of dietary intake is excreted in the human milk as AFM_1 (Zarba *et al.* 1992). The factors affecting the excretion of AFM_1 in human milk have not been studied in detail and therefore very little information is available. Many animal studies have been performed on the excretion of AFM_1 in milk as a result of AFB_1 exposures, but they have mainly focused on the extent of conversion of AFB_1 to AFM_1. The carry-over values of AFB_1 from feed into AFM_1 in milk in dairy cattle or sheep vary from 0.3 to 6.2 % (Patterson *et al.* 1980, Veldman *et al.* 1992, Battacone *et al.* 2005). The concentrations of aflatoxin M_1 in milk appear to vary widely from animal to animal, from day to day, even from one milking to the next (Van Egmond 1994).

AFM_1 can be found in milk within 12–24 h after the first ingestion of AFB_1 (Battacone *et al.* 2003) and it is found up to three to seven days after last ingestion of the mycotoxin. The carry-over (percentage) of AFM_1 in milk is not dependent on the dosage of AFB_1. The levels of AFM_1 in the milk however, are significantly influenced by AFB_1 dosage (Battacone *et al.* 2003).

Veldman et al. (1992), conducted a study in which dairy cows in the early lactation stage (2-4 weeks) and dairy cows in late lactation weeks (34-36 weeks) were fed daily AFB_1 contaminated feed. After 12 days, the carry-over of AFM_1 in the milk was 6.2% in the early stage, but it declined to 1.8% in the late lactation stage.

2.3. Methods of assessing exposure to aflatoxins

It is extremely difficult to obtain an accurate estimation of individual aflatoxin exposure by conventional epidemiological approaches based on questionnaires estimating food intake. This is mainly due to the minute aflatoxin quantity in foodstuffs, the striking daily and seasonal variations, and the practical difficulties in assessing food intake in rural agricultural societies where aflatoxin exposure is more of a problem (Hall and Wild 1994, Chen and Chen 2002). Molecular dosimetry methods have been used to develop biomarkers to quantify the biologically effective dose of aflatoxin exposure. The following sections will review briefly the different methods of assessment of individual aflatoxin exposures: the use of questionnaires and their limitations, and the application of biomarker measurements in human biological fluids.

2.3.1. Dietary questionnaires in aflatoxin exposure assessments

Human exposure to aflatoxins has been assessed in the context of conventional epidemiological studies in two ways: either by the use of questionnaires estimating quantity and frequency of intake of dietary items assumed to be commonly contaminated with aflatoxin, or by a combination of questionnaire and laboratory measurements on representative food samples collected from the population concerned. The limitations by either of these two approaches are summarized below:

Firstly, aflatoxin contamination of foods is extremely variable and estimation of aflatoxin levels in foods may be difficult. Efforts have been made to achieve reliable sampling and a review of sample preparation methods was published by the AOAC International (Scott 1990). However, estimates on human exposure based on food analysis can easily lead to misclassification of individuals with respect to exposure (Hall and Wild 1994). Misclassification of exposure status is claimed to be a major contributor to the insensitivity of many epidemiological investigations (Groopman and Kensler 1999).

Second, an additional confounding factor is the marked seasonal variation in aflatoxin levels (Wild *et al.* 2000), reflecting both climatic changes and food harvesting and storage practices. High temperature and humidity in the wet and hot seasons increase the likelihood that the *Aspergillus* fungi will grow on the crop, resulting in higher aflatoxin contamination. Initially after harvest, the levels of AFB_1 in foodstuffs are expected to be low. However, as the food is stored for longer periods, the likelihood of *Aspergillus* contamination increases as does the potential level of AFB_1 (Hall and Wild 1994).

Third, the exposure linked to a disease outcome may have happened at a different time than the measurements of the food intake, perhaps even months or years apart. In addition, the development of a disease may affect the diet and thus food intake measurements of diseased individuals may be misleading. Furthermore, the actual recording of food intakes may also lead to abnormal eating behaviors (Hall and Wild 1994).

Finally, there are practical difficulties in assessing food intake in rural agricultural societies where aflatoxin exposure mostly occurs (Qian *et al*. 1994, Groopman *et al*, 1992a). Measurements of individual portions may be challenging when individuals eat from a common food bowl either by hand or with a spoon, or as is the case in groundnut producing societies, where they consume nuts throughout the entire day.

In view of these limitations, the preferable method for allowing individual exposure evaluation is that of biomarker assessment and thus measurement of biomarkers in human biological fluids has been used increasingly to confirm and quantify exposure to aflatoxins (Wild and Hall 1994).

2.3.2. Biomarkers of aflatoxin exposure

Biomarkers in general

Biomarkers are a measure of a cellular, biochemical or molecular change in biological media (human tissues, cells or fluids), which is informative with respect to assessing an exposure, or a change relevant to a disease pathway indicating the presence or extent of a disease (Groopman 1994). Biomarkers are used to indicate or measure a biological process, and therefore the detection of biomarkers specific to a disease can aid in the identification, diagnosis, and treatment of affected individuals who may be at risk but do not yet exhibit symptoms.

The main goals of biomarker research are to develop and validate chemical-specific biomarkers that reflect specific exposures and predict disease risks in individuals, to provide an objective measure for determining the effectiveness of interventions to lower exposure and risks, and to clarify the interactions of multiple agents and multiple exposures and their relation to disease outcomes (Groopman and Kensler 1999).

The distinct steps in the progressive nature of many chronic diseases and the alterations in the molecular mechanisms underlying the association between exposure and disease facilitate the development of biomarkers.

The biomarkers can be classified into those reflecting susceptibility, internal dose, biologically effective dose, early biological effect, altered structure/ function and clinical disease (Figure 9).

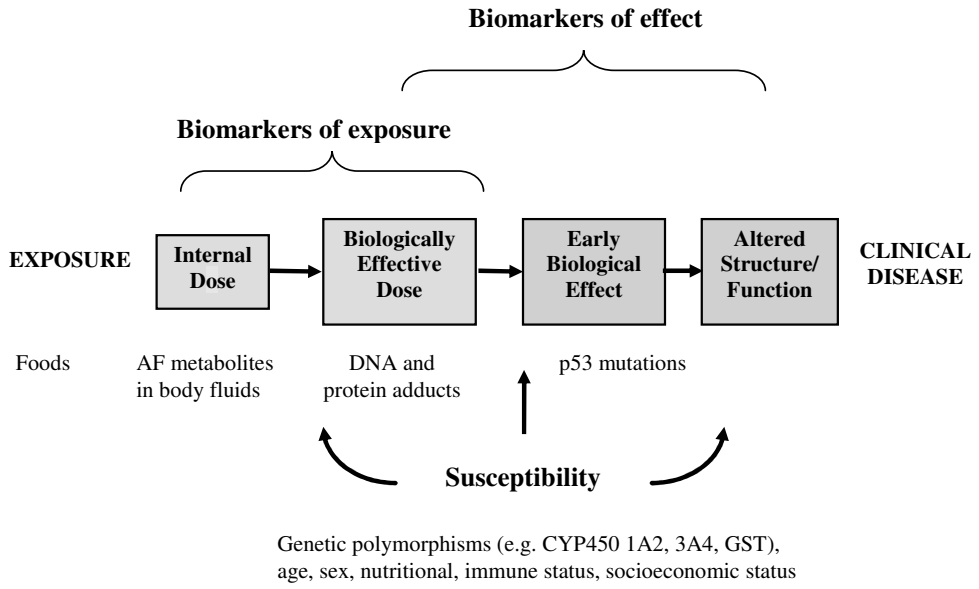


Figure 9: Classification of biomarkers (Groopman and Kensler 1999).

Internal dose is the amount of material which is internalised into the body and metabolised. The level of this dose may be subject to interindividual variation through differences in metabolism, activation and/or detoxification of a compound. The biologically effective dose is the dose that reaches the target tissue and produces an effect. Certain changes are termed as early biological effects e.g. increased proliferation, somatic cell mutations, genetic alterations that result in altered cell structure and function. A range of factors within the individual determine its susceptibility to the exposure, e.g. the ability to activate/detoxify carcinogens, the ability to repair DNA damage, its nutritional and immune status, age, sex, socio-economic status (WHO 1993).

The biomarkers can be also categorized as biomarkers of exposure, effect and susceptibility. Biomarkers of exposure indicate the exposure to the parent compound, the biomarkers of effect signal biological responses to an exposure and the biomarkers of susceptibility provide information about the inherent sensitivity of a person to an environmental agent, as mentioned above (WHO 1993, Groopman 1994).

Biomarkers of aflatoxin exposure

An understanding of the mechanisms of aflatoxin actions provides a rationale for using some of the aflatoxin metabolites found in urine or blood as biomarkers of aflatoxin exposure and effect (Wild and Turner 2002). The use of aflatoxin metabolites as biomarkers reflects not only the dietary exposure of the individual but also the characteristics of that individual with respect to the uptake, distribution, metabolism, detoxification and removal of aflatoxin adducts. This double contribution is a key point to consider when interpreting the data obtained in epidemiological studies (Wild and Pisani 1998).

Biomarkers of aflatoxin exposure and effect have been validated in detail in studies in experimental animals and humans. Dose-response relationships between biomarker levels and liver tumor incidence were first established in experimental animals (Groopman *et al.* 1992b). The biomarkers were then used in pilot studies in humans to define the sensitivity, specificity, accuracy, and reliability parameters. Further validation in transitional epidemiological studies assessed intra and inter-individual variability, background levels, external dose-marker relationship, and the feasibility of use in larger population based studies (Groopman *et al.* 1992a, 1992c). Finally prospective epidemiological studies were conducted to evaluate biomarker effectiveness in identifying the risk of developing hepatocellular carcinoma (HCC) associated with aflatoxin exposure (Qian *et al.* 1994, Sun *et al.* 1999). Currently, the most detailed epidemiological description linking a certain DNA adduct to development of a particular form of cancer exists for AFB_1 (Sharma and Farmer 2004). Few carcinogens have undergone such extensive validation. In fact these studies can be viewed as providing a model for the development, validation and application of chemical-specific biomarkers for evaluating the molecular epidemiology of other cancers in which environmental carcinogens are thought to play etiologic roles (Wogan 2000).

Biomarkers of aflatoxin exposure have been used in studies of the etiology of HCC, in assessments of the role of polymorphism in aflatoxin metabolizing enzymes and the altered levels of metabolite formation in some individuals, and in the assessments of the effect of DNA repair enzyme polymorphisms on the levels of AFB_1-N^7-guanine adducts (Wild and Turner 2002).

Detection of aflatoxins in human biological fluids

Biomarkers of aflatoxin exposure include urinary aflatoxin metabolites, such as AFB_1-N^7-guanine and AFM_1, serum AF-albumin (Wild and Turner 2002), and AFM_1 in milk (Van Egmond 1994).

AFM_1 is the major unconjugated urinary aflatoxin metabolite in humans (Groopman *et al.* 1985). Numerous observational reports of the presence of AFM_1 and other metabolites in urine are available reflecting aflatoxin exposure (Table 2). Zhu et al (1987) analyzed 252 urine samples from 32 households from the Guangxi region in China and reported a good correlation between total dietary AFB_1 intake and AFM_1 excretion. Between 1.2 and 2.2% of dietary AFB_1 was present as AFM_1 in the urine. Groopman et al (Groopman *et al.* 1992c) analyzed the same urine samples, confirmed the findings on AFM_1 and also demonstrated the presence of AFB_1, AFQ_1, AFP_1 and AFB_1-N^7-guanine in urine. In that study also the levels of AFB_1-N^7-guanine adducts were correlated with AFB_1 intake. The percentage of AFB_1 excreted as the above metabolites was 4.4% in women and 7.6% in men.

The levels of AFB_1-N^7-guanine adducts in urine (Groopman *et al.* 1992a, 1992c) and AF-albumin adducts in the blood (Gan *et al.* 1988, Wild *et al.* 1992) reflect the biologically effective aflatoxin dose to which an individual has been exposed and they are both well validated as biomarkers of exposure. Gan et al. (1988) showed that the level of AF-albumin adducts in the blood were correlated not only with the dietary intake of AFB_1, but also with the urinary excretion of AFM_1 in individuals. Wild et al. (1992) confirmed the validity of AF-albumin adducts as a biomarker of aflatoxin exposure, and Groopman et al (Groopman *et al.* 1992c, 1994) demonstrated a correlation between the levels of serum AF-albumin adduct and urinary AFB_1-N^7-guanine adduct. Urinary AFB_1-N^7-guanine adducts reflect dietary exposure over the previous 1-2 days (Groopman *et al.* 1992a, 1992c), while the level of AF-albumin adduct in serum reflects exposure over the previous 2-3 months (Wild *et al.* 1992). It has been estimated that 1.4-2.3% of ingested AFB_1 is covalently bound to albumin (Gan *et al.* 1988) and 0.2% of the AFB_1 is excreted as AFB_1-N^7-guanine in urine (Groopman *et al.* 1992c). An AFG_1-albumin adduct has also been identified (Sabbioni and Wild 1991).

Table 2: Aflatoxins and their metabolites in human biological fluids.

Sample	Country	Aflatoxins and metabolites	n of subjects	% positive samples	Level*		Reference
					mean	range	
Urine	China	AFM_1	145	54	NS[§] ng/ml	0.003-0.243	(Sun *et al.* 1999)
Urine	China	AFM_1	42	NS[§]	930 ng/ml NS[§] NS[§] ng/day	0.01-3.2 40-4800	(Zhu *et al.* 1987)
Urine	China	AFM_1	29	89	192 ng/day	0.9-3569	(Wang *et al.* 2001)
		AFB_1-N^7-Gua		41	407	64.9-1789	
		AFB-mercapturate		89	103	6.6-494	
		AFQ_1		26	92.2	77.3-137	
		AFP_1		30	664	80.4-3569	
Urine	China	AFM_1	317	67	NS[§] ng/ml	0.17-5.2	(Qian *et al.* 1994)
		AFB_1-N^7-Gua		49	NS[§]	0.3-1.81	
		AFP_1		53	NS[§]	0.59-16	
		AFB_1		71	NS[§]	NS[§]	
Urine	China	Total aflatoxins (AFB_1-N^7-Gua, AFB_1, AFQ_1, AFP_1)	42	NS[§]	NS[§] ng**/ml NS[§] ng**/day	1.5-2.3 3300-6600	(Groopman *et al.* 1992c)
Urine	Gambia	AFB_1-N^7-Gua (AFG_1, AFM_1, AFP_1 AFQ_1 also detected)	20	NS[§]	NS[§] ng/day	48.2-7099	(Groopman *et al.* 1992a)
Urine	Egypt	AFB_1	20	30	NS[§] ng/ml	<1.5	(Al-Saadany 1993)
		AFM_1			NS[§]	<2.5	
		AFG_1			1.1[##]	-	
Urine	Egypt	AFB_1	60	61	NS[§] ng/ml	0.01-0.15	(Hatem *et al.* 2005)
Blood	Egypt	AFB_1	60	61	NS[§] ng/ml	0.04-0.69	(Hatem *et al.* 2005)
Blood	Egypt	AFB_1	20	55	NS[§] ng/ml	<4.5	(Al-Saadany 1993)
		AFM_1			NS[§]	<0.5	
		AFM_2			0.2[##]	-	
Blood	China	AF-alb	42	NS[§]	NS[§] pg/mg^	30-340	(Gan *et al.* 1988)
Blood	Gambia	AF-alb	20	NS[§]	44 pg /mg^^	NS[§]	(Wild *et al.* 1992)
Blood	Gambia Feb/March July/Aug	AF-alb	357	100	83.2 pg/mg^ 34.9 pg/mg^	NS[§]	(Wild *et al.* 2000)
Blood	Gambia	AF-alb	444	100	NS[§] pg/mg^	2.2-459	(Turner *et al.* 2000)
Blood	Gambia	AF-alb	117	100	29.3 pg/mg^	2.2-250.4	(Wild *et al.* 1993)
Blood	Guinea	AF-alb	124	96	9.9 pg/mg^	NS[§]	(Turner *et al.* 2005b)
Blood	Guinea	AF-alb	600	95	NS[§] pg/mg^^^	9.4-22	(Sylla *et al.* 1999)

Table 2: Aflatoxins and their metabolites in human biological fluids. *Continued.*

Sample	Country	Aflatoxins and metabolites	n of subjects	% positive samples	Level*		Reference
Blood	Benin	AF-alb	480	99	NS[§] pg/mg^	5-1064	(Gong *et al.* 2002)
Breast milk	United Arab Emirates	AFM_1	140	92	560[#] pg/ml	5-3400	(Abdulrazzaq *et al.* 2003)
Breast milk	United Arab Emirates	AFM_1	445	100	NS[§]pg/ml	1.7-3000	(Saad *et al.* 1995)
Breast milk	Australia Thailand	AFM_1	73 11	15 44	71[#] pg/ml 664[#] pg/ml	28-1031 39-1736	(El-Nezami *et al.* 1995)
Breast milk	Egypt	AFM_1 AFG_1 (AFB_1, AFB_2, AFG_2 also detected)	200	11	160 pg/ml NS[§] pg/ml	120-200 38-47	(El-Shewey 1992)
Breast milk	Gambia	AFM_1	5	100	NS[§] pg/ml	≤ 1.4	(Zarba *et al.* 1992)
Breast milk	Ghana	AFM_1	264	22	NS[§] pg/ml	20-1816	(Lamplugh *et al.* 1988)
Breast milk	Zimbabwe	AFM_1	54	11	NS[§] pg/ml	14-50	(Wild *et al.* 1987)
Breast milk	Sudan	AFM_1	99	23	NS[§] pg/ml	5-64	(Coulter *et al.* 1984)
Cord blood	United Arab Emirates	AFM_1, AFM_2,AFB_1 also	201	53	1229[#] pg/ml	110-4060	(Abdulrazzaq *et al.* 2002)
Cord blood	Thailand	Total aflatoxins (AFB_1, AFQ_1, AFG_1)	35	48	3.1 nmol/ml	0.064-13.6	(Denning *et al.* 1990)
Cord blood	Gambia	AF-alb	30	97	NS[§]pg/mg	5-30	(Wild *et al.* 1991)

*The levels are presented in comparable units whenever possible. The presented means for the AF-albumin adducts are geometric means, [§]not specified
**AFB_1 equivalents, ^pg AFB-alb/mg albumin, ^^this is an arithmetic mean
^^^this is the range of geometric means from 8 different villages in Guinea
[#]This is a median, [##]only one positive sample

AFM_1 is excreted also in breast milk during lactation. Several studies have demonstrated the presence of AFM_1 in human milk (Table 2). For example, in the United Arab Emirates, AFM_1 was detected in milk at levels of 5-3400 pg/ml (Abdulrazzaq *et al.* 2003), in Victoria, Australia in the range 28-1031 pg/ml, and from 39 to 1736 pg/ml in Thailand (El-Nezami *et al.* 1995). In one report of five lactating women in the Gambia (Zarba *et al.* 1992), 0.09-0.43% of dietary intake was excreted in the milk as AFM_1.

Aflatoxins have also been detected in umbilical cord blood samples demonstrating that aflatoxin can cross the placenta barrier and exposure to these carcinogens can start already in utero (Wild *et al.* 1991)**.**

2.4. Aflatoxins and health effects

Aflatoxins are toxic, carcinogenic and mutagenic in humans and animals and AFB_1, the most prevalent and potent form of the aflatoxins, has been classified as a class 1A human carcinogen by the IARC (1993). Recently, aflatoxins have been strongly associated also with early childhood growth faltering (Gong *et al.* 2003, Turner *et al.* 2003, Gong *et al.* 2004) and reduced levels of salivary IgA (Turner *et al.* 2003). Susceptibility to aflatoxins is greater in the young (Williams *et al.* 2004), and aflatoxin exposure can start in utero (Wild *et al.* 1991) and continue throughout early infancy and childhood. AFM_1 provides a source of aflatoxin exposure to the lactating infant (Wild *et al.* 1987, Zarba *et al.* 1992, El-Nezami *et al.* 1995), and thereafter the level of exposure increases significantly with the introduction of adult foods after weaning (Gong *et al.* 2003, 2004). AFM_1 has cytotoxic properties in human hepatocytes (Neal *et al.* 1998) that may have important implications for immunocompetence and growth. This section will address the main health problems associated with human aflatoxin exposure.

2.4.1. Toxicity of aflatoxins

Aflatoxicosis is the poisoning that results from ingesting aflatoxins. Two forms of aflatoxicosis have been identified: 1) acute severe intoxication which results in direct liver damage and subsequent illness or death, and 2) chronic sub-symptomatic exposure that has nutritional and immunological consequences. All doses of exposure are considered to have a cumulative effect on the risk of cancer.

The order of potency for both acute and chronic toxicity of aflatoxins is AFB_1> AFG_1> AFB_2> AFG_2 (IARC 1993). In ducklings and rats, the toxicity of AFM_1 was similar or slightly less than that of AFB_1 (JEFCA 1998). Although AFM_1 can be activated to form AFM_1-8,9-epoxide as shown from recent studies on the urinary AFM_1-N^7-guanine excretion of tree shrews and rats (Egner *et al.* 2003), metabolic epoxidation is not necessary for AFM_1 to exert cytotoxicity as demonstrated by the results of *in vitro*

studies (Neal *et al.* 1998), where AFM_1 was directly toxic in the absence of metabolic activation compared to AFB_1. While it is widely accepted that both the carcinogenic and acutely toxic responses of AFB_1 are dependent on metabolic activation, the role of metabolism in the cytotoxicity of AFM_1 is unclear.

The liver is usually the target organ for both acute and chronic aflatoxin toxicity, but lesions in the kidney and stomach have also been reported in rats (IARC 1993).

The differences in susceptibility to aflatoxins across species and between persons depend largely on the fraction of the dose that is directed into the various possible metabolic pathways, with harmful biological exposure being the result of activation to the epoxide and the reaction of the epoxide with proteins (Figure 10).

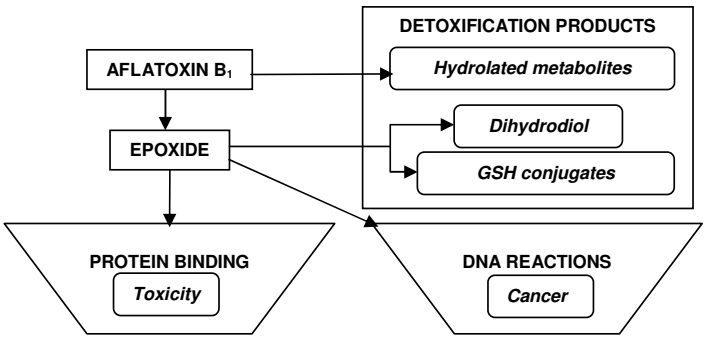


Figure 10. Pathways and consequences of aflatoxin metabolism (Williams *et al.* 2004).

Acute toxicity

The symptoms of severe aflatoxicosis include fever, edema, lethargy, vomiting, abdominal pain, and potentially fatal liver failure. In animal experiments, rabbits, ducks and pigs are more susceptible species with a low median lethal dose (0.3-0.6 mg/kg body weight), whereas Fisher rats (2 mg/kg body weight), chickens (18 mg/kg body weight), and mice 15 mg/kg body weight) display greater tolerance. Adult humans usually have a high tolerance to aflatoxin, and in the reported acute poisonings, it is usually the children who are affected and die (Williams *et al.* 2004).

Reported incidents of acute human aflatoxin poisonings are relatively infrequent, probably because people usually avoid eating obviously mouldy foods. However, in conditions of poverty and food shortages, then people have no option but to use any available food irrespective of quality.

Reports of death and serious illnesses usually originate from the developing countries that are the countries at risk (Table 3). An early case report linked fatal hepatic toxicity of a 15- year-old boy to consumption of mouldy cassava and subsequent analysis of the mouldy cassava in the family's store revealed that it had a high aflatoxin content (1.7 mg/kg) (Serck-Hanssen 1970). More recently, an acute aflatoxicosis outbreak was reported in young adults in Kenya (Barrett 2005). The outbreak followed a poor harvest of maize that had been damaged and rendered susceptible to mold by drought. Furthermore, to guard the inadequate harvest against theft, the people stored the maize in their homes, which were warmer and more damp than the centralized granaries where maize was usually stored. From January to June 2004, 317 people sought hospital treatment for the symptoms of liver failure, and 125 of them died. Health officials examined maize samples and found AFB_1 concentrations as high as 4.4 mg/kg, which is 220 times the Kenyan limit for aflatoxin in foods. In Kenya, a previous outbreak of jaundice accompanied by fatalities, was also associated with the consumption of maize that contained up to 12 mg/kg AFB_1 (Ngindu *et al.* 1982).

In 1988, 13 Chinese children died of acute hepatic encephalopathy (brain and nervous system damage that occurs as a complication of liver disorders) as a result of severe aflatoxicosis (Chao *et al.* 1991, Lye *et al.* 1995). The disease was described to cause symptoms similar to Reye's syndrome. Reye's syndrome primarily targets the brain and liver, and the symptoms involve drowsiness, confusion, seizures, coma, and in severe cases, death. It is a rare but serious disease that most often affects children 6 to 12 years of age and it seems to be related to the use of aspirin to treat some viral illnesses, such as chickenpox.

A number of case reports linking AFB_1 to Reye's syndrome exist in the literature. Although Reye's syndrome occurs throughout the world and there is no apparent geographical association with aflatoxin exposure, the disease has been associated with aflatoxins because AFB_1 has been detected in Reye's syndrome patients in New Zealand (Becroft 1966), Thailand (Shank *et al.* 1971), the former Czechoslovakia (Dvorackova *et al.* 1977), and the United States (Ryan *et al.* 1979). The accumulation of aflatoxin in these cases however, could be a result of the disease affecting metabolism rather than an etiological factor (Hall and Wild 1994).

Table 3: Outbreaks of aflatoxicosis and aflatoxin association with Reye's syndrome

Country	Number of subjects/ deaths	Symptoms	Source and level of exposure	Aflatoxins in biological samples and post-mortem liver examinations	Reference
Kenya	317/125	vomiting, abdominal pain, fatal liver failure	Maize, AFB_1 4.4 mg/kg	AF-alb in blood	(Barrett 2005)
Malaysia	-/13	Vomiting, diarrhoea, abdominal pain, anorexia, seizures, and eventual coma. Iniatially many presented with a Reye like syndrome (hepatic encephalopathy)	Chinese noodles	Necrosis of the liver, giant cell formation, central vein sclerosis, bile stasis, and steatosis. Presence of acute tubular necrosis, superficial upper gastrointestinal erosions, and ensuing encephalopathy. The eventual cause of death was acute hepatic and renal failure.	(Chao *et al.* 1991) (Lye *et al.* 1995)
Kenya	20/12	Vomiting, abdominal discomfort, anorexia, jaundice, leg edema, tachycardia, liver tenderness, gastrointestinal bleeding	Maize, AFB_1 3.2-12 mg/kg, AFB2 1.6-2.7 mg/kg	AFB_1 in liver (autopsy), centrilobular necrosis, fatty infiltration	(Ngindu *et al.* 1982)
India	994/97	Fever, jaundice, hepatomegaly, splenomegaly	Maize, AFB_1 0.01-1.1 mg/kg	AFB_1 in serum	(Tandon *et al.* 1978)
India	397/106	Vomiting, anorexia, jaundice, leg edema, gastrointestinal bleeding	Maize, AFB_1 6.25- 15.6 mg/kg	Bile duct proliferation and giant liver cells	(Krishnamachari *et al.* 1975)
Uganda	1/1	Abdominal pain, leg edema, palpable liver	Cassava, AFB_1 1.7 mg/kg	Centrilobular necrosis, fatty changes	(Serck-Hanssen 1970)
USA	7/7	Reye's syndrome	-	AFB_1 in blood and liver	(Ryan *et al.* 1979)
Czecho-slovakia	27/27	Reye's syndrome	Aflatoxin contaminated milk food (5 cases)	AFB_1 and AFM_1 in liver, liver fatty degeneration, liver fibrosis with bile duct proliferation and steatosis, liver cirrhosis	(Dvorackova *et al.* 1977)
Thailand	23/23	Reye's syndrome	-	AFB_1 and AFB_2 in brain, liver, kidney, stool (autopsy)	(Shank *et al.* 1971)
New Zealand	2/2	Reye's syndrome	-	AFB_1 in liver (autopsy)	(Becroft 1966)

Immunosuppressive effects

AFB_1 has been reported to affect both humoral and cell mediated immunity through the results on cell mediated immunity have been more consistent in studies conducted in poultry, pigs and rats (IARC 1993, Raisuddin *et al.* 1993, Williams *et al.* 2004).

AFM_1 has cytotoxic properties in human hepatocytes (Neal *et al.* 1998) that may have important implications for the effects of AFM_1 on immunocompetence. In an *in vitro* study, AFM_1 and AFB_1 were particularly potent in reducing phagocytosis in rat peritoneal macrophages (Cusumano *et al.* 1995).

There is limited evidence for any immunosuppressive effects of aflatoxins in humans. Turner et al (2003) examined whether aflatoxin exposure was associated with several immune parameters in a cross-sectional study of 478 Gambian children. In multivariable analysis, secretory immunoglobulin A (IgA) was lower in children with detectable AF-albumin concentrations in comparison with those with non detectable concentrations. Additionally, the immune status of Ghanaian adults has been reported to be affected by aflatoxin exposure, with significant suppression of cellular immune system components when the adults with AF-albumin concentrations above the median were compared to those with concentrations below the median (Jiang *et al.* 2005).

In animals, aflatoxin exposure has been also shown to modify the effectiveness of vaccination by reducing the antibody response to vaccines, a finding that is not surprising given the immunosuppressive effect of aflatoxins (Wild and Hall 2000).

Considering the high morbidity and mortality from infectious diseases in the developing countries, an immunosuppressive effect of aflatoxins that would modulate susceptibility to infectious agents may be of major importance.

Nutritional status and growth retardation

Chronic exposure to aflatoxins has major effects on nutritional status and rate of growth in animals. The efficiency of food use is consistently less in animals that are exposed to aflatoxins compared to in those that are not exposed. In poultry and pigs, a 7-10% drop in food conversion efficiency has been observed, in fact decreased growth rates are often viewed as signs of chronic aflatoxin exposure (Shane 1994, Williams *et al.* 2004). Malformations and reduced fetal weight have been seen after mice were injected

intraperitoneally with high doses of aflatoxin. In rats, decreased pup weight and behavioral changes have been reported at low doses (IARC 2002).

Recent studies suggest that these effects may also apply to humans, with aflatoxin exposure associated with growth impairment in young children (1-3 years). In a cross-sectional study in Benin and Togo, young children showed a relationship between stunted growth and the level of AF-albumin in serum. This biomarker of aflatoxin exposure was detected in 99% of the children. The level of serum AF-albumin was strongly associated with growth faltering, particularly stunting (Gong *et al.* 2002, 2003). A longitudinal study in the same population confirmed the association between aflatoxin exposure and growth stunting (Gong *et al.* 2004). An association between AF-albumin adduct level and wasting was also observed in a study in Gambian children (Turner *et al.* 2003). In that study, a negative correlation of AF-albumin levels with vitamin C concentrations in serum was also observed.

Aflatoxin has also been suggested to be a factor modulating the rate of recovery from protein malnutrition (kwashiorkor) (Hendrickse 1997), and in a recent study of 60 Egyptian infants, kwashiorkor strongly associated with both the serum and urinary aflatoxin levels (Hatem *et al.* 2005). In another study from Egypt it was reported that aflatoxins AFB_1, AFG_1 and AFM_1, AFM_2 were detected in blood of 11/20 (55 %) Egyptian children with kwashiorkor and urine samples of 6 children were aflatoxin positive (Al-Saadany 1993). However, aflatoxins have not been suggested to be responsible for the initiation of this condition (Househam and Hundt 1991).

2.4.2. Mutagenicity and carcinogenicity

Aflatoxins are among the most potent mutagenic and carcinogenic substances known (JEFCA 1998) and AFB_1 has been classified as a class 1A human carcinogen by IARC (1993).

Aflatoxin carcinogenicity has now been well established in many *in vitro* and animal experiments, and in addition, most of the epidemiological studies have provided sufficiently strong evidence for a correlation between AFB_1 exposure and increased incidence of hepatocellular carcinoma (HCC) in individuals with a concomitant infection of hepatitis B virus (HBV) (Chen *et al.* 1997, Wild and Turner 2002). AFM_1 carcinogenicity is proven in animals, but as yet there are insufficient epidemiological

data for AFM_1 carcinogenicity to humans. Thus, IARC has classified AFM_1 as a possible human carcinogen, group 2B (IARC 1993).

In a study on weanling rats given either AFM_1 or AFB_1 by intubation, 3% of the rats given AFM_1 developed HCC and 28% had pro-neoplastic lesions. All AFB_1 fed rats developed tumours, while the controls showed no significant liver lesions (Wogan and Paglialunga 1974, Van Egmond 1994). In another study, rats were fed diets containing either AFB_1 or different concentrations of AFM_1. The highest AFM_1 dose produced liver lesions in 15% of the rats and 5% developed HCC, while 95% of rats receiving a similar dose of AFB_1 developed HCC. Some of the AFM_1 fed rats developed intestinal carcinomas (Cullen *et al.* 1987). These and subsequent studies have indicated that the carcinogenic potency of AFM_1 is one to two order of magnitude less than that of AFB_1 (Van Egmond 1994). Milk naturally contaminated with AFM_1 produced fewer lesions than artificially contaminated milk, pointing to differences in the bioavailability of naturally and artificially occurring AFM_1 (JEFCA 2000).

A mutation is one possible consequence of binding of carcinogens to DNA, and it is a step in the carcinogenic process evoked by genotoxic carcinogens (Sharma and Farmer 2004). The pathway of AFB_1 metabolism that apparently accounts for its mutagenic effects is a consequence of the metabolic activation of AFB_1 to the aflatoxin *exo*-epoxide, the subsequent covalent reaction with DNA, predominantly with the formation of the AFB_1-N^7-guanine adduct. The formed adduct is relatively unstable within the DNA structure and it can lead to its release from the DNA giving rise to an apurinic DNA site, or to the formation of the chemically and biologically more stable AFB_1-formamidopyrimidine structure (AFB_1-FAPY).

The AFB_1-N^7-guanine, AFB_1-FAPY and apurinic lesions are thought to be the precursors to the mutations induced by aflatoxin. Bailey et al (1996) assessed the level of aflatoxin induced mutations within *Escherichia coli* and indicated that AFB_1-N^7-guanine gives rise to a significant proportion of the observed AFB_1-induced mutations with the most frequently observed mutation induced to be a G to T transversion. Smela et al (2002) found that AFB_1-FAPY caused a G to T mutation frequency in *Escherichia coli* approximately 6 times more often than that of AFB_1-N^7-guanine. Furthermore, the G to T transversion mutation in codon 249 of the p53 tumour suppressor gene was identified in HCC patients from regions of the world with high aflatoxin exposures

(Bressac *et al.* 1991, Hsu *et al.* 1991). Kirk et al. (2000) reported the same mutation in plasma DNA collected from HCC cases in Gambia. Turner *et al.* (2005b) reported the absence of the TP53 mutation of plasma DNA in young Guinean children with aflatoxin exposure, and noted that if the observation is representative of the true absence of TP53 mutation in the liver, then the early childhood may provide a window in which intervention strategies may delay or reduce the incidence of HCC later in life.

Synergistic interaction of aflatoxin and hepatitis B virus (HBV) in hepatocarcinogenesis.

The chronic exposure to aflatoxins together with the chronic carriage of HBV are the two major risk factors involved in the multifactorial etiology of hepatocellular carcinoma (HCC) in countries with the highest incidence and the youngest patients with the disease (McGlynn *et al.* 2001, Kew 2003). Southeast Asia and sub-Saharan Africa have the highest incidences in HCC, are hyperendemic for HBV infection, and have high aflatoxin exposure prevalences. The rates of HBV positivity in Gambia, China and Guinea are 15%, 14-20% and 10%, respectively (Williams *et al.* 2004). In Egypt, 5.6% of the population are chronic carriers of HBV. In most countries hepatitis C virus (HCV) infection is about 1%, but in Egypt the prevalence of HCV is exceptionally high, reaching 20% in the general population. The annual prevalence of HCC in Egypt has increased significantly during the past decade (El-Zayadi *et al.* 2005). HCC is rapidly fatal with a survival rate after diagnosis of approximately one year.

A striking synergistic interaction of aflatoxin exposure (based on urinary and blood biomarker measurements) and HBV in the risk of developing HCC has been revealed in large cohort studies carried out in Shanghai (Ross *et al.* 1992, Qian *et al.* 1994) and Taiwan (Wang *et al.* 1996) with a vast increase in the risk of HCC development when both risk factors were present (Table 4).

Table 4: Findings demonstrating the synergistic interaction between HBV infection and aflatoxin exposure in the risk of developing hepatocellular carcinoma (HCC).

HBV alone	AFB_1 alone	HBV and AFB_1	Reference
4.8 (1.2, 19.7)	1.9 (0.5-7.5)	60.1 (6.4-561.8)	Ross et al, 1992
7.3 (2.2, 24.4)	3.4 (1.1-10)	59.4 (15.6-212)	Qian et al, 1994
17.4 (3.6, 143.4)	0.3 (0-3.6)	70.0 (11.5-425.4)	Wang et al 1996

Values indicate the relative risk (95% confidence intervals) of developing hepatocellular cancer (HCC).

A synergistic interaction of HCV and aflatoxins in the development of HCC in men with chronic HBV hepatitis has also been reported (Sun *et al.* 1999). The risk of HCC development in the subjects with detectable urinary AFM_1 levels was 3.3-fold higher (95% CI: 1.2, 8.7) compared to controls. Co-infection with HCV increased the risk of HCC to 5.8 (95% CI: 2, 17). This study importantly added to the evidence that HCV can increase the risk of HCC in men with chronic HBV hepatitis. However, there were too few cases to demonstrate the effect conclusively.

Thus, if one wishes to minimize the risk of developing HCC, it is important to minimize the aflatoxin exposure of HBV and HCV infected individuals.

2.5. Methods for reduction of human aflatoxin exposure

Strict food regulations are intended to minimize the aflatoxin exposure of populations in the developed countries, ensuring low levels of contamination in the produced/imported material. These control measures have failed in developing countries which often can be traced to inability to implement regulations, and even if there are restrictions, these often apply only to trade, resulting in exports of the higher quality materials leaving the low quality produce for local consumption (Williams *et al.* 2004). Therefore in the developing countries, economically feasible measures need to be considered to counteract the aflatoxin problem.

Intervention strategies for aflatoxin exposure reduction are described as those applicable at the individual or at the community level (Wild and Hall 2000). At the community

level, a reduction of exposure by limiting fungal contamination of crops can be addressed either as pre-harvest or post-harvest measures. At the individual level, dietary or post-ingestion interventions are considered (Figure 11).

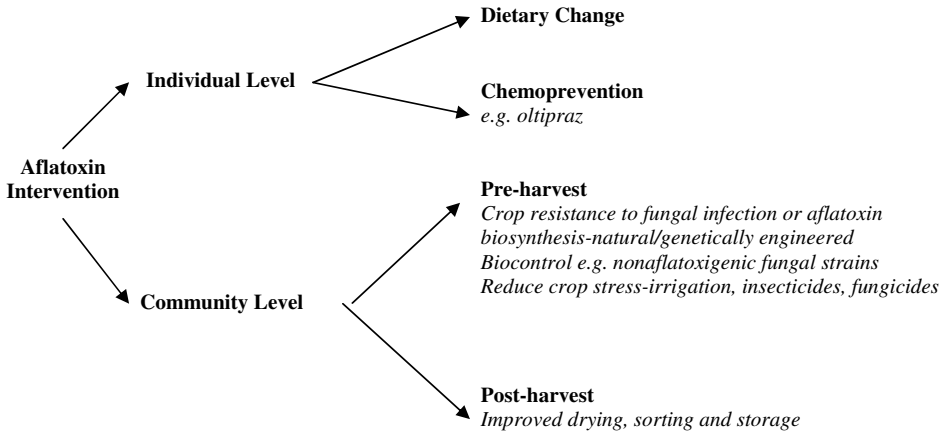


Figure 11: Approaches to primary prevention of hepatocellular carcinoma in developing countries (Wild and Hall 2000).

2.5.1. Interventions at the community level

Pre-harvest interventions involve measures aiming to reduce *Aspergillus* infection of crops and subsequent toxin production. Infestation of crops by *Aspergillus* most readily occurs under conditions of stress involving drought, high temperatures, insect induced injury or other processes which lead to damage of the crop. Therefore, appropriate pre-harvest measures include improved irrigation, use of fungicides, pesticides and insecticides, genetic engineering of crops so that they are resistant to fungal infection or toxin biosynthesis, use of cereal strains resistant to fungal colonization, biocontrol by use of competitive non-aflatoxigenic *Aspergillus* strains. Nonetheless, many of these processes may not be economically feasible in high risk populations (Wild and Turner 2002).

Post-harvest measures focusing mostly on methods which could be applicable at the local farming level, ideally involve low technology approaches designed to inhibit further fungal growth and aflatoxin production in storage. The growth of *Aspergillus* post-harvest is influenced most critically by temperature, moisture content and storage time. Recently, a successful intervention based on harvest, drying, sorting, and storage

activities in rural farming communities in West Africa, was reported by Turner *et al.* (2005a).

Other post-harvest methods have been described for removing of aflatoxins from foods. These include 1) physical methods such as thermal inactivation, irradiation, sorting etc., 2) chemical means, including solvent extraction, 3) adsorption for example using minerals, e.g., phyllosilicate clays, 4) a variety of chemical degradations e.g., acids, alkalis, aldehydes, oxidising agents, ammoniation and sodium bisulphate, and 5) biological decontamination. Most of these available methods are appropriate only for feeds as they alter the nutritional and taste properties of the foods, making them unsuitable for human consumption (Wild and Hall 2000)**.**

2.5.2. Interventions at the individual level

One means of intervening to reduce aflatoxin exposure would be by dietary changes aiming to avoidance of foods with high aflatoxin contamination such as maize and groundnuts, and increased consumption of crops like rice with a lower incidence of *Aspergillus flavus* infestation. However, this would mean alteration to the local agricultural practices and the local dietary habits of the population which in most cases would be a rather infeasible task (Sylla *et al.* 1999, Wild and Hall 2000).

For high risk individuals e.g. people with chronic HBV infection, some other approaches could be considered. Drugs (oltipraz) (Kensler *et al.* 1998), dietary constituents like chlorophyllin (Egner *et al.* 2001), or probiotics (El-Nezami *et al.* 2000b) could be used to modulate aflatoxin metabolism once ingested or to prevent their absorption from the gastrointestinal tract.

Oltipraz is effective in blocking aflatoxin adduct formation and hepatocarcinogenesis in rodents by inducing the GST levels which lead to an increased inactivation of aflatoxins (Kensler *et al.* 1994). The ability of oltipraz to modulate aflatoxin metabolism in humans has been demonstrated in China by Kensler *et al.* (1998). However, sustained health therapy is expensive and is not likely to be feasible, given the health budgets of developing countries and their other pressing health problems (Williams *et al.* 2004).

Chlorophyllin, a water-soluble form of chlorophyll, is used extensively as a food colorant, and is thought to form molecular complexes with carcinogens, including aflatoxins, thereby blocking their bioavailability. It may be therefore used to bind

aflatoxins and in that way to reduce the amount of the toxin reaching the liver. Chlorophyllin has been shown to be an effective anticarcinogen against aflatoxin-induced hepatocarcinogenesis in experimental models (Dashwood *et al.* 1998) and it was also recently evaluated in a clinical trial in a population at a high risk of exposure to aflatoxin. Administration of chlorophyllin three times a day led to a 50% reduction in the median level of urinary excretion of AFB_1-N^7-guanine compared to placebo (Egner *et al.* 2001).

Probiotic bacteria are defined as micro-organisms that have a beneficial effect on the health of the host. Some probiotic bacteria strains have the ability to bind a range of fungal toxins including aflatoxins, and this ability has been studied extensively *in vitro* (El-Nezami *et al.* 1998a, 1998b, Haskard *et al.* 2001, Peltonen *et al.* 2001, Gratz *et al.* 2004) and *in vivo* (El-Nezami *et al.* 2000a, Gratz *et al.* 2005). The ability of these bacteria to reduce human aflatoxin exposure has also been tested in a small trial performed in Egypt (El-Nezami *et al.* 2000b). The low price of probiotic bacteria and their incorporation in traditional foods such as fermented milks, fermented vegetables or fermented cereals in many parts of the world makes this approach an appealing one. In the context of this work, the use of probiotic bacteria in reducing human aflatoxin exposure will be given additional attention.

The use of probiotic bacteria as a method for reduction of human aflatoxin exposure

In the dairy industry, the most widely used probiotic bacteria belong to the group of lactic acid bacteria. This is mainly due to their ability to produce lactic acid as the major end product of fermentation of carbohydrates. Lactic acid producing fermentation is certainly one of the oldest methods of food preservation and production known to mankind causing palatable flavor and texture changes, as well as exercising a preservative effect on the fermented product. It has been estimated that 25% of the European diet and 60% of the diet in many developing countries consists of fermented foods (Holzapfel *et al.* 1995). These include not only fermented milks but also fermented vegetables and cereals and other products around the world.

Currently, the best studied probiotic bacteria are the lactic acid bacteria, particularly *Lactobacillus* and *Bifidobacterium* species (Rolfe 2000). The main beneficial effects attributed to lactic acid bacteria are prevention of diarrhoeal and gastrointestinal related

diseases, but antimutagenic and anticarcinogenic effects and beneficial effects on immune disorders have also been reported (Ouwehand *et al.* 2002). Additionally, meta-analysis studies have demonstrated that probiotics indeed do have scientifically demonstratable effects on human health (Sazawal *et al.* 2006, Szajewska *et al.* 2006).

Several studies have examined the ability of specific strains of lactic acid bacteria to remove aflatoxins from contaminated media. It was found that within a given species, not all strains were equivalent in terms of toxin binding. The capacity for AFB_1 removal was a characteristic of only specific strains, with efficacy varying markedly from species to species (El-Nezami *et al.* 1998a, Peltonen *et al.* 2001).

The most efficient strains in binding AFB_1 were shown to be L. *rhamnosus* strain GG (*LGG*) and L. *rhamnosus* strain *LC 705* (*LC705*), but *L. gasseri*, *L. acidophilus*, *L. casei Shirota* and *Propionibacterium freudenreichii* subsp. *shermanii JS* (*PJS*) had also shown some ability to remove AFB_1 (El-Nezami *et al.* 1998a, Peltonen *et al.* 2001). Non-viable bacteria were more efficient at removing AFB_1 than viable bacteria (El-Nezami *et al.* 1998b), implying that metabolic degradation of the toxin is not the mechanism responsible for the removal of the toxin. It has been proposed that AFB_1 is bound by the bacterial cell wall polysaccharides and peptidoglycanes (Haskard *et al.* 2001). Bacterial concentrations must exceed 10^9 bacteria/ml to achieve effective removal of AFB_1 (El-Nezami *et al.* 1998a). The total number of AFB_1 molecules that can be bound to a single viable bacterium has been estimated to exceed 10^7. An important consideration is the demonstration that once bacteria bind aflatoxin they appear to lose their ability to bind to the intestinal mucosa thus preventing further prolonged contact between the absorptive surface and the toxin leading to removal of the toxin by the fecal route (Kankaanpää *et al.* 2000).

A mixture of *LC-705* and *PJS* is currently used by the food and feed industry as bio-preservative and probiotic preparation (Bioprofit), making it a promising candidate for future applications. Bioprofit, is a patented combination of starter bacteria that efficiently prevents the growth of yeasts, moulds and some contaminant bacteria, and can be used for the preservation of different foods such as cheese and bread. It has been claimed that this mixture has the ability to inhibit *Aspergillus* growth (Valio Ltd).

The *in vitro* ability of this probiotic mixture to bind AFB_1 has also been demonstrated (Gratz *et al.* 2005), and *in vivo* tests have suggested that the mixture could reduce AFB_1

bioavailability from ligated duodenal loops of one week old chickens by reducing toxin absorption (El-Nezami *et al.* 2000a, Gratz *et al.* 2005). To clarify the benefit of this probiotic mixture in individuals exposed to aflatoxin via their diet, a pilot clinical trial on aflatoxin-probiotic interaction was carried out in Egypt. Twenty Egyptian volunteers were recruited for an intervention using the probiotic mixture as a supplement (El-Nezami *et al.* 2000b). Consumption of the probiotics significantly influenced the fecal content of AFB_1.

2.6. Rationale of the present study

Aflatoxins are produced by *Aspergillus* moulds contaminating dietary staples in the developing countries. They are toxic, carcinogenic and mutagenic in humans, and recent evidence suggests that their effects extend to immunosuppression and impaired growth in children. AFB_1 is the most prevalent and potent form of aflatoxins and is classified as a class 1A human carcinogen by the International Agency for Research on Cancer. Following ingestion of contaminated food, AFB_1 is metabolized by specific enzymes of the cytochrome P-450 enzyme superfamily to produce various hydroxylated derivatives and a highly reactive epoxide, reacting with cellular proteins or DNA. Aflatoxin metabolites are excreted in urine and feces, as well as into the milk of nursing mothers if they have consumed aflatoxin contaminated food. A number of molecular dosimetry methods have been developed for assessing aflatoxin exposure by monitoring levels of aflatoxin metabolites in biological samples such as blood, urine and milk.

Chronic carriage of hepatitis B (HBV) and C viruses (HCV) and chronic exposure to aflatoxins are considered to be the major risk factors in the development of hepatocellular carcinoma (HCC) in countries with a high incidence of the disease. In many of these areas, up to 20% of the population is chronically infected with hepatitis viruses, and the aflatoxin exposure is also reported to occur at high levels. Egypt is an African country where the annual prevalence of HCC has increased significantly during the past decade. Aflatoxins have been detected in a number of foods like corn, wheat, peanuts, corn oil, different spices and dairy products that are an essential part of the Egyptian diet. In all, 5.6% of the population are HBV chronic carriers, and the prevalence of HCV infection (20%) is the highest in the world. Reducing or eliminating

aflatoxin exposure in countries where both aflatoxin exposure and chronic hepatitis virus infections are prevalent could potentially reduce the risk of HCC development.

Considering that the early life environment is crucial for growth and development in later life, long term pre- and postnatal exposure to aflatoxins could be one of the factors contributing to growth faltering and/or the early onset of HCC in countries with a high incidence of the disease. In West Africa, elevated biomarker levels in children have been repeatedly reported with those children displaying higher biomarker levels when they are infected with HBV. A number of studies suggest that susceptibility to aflatoxin is greatest in the young, and therefore aflatoxin exposure occurring via mother's milk or weaning foods may be a major factor contributing to the development of HCC in later life.

Therefore, a reduction of early life exposures and potentially of the risk of HCC development later in life is a goal worth achieving. One approach may be manipulation of aflatoxin absorption in lactating mothers by providing them with probiotic bacteria able to bind aflatoxins. The design of such a project demands an understanding of the aflatoxin exposure levels and patterns in the population of interest, for example, this kind of information for Egypt was lacking from the literature.

Specific strains of probiotic bacteria, in addition to having by definition potential benefits for the health of the host, have been shown to possess the ability to bind aflatoxins and remove them from contaminated media *(in vitro)*. The effectiveness of probiotics in binding aflatoxins has been investigated also *in vivo*, but the potential of reducing the biologically effective dose of aflatoxin exposure in human populations has never been assessed via an intervention in a region with known high aflatoxin exposure such as Southeast China.

3. AIMS OF THE STUDY

The overall aim of this study was to evaluate the need for and feasibility of developing a method to reduce aflatoxin exposure in infants, young children, and young adults in the developing countries. For this purpose, the study investigated the patterns and levels of aflatoxin exposure in different study groups from Egypt, Guinea, or China, and characterized the potential of specific probiotics as a dietary intervention approach to reduce the risk of exposure to aflatoxins.

Consequently, the specific research questions in this dissertation were:

- How serious is the problem of aflatoxin exposure in a group of nursing Egyptian mothers and their children? Which factors possibly affect the AFM_1 levels in maternal milk? These research questions were clarified by determining the levels of aflatoxin M_1 (AFM_1) in mother's milk in Egypt and by identifying associations of AFM_1 levels with several socioeconomic, demographic, dietary and environmental factors (Studies I and II).

- How high or low is the exposure of the Egyptian children compared to children living in an area with known high aflatoxin exposure? This research question was addressed by determining the urinary levels of aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2), M_1 (AFM_1), Q_1 (AFQ_1) in children from Egypt and Guinea (Study III)

- Can probiotic supplementation reduce the biologically effective dose of AFB_1? This research question was examined by determining the changes in urinary levels of aflatoxin B_1 N^7-Guanine (AFB_1-N^7-guanine) in male students from China following a dietary supplementation with specific probiotics (Study IV and V).

4. SUBJECTS AND METHODS

4.1. Study subjects

4.1.1. Egypt and Guinea Studies (Studies I, II and III)

Study subjects and design

The Egypt Study was conducted in Qalyubiyah governorate, Egypt. Qalyubiyah governorate is located in the eastern Nile region near the head of the Nile Delta. The area is bordered in the south by Cairo and Giza governorates. The governorate is famous for its agricultural production of maize, cotton, wheat, as well as fruits and vegetables. In the Qalyubiyah governorate approximately two thirds of the population live in rural areas, and the humid and hot environment promotes mould growth and aflatoxin production (El-Shewey 1992).

The Egypt Study consisted of a cross-sectional part and a cohort follow up. In the cross-sectional part (Study I) 388 Egyptian mothers from Qalyubiyah governorate provided breast milk samples for determination of AFM_1 level and its associations with factors affecting AFM_1 presence (dietary, socioeconomic, demographic, environmental factors). In the cohort follow up (Study II), 50 mothers from the above sample with significantly increased AFM_1 levels were selected for longitudinal assessment of breast milk AFM_1 levels at monthly intervals over a one year period. Since the cohort was selected on the basis of breast milk AFM_1 level, it does not represent the general aflatoxin exposure of the population in the region, instead it deliberately illustrates a group exposed to aflatoxins. At the end of the follow up period, the children of these 50 Egyptian mothers provided a single urine sample for aflatoxin biomarker analysis and comparisons with urine samples provided from 50 children of Guinea, a country with a verified high aflatoxin exposure (Study III). The Guinean children (25 boys and 25 girls) were selected randomly out of a group of 100 HBV positive children who were taking part in a study in lower Kindia, Guinea, being conducted by the University of Leeds, UK in cooperation with the Institut Pasteur de Guinée (IPG), Kindia, Republic of Guinea. The children were selected from a defined area in lower Kindia, Guinea, because of the previous observations of high aflatoxin exposure levels (Diallo *et al.*

1995, Sylla *et al.* 1999). The samples of the Guinean children were kindly provided by the Molecular Epidemiology Unit, University of Leeds, UK.

The design of the Egyptian Study is presented in Figure 12.

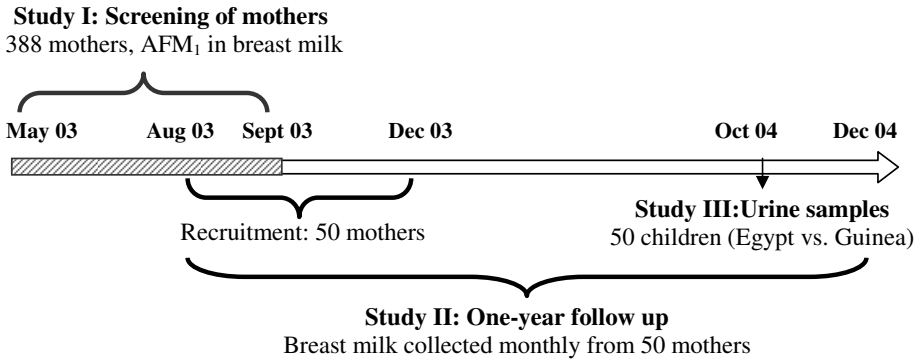


Figure 12: The design of the Egypt Study and samples collected for Studies I, II and III.

Sample size estimations

The Egypt Study was designed to provide the necessary background data for the design of an intervention study aimed at reducing maternal and infant aflatoxin exposure in the Egyptian population. A previous study in the same region (Qalyubiyah governorate) (El-Shewey 1992) reported that 11% of mothers screened for aflatoxins in their breast milk were found to be positive. We anticipated that a group of 50 mothers with monthly collections of breast milk samples for a year of follow up would provide a reasonably large number of samples (50 mothers x 12 months= 600 samples), even after possible drop outs (20%), for identification of factors affecting AFM_1 presence in breast milk. Thus, to obtain a group of 50 mothers, it was originally planned to screen approximately 500 mothers for aflatoxins in their breast milk. Nevertheless, while the study was being conducted, it transpired that the percentage of AFM_1 positive mothers was considerably higher than expected, and therefore the final number of screened mothers was 388 of whom 138 (36%) were positive for breast milk AFM_1.

Data collection

The mothers had given birth in the New El-Qalyub Hospital and were recruited and followed up by the nurses in the hospital. Dietary, socioeconomic, demographic and clinical data were obtained by an interview conducted by the health care nurse and the use of questionnaires. The dietary questionnaires were used to assess the intake of potential dietary sources of aflatoxins during the initial screening and also the follow up periods. During the screening period, the structured food frequency questionnaire (FFQ) was used to record the intake of the most common foods consumed over the one month period before collection of the breast milk samples from 388 mothers. The FFQ consisted of two components, a food list and a frequency response section. The data on food use were expressed as frequencies ranging from several times a day to never, while the amounts of foods used were fixed in serving sizes described by standard weight and volume measures of the servings commonly consumed by this population. During the one year follow up period, a structured questionnaire was used each month to record the usual food intake over the previous week before the breast milk sample collection from the cohort of 50 mothers. The questionnaire of the follow up period was less detailed than the FFQ covering the previous week's consumption with responses designated as either yes or no. The questionnaires focused on foods more likely to contribute to the dietary intake of aflatoxins, i.e. grain products (wheat bread, corn bread, pasta, rice, corn), milk and milk products (hard cheese, feta cheese, cream), legumes (beans, lentils), meat (chicken, beef, lamb), fish, corn oil, cotton seed oil, dried fruits, and peanuts (Selim *et al.* 1996, El-Nezami *et al.* 2000b, El-Sayed *et al.* 2003).

Other relevant information (age, working status, level of education, number of miscarriages, health status, medication) was also recorded during the initial screening. The socioeconomic status of the households was dichotomized in categories of either higher or lower (HSES/ LSES). HSES included families with one or both parents working and at least one occupation demanding higher education (teachers, nurses, government employees, police officers etc.), whereas for LSES one or neither of parents were working and the work did not demand higher education (butcher, carpenter, tailor, salesman, conductor, driver, farmer etc.), respectively.

Maternal HBV status, child's birth date and the anthropometrical data (mother's weight and height, baby's weight and height at the time of the initial screening and at delivery)

were taken from the reports of the New El-Qalyub Hospital where the mothers had been followed since the delivery. Children's body weights were measured by the nursing staff using a Tanita 1584, Precision weighing balance, Bradford, MA (max weight 20 kg, accuracy 50 g).

Descriptive information

In the first part of the Egyptian Study (Study I), the mothers were screened for AFM_1 in their breast milk. From 409 contacted mothers, 388 (participation rate 95%) provided one breast milk sample during May-September 2003. The median age of the mothers was 25 years (range 15- 47), most mothers (71.6%) already had one or more children, and 14% had previously had a miscarriage (Table 5). Most households (76%) belonged to LSES category while 89% of the mothers were not employed and were either at home or involved in farming. Fifteen percent of the mothers were obese (BMI>30), and obese mothers were consumers of corn oil more frequently than the non obese mothers (84 vs. 67%, p= 0.020). A few of mothers (6.2%) reported a health problem associated with liver or intestine function, while two percent of the mothers reported consumption of medicines such as blood pressure reducing tablets and anti-depressant tablets, or dietary supplements such as iron and calcium tablets.

Beans, corn bread, corn oil, wheat bread and cotton seed oil were used by most of the mothers on a daily basis (Figure 13), but mothers belonging to the LSES category consumed beans (p= 0.009), corn oil (p= 0.001), and cotton seed oil (p= 0.001) more frequently than mothers belonging to the HSES category.

Table 5. Descriptive maternal data in the cross-sectional part of the Egyptian Study (Study I).

		% of mothers (n) [§]	Median (IQR)	Range
Lactation stage* (months)		100 (384)	5 (2-8)	0.2-33.0
Age (years)		100 (381)	25 (22-30)	15-47
Number of miscarriages[#]	Total	100 (383)	-	-
	0	86 (331)		
	1-3	14 (52)		
Number of children[##]	Total	100 (384)	-	-
	1	28 (109)		
	2-7	72 (275)		
BMI	Total	100 (336)	26.0 (24-28.5)	15.8-68.9
	<20	2 (8)		
	20-<25	32 (106)		
	25-<30	51 (171)		
	>30	15 (51)		
Employment	Total	100 (382)	-	-
	unemployed	89 (339)		
	employed	11 (43)		
Socioeconomic status	Total	100 (384)	-	-
	LSES[§§]	76 (293)		
	HSES[§]	24 (91)		

[§]Mothers who provided the required information
*The number of months that mother has been lactating
[#]Prior to index child, [##]Including index child,
[§§] (LSES) Low socioeconomic status, (HSES) High socioeconomic status

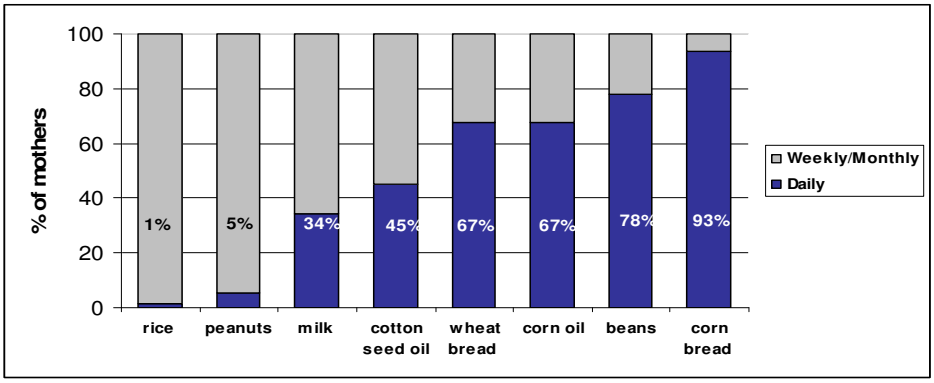


Figure 13. Food use among lactating Egyptian mothers. Frequency distributions of responses to selected foods grouped according to either daily or weekly/ monthly consumption.

The children in the cross-sectional part of the Egyptian Study included 227 boys and 159 girls (Table 6). The mean age of the children was 5.9 months. There were no reported illnesses within any of the infants. For assessing children's growth status, the measured anthropometric values (baby's weight and height) were expressed as standard deviations (z-scores) below or above the median value of the international reference population as recommended by the National Centre of Health Statistics/World Health Organization (WHO) (WHO 1995). Weight for height z-score (WHZ), weight for age z-score (WAZ), and height for age z-score (HAZ) below -2 reflects malnutrition, while a value below -3 refers to severe malnutrition. Among the children, the prevalence rate for stunting (HAZ≤-2) was 35.7%, for being underweight (WAZ≤-2) 14.4%, and for wasting (WHZ≤-2) 0.6%. According to the WHO criteria (WHO 1995) these frequencies are high, moderate and infrequent, respectively. A total of 78 (23.4%) children had a z-score of ≤-3 in at least one parameter (extreme malnutrition). Median z-scores (IQR) for the infants were below zero for stunting and being underweight, but not for wasting.

Table 6. Sex, age and growth status of the infants in the cross-sectional part of the Egyptian Study (Study I).

		% of children (n)	Median (IQR)	Range
Sex*	boys	58 (227)	-	
	girls	42 (159)		
Age (months)			5 (2 to 8)	0.2 to 33.0
Height for age z-score (HAZ)**	total	100 (322)	-1.49 (-2.63 to 0.02)	- 4.2 to 2.9
	> -2	64 (207)		
	-3 to -2	18 (56)		
	≤ -3	18 (59)		
Weight for age z-score (WAZ)**	total	100 (375)	-0.45 (-1.47 to 0.43)	- 4.5 to 4.6
	> -2	86 (321)		
	-3 to -2	9 (35)		
	≤ -3	5 (19)		
Weight for height z-score (WHZ)**	total	100 (351)	0.82 (0.17 to 1.66)	-2.3 to 4.8
	> -2	99 (349)		
	-3 to -2	1 (2)		
	≤ -3	0 (0)		

*Sex information were missing for two children

**Z score less than -2 for HAZ (stunting), WAZ (being underweight) or WHZ (wasting) is defined as malnutrition, while smaller than -3 as severe malnutrition. Malnutrition status is based on World Health Organization Guidelines.

Fifty mothers who were positive for AFM_1 in breast milk participated in the one year of follow up (Study II). Of these, 26 (52%) gave 12 monthly breast milk samples, while 20 (40%), 3 (6%) and one (2%) mothers gave 5, 9, and 4 monthly samples, respectively, because they had terminated breastfeeding. A total of 443 breast milk samples were collected during the 12 month follow up period. Most mothers were not employed (92%) but belonged to small farming communities and were therefore undertaking some farm work and domestic chores (Table 7). The employed mothers (8%) were either in nursing or teaching. For 17 mothers (34%) this was their first child, while the rest had 2-5 children. Seven mothers (14%) had previously had one miscarriage, and one mother (2%) had experienced three miscarriages. At the outset of the follow up period, the average infant age (same as lactation time) was 9.2 months old (range 3–20), while at the end of the follow up it was 17.2 (range 9–29). All mothers were healthy.

Table 7. Descriptive maternal data of the follow upstage of the Egyptian Study (Study II).

		% of mothers (n)	Median (IQR)	Range
Lactation stage* (months)	beginning of follow up	100 (50)	8.5 (6-11)	3-20
	end	100 (50)	17 (14-22)	9-29
Age (years)		100 (50)	24.5 (21-28)	15-40
Number of miscarriages[#]	Total	100 (50)	-	-
	0	84 (42)		
	1	14 (7)		
	3	2 (1)		
Number of children[##]	Total	100 (50)	-	-
	1	34 (17)		
	2-5	66 (33)		
BMI	Total	100 (42)	25.3 (24.0-28.0)	19.5-36.4
	<20	2 (1)		
	20-<25	43 (18)		
	25-<30	41 (17)		
	>30	14 (6)		
Employment	Total	100 (50)	-	-
	unemployed	92 (46)		
	employed	8 (4)		
Socioeconomic status	Total	100 (49)	-	-
	LSES[§]	78 (38)		
	HSES[§]	22 (11)		

*The number of months that a mother has been lactating
[#]Prior to index child, [##]Including index child
[§](LSES) Low socioeconomic status, (HSES) High socioeconomic status

At the end of the follow up period (October 2004), a single urine sample was collected from the children of the Egyptian Study (Study III). In all, 30 children were fully weaned (had stopped breast feeding) and 20 were partially weaned (Table 8). All Egyptian children were diagnosed to be HBV negative as determined by HBsAg in serum. The median age of the children was 1.5 years. The median age of the Guinean children was 3 years, and they all were fully weaned. All Guinean children had been exposed to aflatoxins as determined by the aflatoxin albumin adduct concentrations in their serum.

Table 8. Descriptive data on the Egyptian and the Guinean children at the time of urine sample collection (Study III)

		Egyptian children*		Guinean children*	
		% of children (n)	Median (IQR) Range	% of children (n)	Median (IQR) Range
Sex	boys	68 (34)		50 (25)	
	girls	32 (16)		50 (25)	
Age (years)**			1.5 (1.3-1.75) 1.08-2.5		3 (2-3) 2-10
HBV	negative	100 (50)			
	positive			100 (50)	
Weaning status					
	partly-weaned	40 (20)		-	
	fully-weaned	60(30)		100 (50)	

*The collection of the urine samples of the Egyptian children took place during October, and of the Guinean children during July/August
**The age of the Egyptian children was calculated based on the date of birth, while the age of the Guinean children was that reported by the parents

4.1.2. China Study (Study IV and V)

Study subjects and design

The study population was selected from an area of Guangzhou in Guangdong province which is located in the southern China. In this area the exposure to aflatoxin via foods and diet is known to be common.

The study was conducted in two parts. First, 300 male students at the Sun Yat- Sen University were screened for AFM_1 from a spot urine sample (Study IV). Of those with a detectable level of AFM_1 (>0.08 ng/ml) (142/300, 47%), 90 were selected for the

second part of the study (Study V) which was a randomized, double-blind, placebo-controlled trial with two parallel groups (Figure 14). Exclusion of individuals was based on abnormal hematological values and outlying AFM_1 (< 0.008 or > 20 ng/ml). The subjects were randomly assigned to two groups (n=45/group), one receiving the probiotic preparation and the other receiving the placebo preparation. The subjects received written and oral directions to take two capsules per day immediately prior to their main meals (breakfast and dinner). The probiotic preparation contained a mixture of the strains *Lactobacillus rhamnosus* LC705 and *Propionibacterium freudenreichii ssp shermanii* (1:1 wt/wt) at a dose level of 2-5 x 10^{10} CFU/day. The placebo capsules contained only cellulose. The probiotic strains used are of GRAS grade (generally recognized as safe) and are commonly used in the manufacturing of many dairy products. The compliance to the study protocol was assessed based on the presence of *Lactobacillus rhamnosus* LC705 in fecal samples taken at the same time points as the urine samples. The subjects provided an early morning single urine sample on the 3rd and 5th week of the intervention period, and after a 5-week post intervention period.

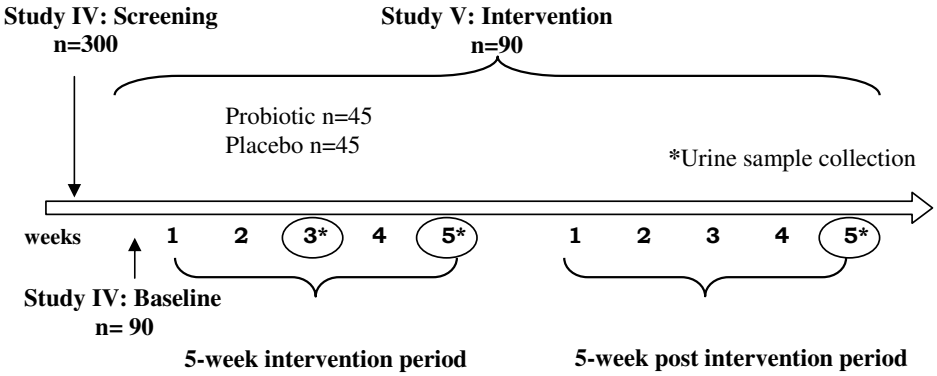


Figure 14: Design of the China Study (Studies IV and V). A total of 300 male students were screened for AFM_1 in a spot urine sample. Of those with a detectable level of AFM_1 (>0.08 ng/ml), 90 were selected for a 5-week intervention study. The subjects were randomly assigned to two groups, one receiving the probiotic preparation and the other receiving placebo preparation. The subjects provided an early morning single urine sample in the 3rd and 5th week of the intervention period, and after a 5-week post intervention period.

Sample size calculation

The calculation of the sample size of 90 subjects with 45 subjects in each arm was based on the assumption that intervention with probiotic bacteria could reduce the level of AFB_1-N^7-guanine by at least 25% when compared with the placebo, giving a standardized effect of $\Delta = 0.5$. Using these assumptions the required sample size for two-sided significance of 5% and power of 80% was 86 subjects per arm. Using 0.5 as the correlation between repeated AFB_1-N^7-guanine measurements (baseline + 2 intervention measurements + post intervention), the required number of subjects was reduced to 33 per arm. The two phases of the study (intervention and post intervention) are considered as separate phases. To compensate for the anticipated drop-out (20%), a total of 90 subjects was randomized, 45 in each arm.

Data collection and descriptive information

Students that failed to provide all of the required samples during the intervention and post intervention periods were excluded from the study. Overall 90% of the subjects (n=81) delivered the required urine samples (39 in the probiotic group and 42 in the placebo) (Figure 15).

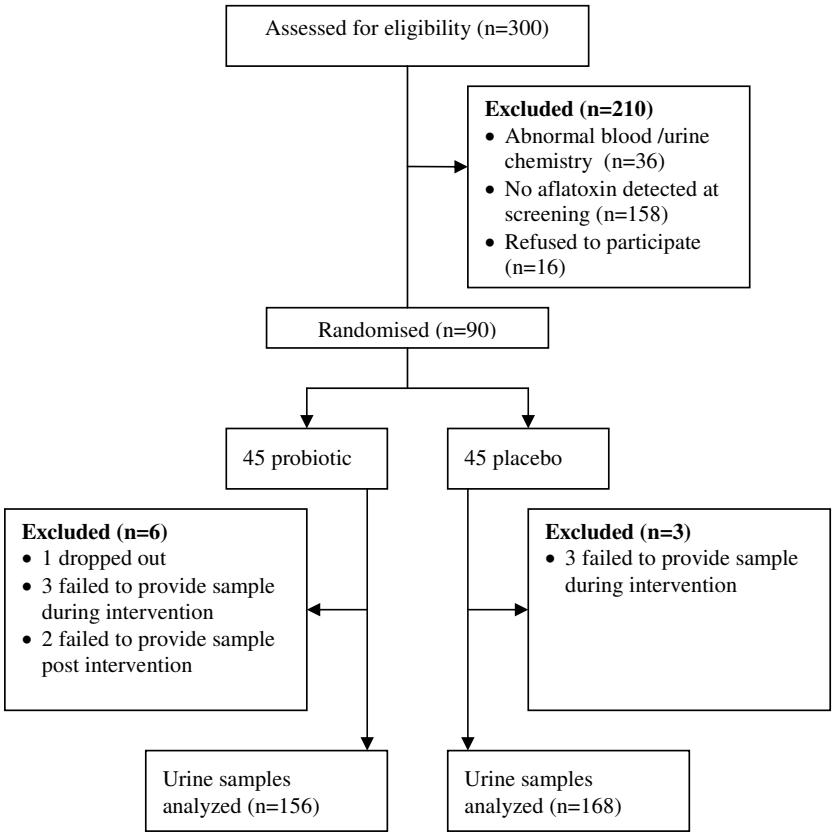


Figure 15. Flow diagram of the progress through the phases of the probiotic randomised trial (Study V)

The 90 selected Chinese students underwent a complete health examination: medical history, smoking status, physical examination, routine hematological and clinical biochemistry tests on hepatic and renal function, and antigens to hepatitis B virus (HBsAg) (14 subjects were tested as being positive for HBsAg) (Table 9). All students were clinically healthy according to all clinical chemistry values which were within the reference range for normal values and no other differences were noted in liver function parameters. Eleven HBsAg positive subjects (6 in the probiotic and 8 in the placebo group) were included in the study. The clinical chemistry measurements and HBsAg determination were carried out at the Sun Yat-Sen University according to standard protocols. Antibiotics and/or traditional Chinese medicines and herbs had been used

during the preceding two months by 21 students (25%), evenly distributed between the two study groups.

Table 9: Descriptive data on the male students participating in the China study (Study IV).

	Range
Age (years)	18 - 24
Body weight (kg)	45 - 85
Body height (cm)	158-182
HBV status positive, n (%)	14 (17)
Herbal chinese medicine users, n (%)	21 (25)

83 subjects provided all urine samples during the intervention

Consumption of foods known to be the most common sources of dietary aflatoxins in China was assessed using a structured FFQ filled at the same time as the interview at the baseline period. All subjects resided in student accommodation and consumed their main meals (breakfast, lunch and dinner) at the student restaurant operated by the university. During the 5-week intervention period, the subjects consumed their normal diets. With respect to the consumption of possible sources of dietary aflatoxins, the subjects represented a homogenous population of students eating their main meals at the same cafeteria. There were no significant differences between the two intervention groups in the intakes of foods known to be the most common sources of dietary aflatoxins in China (Table 10).

Table 10. Weekly intake of major foods in the diet of healthy Chinese male students receiving either a probiotic or placebo preparation. (Study IV)

	Weekly intake (mean ± SD)	
Foods (servings)	**Probiotic group (n = 39)**	**Placebo group (n = 42)**
Rice (scoops)	157 ± 36	164 ± 35
Bread (slices)	9.9 ± 5.1	10.0 ± 4.6
Pork (chops)	6.5 ± 4.7	6.9 ± 3.8
Chicken(chops)	6.4 ± 4.4	4.7 ± 4.1
Beef (chops)	2.8 ± 4.3	2.8 ± 4.0
Fish (chops)	2.1 ± 3.3	2.2 ± 3.6
Beans (scoops)	5.5 ± 2.8	5.1 ± 2.9
Noodles (scoops)	1.3 ± 1.8	1.5 ± 2.1
Peanuts, total (handfuls)	0.4 ± 1.0	0.3 ± 0.6

No differences were observed between the probiotic and placebo arms in the intakes of foods known to be the most common sources of dietary aflatoxins in China ($p>0.05$, Mann-Whitney).

4.2. Methods

4.2.1. Aflatoxin standards

The AFB_1-N^7-guanine standard was synthesized in the laboratories of the Department of Clinical Nutrition, University of Kuopio since it was not available commercially. The experimental procedure for synthesis of the AFB_1-N^7-guanine standard is described in detail below. The other aflatoxin standards, AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFQ_1 were purchased from Sigma/Aldrich Chemical Co (St Louis, MO, USA), dissolved in methanol and stored at -20 ^oC wrapped in aluminium foil before use. Aflatoxins are human carcinogens and care was exercised to avoid personal exposure. Appropriate decontamination procedures with 10 % sodium hypochlorite were used.

In vitro synthesis of AFB_1-N^7-guanine adduct

For the synthesis of the AFB_1-N^7-guanine, AFB_1 was microsomally activated into the epoxide form and adducted to double stranded oligodeoxynucleotide. Subsequent acid hydrolysis of the formed AFB_1 oligodeoxynucleotide adduct, yielded AFB_1-N^7-guanine.

AFB_1-N^7-guanine which was purified, identified and quantified by HPLC, LC-MS/MS and spectrophotometric techniques.

For the activation of AFB_1 and its adduction to the oligodeoxynucleotide, AFB_1 was incubated with pyrazole induced mice liver microsomes (prepared by the Department of Pharmacology and Toxicology, University of Kuopio) and annealed oligonucleotides 5´-TCA ATC TGC ATC ACT CAA C-3´and 5´-GAT GCA G-3´ (Johnston and Stone 2000) (synthesized by the A.I.Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland) in aqueous phosphate buffer and magnesium chloride solution containing NADPH (Sigma/Aldrich, St Louis, MO, USA). NADPH was replaced with an equal volume of phosphate buffer (pH 6.8) in the mixtures used as the control. Reactions with AFB_1 were performed under subdued light conditions to minimize potential photodecomposition of the resulting oligo adduct (Gopalakrishnan *et al.* 1989). The reaction mixture was incubated overnight at room temperature (25^{o}C), and thereafter was stopped by boiling (5 min). The microsomal pellet was discarded after centrifugation (16000 *g,* 20 min, 4 ^{o}C). The AFB_1 adducted oligos were precipitated from the supernatant by addition of cold ethanol. The supernatant was removed after centrifugation (16000 *g*, 20 min, 0-4^{o}C) and the precipitated adducted oligonucleotides were washed with 80% cold ethanol. The adducted oligonucleotides were then hydrolysed with formic acid for 1 hr at room temperature. This resulted in sufficient depurination of the nucleic acids, and the release of AFB_1-N^7-guanine (Essigmann *et al.* 1977). Prior to HPLC analysis, potassium acetate was added for pH adjustment to 4.0, which is the optimal condition for the HPLC system.

HPLC purification and spectrophotometric quantitation

Reverse phase HPLC (model LC-10ADvp solvent delivery system and model SIL-10Advp auto injector, Shimadzu, Japan) was used to separate the oligo-AFB_1 hydrolysates on an ODS-5 Brownlee column (220 by 4.6 mm, particle size 5 µm) fitted with a C18 guard column (Perkin Elmer, Norwalk, Conn.). The assay was carried out using an isocratic system of 70 % 20 mM ammonium acetate (pH 3.9) and 30 % methanol/acetonitrile (1:1) (Iyer and Harris 1993) with a flow rate of 1.5 ml/min, oven temperature 40 ^{o}C and injection volume of 20 µl. This method was determined to separate efficiently acid hydrolyzed products of the adducted oligo detected at 360 nm

(UV-10 Shimadzu detector). Repetitive collections of the predominant eluting compound accumulated sufficient amounts for LC-MS/MS identification. LC-MS/MS confirmed the identity of the compound to be AFB_1-N^7-guanine. The LC-MS/MS conditions are described in detail in the paragraph below. Afterwards, the adduct was purified again in HPLC with a different mobile phase [95% ethanol: water: acetic acid) (5:15:0.001, v/v/v), flow rate 1 ml/min]. The adduct (in 25% ethanol) was quantitated spectrophotometrically at 360nm (ε_{360}= 18000 $M^{-1}cm^{-1}$) (Lin *et al.* 1977). The concentration of the adduct was calculated using the equation: C= (A x 1000 x M*r*)/(18000 x b) where C is the concentration of the adduct (μg/ml), A the mean of three repetitive absorbance values, M*r* the relative molecular mass of AFB_1-N^7-Gua (M*r* = 479), and b the cell path length (1 cm). UV spectra in the range of 200-400 nm were measured by a Hitachi U-2000 spectrophotometer. The adduct was stored at -20°C under acidified conditions. Figure 16 displays the HPLC chromatogram of the AFB_1-N^7-guanine final standard. The experimental procedures are summarized in figure 17. All chromatographic solvents were of HPLC grade quality and all reagents were of analytical grade or higher.

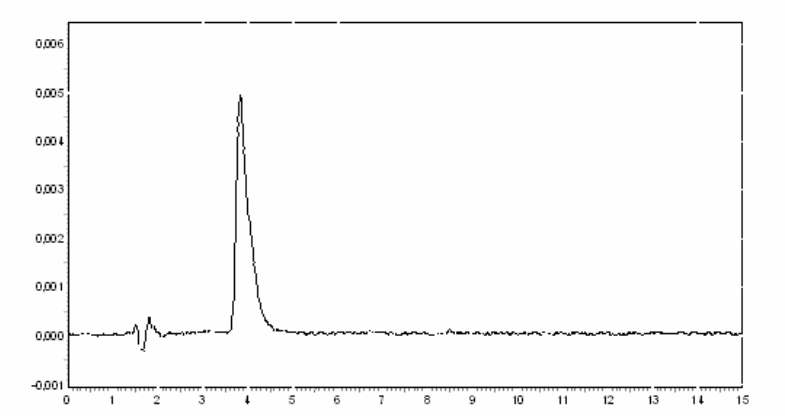


Figure 16. HPLC chromatogram of AFB_1-N^7-guanine final standard (1,450 μg/ml) in 25% ethanol, eluting at 4 min. retention time from the HPLC system. [mobile phase: 70% 20 mM ammonium acetate, pH 3.9 and 30% methanol/acetonitrile (1/1); flow rate: 1,5 ml/min; column: ODS-5 C18 220 x 4.6 mm].

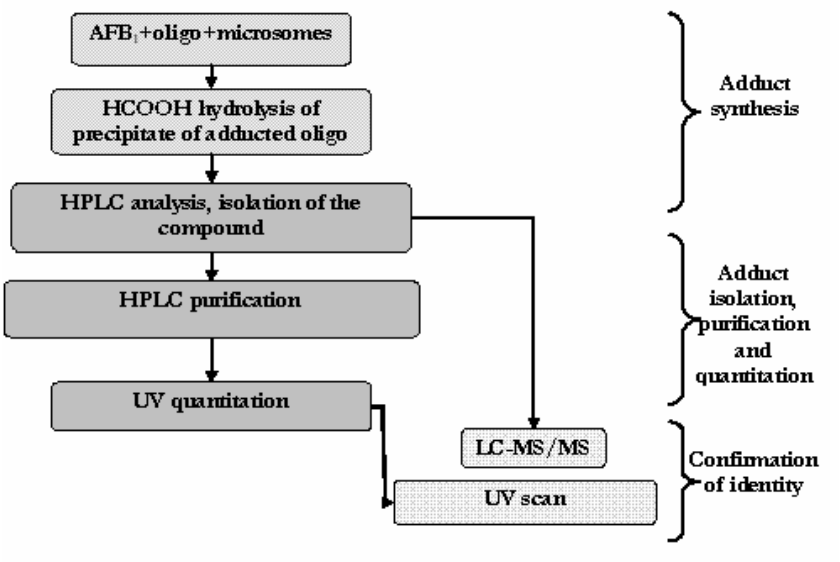


Figure 17. Schematic presentation of the experimental procedures for the synthesis of the AFB_1-N^7-guanine adduct standard

Identification of AFB_1-N^7-guanine by LC-MS/MS

The AFB_1-N^7-guanine adduct eluents collected by HPLC, were concentrated to a smaller volume (1 ml) under a gentle steam of nitrogen at room temperature and were analysed using an Ultimate/Famos LC system (LC Packings, Amsterdam, NL) with a gradient from 0% to 80% B over A in 8 min (A: 5 mM ammonium acetate in H_2O, 0.1% acetic acid, B: 5 mM ammonium acetate in ethanol, 0.1% acetic acid) (Figure 18). The flow rate was 180 µl/ min. The samples were injected into a Genesis C18 column (50 x 2.1 mm, particle size 4 µm) (Jones Chromatography, Hengoed, UK), with an injection volume of 30 µl. The LC mass spectra were recorded using a LCQ quadrupole ion trap mass spectrometer (Thermoquest, San Jose, CA). Full scan mass spectrum was measured for range m/z 300-600 and a second scan was used to measure the collision induced MS/MS spectrum of the AFB_1 adducts. The spray needle was set to 4.00 kV in the positive ion mode. The inlet capillary temperature was maintained at 220 ^oC.

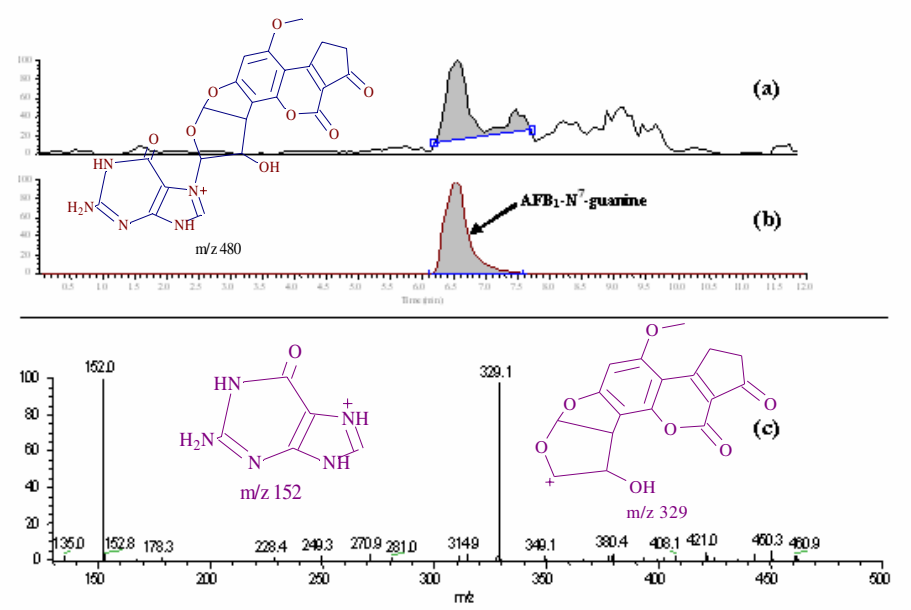


Figure 18. Liquid chromatography ion trap mass spectrometry of AFB_1-N^7-guanine. (a) elution of m/z 480 molecular ions including the adduct at 6.5 min. (b) Selected reaction chromatogram of the adduct by monitoring m/z 329 and 152 fragments obtained by MS/MS of m/z 480. (c) the MS/MS spectrum resulting from colliding the major peak found in panel a. [mobile phase: gradient 0% to 80 % B in 8 min (A: 5 mM ammonium acetate in water, 0.1 % acetic acid, B: 5 mM ammonium acetate in ethanol, 0.1 % acetic acid); flow rate: 150 µl/min; column: Genesis C18 50 x 2.1 mm]

4.2.2. Biomarker measurements

Breast milk samples

Aflatoxin extraction

Breast milk was collected into a sterile plastic container by self-expression before nursing the baby. The sample was kept at 4 ^{o}C and frozen within one day at -20 ^{o}C prior to sample processing. Extraction of aflatoxins from milk samples was achieved by the modified the method of El-Nezami et al (1995). Briefly, 10 ml breast milk samples were warmed to 37^{o}C and were shaken to distribute fat. For defatting, the samples were centrifuged (3000 *g*, 15 min, 5^{o}C) and filtered through glass wool. To facilitate the passage through a C18 cartridge (Strata C18-E, 50 um, 70A, Phenomenex, USA), the samples were diluted 1:1 with milli-Q water. The cartridge was pre-activated with 10 ml

acetonitrile and then 10 ml of water, prior to passage of diluted breast milk at a flow rate of 3.5 ml/min. The loaded cartridge was then washed with 10 ml water, 10 ml basic acetonitrile/ water (1% ammonia, 10% acetonitrile) and 10 ml acidic acetonitrile/water (1% acetic acid, 10% acetonitrile). AFM_1 was eluted with 5 ml acidic acetonitrile (1% acetic acid, 40% acetonitrile). AFM_1 was extracted twice from the eluent with 2 ml dichloromethane. Following centrifugation (3000g, 15 min) to separate the layers, the two dichloromethane fractions were pooled and dried under nitrogen gas. The residue was dissolved in 0.7 ml of methanol.

HPLC analysis

Reverse phase HPLC (model LC-10ADvp solvent delivery system; model SIL-10Advp auto injector, Shimadzu, Japan) was used to determine the level of AFM_1 in breast milk extracts. The columns used were ODS-5 C18 Brownlee reverse phase column (220 x 4.6 mm, particle size 5 μm) with a C18 guard column (Perkin Elmer, Norwalk, Conn.). An isocratic system with water: methanol: acetonitrile 66:17:17, flow rate 1 ml/min, oven temperature 40 ^{o}C and injection volume of 30 μl were used. HPLC grade methanol, acetonitrile and dichloromethane were purchased from Sigma/Aldrich Chemical Co. (Kemia Oy, Helsinki, Finland).

Detection was by excitation at 360 nm and emission at 440 nm (Fluorescence-10A XL, Shimadzu detector) (Figure 19). The concentrations of AFM_1 in milk were estimated from a standard curve 0.04-10 ng/ml, prepared from AFM_1 in chloroform (9.93 ug/ml) reference material RM 423 (LGC Promochem AB, Borås, Sweden). An AFM_1 standard was injected every 10 injections as the quality control. The limit of detection of this method was 0.06 ng AFM_1/ml methanol or 4.2 pg AFM_1/ml of breast milk based on a 10-ml breast milk sample, and reconstitution of extracts with 0.7 ml of methanol.

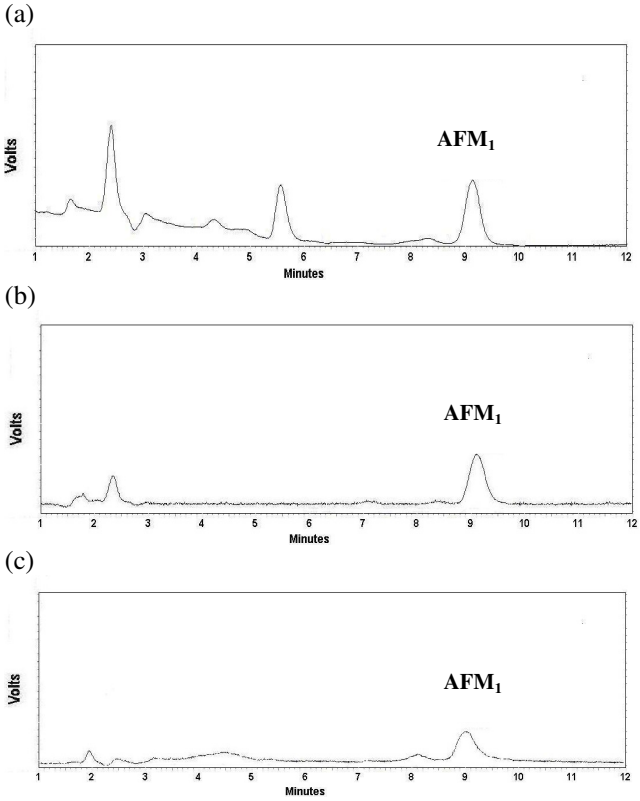


Figure 19. HPLC chromatograms of AFM_1. (a) AFM_1 extracted from an Egyptian breast milk sample (1004 pg/ml breast milk or 14 ng/ml in methanol after extraction); (b) AFM_1 standard 10 ng/ml in methanol; (c) AFM_1 extracted from spiked water (250 pg/ml water or 5 ng/ml methanol). [Mobile phase; water: methanol: acetonitrile, 66:17:17; flow rate: 1ml/min; column: ODS-5 C18 220 x 4.6, fluorescence detection (ex. 360 nm, em. 440 nm)].

Method validation and confirmation of the breast milk AFM_1 identity

Validation and repeatability of the measurement of breast milk aflatoxin was carried out using breast milk samples kindly provided by the Breast Milk Centre of Kuopio University Hospital (Kuopio, Finland). AFM_1 was not detected in these samples. In the recovery studies, seven breast milk samples (triplicates) were spiked with 12.5, 25, 50, 125, 250, 375, 500 pg AFM_1/ml milk. One water sample (triplicates) was spiked with 250 pg AFM_1/ml water. AFM_1 was extracted from the samples as previously described. The average percentages of aflatoxin recovered were 88 ±28 and 94 ±8 from breast milk

and milli-Q water respectively. The correlation coefficient between AFM_1 added and AFM_1 recovered from spiked breast milk was 0.993.

To assess the repeatability of the method, breast milk samples were spiked with 250 pg AFM_1/ml milk and the measurement was replicated four times. The analysis gave a mean value of 205 ±15 pg/ml milk and 8% coefficient of variation.

The identity of AFM_1 was confirmed through spiking of positive breast milk samples with AFM_1, and by derivatization of AFM_1 to form AFM_{2a} according to the method of the AOAC International (AOAC 1995) (Figure 20)**.** Briefly, the breast milk extract containing AFM_1 was evaporated to dryness and redissolved in 200 ul of hexane and 200 ul of trifluoroacetic acid. After 10 min incubation at 40^{o}C, the solvents were again dried under nitrogen gas, reconstituted in methanol and analyzed in an HPLC using the described method.

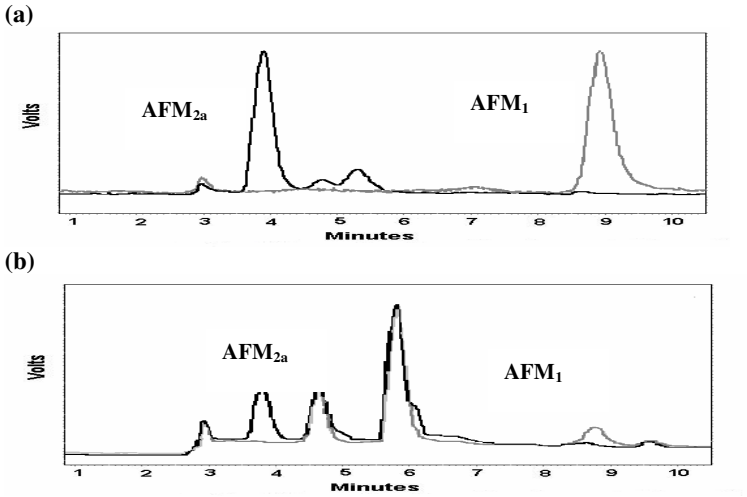


Figure 20. HPLC chromatograms of AFM_1 before (▬) and after derivatization (▬) to AFM_{2a}. (a) AFM_1 standard 10 ng/ml methanol, (b) AFM_1 extracted from an Egyptian breast milk sample (50 pg/ml breast milk or 0.7 ng/ml methanol). [Mobile phase water: methanol: acetonitritle, 66:17:17; flow rate 1 ml/min, column PDS-5 C18 220x 4.6, fluorescence detection (ex. 360nm, em. 440nm)].

Urine samples

The main focus in the analysis of the urine samples from the China Study (Studies IV and V) was on AFB_1-N^7-guanine, while in the urine samples from children (Study III) the primary aflatoxins and the hydroxylated AFB_1 metabolites were determined.

Therefore, two different extraction methods were used for the analysis of the urine samples, one requiring the use of C18 cartridges, and another a combination of C18 and immunoaffinity columns for more thorough sample cleanup. The analysis of the Chinese student urine samples was performed in the University of Kuopio, and those of the children in the University of Leeds.

Aflatoxin extraction

To measure the AFB_1-N^7-guanine adduct in the samples from the Chinese students, after collection of the urine samples in Sun-Yat Sen University, China, an aliquot (25 ml) was acidified with 1 N hydrochloric acid or 1 M ammonium formate, centrifuged, and passed through Bond Elut C18 cartridge (6 ml, 500 mg silica sorbent) (Varian, Middelburg, the Netherlands). The cartridge was washed with milli-Q water and 5% methanol. The ends of the cartridge were sealed with parafilm and the cartridges were shipped to the University of Kuopio for the analysis of the urinary levels of AFB_1-N^7-guanine. In the University of Kuopio, the cartridges were washed with milli-Q water followed by 10% basic acetonitrile (ammonia: acetonitrile: water 1:10:90) and 10% acidic acetonitrile (acetic acid:acetonitrile:water 1:10:90). AFB_1-N^7-guanine was eluted from the cartridge using 40% acidic acetonitrile (acetic acid:acetonitrile:water 1:40:60), the eluent was extracted twice with two volumes of dichloromethane, and the extracts were pooled and dried in a vacuum. The vacuum-dried extracts were reconstituted in 30% acetonitrile/methanol (1:1, v/v) in 20 mM ammonium acetate buffer (pH 3.9) for HPLC analysis.

To measure the urinary aflatoxins in the samples from the children, after collection of the urine samples in the New El-Qalyub Hospital, Egypt, 20 ml of urine were treated as described above prior to shipment. The ends of the cartridge were sealed with parafilm and the cartridges were shipped to the University of Leeds (Leeds, UK). In the case of the samples from Guinea, the urine samples were shipped as such and the entire extraction procedure was performed in the University of Leeds. In the University of Leeds, the cartridges were washed with 5% methanol, and the aflatoxin residues were eluted with 3 ml of 1:1 methanol: 1% acetic acid followed by 5 ml methanol. The urine samples were extracted in batches of eight, and between each batch, the pump tubes were washed with methanol to avoid cross-contamination. The eluent was dried in a

vacuum to approximately 200 µl and was reconstituted in 2 ml of water for passing through AflaTest P immunoaffinity columns (IAC) (Vicam, Hallmark Analytical Ventures Limited, Chester, UK) under gravity. The IAC columns were attached to syringes to facilitate the procedure. The IAC were washed with 10 ml PBS followed by 10 ml of water, were loaded with the 2 ml samples, and then were washed with 5 ml PBS followed by 10 ml of water. The aflatoxin residues were eluted with 5 ml of 95% methanol and were dried overnight in vacuum. The vacuum-dried extracts were reconstituted in 100 µl of methanol for HPLC analysis.

HPLC analysis

The analysis of the urine samples from the children (University of Leeds) was performed as follows: Reverse phase HPLC (Prostar 240 quaternary gradient solvent delivery module and Prostar 410 autosampler model, Varian, Inc.) was used to separate the primary aflatoxins and aflatoxin metabolites on an C18 Microsorb-MV column (150 by 4.6 mm, particle size 5 µm) fitted with a C18 metaguard column (Microsorb A104MG, Varian, Inc.). Chromatographic separation was obtained by an isocratic system with a mobile phase of 30% acetonitrile/ methanol (1:1, v/v) in 20mM ammonium acetate buffer (pH 3.9) at a flow rate of 1.2 ml/ min. The assay oven temperature was 40 ^{o}C and the sample injection volume 10 µl.

The concentrations of urinary aflatoxins were estimated from the respective standard curves: 8- 125 ng AFB_1, 0.15- 2.5 ng B_2, 15- 250 ng G_1, 0.3- 5 ng G_2, 1.5- 25 ng M_1, 25- 400 ng Q_1/ml in methanol (regression coefficient >0.995 for all curves) corresponding to 40- 625, 0.75-12.5, 75- 1250, 1.55- 25, 7.5- 250, 125- 2000 pg/ml urine, respectively**.** The HPLC run (isocratic system: 30% acetonitrile:methanol 1:1, v/v, in 20 mM ammonium acetate buffer, pH 3.9, flow rate of 1.2 ml/ min) was repeated twice, and the results presented are mean values of the two runs. A mixture of all aflatoxin standards was injected after every 6 injections to assure the correct identification of the urine sample peaks based on their retention times.

The limits of detection were 25, 0.35, 50, 0.8, 5, 100 pg/ml of urine for AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFQ_1, respectively. The variation in the levels of the detection limit was due to the different fluorescent properties of the aflatoxins.

AFQ_1 standard (Sigma/ Aldrich, Helsinki, Finland) appeared always as a double peak (Figure 21) possibly being a mixture of two AFQ1 epimers (Buchi *et al.* 1975)**.**

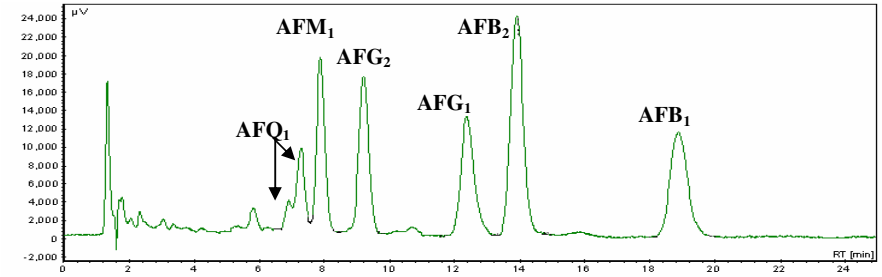


Figure 21: Mixture of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFQ_1 standards in concentrations of 125, 2.5, 250, 5, 25, and 400 pg/ml urine, respectively. The AFQ_1 standard appeared always as a double peak.

The analysis of the urine samples from the young male students (University of Kuopio) was performed as follows: Reverse-phase HPLC, a Shimadzu model SPD-10 Avp UV-Vis detector (360 nm) in series with a Shimadzu RF-10AXL fluorescence detector (excitation 366 nm, emission 440 nm) was used to quantify aflatoxins. The HPLC column used was the ODS Spheri-5 Brownlee column (220 x 4.6 mm, 5 µm; Perkin Elmer) fitted with a C_{18} guard column (Perkin Elmer). Chromatographic separation was achived within 40 min with an isocratic mobile phase of 30% acetonitrile:methanol (1:1, v/v) in 20 mM ammonium acetate buffer (pH 3.9) at a flow rate of 1.5 ml/min. The assay temperature was 40°C, and the sample volume injected 10 µl. Samples were run in batches with authentic standards running between every 10 samples to control any changes to the retention time. The limit of detection for AFB_1-N^7-guanine was 5 pg/ml of urine.

Method validation and confirmation of urinary aflatoxin identities

The method validation was carried out using blank urine samples spiked with a mixture of known amounts of aflatoxin standards. In the University of Leeds, blank urine samples (n=8) were spiked with 625 pg AFB_1, 12.5 pg AFB_2, 1250 pg AFG_1, 25 pg AFG_2 and 125 pg AFM_1/ml urine. The spiked urine samples were extracted with each

batch of samples. The method gave average recovery rates of 33±14, 46±16, 17±11, 23±12, 42±22% for AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1 respectively.

In the University of Kuopio, the method was evaluated based on the recovery of AFM_1 from spiked urine samples (n=3) and this was found to be 88±5%. The recovery of AFB_1-N^7-guanine was not determined due to the limited availability of the standard.

Confirmation of aflatoxin identities in urine samples from the children was performed in 10 (24%) out of the 42 positive Guinean samples and in 7 (30%) out of the 23 positive Egyptian samples by spiking with aflatoxin standards and co-chromatography using two different HPLC methods; an isocratic (mentioned above) and a linear 15% - 40% gradient system of B over A in 25 minutes (A: water/ acetonitrile/ phosphoric acid 100/ 8/ 0.01 v/v/v and B: methanol/ acetonitrile/ phosphoric acid 100/ 8/ 0.01 v/v/v) at a flow rate of 1.2 ml/ min, oven temperature 37 ^oC and injection volume 10 µl.

The identities of urinary AFB_1 AFB_2, AFG_1, AFG_2 and AFM_1 were confirmed successfully by co-chromatography using both HPLC methods, while AFQ_1 identification gave controversial results (Figure 22). The peak thought to be AFQ_1 in the urine samples co-eluted with the first peak of the AFQ_1 standard when analyzed by the isocratic HPLC system, while it co-eluted with the second AFQ_1 peak when analyzed with the gradient system. This was assessed by calculating the ratios of the two areas of the AFQ_1 peaks for each spiked urine sample. The presumed urinary AFQ_1 was detected in 15/50 Egyptian children (mean 159 pg/ml urine, range 75- 296) and 14/50 Guinean children (mean 946 pg/ml urine, range 167- 6352). However, the presence and levels of urinary AFQ_1 are not discussed further in the following sections, since its identity was never successfully confirmed.

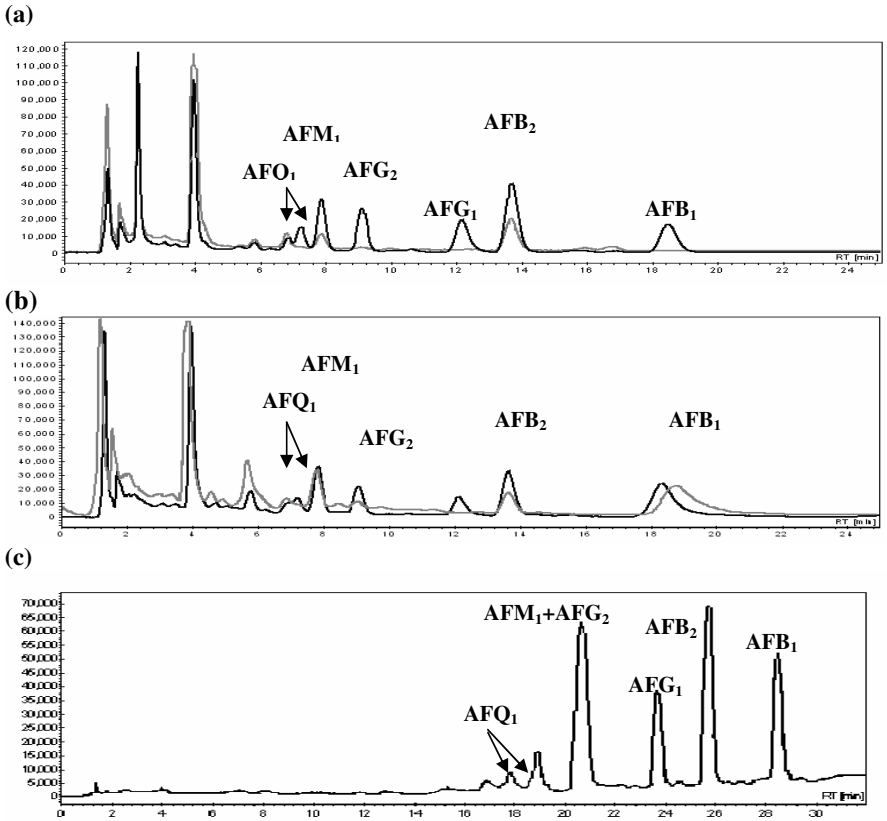


Figure 22. Aflatoxins extracted from urine samples before (▬) and after spiking (▬) with a mixture of aflatoxin standards. (a) 290 pg AFQ_1/ml urine, 12 pg AFM_1/ml urine, 2 pg AFB_2/ml urine detected in urine sample (isocratic HPLC conditions) (b): 378 pg AFQ_1/ml urine, 49 pg AFM_1/ml urine, 3 pg AFG_2/ml urine, 1.7 AFB_2/ml urine, 436 AFB_1/ml urine detected in urine sample (isocratic HPLC conditions). (c): The spiked urine sample displayed in (a) analyzed with the gradient HPLC conditions.

Confirmation of the AFB_1-N^7-guanine peaks in urine samples from the young Chinese males was performed using a gradient from 22% to 34% of methanol: acetonitrile (1:2, v/v) over 55 min and flow rate 0.2 ml/min. The aqueous buffer used was 0.1% formic acid: water.

4.3. Statistical treatment of the data

4.3.1. Egypt and Guinea Studies (I, II and III)

Screening phase (Study I)

SPSS 11.0 for windows was used in the statistical analysis of the data. AFM_1 values did not follow a normal distribution even after logarithmic transformation (p=0.005, Kolmogorov-Smirnov normality test). Therefore, to identify possible associations between AFM_1 levels and related factors, the nonparametric Mann-Whitney U (or Kruskal-Wallis) test or Spearman correlation were used for the continuous variables. The mothers were dichotomized depending on the presence/absence of AFM_1 in their breast milk, and the chi-square test was used to associate the presence of AFM_1 in breast milk with categorical variables. Logistic regression models were used to analyse the association of each potential factor and AFM_1 presence/absence. For the analysis of the FFQ data, the food consumption data were either regrouped into the two categories of "daily consumption" and "weekly or monthly consumption" (the category "never" was included into the "weekly or monthly consumption" category) or treated as a continuous variable, i.e. the reported frequencies of consumption were expressed as a number representing the frequency of consumption. eg. 1/ month, 1/ fortnight, 1/ week or 3-4 times/ week calculated as 1/30 or 0.03, 1/14 or 0.06, 1/7 or 0.21, 3.5/7 or 0.5, respectively (Willet 1990).

Follow up data on mothers (Study II)

An exploratory approach was taken in the statistical analysis of the data. The outcome measure was taken to be the presence or absence of a detectable level of AFM_1 in the breast milk sample. A logistic regression was performed to explore factors that were associated with the presence of AFM_1. To account for clustering of samples within mothers, a multi-level logistic regression model was fitted using MLwiN version 2.02 (Multilevel Models Project, Institute of Education, University of London) statistical software, with the Markov Chain Monte Carlo (MCMC) option for model fitting. This permits variation between mothers as well as variation between milk samples using a two-level model. All explanatory variables (mother's age, working status, and number of children, month of the year on which the sample was collected and number of months

of lactation, consumption of corn oil, cotton seed oil and peanuts) were first included in the model and then variables removed stepwise if not significant. Care was taken to investigate various combinations of variables in the model and the model best describing the data was retained. A chi-square test was used to explore associations between the presence of AFM_1 and the presence of AFB_1, AFB_2, AFG_1 and AFG_2.

Data on children (Study III)

Two-tailed Fisher's exact test was used to explore associations between the presence of each toxin in Guinean compared to Egyptian children. The urinary levels of each aflatoxin species were also investigated. For samples below the detection limit for a given aflatoxin metabolite, a mid point value between the limit of detection and zero was assigned. Data were then log transformed to provide a normal distribution and the Students t-test (two-tailed, unpaired, equal variance was not assumed) was performed to compare the levels measured between the Guinean and Egyptian samples. Data were then back-transformed and are presented as geometric means with 95%CI.

4.3.2. China Study (Studies IV and V)

The data were analyzed using SPSS (Version 12.0) and SAS (Version 9.1.3). Urinary AFB_1-N^7-guanine during the 5 weeks intervention period was the primary variable. The study groups were compared during the intervention period at week 3 and at week 5. In addition, a comparison was made 5 weeks after the cessation of the intervention to determine if the urinary AFB_1-N^7-guanine had returned to the baseline level. Urinary AFB_1-N^7-guanine was first dichotomised (negative vs. positive). Adjusted ORs, with the corresponding baseline urinary AFB_1-N^7-guanine included as a categorical covariate, were calculated from logistic regression models for each time point separately. Since each subject contributed to two binary outcomes during treatment, as a primary analysis models using GEE (Generalized Estimating Equation) methods were fitted, assuming an unstructured covariance matrix and a logit link. The analysis started from the model including treatment, baseline AFB_1-N^7-guanine, time and interaction between treatment and time as factors. The factors were retained in the model if they were significant at the 0.10 level. The time effect and the interaction between treatment and time were non-

significant (p=0.280 and p=0.860, respectively), and were excluded from the final model. The final model was used to estimate the OR and the confidence interval.

Due to the high rate of non-detectable values (values below the detection limit), the analysis of the mean levels of urinary AFB_1-N^7-guanine was conducted as a secondary analysis. The zero concentrations were transformed to the observed minimum value divided by two. The final distributions of AFB_1-N^7-guanine were skewed to the right, and therefore, all values were logarithmically (ln) transformed before the analysis. Analysis of covariance (ANCOVA) was applied with respect to the level of AFB_1-N^7-guanine concentrations in urine, at weeks 3 and 5, and after the cessation of the intervention, separately. The corresponding ln-transformed baseline concentration was included as a continuous covariate. The baseline-adjusted group means and their differences were then back-transformed to the original units and the results are given as geometric means and ratios (probiotic/placebo) with 95% confidence intervals, respectively. To account for multiple comparisons in repeated measurements, the urinary concentration of AFB_1-N^7-guanine was analyzed using ANOVA for repeated measurements. Concentrations at weeks 3 and 5 were included as dependent variables and the baseline concentration was included as a continuous covariate. ANOVA for repeated measurements was applied to study 1) difference between study groups, 2) time-effect i.e. change during the intervention period and 3) interaction between treatment and time.

Mann-Whitney U test was used to examine the differences of AFB-N^7-guanine between the HBV-positive and the HBV-negative subjects.

4.4. Ethical considerations

The Egypt Study was approved by the Ethics Committee of the University of Kuopio and Kuopio University Hospital in Finland and by the National Research Centre Ethical Committee. The study was approved by the Comité National d'Ethique pour la Recherche en Santé in Guinea and by the ethics committee at the London School of Hygiene and Tropical Medicine, UK.

The China Study was approved by the Ethics Committee of the University of Kuopio and Kuopio University Hospital in Finland and Ethical Committee of Sun Yat-Sen University in China.

All subjects were made aware of the content of the study and if they agreed to participate, a written informed consent was obtained. In Egypt, the mothers were approached by the nurses of the New El-Qalyub hospital, and in Guinea, researchers of the Institute Pasteur de Guinée conducted a series of public meetings to explain the project and to discuss the parental concerns. The mothers of all children participating in the study gave informed consent. In China, the students were approached by researchers of the Sun Yat Sen University. Each subject was informed that they could discontinue the study at any time.

5. RESULTS

5.1. AFM_1 in breast milk of selected Egyptian mothers and determining factors (Studies I and II)

Presence of AFM_1 and dietary, socioeconomic and demographic factors affecting the levels of AFM_1 in breast milk (Study I)

AFM_1 was detected in the breast milk of 138 from the 388 screened Egyptian mothers (35.5%). The mean level of AFM_1 was 27 pg/ml milk (range 5.6-5131). The presence of AFM_1 in breast milk was influenced by the frequency of corn oil consumption, the working status of the mothers, the stage of lactation, the number of children and the body mass index (BMI) of the mothers (Table 11).

AFM_1 in breast milk was detected significantly more frequently (p=0.016) in mothers consuming corn oil on a daily basis compared to less frequent corn oil consumers. Out of the 262 mothers who were consuming corn oil on a daily basis, 104 (40%) tested positive for AFM_1, while out of the 125 who were consuming corn oil on a weekly or monthly basis, only 34 (27%) were AFM_1 positive.

AFM_1 was detected most frequently in breast milk during the first month of lactation. Fourteen of 24 mothers who were in their first lactation month were found to be AFM_1 positive (58%), while out of the 360 mothers at lactation stage > 1 month only 123 (34%) were AFM_1 positive. The frequency of detection of AFM_1 in breast milk during the first month of lactation was significantly greater (p = 0.017) than that in subsequent months combined.

Out of the 339 mothers who did not work, 129 (38%) were found to have detectable AFM_1 in their breast milk, while out of the 43 mothers who were employed, only 7 (16%) had detectable breast milk AFM_1 levels (p = 0.005). AFM_1 was detected more frequently in obese women (BMI >30) (25 AFM_1 positive out of 51 obese women, 49%) compared to those who were either overweight (BMI>25) (58 out of 171 overweight women, 34%) and those whose BMI was <25 (34 out of 114 women, 30%) (p=0.031).

Table 11. Factors significantly associated with the presence of AFM_1 in breast milk of Egyptian mothers

Mothers (n)	AFM_1 positive % (n)	Univariate	Multivariate model			
			Without BMI		Without corn oil	
		Unadjusted p-values	Adjusted p-values	Adjusted OR (95%CI)	Adjusted p-values	Adjusted OR (95%CI)
Mother's age	-	0.460	0.051	0.95 (0.91-1.00)	0.014	0.94 (0.89-0.99)
Working status						
non-working (339)	38 (129)	0.005	0.012	3.01 (0.14-0.78)	0.018	2.87 (1.2-6.87)
working (43)	16 (7)					
Number of children[#]						
2-7 (275)	38 (105)	0.104	0.036	1.81 (0.31-0.96)	0.025	1.99 (1.09-3.64)
1 (109)	29 (32)					
Lactation stage*						
<1month (24)	58 (14)	0.017	0.004	3.94 (0.10-0.63)	0.028	3.57 (1.15-1.09)
≥1 months (360)	34 (123)					
Corn oil consumption						
daily (262)	40 (104)	0.016	0.002	2.21 (0.27-0.74)	-	-
weekly/monthly (125)	27 (34)					
BMI			-	-	0.011**	
≤25 (not overweight) (114)	30 (34)	0.031				
25-30 (overweight) (171)	34 (58)		-	-	0.578	1.16 (0.68-1.99)
≥30 (obese) (51)	49 (25)		-	-	0.004	3.01 (1.43-6.33)

[#]including the newborn child, *the number of months that mother has been breastfeeding, **overall effect of BMI to AFM_1 presence

In multivariable models however, BMI and corn oil consumption were collinear to each other which violated the assumptions of the model and excluded them from being present in the same model. Therefore, separate models including either of these two factors were generated. When BMI was taken into consideration, mother's age, non-working status, having more than one child, early lactation stage (<1 month), and obesity appeared to be strong predictors of the presence of AFM_1. Non-working mothers were 2.87 times more likely to be AFM_1 positive than the working mothers (p= 0.018), having more than one child almost doubled the risk of being AFM_1 positive (p= 0.025), and early lactation stage (<1 month) seemed to be a strong predictor of the presence of AFM_1 with an OR of 3.57 (p= 0.028). BMI had a significant overall effect to the AFM_1 presence (p= 0.011). There was no difference in AFM_1 frequency for overweight mothers (BMI 25-30) compared to those with BMI ≤25 (p= 0.57), though obese mothers (BMI ≥30) had a three times greater risk of being positive for AFM_1 than

those with BMI ≤25 (p= 0.004). In the multivariate corn oil consumption model (replacing BMI), mothers with daily corn oil consumption had a 2.21 times greater risk of being AFM_1 positive than the non-frequent consumers (p= 0.002). Replacing BMI with corn oil in the model did not alter significantly the strengths of the associations between the other variables and the presence of AFM_1 (Table 11).

Consumption of other grain products, milk and milk products, meat, fish, legumes, cotton seed oil, dried fruits and peanuts did not exhibit any significant associations with the presence of AFM_1 in breast milk (p >0.05).

Longitudinal assessment of AFM_1 in breast milk of Egyptian mothers (Study II)

Fifty mothers who were selected for the follow up had a mean AFM_1 level of 137 pg/ml (range 10-5131) in their breast milk. A total of 443 samples were collected during the 12 months follow up period, and AFM_1 was detected in 248 (56%) of the samples (mean for all samples 24 pg/ml milk, range 5.6-5131). During the summer months (May- September, n=136 breast milk samples), 121 (89%) samples had detectable AFM_1 levels (mean 31 pg/ml, range 4.3-609) (Table 12). The highest percentage of positive samples occurred in June (96%) and the lowest in February (16%).

The AFM_1 levels followed the same pattern, showing higher levels during the summer months, highest mean AFM_1 level in July (64 pg ml^{-1} milk, range 6.3-497) and the lowest mean level in January (8 pg ml^{-1} milk, range 4.2-108). However, the individual sample with the greatest AFM_1 level (889 pg/ml milk) was obtained in April.

Table 12: The percentage of positive samples and the levels of AFM_1 in breast milk of Egyptian mothers during the 12 months of follow up

	n total	number of AFM_1 positive samples (%)	AFM_1 levels (pg/ml milk) mean*	range
January	50	12 (24)	8	4.2–108
February	49	8 (16)	12	4.8–275
March	50	28 (56)	18	5.0–181
April	50	20 (40)	36	5.7–889
May	26	23 (88)	40	4.6–609
June	26	25 (96)	28	4.5–228
July	26	24 (92)	60	6.3–497
August	29	22 (86)	15	4.5–127
September	29	24 (83)	14	4.3–63
October	29	22 (76)	13	5.3–110
November	29	21 (72)	28	4.9–360
December	50	16 (32)	12	9.2–61

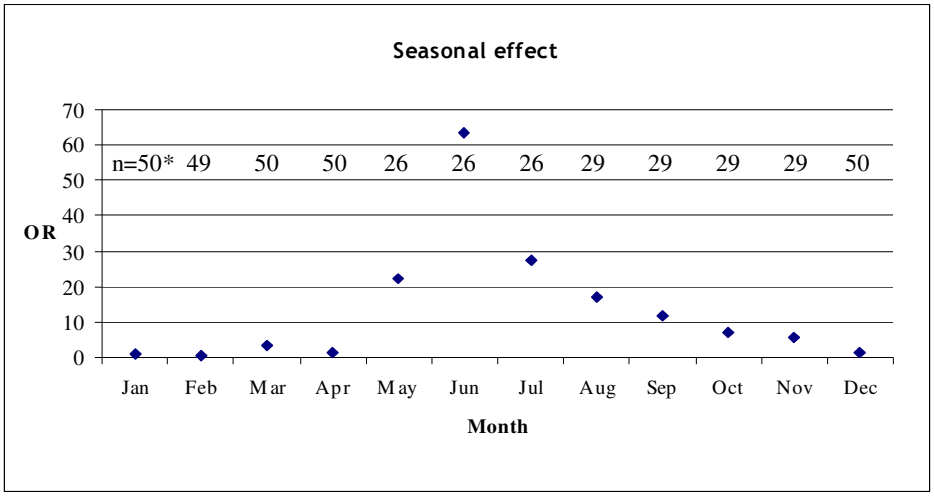
*The zero values have been replaced by the detection limit value divided by 2 (2.1 pg/ml milk)

During the follow up period, the factors determining the temporal variation of AFM_1 were the months of breast milk sample collection (January to December) ($p< 0.001$), the duration of lactation in months (lactation) ($p= 0.003$), and the peanut consumption over the week prior to sample collection (peanuts) ($p= 0.061$) (Table 13). The month where the breast milk sample collection took place appeared to be the dominant factor determining AFM_1 presence. Summer months (May-September) were the months with the highest risk of aflatoxin presence, with June presenting an OR of 63 [95% CI: (7.7, 522)] (Figure 23). Winter months (December–February) had the lowest AFM_1 presence risk [OR December 1.59, 95% CI: (0.65, 3.8), January (reference month) 1, February 0.51, 95% CI: (0.18 1.4)]. The duration of lactation was fitted as a linear term into the model. Every month increase in the lactation stage contributed to a significant 8% increased risk of AFM_1 presence [OR= 1.08, 95% CI: (1.02, 1.13)]. Peanut consumption was weakly related to AFM_1 ($p= 0.061$) with mothers consuming peanuts during the week prior to sample collection having a 69% elevated risk of being AFM_1 positive [OR= 1.69, 95% CI: (0.9, 2.9)].

Table 13. Factors affecting the temporal variation in the level of AFM_1* (multilevel modeling results).

Covariate	p	OR	CI lower	CI upper
Lactation	0.003	1.08	1.02	1.12
Peanuts	0.061	1.69	0.98	2.91
January**		1.00		
February	0.190	0.51	0.18	1.40
March	0.005	3.42	1.43	8.18
April	0.268	1.65	0.68	3.97
May	0.000	22.38	5.63	88.93
June	0.000	63.31	7.67	522.65
July	0.000	27.55	5.57	136.37
August	0.000	17.24	4.91	60.54
September	0.000	11.60	3.54	37.97
October	0.000	6.94	2.29	21.02
November	0.001	5.80	1.97	17.11
December	0.308	1.59	0.65	3.88

*The outcome measure was taken to be the presence or absence of a detectable level of AFM_1 in the breast milk samples, **January was used as the reference month.



*number of mothers

Figure 23. Seasonal effect on the risk of AFM_1 presence in breast milk of Egyptian mothers.

During the follow up period, aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) were occasionally observed in the breast milk samples (Table 14) with AFG_1 being the compound most frequently detected (6.8%, 30/443).

Table 14. Frequency of aflatoxin detection in breast milk

Aflatoxins	n total	% of positive samples (n)
AFM_1	443	56 (248)
AFB_1	443	2 (9)
AFB_2	443	2.5 (11)
AFG_1	443	6.8 (30)
AFG_2	443	1.6 (7)

Most of the positive samples were collected during the summer months (May-September): out of the 9 samples that contained AFB_1, 7 were detected during summer (78%), with the respective frequencies for AFB_2, AFG_1 and AFG_2 being 54% (6/11), 63% (19/30), and 43% (3/7). Winter months (December-February) had rather low frequencies of detection (AFB_1 1/9, AFB_2 2/11, AFG_1 3/30, AFG_2 2/7). These aflatoxins generally occurred in samples positive for AFM_1. Out of the 9 samples that were AFB_1 positive, 7 were also positive in AFM_1 (78%), while out of the 11 AFB_2 and 7 AFG_2 positive samples, 8 (73%) and 4 (57%) were AFM_1 positive, respectively. These associations were not significant, whereas the presence of AFM_1 was significantly correlated ($p<0.001$) with AFG_1 presence; 27 out of the 30 AFG_1 positive samples were also AFM_1 positive (90%). There was no significant association between the presence of any other pair of toxins (e.g. AFG_1 and AFG_2).

5.2. Urinary biomarkers of aflatoxin exposure in young children from Egypt and Guinea (Study III)

The urinary levels of different aflatoxins were measured in samples collected from Egyptian children who were subjected to a moderate aflatoxin exposure, and from Guinean children representing a high aflatoxin exposure. Aflatoxins were less frequently present in urine of the Egyptian than Guinean children (38% vs. 86%). The difference in prevalence was statistically significant for most of the detected toxins: AFB_1 (2 vs. 16%,

p= 0.016), AFB_2 (10 vs. 58%, p= 0.000), AFG_1 (4 vs. 2%), AFG_2 (24 vs. 36%, p= 0.275) and AFM_1 (8 vs. 64%, p= 0.000) (Table 15).

Table 15. Frequencies of detection of different aflatoxins in the urine samples collected from the Egyptian and Guinean children

	n of positive samples (%)			Limit of detection**
	Egypt (n=50)	Guinea (n=50)	P value*	pg/ml
AFB_1	1 (2)	8 (16)	0.016	25
AFB_2	5 (10)	29 (58)	0.000	0.35
AFG_1	2 (4)	1 (2)	1	50
AFG_2	12 (24)	18 (36)	0.275	0.8
AFM_1	4 (8)	32 (64)	0.000	5

*two-tailed Fishers exact test, **The limit of detection was determined by spiking blank urines with aflatoxin standards. The value varies due to the different fluorescent properties of the aflatoxin metabolites.

Also the levels of the aflatoxins excreted in the urine were higher in the Guinean children. The geometric mean level of AFM_1 was 6-fold higher in the urine from the Guinean children, and the other aflatoxins, AFB_1, AFB_2, and AFG_2, but not AFG_1 were also observed at higher geometric mean levels (Table 16).

Table 16. Levels of different aflatoxins (pg/ml) in the urine samples of the Egyptian and Guinean children.

	Average (range)[#] Geometric mean (95% CI)[##]		
	Egypt (n=50)	Guinea (n=50)	P value*
AFB_1	189	2682 (179-18000)	
	13.2 (11.8, 14.6)	**26.6 (16.3, 42.9)**	**0.007**
AFB_2	1.4 (0.8-2.2)	5.7 (0.6-43)	
	0.2 (0.2, 0.3)	0.8 (0.5, 1.3)	**<0.001**
AFG_1	76 (72- 81)	709	
	26.0 (24.5, 27.7)	**26.6 (23.3, 30.6)**	**0.77**
AFG_2	2.2 (0.85-8)	19 (1.4-199)	
	26.0 (24.5, 27.7)	26.6 (23.3, 30.6)	**0.008**

[#]The arithmetic mean and range refer to those samples that are positive only. [##]The geometric mean values are calculated including all samples. A mid-point value between the limit of detection and zero was assigned to those samples that were below the limit of detection. [*]P values are based on natural log transformed data, using two tailed unpaired student T-test. Equal variance was not assumed.

5.3. Reduction of aflatoxin exposure by probiotic supplementation in young male students from China (Studies IV and V)

300 young male students from Southern China were screened for the presence of AFM_1 in urine and of these, 90 with detectable AFM_1 in urine were selected for an intervention aiming to determine whether administration of probiotic bacteria could reduce aflatoxin exposure by preventing its absorption from the small intestine. The urinary excretion of AFB_1-N^7-guanine was used as a biomarker for reduced absorption of AFB_1.

The median urinary AFM_1 screening concentration of those participating in the intervention (n=83) was 0.08 ng/ml (IQR 0.06 to 0.12), and the median baseline value (prior to the intervention) of AFB_1-N^7-guanine 0.38 ng/ml (IQR 0.0 to 2.15) (study IV). The percentage of subjects with detectable (positive) AFB_1-N^7-guanine levels was not significantly different between the treatment arms at baseline: 51.3% (20 of 39 samples) of probiotic and 59.5% (25 of 42 samples) of placebo samples (study V). The percentage of samples with negative values tended to be higher in the probiotic arm than in the placebo arm during the intervention period of 5 weeks (OR=2.63, p=0.052) (Table 17) but the effect of probiotic was no longer seen 5 weeks after the cessation of the intervention (post-intervention), where the difference between groups was not significant (p=0.289).

Table 17. Effect of the probiotic intervention on the proportion of urinary AFB_1-N^7-guanine values below the detection limit*

	Probiotic (n=39) % negative	Placebo (n=42) % negative	Probiotic vs Placebo** OR (95% CI)	P value
Baseline	49	41		
Intervention-week 3	56	36	2.88 (0.89, 9.39)	
Intervention-week 5	62	43	2.43 (0.78, 7.61)	
Total* **			2.63 (0.99, 6.95)	0.052
Post-intervention period	39	45	0.58 (0.21, 1.59)	0.289

*The probiotic group received *Lactobacillus rhamnosus* LC705 and *Propionibacterium freudenreichii ssp shermanii* (1:1 wt/wt) at a dose level of 2-5 x 10^{10} colony forming units/day

**ORs were calculated with the use of logistic regression analysis, where baseline AFB_1-N^7-guanine was included as a categorical covariate

***Data was corrected by generalized equation equations before analysis.

Probiotic administration led to a decrease in the urinary level of AFB_1-N^7-guanine. The reduction was 36% at week 3 and 55% at week 5, but had disappeared during the 5-week post intervention period (Figure 13). The geometric mean during the intervention period for the probiotic group was 0.24 and that for the placebo group 0.49 ng/ml, and the ratio probiotic:placebo was 0.49 (95% CI: 0.30, 0.80, p=0.005). After the cessation of the intervention, the difference between groups was no longer statistically significant (p=0.703).

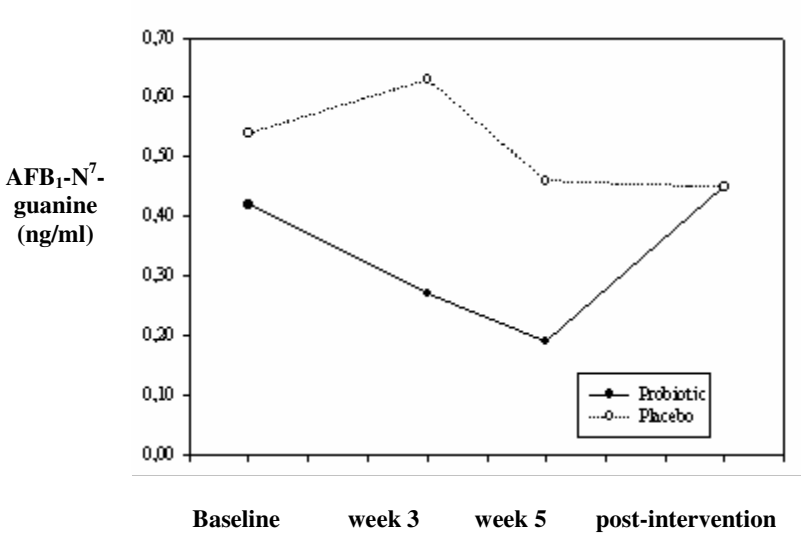


Figure 24. Urinary levels of AFB_1-N^7-guanine in healthy Chinese males during the probiotic intervention, and post intervention period. The values are geometric means of 39 and 44 subjects in the probiotic and placebo groups, respectively.

6. DISCUSSION

Our study demonstrated the presence of aflatoxins or their metabolites in milk samples of mothers from Egypt and urine samples of young children from Egypt and Guinea as well as in young Chinese adults. Children are a highly susceptible population to environmental toxicants (Landrigan 1999), and early life exposure to carcinogens may result in a greater lifetime risk of cancer (Miller *et al.* 2002). In addition, the result of exposure to xenobiotics may be different in children from those encountered in adults, both in the degree of severity of effect and in the nature of the effect (Miller *et al.* 2002). An example of such an outcome for aflatoxins is postnatal growth retardation. Aflatoxins can cross the human placenta (Denning *et al.* 1990, Wild *et al.* 1991) and young infants may be exposed to aflatoxins from breast milk (Wild *et al.* 1987, El-Nezami *et al.* 1995) or when they are weaned onto solid foods (Gong *et al.* 2003).

6.1. AFM_1 in breast milk and maternal aflatoxin exposure (Studies I and II)

AFM_1, which is carcinogenic and cytotoxic (IARC 1993) with potentially important implications in immunocompetence and growth, was detected in 138 out of the 388 breast milk samples from individual mothers (35.5%) during May-September 2003. The median AFM_1 level of the positive samples was 13.5 pg/ml of milk (range 5.6- 5131 pg/ml).

The frequency of AFM_1 detection in these mothers was higher than that previously reported from this region (El-Shewey 1992), although this may partly reflect improved analytical sensitivity. El-Shewey et al. (1992), reported 11% positive samples (22 out of 200) with mean levels 160 pg/ml (range 120-200 pg/ml). In that previous study, the determination of aflatoxins in human milk was performed by thin layer chromatography (TLC), a much less sensitive method than the HPLC technique used in our study. According to our study as well as the results of El-Shewey et al, the AFM_1 level in the breast milk of the Egyptian mothers is lower than the levels reported elsewhere. For example, the median levels of positive samples and the detection rates of two of the most recently published studies on AFM_1 in breast milk levels were 560 pg/ml and 92%

in the United Arab Emirates (Abdulrazzaq *et al.* 2003), and 664 pg/ml and 44% in Thailand (El-Nezami *et al.* 1995).

Fifty mothers identified as being AFM_1 positive in the screening phase of the study were recruited for a year of follow up with monthly collections of breast milk samples. AFM_1 was detected in 56% of the collected breast milk samples with the median levels of all samples being 5.76 pg/ml (range 4.2-889). The highest detection rates of AFM_1 in the breast milk occurred during the summer months (May-September) (89%, 121/136), with the highest rate found in June (96%). The median AFM_1 concentration of the positive samples during May-September was 11.44 pg/ml milk (range 4.3-609), while that in June was 12.06 pg/ml. However, since the mothers were selected on the basis of AFM_1 breast milk levels, they do not represent the general aflatoxin exposure of the population in this region but that of an exposed group.

Calculations of maternal AFB_1 and child AFM_1 daily exposures

AFM_1 in the breast milk is a biomarker of children's exposure to this carcinogen but can also be considered as a biomarker of maternal exposure to AFB_1. However, there are certain limitations in estimating the average daily aflatoxin intake of the mother and the infant based on breast milk aflatoxin levels due to factors influencing the amount of the aflatoxin that passes into the milk. These factors include the variation of the maternal dietary exposures, the total milk volume, the fat and protein content of the milk, the contribution, if any, of aflatoxin or its metabolites potentially stored in fat reserves, the intra-individual differences in conversion of AFB_1 to AFM_1 (e.g. CYP 1A2 polymorphisms), and the amount of milk consumed by the infant. Despite these difficulties, estimates can be made based on the assumptions that the average daily milk volume is 500 ml and AFM_1 in human milk represents 0.09%–0.43% of dietary intake of AFB_1 (Zarba *et al.* 1992). Using the median AFM_1 levels during the screening period (Study I) (13.5 pg/ml milk), the average intake of AFM_1 by the infants can be calculated as being 6.75 ng AFM_1/day, while the intake by mothers is estimated to be 1.5-7.5 µg AFB_1/day (Table 18). For the follow up period (Study II), using the median AFM_1 levels during June (12.06 pg/ml) to reflect consumption of contaminated food at peak exposure, the estimated AFM_1 intake by the infants was 6.03 ng/day infant whareas that of AFB_1 by the mothers was in the range of 1.4-6.7 µg/day. These levels from the two

studies are very similar to each other, reflecting the high exposures during the summer months. However, the median level of all samples collected over the year (5.76 pg/ml) represents perhaps a more realistic value, giving estimated intakes of 2.88 ng AFM_1/day and 0.7-3.2 µg AFB_1/day for the average infant and mother, respectively. It must be emphasized that , since the mothers were pre-selected on the basis of AFM_1 breast milk levels, they do not represent the general aflatoxin exposure of the population in the region but that of an exposed group, and thus the average exposure in this region will be lower than the above estimates.

Table 18: Estimations of the maternal and infant daily aflatoxin intakes

AFM_1 levels in breast milk median (pg/ml milk)		**Infant intake of AFM_1* average (ng/day)**	**Maternal intake of AFB_1** range**	
			µg AFB_1/day)	**(ng AFB_1/kg bw/day)**
(Study I, May- September)	13.5	6.75	1.5-7.5	23-115
(Study II, June)	12.6	6.03	1.4-6.7	21-103
(Study II, January-December)	5.76	2.88	0.7-3.2	10-49

* Based on the assumption that average daily milk volume is 500 ml
**Based on the calculation that AFM_1 in human milk represents 0.09%–0.43% of dietary AFB_1 intake

To obtain some perspective of the intakes of the Egyptian mothers, they can be compared with the average daily intake as calculated by the average contamination in the diet multiplied by the amount of food consumed by women in two high risk regions for aflatoxin exposure i.e. 8 µg AFB_1 in Zhuqing, China (Wang *et al.* 2001), and 15.7 µg total aflatoxins in The Gambia, West Africa (Groopman *et al.* 1992a). In comparison to these values, the dietary aflatoxin exposure level of the Egyptian mothers can be viewed as modest.

Data assembled by Hall and Wild (Hall and Wild 1994) indicate that the range of estimated aflatoxin exposure based on the analysis of aflatoxins in foods was 3.5-14.8 ng/kg bw/day in Kenya, 38.6-183.7 in Mozambique, 16.5 in South Africa, 4-115 in The Gambia, 11.7-2027 in southern Guangxi province of China, and 6.5-53 ng/kg bw /day in Thailand. These exposure rates however, are average estimations based on annual grain consumption, which is appropriate for cancer risk estimates because of the cumulative nature of this response. Doses in the short term may vary significantly from the average

either because of seasonal variability or with variability associated with testing grain products for aflatoxins. The levels of maternal AFB_1 exposure based on median levels of all milk samples in our study (10-49 ng/kg bw/day) are however at comparable levels.

Estimations of potential health risk for the mothers and the breast feeding infants

The results of epidemiological studies have indicated that the dose considered as "virtually safe" or representing a risk level of 1 in 10^5 (giving a single HCC lifetime risk per 100 000 persons from the consumption of AFB_1 contaminated foodstuffs) is 0.19 ng/kg bw/day (Kuiper-Goodman. 1991). Lactating mothers fall into a special category of the population, and in the present study, the mothers were from a selected area known to have significant aflatoxin exposure. Consequently, the exposure estimates cannot be generalized to the general population. However, the potential health risk based on the above carcinogenic potency estimate cannot be assessed as negligible since the above reported safe level is clearly less than the estimated AFB_1 exposure levels of the mothers in our study.

Nevertheless, due to the limited passage of aflatoxins into the milk [0.09%–0.43% of dietary AFB_1 intake is excreted as AFM_1 into the milk (Zarba *et al.* 1992)] the levels of AFM_1 detected in this study fall within the EU maximum permissible levels of AFM_1 in infant ready-to-use milk formula (25 pg AFM_1/ml). The Commission has stated that it is appropriate for the health protection of the infants and young children, a vulnerable population group, to establish the lowest maximum level, that is achievable through a strict selection of the raw materials used for the manufacturing of the infant milk formulae and baby foods (EC 2004). However, for genotoxic carcinogenic substances, such as aflatoxins, there is no threshold value below which the risk value for human health is equal to zero.

6.2. Determinants of AFM_1 in breast milk *(Studies I and II)*

The presence of AFM_1 in the breast milk of the 388 mothers was affected by the working status of the mothers, the number of children, the frequency of corn oil consumption, the body mass index (BMI) of the mothers, and their stage of lactation.

Furthermore, factors affecting the temporal variation of AFM_1 were the month of breast milk sample collection, and the stage of lactation. The mothers that took part in the follow up were initially selected to be AFM_1 positive and subsequently several other factors were found in these women i.e. non working, more than one child already (>1), and a high BMI were also found in the vast majority of these women.

Working status and seasonal effect

The non-working mothers were not employed, but mostly belonged in small rural or share-cropping communities; they were doing farm work and domestic chores. In share-cropping communities, after the crops are harvested, they are left to dry over plastic or synthetic sheets, practices that may promote *Aspergillus* growth and toxin production (Turner *et al.* 2005a). Most of these crops are consumed locally by either the household or by others in the immediate community. In our study, a very strong seasonal effect was evident i.e. the high exposure period of summer corresponds to the hottest season. *Aspergillus* fungi are ubiquitous and they can grow on many foodstuffss whenever conditions allow. Food contamination frequently occurs during post harvest storage (Sylla *et al.* 1999, Turner *et al.* 2000, Wild *et al.* 2000) and presumably, the high risk of AFM_1 presence during summer may reflect more severe aflatoxin exposure due to the accumulation of aflatoxin during storage under conditions promoting growth of *Aspergillus*.

Food consumption

A high BMI seemed to act as an indicator of the amount and type of food consumed including corn oil, with obese mothers consuming corn oil more frequently than the non-obese mothers (Study I). Worldwide, two of the major sources of aflatoxin exposure are groundnuts and corn, thus corn oil must be considered as a potential source of aflatoxin exposure in this region (El-Sayed *et al.* 2003, El-Shanawany *et al.* 2005). The fact that obese mothers had higher AFM_1 levels than the non-obese possibly reflects the frequency of consumption of corn oil. This complicates understanding the contribution of corn oil to breast milk AFM_1 level in our analysis when both factors were included in the analysis. Another theoretical explanation could be that aflatoxins also would be stored in body fat reserves, contributing in this way to higher aflatoxin

levels in breast milk. However, the presence of aflatoxins in animal fat tissue has not been reported (Hayes *et al.* 1977).

During the follow up (Study II), the consumption of specific foods possibly contaminated with aflatoxin (corn and corn oil, cottonseed oil, peanuts) did not significantly relate to AFM_1 presence. This may be due to a relatively steady pattern of food consumption throughout the year with the presence of AFM_1 in breast milk reflecting the level of food contamination and not the frequency of food consumption. In addition, it could be the amount of consumption of specific foods rather than the frequency which may vary throughout the year, though we did not collect this additional dietary information in our questionnaire.

Duration of lactation and number of children

In the cross-sectional part of the study (Study I), the mothers at an early lactation stage (<1 month) were more frequently found to be positive of AFM_1 than the other mothers. These mothers had given birth no more than 15 days before providing the breast milk sample. The relative proportions of milk components is known to vary significantly with the stage of lactation, especially at the beginning of lactation where proteins, fat-soluble vitamins, phospholipids and cholesterol are present in much higher concentrations than they are in the mature milk (Lawrence 1999). It is possible that the changes in fat and protein content are important in terms of accumulation and mobilization of aflatoxins. However, during the follow up period (Study II), the risk of AFM_1 presence was increased when the duration of lactation was longer (range of lactation months 3-29). This observation may reflect time-dependent changes in maternal physiology that alter the amount of toxin passing to the infant (Clewell and Gearhart 2002). These include adipose tissue levels, age, milk composition and volume, gradual weaning, and breast-feeding patterns. The presence of other children in the family (number of children >1) was also associated with a higher risk of AFM_1 presence in milk. Previous childbirths, the number and length of previous lactations, and the period between childbirths, has been reported to affect significantly the concentrations of the highly lipophilic organochlorines stored in human fat (Harris *et al.* 2001). Unfortunately, for aflatoxins this kind of information is lacking in the literature. The

possibility that a high number of children is likely to be an indicator of lower socio-economic status and therefore of a less varied diet cannot be excluded.

6.3. Children's aflatoxin exposure (Studies I and III)

In developing countries, growth faltering is often associated with the quantity and/ or poor quality of foods, in addition to multiple other hazards (WHO 1995). In common with many developing countries (Shrimpton *et al.* 2001), Egyptian infants had a high frequency of stunting (height to age z-score ≤ -2, length faltering) (35.7%) and a moderate frequency of being underweight (weight to age z-score ≤ -2, weight faltering) (14.4%), based on WHO (1995) criteria. Older infants had lower HAZ and WAZ scores than the young children (Figure 25). Length faltering started immediately after birth, while weight faltering started at 3 months of age, after having been parallel to the reference for the first three months. This observed strong positive association between impaired growth and age is to be expected in developing country setting (Shrimpton *et al.* 2001).

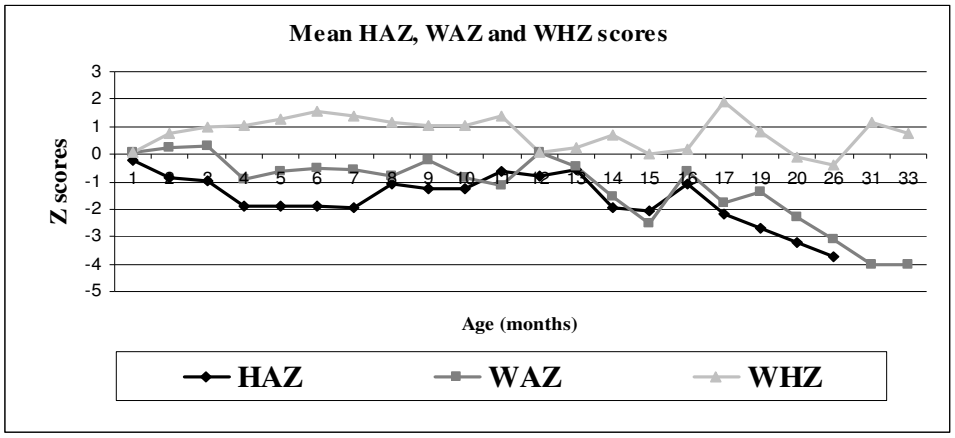


Figure 25. Egyptian children's growth. Mean weight for height Z-score (WHZ) (n= 351), weight for age Z-score (WAZ) (n= 375), and height for age Z-score (HAZ) (n= 322) by age (Study I).

It has been claimed that high levels of aflatoxin-albumin adducts may be associated with growth faltering in Beninese children, with a marked increase in aflatoxin exposure

occurring when children were weaned onto solid foods (Gong *et al.* 2003, 2004). Unfortunately, in the cross-sectional study (*Study I*), we did not have access to biomarkers of infant aflatoxin exposure in order to evaluate the contribution of aflatoxins to the growth impairment in the Egyptian fully breast fed (n=182) or partially breast fed (n=202) children. The modest maternal breast milk levels of AFM_1 would indicate that overall exposures from milk or weaning foods appear to be relatively low.

The levels of aflatoxin exposure of 50 of the above children was assessed and compared to the exposure of 50 Guinean children by measurement of aflatoxin metabolites in urine. Aflatoxins were less frequently present in Egyptian than in Guinean children (38% vs. 86%) with statistically significant differences in the prevalence for most of the detected aflatoxins: AFB_1 (2% vs. 16%, p= 0.016), AFB_2 (10% vs. 58%, p= 0.000), AFG_1 (4% vs. 2%), AFG_2 (24% vs. 36%, p= 0.190) and AFM_1 (8% vs. 64%, p= 0.000). For AFM_1 the mean level in Guinean samples was 18-fold higher than the corresponding value in Egyptian samples.

The Guinean children were all HBV positive while the Egyptian children were all HBV negative as determined from HBsAg in serum. It has been previously reported that HBV infection significantly influences the AF-albumin adduct levels; HBV positive Gambian children having a higher level of AF-albumin than HBV negative children (Turner *et al.* 2000). These authors proposed that liver injury could be an important modifier of aflatoxin metabolism and thus young children may be more severely affected than adults by HBV infection. However, in our study, the highly significant difference in the frequency and levels of exposures between these two regions is probably unlikely to be explained simply by a difference in HBV status of these children since though HBV status might possibly have a modest effect on the AFM_1 metabolite, it is most unlikely to alter the levels of excretion of the parent compounds AFB_1, AFB_2, AFG_1, AFG_2 in urine.

From the 50 Egyptian children, 30 children were fully weaned, and 20 were partially receiving breast feeding. 30% (6/20) of the partially breast-fed children were positive for aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2), with all children being negative with respect of AFM_1 in urine. In all, 43% (13/30) of the fully weaned were positive to aflatoxins. All Guinean children were fully weaned. Weaning status has been previously reported to contribute significantly towards higher aflatoxin exposures (Gong *et al.* 2003, 2004)

and partial breast-feeding may to some extent have protected the Egyptian children against higher exposures, due to the limited passage of aflatoxins into breast milk.

A good correlation has been reported between AFB_1 intake and urinary excretion of AFM_1 (Zhu *et al.* 1987, Groopman *et al.* 1992a) and AFM_1 has been used as an index for human exposure of AFB_1 in molecular epidemiology studies. Based on average AFM_1 levels [arithmetic means; Egypt 5.2 pg/ml urine (range 4-6.2) and Guinea 97 pg/ml urine, (range 8-801)], the AFB_1 daily exposure was estimated to be 0.2 and 3.8 µg for Egypt and Guinea, respectively. This calculation was based on the assumption that 1.5% of AFB_1 intake is excreted in the urine in the form of AFM_1 (Zhu *et al.* 1987) and that the average daily urinary volume of a 1.5-2 year old child is approximately 600 ml (Goellner *et al*., 1981) In the literature, there is a scarcity of urinary biomarker data and levels of aflatoxin exposure in children. Therefore comparisons can only be made with levels of adult aflatoxin exposure (Table 19).

Table 19. Comparison of the calculated AFB_1 children exposure with the reported AFB_1 exposure in adults.

Country	Aflatoxin daily exposure	Reference
Egypt	0.2*µg AFB_1 Egypt, children	Study I
Guinea	3.8* µg AFB_1 Guinea, children	Study III
Gambia	8.2 µg, adult men (total aflatoxins) 15.7 µg, adult women (total aflatoxins)	(Groopman *et al.* 1992a)
China	48.4 µg AFB_1, adult men 77.4 µg AFB_1, adult women	(Groopman *et al.* 1992c)
China	14 µg AFB_1, adult men 8 µg AFB_1, adult women	(Wang *et al.* 2001)

*calculations are based on average urinary AFM_1 levels (arithmetic mean), on the assumption that 1.5% of AFB_1 is excreted as AFM_1 in urine (Zhu *et al.* 1987) and that the total daily urinary volume for children aged 1.5-2 years is 600 ml (Goellner *et al*. 1981).

The presence of AFB_1 and AFG_1 was lower for both Guinea (16 and 2%, respectively) and Egypt (2 and 4%) in comparison to AFB_2 and AFG_2 (58 and 36% in Guinea and 10 and 24% in Egypt). The lower frequencies of both AFB_1 and AFG_1 in the urine mostly likely reflect the higher conversion of these dietary aflatoxins to other metabolites in the liver. However, the arithmetic mean urinary levels of AFB_1 and AFG_1 (2682 and 709

pg/ml in Guinea, 189 and 76 pg/ml in Egypt) were higher than those of AFB_2 and AFG_2 (5.7 and 19 pg/ml in Guinea, 1.4 and 2.2 pg/ml in Egypt). This may reflect the fact that in aflatoxin contaminated samples, AFB_1 is the most frequent type of toxin being present in high amounts, followed by AFG_1. AFB_2 and AFG_2 are typically present in much lower quantities (IARC 1993). The *A. paraciticus* species producing AFB_1, AFB_2, AFG_1 and AFG_2 are known to be widely distributed in Africa (IARC 1993). Nevertheless, the difference in urinary frequency will also be significantly influenced by the greater sensitivity of detection of AFB_2 and AFG_2 compared to AFB_1 and AFG_1 (see materials and methods for the limit of detection of each aflatoxin metabolite).

6.4. Probiotic intervention (Studies IV and V)

The primary goal of the intervention study was to determine whether administration of a commercially available mixture of probiotic bacteria could prevent/reduce the absorption of AFB_1 from the small intestine (*Study V*). The results of the intervention were assessed on the basis of AFB_1-N^7-guanine in urine that was used as a marker of the biologically effective dose of AFB_1. The median baseline AFB_1-N^7-guanine values were 0.38 ng/ml (IQR 0.0 to 2.15) (*Study IV*); these levels are similar to those reported from other studies (Table 20).

Table 20. The AFB_1-N^7-guanine excreted in the urine of young Chinese males in comparison to other reported urinary levels in adults.

Country	AFB_1-N^7-guanine Range (ng/ml)	Reference
China	0.0-2.15	Study IV
China	0.3-1.81	(Qian *et al.* 1994)
China	0.04-1.27*	(Wang *et al.* 2001)
Gambia	0.03-5*	(Groopman *et al.* 1992a)

*values were given as ng/day and expression as ng/ml are based on the assumption that the average adult daily urinary volume is 1600 ml (JEFCA 1998).

Studies on the correspondence between individual dietary AFB_1 exposure and the excretion of AFB_1-N^7-guanine in the urine have shown a dose-dependent relationship

between these two parameters (Groopman *et al.* 1992a, 1992b, 1992c) with about 0.2% of the oral AFB_1 dose being excreted as AFB_1-N^7-guanine in urine (Groopman *et al.* 1992c). Based on this estimation and on the assumption that average adult daily volume is 1600 ml (JEFCA 1998), the average dietary AFB_1 intake of the Chinese students is calculated to be 304 µg/day or 5µg/kg bw/day. This value represents the exposure level of a group of selected Chinese subjects with verified aflatoxin exposure as indicated by the presence of AFM_1 in their urine, and therefore is higher than other reported values for the Chinese population (Table 20).

The levels of AFB_1-N^7-guanine adducts in the urine reflect the formation of AF-DNA promutagenic liver lesions involved in the cancer initiation process (Smela *et al.* 2001). Elevated urinary excretion of this aflatoxin-DNA adduct has been associated with an increased risk of HCC (Qian *et al.* 1994) and the importance of AFB_1-N^7-guanine relative to other urinary aflatoxin biomarkers as a predictor of HCC risk has been clearly shown from Qian et al (1994). The presence of the AFB_1-N^7-guanine adduct together with other AFB_1 metabolites (AFM_1, AFQ_1, AFP_1) significantly elevated the risk of HCC compared to the presence of the other metabolites alone.

Diminished concentrations of AFB_1-N^7-guanine have been associated with a reduced risk in HCC in chemoprevention studies in animals (Roebuck *et al.* 1991). Loeb (2001) stated that a 2-fold reduction in mutation rates (as could be anticipated from the decline in DNA adduct burden achieved in the current study) could prolong the time between initiation and clinical manifestations of cancer from 20 or more years to 40 or more years. Given that the median age of diagnosis for HCC in many developing countries is less than 50 years (Hall and Wild 2003), such a delay could have a major impact in high risk areas.

The results of this intervention clearly revealed probiotic supplementation as a feasible means to reduce the biologically effective dose of aflatoxin in individuals with detectable aflatoxin exposure, providing a potentially effective approach in the reduction of the risk of HCC development in high risk regions. In addition, probiotic bacteria have by definition a beneficial impact on the health of the host that may be advantageous especially in a developing country setting where the population is exposed to multiple infectious and nutritional hazards.

7. SUMMARY AND CONCLUSIONS

In the present work, we 1) investigated the patterns and levels of maternal and child aflatoxin exposure in a selected group of mothers and children in Egypt, 2) compared the exposure levels of Egyptian children with children from Guinea, a country with a recognized high aflatoxin exposure, and 3) assessed the effectiveness of probiotic supplementation in reduction of the biologically effective dose of aflatoxin exposure in a highly exposed region of southeastern China. From this work, the following conclusions can be drawn:

- Aflatoxin contamination of breast milk in the Egyptian mothers was frequent although at moderate levels. Several socioeconomic, demographic, dietary or environmental factors affected the presence and levels of AFM_1 in breast milk, with the most dominant being a seasonal effect. This kind of seasonality of aflatoxin exposure has been repeatedly reported in the literature.

- In the comparison of the Egyptian and Guinean children, the aflatoxin exposure of the Guinean children was more prevalent and occured at higher levels.

- The results of the intervention in young Chinese adults clearly showed that probiotic supplementation could impair the bioavailability of dietary aflatoxins and reduce their biologically effective dose.

Overall, these results highlight that measures to reduce aflatoxin exposure in infants, young children, and young adults in developing countries are important, while probiotic supplementation could provide an affordable and feasible means to reduce the aflatoxin exposure and possibly the incidence of liver cancer in aflatoxin endemic areas.
Breast milk is without doubt the best type of infant nutrition, providing multiple advantages for the developing child. Additionally, extended breast feeding may play a protective role against higher aflatoxin exposure through the delayed introduction of weaning foods which may have a higher aflatoxin content. In Egypt, weaning foods are the foods consumed by the rest of the household, and the fact that the mothers were

exposed means also that children would also be exposed when they start to consume the same diet as the other family members. However, AFM_1 is carcinogenic and cytotoxic with potentially important implications on immunocompetence and growth and possibly also on disease risk later in life. It is therefore advantageous if the AFM_1 levels in breast milk could be kept as low as possible. For genotoxic carcinogenic substances such as aflatoxins, there is no threshold value below which the risk value for human health is equal to zero. Moreover, babies, infants and young children are critical groups when considering exposure to contaminants such as aflatoxins, since they consume large quantities of food in comparison to their body weight.

The identification and understanding of factors determining the presence of toxicants in human milk is important for the implementation of intervention strategies aimed at reducing aflatoxin exposure. An intervention utilizing probiotic bacteria to reduce maternal exposure would be predicted to have a beneficial impact on the fetus and on the infant's exposure to AFM_1 in breast milk. This may be best achieved by incorporating into the diet traditional foods fermented with selected aflatoxin binding starter bacteria. The results of this present work have been utilized in the planning and design of a probiotic intervention in mothers and young infants currently ongoing in Egypt.

8. REFERENCES

Abdelhamid AM. Occurrence of some mycotoxins (aflatoxin, ochratoxin A, citrinin, zearalenone and vomitoxin) in various Egyptian feeds. Arch Tierernahr 1990; 40(7): 647-64.

Abdulrazzaq YM, Osman N, Ibrahim A. Fetal exposure to aflatoxins in the United Arab Emirates. Ann Trop Paediatr 2002; 22(1): 3-9.

Abdulrazzaq YM, Osman N, Yousif ZM, Al-Falahi S. Aflatoxin M1 in breast-milk of UAE women. Ann Trop Paediatr 2003; 23(3): 173-9.

Allcroft R, Carnaghan RBA. Groundnut toxicity: An examination for toxin in human food products from animals fed toxic groundnut meal. Vet. Rec. 1963; 75: 259.

Al-Saadany A, El-Hennawy S, Amra H, Iman Abd El-Reheim and Ezzat, S A study of aflatoxins and Kwashiorkor in Benha. Benha Medical Journal 1993; 10(3): 125-138.

AOAC. Aflatoxins M_1 and M_2 in fluid milk. Natural Toxins. Scott PM. Arlington, VA, J Assoc Off Anal Chem 1995;49: 34-35.

Bailey EA, Iyer RS, Stone MP, Harris TM, Essigmann JM. Mutational properties of the primary aflatoxin B1-DNA adduct. Proc Natl Acad Sci USA 1996; 93(4): 1535-9.

Barrett J. Liver Cancer and Aflatoxin: New Information from the Kenyan Outbreak. Environ Health Perspect 2005; 113(12): A837–A838.

Battacone G, Nudda A, Cannas A, Cappio Borlino A, Bomboi G, Pulina G. Excretion of aflatoxin M_1 in milk of dairy ewes treated with different doses of aflatoxin B_1. J Dairy Sci 2003; 86(8): 2667-75.

Battacone G, Nudda A, Palomba M, Pascale M, Nicolussi P, Pulina G. Transfer of aflatoxin B_1 from feed to milk and from milk to curd and whey in dairy sheep fed artificially contaminated concentrates. J Dairy Sci 2005; 88(9): 3063-9.

Becroft DM. Syndrome of encephalopathy and fatty degeneration of viscera in New Zealand children. Br Med J 1966; 5506: 135-40.

Bennett JW, Klich M. Mycotoxins. Clin Microbiol Rev 2003; 16(3): 497-516.

Bressac B, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. Nature 1991; 350(6317): 429-31.

Buchi G, Luk K, Muller PM. Synthesis of aflatoxin Q_1. J Org Chem 1975; 40(23): 3458-9.

Busby WF, Wogan GN. Aflatoxins (Chapter 16). Chemical Carcinogens. Seattle CE. Washington DC, American Chemical Society. 1984;2: 946-1135.

Chao TC, Maxwell SM, Wong SY. An outbreak of aflatoxicosis and boric acid poisoning in Malaysia: a clinicopathological study. J Pathol 1991; 164(3): 225-33.

Chen CJ, Chen DS. Interaction of hepatitis B virus, chemical carcinogen, and genetic susceptibility: multistage hepatocarcinogenesis with multifactorial etiology. Hepatology 2002; 36(5): 1046-9.

Chen CJ, Yu MW, Liaw YF. Epidemiological characteristics and risk factors of hepatocellular carcinoma. J Gastroenterol Hepatol 1997; 12(9-10): S294-308.

Clewell RA, Gearhart JM. Pharmacokinetics of toxic chemicals in breast milk: use of PBPK models to predict infant exposure. Environ Health Perspect 2002; 110(6): A333-7.

Coulombe RA, Sharma RP. Clearance and excretion of intratracheally and orally administered aflatoxin B1 in the rat. Food Chem Toxicol 1985; 23(9): 827-30.

Coulombe RA, Shelton DW, Sinnhuber RO, Nixon JE. Comparative mutagenicity of aflatoxins using a Salmonella/trout hepatic enzyme activation system. Carcinogenesis 1982; 3(11): 1261-4.

Coulter JB, Lamplugh SM, Suliman GI, Omer MI, Hendrickse RG. Aflatoxins in human breast milk. Ann Trop Paediatr 1984; 4(2): 61-6.

Cullen JM, Ruebner BH, Hsieh LS, Hyde DM, Hsieh DP. Carcinogenicity of dietary aflatoxin M1 in male Fischer rats compared to aflatoxin B_1. Cancer Res 1987; 47(7): 1913-7.

Cusumano V, Costa GB, Trifiletti R, Merendino RA, Mancuso G. Functional impairment of rat Kupffer cells induced by aflatoxin B1 and its metabolites. FEMS Immunol Med Microbiol 1995; 10(2): 151-5.

Dashwood R, Negishi T, Hayatsu H, Breinholt V, Hendricks J, Bailey G. Chemopreventive properties of chlorophylls towards aflatoxin B1: a review of the antimutagenicity and anticarcinogenicity data in rainbow trout. Mutat Res 1998; 399(2): 245-53.

De Longh H, Vles RO, Van Pelt JG. Investigation of the milk in mammals fed on aflatoxin containing diet. Nature 1964; 202: 466.

Denning DW, Allen R, Wilkinson AP, Morgan MR. Transplacental transfer of aflatoxin in humans. Carcinogenesis 1990; 11(6): 1033-5.

Diallo MS, Sylla A, Sidibe K, Sylla BS, Trepo CR, Wild CP. Prevalence of exposure to aflatoxin and hepatitis B and C viruses in Guinea, West Africa. Nat Toxins 1995; 3(1): 6-9.

Dvorackova I, Kusak V, Vesely D, Vesela J, Nesnidal P. Aflatoxin and encephalopathy with fatty degeneration of viscera (Reye). Ann Nutr Aliment 1977; 31(4-6): 977-89.

Eaton DL, Bammler TK, Kelly EJ. Interindividual differences in response to chemoprotection against aflatoxin-induced hepatocarcinogenesis: implications for human biotransformation enzyme polymorphisms. Adv Exp Med Biol 2001; 500: 559-76

Eaton DL, Ramsdell HS, Neal GE. Biotransformation of Aflatoxins. Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance. Eaton DL and Groopman JD. San Diego, Academic Press, Inc. 1994: 45-71.

(EC). COMMISSION REGULATION No 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union 2001.

(EC). COMMISSION REGULATION No 683/2004. Amending Regulation No 466/2001 as regards aflatoxins and ochratoxin A in foods for infants and young children. Official Journal of the European Union 2004.

(EC). COUNCIL DIRECTIVE 1999/29/EC of 22 April 1999 on the undesirable substances and products in animal nutrition. Official Journal of the European Union 1999.

Egal S, Hounsa A, Gong YY, Turner PC, Wild CP, Hall AJ, Hell K, Cardwell KF. Dietary exposure to aflatoxin from maize and groundnut in young children from Benin and Togo, West Africa. Int J Food Microbiol 2005; 104(2): 215-24.

Egner PA, Wang JB, Zhu YR, Zhang BC, Wu Y, Zhang QN, Qian GS, Kuang SY, Gange SJ, Jacobson LP, Helzlsouer KJ, Bailey GS, Groopman JD, Kensler TW. Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer. Proc Natl Acad Sci U S A 2001; 98(25): 14601-6.

Egner PA, Yu X, Johnson JK, Nathasingh CK, Groopman JD, Kensler TW, Roebuck BD. Identification of aflatoxin M_1-N^7-guanine in liver and urine of tree shrews and rats following administration of aflatoxin B1. Chem Res Toxicol 2003; 16(9): 1174-80.

El-Kady IA, El-Maraghy SS, Eman Mostafa M. Natural occurrence of mycotoxins in different spices in Egypt. Folia Microbiol (Prague) 1995; 40(3): 297-300.

El-Nezami H, Kankaanpaa P, Salminen S, Ahokas J. Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B1. Food Chem Toxicol 1998a; 36(4): 321-6.

El-Nezami H, Kankaanpaa P, Salminen S, Ahokas J. Physicochemical alterations enhance the ability of dairy strains of lactic acid bacteria to remove aflatoxin from contaminated media. J Food Prot 1998b; 61(4): 466-8.

El-Nezami H, Mykkänen H, Kankaanpaa P, Salminen S, Ahokas J. Ability of Lactobacillus and Propionibacterium strains to remove aflatoxin B, from the chicken duodenum. J Food Prot 2000a; 63(4): 549-52.

El-Nezami H, Mykkänen H, Kankaanpaa P, Suomalainen T, Salminen S, Ahokas J. Ability of a mixture of *lactobacillus* and *propionibacterium* to influence the faecal aflatoxin content in healthy Egyptian volunteers: a pilot clinical study. Bioscience Microflora 2000b; 19(1): 41-5.

El-Nezami H, Nicoletti G, Neal GE, Donohue DC, Ahokas JT. Aflatoxin M1 in human breast milk samples from Victoria, Australia and Thailand. Food Chem Toxicol 1995; 33(3): 173-9.

El-Sayed AM, Soher EA, Sahab AF. Occurrence of certain mycotoxins in corn and corn-based products and thermostability of fumonisin B1 during processing. Nahrung 2003; 47(4): 222-5.

El-Shanawany AA, Mostafa ME, Barakat A. Fungal populations and mycotoxins in silage in Assiut and Sohag governorates in Egypt, with a special reference to characteristic Aspergilli toxins. Mycopathologia 2005; 159(2): 281-9.

El-Shewey EA. Some Toxicological Studies on contaminated breast milk by aflatoxins. Faculty of Medicine. Egypt, Zagazig University, Benha Branch. 1992: 170.

El-Zayadi AR, Badran HM, Barakat EM, Attia Mel D, Shawky S, Mohamed MK, Selim O, Saeid A. Hepatocellular carcinoma in Egypt: a single center study over a decade. World J Gastroenterol 2005; 11(33): 5193-8.

Essigmann JM, Croy RG, Bennett RA, Wogan GN. Metabolic activation of aflatoxin B1: patterns of DNA adduct formation, removal, and excretion in relation to carcinogenesis. Drug Metab Rev 1982; 13(4): 581-602.

Essigmann JM, Croy RG, Nadzan AM, Busby WF, Reinhold VN, Buchi G, Wogan GN. Structural identification of the major DNA adduct formed by aflatoxin B1 in vitro. Proc Natl Acad Sci USA 1977; 74(5): 1870-4.

Fandohan P, Zoumenou D, Hounhouigan DJ, Marasas WF, Wingfield MJ, Hell K. Fate of aflatoxins and fumonisins during the processing of maize into food products in Benin. Int J Food Microbiol 2005; 98(3): 249-59.

Forrester LM, Neal GE, Judah DJ, Glancey MJ, Wolf CR. Evidence for involvement of multiple forms of cytochrome P-450 in aflatoxin B_1 metabolism in human liver. Proc Natl Acad Sci USA 1990; 87(21): 8306-10.

Gan LS, Skipper PL, Peng XC, Groopman JD, Chen JS, Wogan GN, Tannenbaum SR. Serum albumin adducts in the molecular epidemiology of aflatoxin carcinogenesis: correlation with aflatoxin B_1 intake and urinary excretion of aflatoxin M1. Carcinogenesis 1988; 9(7): 1323-5.

Girgis AN, El-Sherif S, Rofael N, Nesheim S. Aflatoxins in Egyptian foodstuffs. J Assoc Off Anal Chem 1977; 60(3): 746-7.

Goellner M, Ziegler E, Fomon S. Urination during the first three years of life. Nephron 1981; 28(4): 174-8.

Gong YY, Cardwell K, Hounsa A, Egal S, Turner PC, Hall AJ, Wild CP. Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: cross sectional study. Br Med J 2002; 325(7354): 20-1.

Gong YY, Egal S, Hounsa A, Turner PC, Hall AJ, Cardwell KF, Wild CP. Determinants of aflatoxin exposure in young children from Benin and Togo, West Africa: the critical role of weaning. Int J Epidemiol 2003; 32(4): 556-62.

Gong YY, Hounsa A, Egal S, Turner PC, Sutcliffe AE, Hall AJ, Cardwell K, Wild CP. Postweaning exposure to aflatoxin results in impaired child growth: a longitudinal study in Benin, West Africa. Environ Health Perspect 2004; 112(13): 1334-8.

Gopalakrishnan S, Stone MP, Harris TM. Preparation and Characterization of an Aflatoxin-B1 Adduct with the Oligodeoxynucleotide D(Atcgat)2. J Am Chem Soc 1989; 111(18): 7232-7239.

Gratz S, Mykkanen H, El-Nezami H. Aflatoxin B1 binding by a mixture of Lactobacillus and Propionibacterium: in vitro versus ex vivo. J Food Prot 2005; 68(11): 2470-4.

Gratz S, Mykkanen H, Ouwehand AC, Juvonen R, Salminen S, El-Nezami H. Intestinal mucus alters the ability of probiotic bacteria to bind aflatoxin B1 in vitro. Appl Environ Microbiol 2004; 70(10): 6306-8.

Groopman J. Molecular dosimetry methods for assessing human aflatoxin exposures. Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance. Eaton DL and Groopman JD. San Diego, Academic Press, Inc. 1994: 259-277.

Groopman JD, Donahue PR, Zhu JQ, Chen JS, Wogan GN. Aflatoxin metabolism in humans: detection of metabolites and nucleic acid adducts in urine by affinity chromatography. Proc Natl Acad Sci USA 1985; 82(19): 6492-6.

Groopman JD, Hall AJ, Whittle H, Hudson GJ, Wogan GN, Montesano R, Wild CP. Molecular dosimetry of aflatoxin-N7-guanine in human urine obtained in The Gambia, West Africa. Cancer Epidemiol Biomarkers Prev 1992a; 1(3): 221-7.

Groopman JD, Hasler JA, Trudel LJ, Pikul A, Donahue PR, Wogan GN. Molecular dosimetry in rat urine of aflatoxin-N7-guanine and other aflatoxin metabolites by multiple monoclonal antibody affinity chromatography and immunoaffinity/high performance liquid chromatography. Cancer Res 1992b; 52(2): 267-74.

Groopman JD, Kensler TW. The light at the end of the tunnel for chemical-specific biomarkers: daylight or headlight? Carcinogenesis 1999; 20(1): 1-11.

Groopman JD, Zhu JQ, Donahue PR, Pikul A, Zhang LS, Chen JS, Wogan GN. Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in Guangxi Autonomous Region, People's Republic of China. Cancer Res 1992c; 52(1): 45-52.

Guengerich FP. Cytochrome P-450 3A4: regulation and role in drug metabolism. Ann Rev Pharmacol Toxicol 1999; 39: 1-17.

Guengerich FP, Arneson KO, Williams KM, Deng Z, Harris TM. Reaction of aflatoxin b(1) oxidation products with lysine. Chem Res Toxicol 2002; 15(6): 780-92.

Guengerich FP, Johnson WW, Shimada T, Ueng YF, Yamazaki H, Langouet S. Activation and detoxication of aflatoxin B1. Mutat Res 1998; 402(1-2): 121-8.

Hall AJ, Wild CP. Epidemiology of Aflatoxin-Related disease. Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance. Eaton DL and Groopman JD. San Diego, Academic Press, Inc. 1994: 233-279.

Hall AJ, Wild CP. Liver cancer in low and middle income countries. Br Med J 2003; 326(7397): 994-5.

Harris CA, Woolridge MW, Hay AW. Factors affecting the transfer of organochlorine pesticide residues to breastmilk. Chemosphere 2001; 43(2): 243-56.

Haskard CA, El-Nezami HS, Kankaanpaa PE, Salminen S, Ahokas JT. Surface binding of aflatoxin B(1) by lactic acid bacteria. Appl Environ Microbiol 2001; 67(7): 3086-91.

Hatem NL, Hassab HM, Abd Al-Rahman EM, El-Deeb SA, El-Sayed Ahmed RL. Prevalence of aflatoxins in blood and urine of Egyptian infants with protein-energy malnutrition. Food Nutr Bull 2005; 26(1): 49-56.

Hayes JR, Polan CE, Campbell TC. Bovine liver metabolism and tissue distribution of aflatoxin B1. J Agric Food Chem 1977; 25(5): 1189-93.

Hendrickse RG. Of sick turkeys, kwashiorkor, malaria, perinatal mortality, heroin addicts and food poisoning: research on the influence of aflatoxins on child health in the tropics. Ann Trop Med Parasitol 1997; 91(7): 787-93.

Holzapfel CW, Steyn PS, Purchase IFH. Isolation and structure of aflatoxins M_1 and M_2. Tetrahedron Lett 1966; 25: 2799.

Holzapfel WH, Geisen R, Schillinger U. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int J Food Microbiol 1995; 24(3): 343-62.

Househam KC, Hundt HK. Aflatoxin exposure and its relationship to kwashiorkor in African children. J Trop Pediatr 1991; 37(6): 300-2.

Hsieh D, Wong JJ. Pharmacokinetics and Excretion of Aflatoxins. The Toxicology of Aflatoxins: Human Health, Veterinary and Agricultural Significance. Eaton DL and Groopman J. New York, Academic Press. 1994.

Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature 1991; 350(6317): 427-8.

IARC. Some naturally occurring substances - food items and constituents, heterocyclic aromatic amines and mycotoxins. World Health Organization, International Agency for Research on Cancer. IARC Monogr Eval Carcinog Risks Hum. Lyon, France. 1993; 56. 245-391.

IARC. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. World Health Organization, International Agency for Research on Cancer. IARC Monogr Eval Carcinog Risks Hum. Lyon, France. 2002; 82. 1-556.

Iyer RS, Coles BF, Raney KD, Thier R, Guengerich FP, Harris TM. DNA adduction by the potent carcinogen Aflatoxin B1: mechanistic studies. J Am Chem Soc 1994; 116(5): 1603-1609.

Iyer RS, Harris TM. Preparation of aflatoxin B1 8,9-epoxide using m-chloroperbenzoic acid. Chem Res Toxicol 1993; 6(3): 313-6.

JEFCA. Aflatoxin M1 (JEFCA evaluation). Joint FAO/WHO Expert Committee on Food Additives. Food additives series 47, Toxicological monographs. Geneva. 2000; 47.

JEFCA. Safety evaluation of certain food additives and contaminants (JEFCA evaluation). Joint FAO/WHO Expert Committee on Food Additives. Food additives series 40, Toxicological monographs. Geneva. 1998; 40.

Jensen AA, Slorach SA. Chemical contaminants into human milk. Florida, United States., CRC Press, Inc. 1991; 1-298.

Jiang Y, Jolly PE, Ellis WO, Wang JS, Phillips TD, Williams JH. Aflatoxin B1 albumin adduct levels and cellular immune status in Ghanaians. Int Immunol 2005; 17(6): 807-14.

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(12): 6121-5.

Johnston DS, Stone MP. Replication of a site-specific trans-8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B(1) adduct by the exonuclease deficient klenow fragment of DNA polymerase I. Chem Res Toxicol 2000; 13(11): 1158-64.

Kankaanpää P, Tuomola E, El-Nezami H, Ahokas J, Salminen SJ. Binding of aflatoxin B1 alters the adhesion properties of Lactobacillus rhamnosus strain GG in a Caco-2 model. J Food Prot 2000; 63(3): 412-4.

Kensler TW, Davies EF, Bolton MG. Strategies for chemoprevention against aflatoxin- induced liver cancer. Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance. Eaton DL and Groopman JD. San Diego, Academic Press, Inc. 1994: 281-306.

Kensler TW, He X, Otieno M, Egner PA, Jacobson LP, Chen B, Wang JS, Zhu YR, Zhang BC, Wang JB, Wu Y, Zhang QN, Qian GS, Kuang SY, Fang X, Li YF, Yu LY, Prochaska HJ, Davidson NE, Gordon GB, Gorman MB, Zarba A, Enger C, Munoz A, Helzlsouer KJ, et al. Oltipraz chemoprevention trial in Qidong, People's Republic of China: modulation of serum aflatoxin albumin adduct biomarkers. Cancer Epidemiol Biomarkers Prev 1998; 7(2): 127-34.

Kew MC. Synergistic interaction between aflatoxin B1 and hepatitis B virus in hepatocarcinogenesis. Liver Int 2003; 23(6): 405-9.

Kirk GD, Camus-Randon AM, Mendy M, Goedert JJ, Merle P, Trepo C, Brechot C, Hainaut P, Montesano R. Ser-249 p53 mutations in plasma DNA of patients with hepatocellular carcinoma from The Gambia. J Natl Cancer Inst 2000; 92(2): 148-53.

Krishna DR, Klotz U. Extrahepatic metabolism of drugs in humans. Clin Pharmacokinet 1994; 26(2): 144-60.

Krishnamachari KA, Bhat RV, Nagarajan V, Tilak TB. Hepatitis due to aflatoxicosis. An outbreak in Western India. Lancet 1975; 1(7915): 1061-3.

Kuiper-Goodman. T. Approaches to the risk assessment of mycotoxins in foods: aflatoxins. Mycotoxins, Cancer, and Health. Bray GA and Ryan DH, Louisiana State University Press. 1991: 352.

Kumagai S. Intestinal absorption and excretion of aflatoxin in rats. Toxicol Appl Pharmacol 1989; 97(1): 88-97.

Lafont P, Platzer N, Siriwardana MG, Sarfati J, Mercier J, Lafont J. Un nouvel hydroxy-derive de l'aflatoxine B_1: l'aflatoxine M_4. Production *in vitro* - Structure. Microbiol Alim Nut 1986; 4: 65-74.

Lamplugh SM, Hendrickse RG, Apeagyei F, Mwanmut DD. Aflatoxins in breast milk, neonatal cord blood, and serum of pregnant women. Br Med J 1988; 296(6627): 968.

Landrigan PJ. Risk assessment for children and other sensitive populations. Ann NY Acad Sci 1999; 895: 1-9.

Lawrence RA. Breastfeeding: a guide for the medical profession. 5th edition. St.Louis, London, Mosby. 1999.

Li FQ, Yoshizawa T, Kawamura O, Luo XY, Li YW. Aflatoxins and fumonisins in corn from the high-incidence area for human hepatocellular carcinoma in Guangxi, China. J Agric Food Chem 2001; 49(8): 4122-6.

Lin JK, Miller JA, Miller EC. 2,3-Dihydro-2-(guan-7-yl)-3-hydroxy-aflatoxin B1, a major acid hydrolysis product of aflatoxin B1-DNA or -ribosomal RNA adducts formed in hepatic microsome-mediated reactions and in rat liver in vivo. Cancer Res 1977; 37(12): 4430-8.

Loeb LA. A mutator phenotype in cancer. Cancer Res 2001; 61(8): 3230-9.

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(1): 68-72.

McGlynn KA, Tsao L, Hsing AW, Devesa SS, Fraumeni JF, Jr. International trends and patterns of primary liver cancer. Int J Cancer 2001; 94(2): 290-6.

Miller MD, Marty MA, Arcus A, Brown J, Morry D, Sandy M. Differences between children and adults: implications for risk assessment at California EPA. Int J Toxicol 2002; 21(5): 403-18.

Muture BN, Ogana G. Aflatoxin levels in maize and maize products during the 2004 food poisoning outbreak in Eastern Province of Kenya. East Afr Med J 2005; 82(6): 275-9.

Neal GE, Eaton DL, Judah DJ, Verma A. Metabolism and toxicity of aflatoxins M1 and B1 in human-derived in vitro systems. Toxicol Appl Pharmacol 1998; 151(1): 152-8.

Neal GE, Nielsch U, Judah DJ, Hulbert PB. Conjugation of model substrates or microsomally-activated aflatoxin B1 with reduced glutathione, catalysed by cytosolic glutathione-S-transferases in livers of rats, mice and guinea pigs. Biochem Pharmacol 1987; 36(24): 4269-76.

Nesbitt BF, O'Kelly J, Sargeant K, Sheridan A. Aspergillus flavus and turkey X disease. Toxic metabolites of Aspergillus flavus. Nature 1962; 195: 1062-3.

Ngindu A, Johnson BK, Kenya PR, Ngira JA, Ocheng DM, Nandwa H, Omondi TN, Jansen AJ, Ngare W, Kaviti JN, Gatei D, Siongok TA. Outbreak of acute hepatitis caused by aflatoxin poisoning in Kenya. Lancet 1982; 1(8285): 1346-8.

Omiecinski CJ, Remmel RP, Hosagrahara VP. Concise review of the cytochrome P450s and their roles in toxicology. Toxicol Sci 1999; 48(2): 151-6.

Ouwehand AC, Salminen S, Isolauri E. Probiotics: an overview of beneficial effects. Antonie Van Leeuwenhoek 2002; 82(1-4): 279-89.

Patterson DS, Glancy EM, Roberts BA. The 'carry over' of aflatoxin M1 into the milk of cows fed rations containing a low concentration of aflatoxin B1. Food Cosmet Toxicol 1980; 18(1): 35-7.

Peltonen K, el-Nezami H, Haskard C, Ahokas J, Salminen S. Aflatoxin B1 binding by dairy strains of lactic acid bacteria and bifidobacteria. J Dairy Sci 2001; 84(10): 2152-6.

Qian GS, Ross RK, Yu MC, Yuan JM, Gao YT, Henderson BE, Wogan GN, Groopman JD. 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(1): 3-10.

Raisuddin S, Singh KP, Zaidi SI, Paul BN, Ray PK. Immunosuppressive effects of aflatoxin in growing rats. Mycopathologia 1993; 124(3): 189-94.

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 detoxication product of aflatoxin B1. Chem Res Toxicol 1992; 5(2): 202-10.

Roebuck BD, Liu YL, Rogers AE, Groopman JD, Kensler TW. Protection against aflatoxin B1-induced hepatocarcinogenesis in F344 rats by 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione (oltipraz): predictive role for short-term molecular dosimetry. Cancer Res 1991; 51(20): 5501-6.

Rolfe RD. The role of probiotic cultures in the control of gastrointestinal health. J Nutr 2000; 130(2S Suppl): 396S-402S.

Ross RK, Yuan JM, Yu MC, Wogan GN, Qian GS, Tu JT, Groopman JD, Gao YT, Henderson BE. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. Lancet 1992; 339(8799): 943-6.

Ryan NJ, Hogan GR, Hayes AW, Unger PD, Siraj MY. Aflatoxin B1; its role in the etiology of Reye's syndrome. Pediatrics 1979; 64(1): 71-5.

Saad AM, Abdelgadir AM, Moss MO. Exposure of infants to aflatoxin M1 from mothers' breast milk in Abu Dhabi, UAE. Food Addit Contam 1995; 12(2): 255-61.

Sabbioni G, Skipper PL, Buchi G, Tannenbaum SR. Isolation and characterization of the major serum albumin adduct formed by aflatoxin B1 in vivo in rats. Carcinogenesis 1987; 8(6): 819-24.

Sabbioni G, Wild CP. Identification of an aflatoxin G1-serum albumin adduct and its relevance to the measurement of human exposure to aflatoxins. Carcinogenesis 1991; 12(1): 97-103.

Sargeant K, Carnaghan RBA, Allcroft R. Toxic products in groundnuts- chemistry and origin of aflatoxins. Chem Ind (London) 1963: 53-55.

Sargeant K, Sheridan A, O'Kelly J, Carnaghan RBA. Toxicity associated with Certain Samples of Groundnuts. 1961; 192(4807): 1096-97.

Sazawal S, Hiremath G, Dhingra U, Malik P, Deb S, Black RE. Efficacy of probiotics in prevention of acute diarrhoea: a meta- analysis of masked, randomised, placebo-controlled trials. Lancet Infect Dis 2006; 6(6): 374-82.

Scholl PF, Musser SM, Groopman JD. Synthesis and characterization of aflatoxin B1 mercapturic acids and their identification in rat urine. Chem Res Toxicol 1997; 10(10): 1144-51.

Scott PM. Natural Poisons. Chapter 49. Official Methods of Analysis. Helrich K. Arlington, VA, J Assoc Off Anal Chem. 1990: 1184-1213.

Selim MI, Popendorf W, Ibrahim MS, El-Sharkawy S, El-Kashory ES. Aflatoxin B1 in common Egyptian foods. J Assoc Off Anal Chem 1996; 79(5): 1124-9.

Serck-Hanssen A. Aflatoxin-induced fatal hepatitis? A case report from Uganda. Arch Environ Health 1970; 20(6): 729-31.

Shane SM. Economic issues related with aflatoxins. Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance. Eaton DL and Groopman JD. San Diego, Academic Press, Inc. 1994: 365-81.

Shank RC, Bourgeois CH, Keschamras N, Chandavimol P. Aflatoxins in autopsy specimens from Thai children with an acute disease of unknown aetiology. Food Cosmet Toxicol 1971; 9(4): 501-7.

Sharma RA, Farmer PB. Biological relevance of adduct detection to the chemoprevention of cancer. Clin Cancer Res 2004; 10(15): 4901-12.

Shrimpton R, Victora CG, de Onis M, Lima RC, Blossner M, Clugston G. Worldwide timing of growth faltering: implications for nutritional interventions. Pediatrics 2001; 107(5): 75-82

Smela ME, Currier SS, Bailey EA, Essigmann JM. The chemistry and biology of aflatoxin B(1): from mutational spectrometry to carcinogenesis. Carcinogenesis 2001; 22(4): 535-45.

Smela ME, Hamm ML, Henderson PT, Harris CM, Harris TM, Essigmann JM. The aflatoxin B(1) formamidopyrimidine adduct plays a major role in causing the types of mutations observed in human hepatocellular carcinoma. Proc Natl Acad Sci USA 2002; 99(10): 6655-60.

Sun Z, Lu P, Gail MH, Pee D, Zhang Q, Ming L, Wang J, Wu Y, Liu G, Zhu Y. 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(2): 379-83.

Sylla A, Diallo MS, Castegnaro J, C.P. W. Interactions between hepatitis B virus infection and exposure to aflatoxins in the development of hepatocellular carcinoma: a molecular epidemiology approach. Mutat Res 1999; 428(1-2): 187-96.

Szajewska H, Ruszczynski M, Radzikowski A. Probiotics in the prevention of antibiotic-associated diarrhea in children: a meta-analysis of randomized controlled trials. J Pediatr 2006; 149(3): 367-372.

Tandon HD, Tandon BN, Ramalingaswami V. Epidemic of toxic hepatitis in India of possible mycotoxic origin. Arch Pathol Lab Med 1978; 102(7): 372-6.

Turner PC, Mendy M, Whittle H, Fortuin M, Hall AJ, Wild CP. Hepatitis B infection and aflatoxin biomarker levels in Gambian children. Trop Med Int Health 2000; 5(12): 837-41.

Turner PC, Moore SE, Hall AJ, Prentice AM, Wild CP. Modification of immune function through exposure to dietary aflatoxin in Gambian children. Environ Health Perspect 2003; 111(2): 217-20.

Turner PC, Sylla A, Diallo MS, Castegnaro JJ, Hall AJ, Wild CP. The role of aflatoxins and hepatitis viruses in the etiopathogenesis of hepatocellular carcinoma: A basis for primary prevention in Guinea-Conakry, West Africa. J Gastroenterol Hepatol 2002; 17 Suppl: S441-8.

Turner PC, Sylla A, Gong YY, Diallo MS, Sutcliffe AE, Hall AJ, Wild CP. Reduction in exposure to carcinogenic aflatoxins by postharvest intervention measures in west Africa: a community-based intervention study. Lancet 2005a; 365(9475): 1950-6.

Turner PC, Sylla A, Kuang SY, Marchant CL, Diallo MS, Hall AJ, Groopman JD, Wild CP. Absence of TP53 codon 249 mutations in young Guinean children with high aflatoxin exposure. Cancer Epidemiol Biomarkers Prev 2005b; 14(8): 2053-5.

Valio Ltd. Production of starter cultures, 2006 Valio Oy, available from http://www.valio.fi/portal/page/portal/valiocom/R_D/Current_operations/starter_production18082006093109, accessed 16/04/07.

Van Egmond HP. Aflatoxins in milk. Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance. Eaton DL and Groopman JD. San Diego, Academic Press, Inc. 1994: 365-81.

Veldman A, Meijs JAC, Borggreve GJ, Heeres-van der Tol JJ. Carryover of aflatoxin from cows' food to milk. Anim Prod 1992; 55: 163-168.

Wang J, Liu XM. [Surveillance on contamination of total aflatoxins in corn, peanut, rice, walnut and pine nut in several areas in China]. Zhonghua Yu Fang Yi Xue Za Zhi 2006; 40(1): 33-7.

Wang JS, Groopman JD. DNA damage by mycotoxins. Mutat Res 1999; 424(1-2): 167-81.

Wang JS, Huang T, Su J, Liang F, Wei Z, Liang Y, Luo H, Kuang SY, Qian GS, Sun G, He X, Kensler TW, Groopman JD. Hepatocellular carcinoma and aflatoxin exposure in Zhuqing Village, Fusui County, People's Republic of China. Cancer Epidemiol Biomarkers Prev 2001; 10(2): 143-6.

Wang LY, Hatch M, Chen CJ, Levin B, You SL, Lu SN, Wu MH, Wu WP, Wang LW, Wang Q, Huang GT, Yang PM, Lee HS, Santella RM. Aflatoxin exposure and risk of hepatocellular carcinoma in Taiwan. Int J Cancer 1996; 67(5): 620-5.

WHO. Biomarkers and risk assessment: concepts and principles. World Health Organization, Environmental Health Criteria Series 155, International Programme on Chemical Safety. Geneva 1993; 1-82.

WHO. Physical status: the use and interpretation of anthropometry. World Health Organization, Technical Report Series 854, Geneva 1995; 1-256.

Wild CP, Fortuin M, Donato F, Whittle HC, Hall AJ, Wolf CR, Montesano R. Aflatoxin, liver enzymes, and hepatitis B virus infection in Gambian children. Cancer Epidemiol Biomarkers Prev 1993; 2(6): 555-61.

Wild CP, Hall AJ. Primary prevention of hepatocellular carcinoma in developing countries. Mutat Res 2000; 462(2-3): 381-93.

Wild CP, Hudson GJ, Sabbioni G, Chapot B, Hall AJ, Wogan GN, Whittle H, Montesano R, Groopman JD. Dietary intake of aflatoxins and the level of albumin-bound aflatoxin in peripheral blood in The Gambia, West Africa. Cancer Epidemiol Biomarkers Prev 1992; 1(3): 229-34.

Wild CP, Pionneau FA, Montesano R, Mutiro CF, Chetsanga CJ. Aflatoxin detected in human breast milk by immunoassay. Int J Cancer 1987; 40(3): 328-33.

Wild CP, Pisani P. Carcinogen DNA and protein adducts as biomarkers of human exposure in environmental cancer epidemiology. Cancer Detect Prev 1998; 22(4): 273-83.

Wild CP, Rasheed FN, Jawla MF, Hall AJ, Jansen LA, Montesano R. In-utero exposure to aflatoxin in west Africa. Lancet 1991; 337(8757): 1602.

Wild CP, Turner PC. The toxicology of aflatoxins as a basis for public health decisions. Mutagenesis 2002; 17(6): 471-81.

Wild CP, Yin F, Turner PC, Chemin I, Chapot B, Mendy M, Whittle H, Kirk GD, Hall AJ. Environmental and genetic determinants of aflatoxin-albumin adducts in the Gambia. Int J Cancer 2000; 86(1): 1-7.

Willett W. Nutritional Epidemiology. Monographs in epidemiology and biostatistics volume 15, 1st Ed, New York, Oxford University Press 1990.

Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr 2004; 80(5): 1106-22.

Wilson DM, Payne GA. Factors affecting *Aspergillus flavous* group infection and aflatoxin contamination in crops. Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance. Eaton DL and Groopman JD. San Diego, Calif, Academic Press, Inc. 1994: 309-325.

Wilson R, Ziprin R, Ragsdale S, Busbee D. Uptake and vascular transport of ingested aflatoxin. Toxicol Lett 1985; 29(2-3): 169-76.

Wogan GN. Impacts of chemicals on liver cancer risk. Semin Cancer Biol 2000; 10(3): 201-10.

Wogan GN, Paglialunga S. Carcinogenicity of synthetic aflatoxin M1 in rats. Food Cosmet Toxicol 1974; 12(3): 381-4.

Zarba A, Wild CP, Hall AJ, Montesano R, Hudson GJ, Groopman JD. Aflatoxin M1 in human breast milk from The Gambia, west Africa, quantified by combined monoclonal antibody immunoaffinity chromatography and HPLC. Carcinogenesis 1992; 13(5): 891-4.

Zhu JQ, Zhang LS, Hu X, Xiao Y, Chen JS, Xu YC, Fremy J, Chu FS. Correlation of dietary aflatoxin B1 levels with excretion of aflatoxin M1 in human urine. Cancer Res 1987; 47(7): 1848-52.

Kuopio University Publications D. Medical Sciences

D 392. Pesonen, Tuula. Trends in Suicidality in Eastern Finland, 1988–1997.
2006. 119 p. Acad. Diss.

D 393. Tuhkanen, Hanna. DNA copy number changes in the stromal and epithelial cells of ovarian and breast tumours.
2006. 112 p. Acad. Diss.

D 394. Koskelo, Reijo. Säädettävien kalusteiden vaikutukset tuki- ja liikuntaelimistön terveyteen lukiolaisilla.
2006. 96 p. Acad. Diss.

D 395. Elo, Mika. Stress-Related Protein Synthesis in Mammalian Cells Exposed to Hydrostatic Pressure.
2006. 74 p. Acad. Diss.

D 396. Remes-Pakarinen, Terhi. Influences of genetic factors and regular exercise on bone in middle-aged men.
2006. 95 p. Acad. Diss.

D 397. Saarela, Tanja. Susceptibility genes of diabetes and endothelial dysfunction in preeclampsia.
2006. 103 p. Acad. Diss.

D 398. Piippo-Savolainen, Eija. Wheezy babies - wheezy adults? Adulthood asthma, bronchial reactivity and lung function after hospitalization for bronchiolitis in early life.
2006. 91 p. Acad. Diss.

D 399. Kauppinen, Anu. Lipocalin Allergen-Induced T Cell Response: Prospects for Peptide-Based Immunotherapy.
2006. 81 p. Acad. Diss.

D 400. Vasara, Anna. Autologous chondrocyte transplantation: Properties of the repair tissue in humans and in animal models.
2007. 92 p. Acad. Diss.

D 401. Andrulionyte, Laura. Transcription factors as candidate genes for type 2 diabetes: studies on peroxisome proliferator-activated receptors, hepatic nuclear factor 4α and PPARγ coactivator Iα.
2007. 112 p. Acad. Diss.

D 402. Raatikainen, Kaisa. Health behavioural and social risks in obstetrics: effect on pregnancy outcome.
2007. 100 p. Acad. Diss.

D 403. Kinnunen, Tuure. The role of T cell recognition in the immune response against lipocalin allergens: prospects for immunotherapy.
2007. 76 p. Acad. Diss.

D 404. Gratz, Silvia. Aflatoxin binding by probiotics : experimental studies on intestinal aflatoxin transport, metabolism and toxicity.
2007. 85 p. Acad. Diss.

D 405. Ming, Zhiyong. Upper limb musculoskeletal disorders with special reference to sympathetic nerve functions and tactile sensation.
2007. 91 p. Acad. Diss.

D 406. Timonen, Leena. Group-based exercise training in mobility impaired older women.
2007. 91 p. Acad. Diss.