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Free and DNA Adducted Aflatoxins in Chronic Liver Diseases that Predispose Patients to Hepatocellular Carcinoma in Mexico

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Abstract

Aflatoxins are mutagenic hepatocarcinogenic fungal metabolites that contribute to chronic hepatitis B and C and viral cirrhosis, which can both evolve into hepatocellular carcinoma.

Objective: To identify and quantify free aflatoxins and AFB_1-N^7-guanine (AFB_1-N^7-Gua) adducts (active carcinogen) in the urine of Mexican patients with chronic liver diseases.

Methods: Urine samples from 210 Mexican patients with chronic liver diseases, hepatitis B or C, or viral cirrhosis and four control groups: 1) patients with alcoholic cirrhosis, 2) patients with no hepatic diseases, 3) patients with kidney failure, and 4) healthy persons, were analyzed for free aflatoxins and AFB_1-N^7-Gua adducts by Inhibitory Indirect ELISA and high performance liquid chromatography, and both methods produced similar results (R^2=0.90). A questionnaire regarding foods with high risk of containing aflatoxins was applied to relate diet and disease.

Results: Aflatoxin-positive samples were found from patients in the following groups: hepatitis B (50%), viral cirrhosis (26%), hepatitis C (16.6%), alcoholic cirrhosis (10%), healthy (10%), kidney failure (0.47%), and chronic non-hepatic diseases (0%), with R^2=0.95. Risk groups had more AFB_1-N^7-Gua adducts than controls. High performance liquid chromatography identified free AFB_1 (exposure), types M_1 and P_1 (detoxification metabolites), and Inhibitory Indirect ELISA quantified AFB_1-N^7-Gua adduct (a DNA repair biomarker).

High-risk foods related (P≤0.001) to hepatic diseases were maize, oil seeds, and dairy products.

Conclusion: Mexican patients with chronic liver diseases exhibited high concentrations of aflatoxins and Aflatoxin-N^7-Gua adducts, both of which showed high exposure and the last are significant biomarkers for the risk of liver diseases that predispose patients to liver cancer.

Keywords: Aflatoxins; AF-N^7-Gua adducts; Hepatitis; Cirrhosis; Hepatic diseases

Abbreviations: AC: Alcoholic Cirrhosis; AFB_1: Aflatoxin B_1; AFB_1-FAPY adduct: AFB_1-formamido Pyrimidine; AFB1-N7-Gua adduct: AFB_1-N^7-guanine Adduct; AFM_1: Aflatoxin M_1; AFP_1: Aflatoxin P_1; AFs: Aflatoxins; BSA: Bovine Serum Albumin; c.o.s.s. = cereals, oil seeds and spices; CII ELISA: Competitive Inhibitory Indirect ELISA; CNHD: Chronic Non Hepatic Diseases; cr: creatinine; CV %: Coefficient of Variation Percentage; CV: Standard Coefficient of Variation; ELISA: Enzyme-Linked Immunosorbent Assay; H: Healthy; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HPLC: High performance liquid chromatography; IA: Total Anti-AF Immunoaffinity Columns; Inhib%: Inhibition Percentage; KF: Kidney Failure; LOD: Limit of Detection; LOQ: Limit of Quantification; Monoclonal AFB_1-BSA conjugate: Aflatoxin B_1- Bovine Serum Albumin Conjugate; R^2: Correlation Coefficient; SD: Standard Deviations; TMB: 3,30-,5,50-tetramethylbenzidine; UV abs: Ultraviolet Absorption; VC: Viral Cirrhosis

Introduction

Aflatoxins (AFs) are secondary metabolites and polyketides of a group of dihydrobisfuran-coumarins, which are divided into two subgroups depending on their chemical structure. The bisfuran-coumarin-cyclopentanones include AFs, which are further categorized into groups labeled B, Q, P and M (AFB_1, AFB_2, AFB_{2a}, AFM_1, AFM_2, AFM_{2a}, AFQ_1, AFP_1), and aflatoxicol (AFL), which corresponds to the subgroup of bisfuran-coumarin-lactones, in which the AFs in the G groups G (AFG_1, AFG_2, AFG_{2a}) are placed [1-3].

AFs are produced primarily by the fungi *Aspergillus flavus* Link, *A. parasiticus* Speare, and *A. nomius* Kurtzman [4] and contaminate many foods worldwide [5]. Other fungi have been reported to be AF producers: *A. tamarii* [6], *A. arachidicola*, *A. bombycis*, *A. minisclerotigenes*, *A. ochraceoroseus*, *A. pseudotamarii*, *A. rambellii*, and *Emericella venezuelensis* [7,8]. Other fungi reported to produce AFs [9] (*A. tamarii, A. oryzae, A. versicolor, Penicillium commune, P. griseofulvum*) have been proven to be misidentified.

AFs are the most toxic of the known mycotoxins and are a significant risk factor for liver and kidney cancer [10]. These potent mutagens and carcinogens [11] are directly implicated in causing or exacerbating liver diseases such as hepatitis B (HBV) [12] and C (HCV) [13], cirrhosis [14,15], various cancers [11,16,17], and primary Hepatocellular Carcinoma (HCC) [18,19], as well as teratogenicity [20], undernutrition, micronutrient malabsorption [10], immune dysfunction [21,22], stunting [23] and protein deficiency syndromes

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[24]. The immunosuppressive effect of AFB_1 in humans has been demonstrated; when human lymphocytes are exposed to AFB_1, they exhibit impaired cellular respiration, provoking necrosis [25].

Global diet differences may lead to heterogeneity in AF-induced morbidity. Although such data would represent a crucial resource for managing food-borne AF risk, data are lacking from many parts of the world.

Mexico is an area with a diet particularly high in AFs and extraordinarily high instances of liver disease. Maize, the dietary staple food in Mexico, has among the highest levels of AFs of all grains [26]. In 2003, hepatic cirrhosis, chronic liver diseases and HCC were the second cause of death among Mexicans of working age (15-64 years), second only to cardiovascular diseases, and representing one of the highest such rates worldwide. In 2013, the fourth cause of mortality in Mexico was liver cirrhosis, with 29,335 deaths [15,27,28]. Despite this exceptionally high incidence of liver disease, little attention has been given to AF levels in foods, tissues or fluids of patients in Mexico [15].

AFs provide an effective system to understand the relationship between diet and disease because they are readily detected in tissues and fluids. Oxidation of AFB_1 links it covalently to DNA, forming adducts that can produce point mutations, activate proto-oncogene to oncogene transformation and initiate the carcinogenic process [29]. AFs covalently bind to albumin, forming AFB_1-albumin adducts, and to DNA, forming aflatoxin-DNA adducts, which primarily exist as 8,9-dihydroxy-8-(N^7) guanyl-9-hydroxy AFB_1 adducts (AFB_1-N^7-Gua) [30]. These adducts are converted to two secondary lesions: an apurinic site and the AFB_1-formamido pyrimidine (AFB_1-FAPY) adduct, which is primarily responsible for the genotoxic, mutagenic and carcinogenic properties of AFB_1 [31]. These adducts are valuable biomarkers for hepatic diseases, such as hepatitis B [32], hepatitis C [13], and HCC [33].

Dietary information from patients with chronic liver diseases and various control groups, along with AF urine levels, were collected, and the data support the hypothesis that high dietary AF levels correlate with chronic liver diseases

Methods

Sampling design and patient selection criteria

Seven groups were studied, each with 30 individuals totaled 210 cases (109 women and 101 men aged 26 to 74) that originated from the National Institute of Medical Sciences and Nutrition "Salvador Zubirán" (Instituto Nacional de Ciencias Médicas y de la Nutrición "Salvador Zubirán"), and the patient pool also contained 12 hepatitis B cases from the National Medical Center "The Race" (Centro Médico Nacional "La Raza"), both in Mexico City.

The selection criteria for the three risk groups of patients were

1. For chronic HBV and HCV: histopathology, presence of the HBsAg-positive marker for HBV or positive result with anti-HCV marker, and abnormal biochemical tests.
2. For post-viral hepatic cirrhosis (VC): histopathology and abnormal hepatic functions and biochemical test results.

The selection criteria for control groups of patients were

1. Alcoholism: history of chronic alcoholism, histopathology of alcoholic cirrhosis, abnormal hepatic function, and negative HBV and HCV markers.
2. Chronic or terminal kidney failure (urea and creatinine tests), blood or chronic peritoneal dialysis substitution, and negative viral markers.
3. Chronic non-hepatic diseases (CNHD) or gastric ulcers.
4. Healthy individuals: normal hepatic functions and serological test.

The sample size was the minimum size to ensure that the sample mean exhibited an approximately normal sampling distribution. Sex and age ranges of the risk and control groups are shown in Table 1.

Collection and urine sample analysis

This study focused on collecting AFB_1-N^7-Gua adducts in urine because this method is non-invasive and because chronic liver diseases are not treated through surgical operations in which samples could be collected; therefore, the collection of sampling tissues is possible only by biopsies and necropsies, and urine is easier to obtain. Each patient answered 200 questions about their diet, which were developed by the Dept. of Epidemiology of the Institute of Medical Sciences and Nutrition, and provided a 24-hour urine sample that was measured and homogenized by vortexing; an aliquot of urine was removed for colorimetric determination. To adjust urine concentrations and make comparable samples, creatinine (cr) test kits were applied to calculate the rate of excretion (mg AF/cr mg) in order to establish kidney function. The remaining urine was adjusted to 5.0 pH, and 25 mL of the urine was concentrated through Sep-Pak C_{18} activated columns, purified with total AF immunoaffinity columns, eluted with 1.5 mL of methanol, dried and re-dissolved in PBS to recover the original urine concentration per case. Each sample, with 3 replicates, was filtered through 1 μm Millipore membranes. Reverse-phase High-Performance Liquid Chromatography (HPLC) was used to verify the purity and to quantify the reference standards and the free and adducted AF urine samples with AFB_1, the hydroxylates AFM_1 and AFP_1, and AFB_1-N^7-Gua adducts by standard methods [34,35]. The HPLC used a pump system with a injector adapted to a 20 mL loop, a data integrator, a spectrophotometer at an absorbance of 360 nm (excitation 360 nm, emission 450 nm), and a fluorescence detector. An HPLC reverse-phase C18 column (25 × 0.460 cm) adapted with a pre-column was used.

All solvents were HPLC purity grade and were degasified with a flask adapted to a vacuum pump. The mobile phase was an isocratic solution of methanol/distilled H_2O (60:40 v/v) mixture filtered over microfiltration membranes (0.2-mm pore size) at a flow rate of 1 mL/min.

Conjugates and AFB_1-N^7-Gua adduct *in vitro* synthesis

Monoclonal AFB_1-BSA conjugates recognized eight types of AFs as well as the AFB_1-N^7-Gua adduct. Two preparations of the anti-aflatoxin monoclonal AFB_1-BSA (AFB_1-BSA) conjugate were tested: 6E9 purified with Sephadex and from tissue culture, each with six concentrations (1:100, 1:200, 1:300, 1:500, 1:1000 and 1:1500) and six replicates.

AFB_1-N^7-Gua synthesis was performed as a control standard for ELISA [16,36,37]. To test the purity of the AFB_1-N^7-Gua adduct, electrophoresis was performed using a 1% agarose gel with Tris-acetate and ethidium bromide buffer. As a standard, lymphocyte DNA and different concentrations of AF-adducted DNA were applied with phenolbromine at 100 volts for 35 min. Bands were visualized with a

transilluminator. Total anti-AF immunoaffinity columns were used to concentrate free AFB_1, AFB_1-N^7-Gua adducts, and AFM_1 and AFP_1 hydroxylates.

AFB_1- chloride-Ovalbumin (AFB_1-Ov) conjugate synthesis

ELISA plates were coated with AFB_1-Ov conjugate and incubated overnight. To obtain a stock concentration of 50 mg/mL, each new synthesized batch of AFB_1-Ov conjugate was tested by preparing seven dilutions (2.5, 5, 10, 20, 30, 40 and 50 ng) with six replicates each. AFB_1-Ov conjugate and AFB_1-N^7-Gua adducts were obtained using standard methodology [16,34-36].

AFB_1 was dissolved in dichloromethane (DCM) and gaseous chloride linked to an AFB_1-Cl-DCM conjugate; DCM was eliminated by roto-evaporation, leaving behind the AFB_1-Cl. Dimethylsulfoxide, ovalbumin and NaOH (5M) were added to form the AFB_1-Cl-Ov conjugate. To ensure the quality of the synthesis, thin layer chromatography was performed at different times, producing an efficiency level of 90% and a concentration of 1.5 mg/mL.

Anti-mouse IgG peroxidase conjugate (α-mouse)

To determine the best concentration for the competitive indirect inhibitory (CII) ELISA test, six dilutions (1:400, 1:500, 1:600, 1:800, 1:1000 and 1:1500) of α-mouse IgG peroxidase antibody conjugate were tested, each with six replicates.

The AFB_1-N^7-Gua adduct was synthesized *in vitro* using standard methodology [29,36,38] and was modified [16] to use it as an ELISA test reference standard. It was obtained in a high-risk laboratory with all possible precautionary measures taken to avoid fatal accidents. The purity of the adduct was determined by electrophoresis [34,38,39].

Aflatoxin competitive indirect inhibition ELISA method

Validation of CII ELISA method was necessary to evaluate the characteristics, quality parameters and the precision of the system [40-43], including the following parameters: the standard deviation (SD), the coefficient of variation (CV %), the intra- and inter-assay, the repeatability, the linearity of the method and of the system (calibration curves), the detection and quantification limits [44], the accuracy and the repeatability of the method [41,44], the recovery percentage [44,45] and the recovery percentage limits [34,35].

The limit of detection (LOD) was 0.1 pg/mg DNA, and the Limit of Quantification (LOQ) was 10 pg/mg DNA; these limits were of adequate sensitivity for the AF assay. In addition, recovery reached nearly 100%. The linearity of the system (calibration curves) was measured, and the standard curves of AFB_1 and AFB_1-N^7-Gua adduct with eight dilutions (0.1, 1, 10, 100, 1000, 1×10^4, 1×10^5, 1×10^6 pg/mg) were run with three replicates to establish the CII ELISA UV absorption curve, the correlation coefficient (R^2), and the values of the ordinate to origin and the slope. The linear regression was calculated and the curves were plotted with Excel software.

Stock solution, type of plate and coating evaluation

To prepare a stock solution of 1 mg/mL AFB_1, which was used as a standard in CII ELISA or to synthesize the AFB_1-N^7-Gua adduct, the molecular weight and molar extinction coefficient were used following a known method (970.44 (A)) [46]. To optimize the system, five different ELISA plates with AFB_1-Cl-Ov adduct optimal coating were simultaneously tested: Flow Laboratories, Inc., Corning Costar plates (low and high adherence), Nunc polysorp (tissue culture) and Maxisorp of different adherence. All plates were tested with the same reactives, conditions of incubation and washing, and they were stored at 4°C [34]. A cross titering of AFB_1-Bovine Serum Albumin (AFB_1-BSA) and second-mouse conjugate coupled with peroxidase was performed to select the highest concentration signal of 1×10^6 pg/mg of the AF antigen.

Tests were performed to evaluate the quality of the system within the international accepted parameters of minimum detectable concentration, accuracy, precision, recovery limits and coefficient of variation.

Levels and purity tests of each aflatoxin and adduct by HPLC were performed. Individual standard curves with 5 dilutions (4, 8, 12, 16, 20 µg/L) for each AF were run to establish retention times, and the linear regression was calculated with Excel software. ELISA conditions were 10 ng/well of ovalbumin-AF adduct for coating, anti-mouse IgG peroxidase conjugate antibody dilution (1:1000), anti-AF monoclonal dilution (1:300) and 3, 3′, 5, 5′ tetramethyl benzidine to dye the reaction. Absorbance (450 nm) was registered in an ELISA automatic plate reader, and a plate washer was also used. Averages, standard deviations, coefficients of variation and an accepted minimum of 80% of inhibition were calculated [16,35].

Aflatoxin Competitive Indirect Inhibition ELISA (CII ELISA)

The CII ELISA test was used to quantify the AFB_1 and AFB_1-N^7-Gua adducts in the samples by comparing the standard curve inhibition of the adducts following known methodology [16,35]. The ELISA's selectivity increased with the concentration of anti-aflatoxin monoclonal AFB_1-BSA conjugate, as well as the total anti-AF immunoaffinity columns (IA), which extract concentrations of different AFs, AF hydroxylates and AFB_1-DNA adducts from samples, as certified in our laboratory and in previous research laboratories [47,48]. Included on all plates as negative controls were the following: triplicate wells of blank samples diluted in buffer B only, unadducted BSA and calf thymus DNA, to account for any nonspecific inhibition, and the enzyme substrate color indicator 3, 30-, 5, 50-tetramethylbenzidine (TMB); sulfuric acid was used to stop the reaction and an ELISA plate Multiwash and ELISA automatic plate reader were used to obtain the inhibition percentage. The automatic ELISA plate reader provided media absorbances, from which the standard deviations of each well from the standard coefficient of variation (CV) and inhibition percentage (Inhib %) were calculated. If the CV was higher than 10%, the sample measurement was repeated.

Food survey questionnaire

The 200 questions provided to each of the 210 patients in this study were chosen by the Departments of Epidemiology of Social Health and of Nutrition, which designed the food questionnaire; both departments are located at the National Institute of Nutrition Sciences "Salvador Zubirán".

The food survey questionnaire was based on the information provided by the patients; no food was analyzed for AF contamination. The types of food considered in the questionnaire were those considered at risk for AF contamination [47].

The risk foods were grouped as cereals, oil seeds, spices, dairy products, meats, alcoholic fermented beverages and their derivatives, and sauces.

The number of ingested rations, types of food susceptible to AF contamination, and the diet during the 24 to 48 hours prior to urine sampling, were summarized weekly, monthly and annually in the

applied questionnaire and were based on the information provided by the patients in the questionnaire. No food was weighed, nor was food intake measured.

These data were checked against a total of 42,000 reported ingested foods with a risk of AF contamination, based on a questionnaire including approximately 200 different foods and the amount of reported ingested amounts by the monitored patients.

Statistical analysis

The data set was assembled using Fox Plus and Excel and was analyzed with the JMP and SAS (Statistical Analysis System) packages.

The dependent variables (consumption per food and AF-logarithm) had nearly normal distributions. Analysis of variance was used to evaluate whether AF-log media changed between groups of disease. The Box-Cox transformation was applied to some variables and the Kruskall-Wallis test was used for non-normal data. A Tukey test was applied to significant differences in the F test. These analyses included the seven groups of diseases and controls and another analysis excluding alcoholics.

Cluster analysis was used to analyze types of food consumption (eliminating alcohol) represented as categorical variables, followed by correspondence and discriminant analyses (canonical correlation) of viral hepatic diseases with the healthy control group to evaluate the association between food consumption and diseases [39].

Only positive tests of free AFB_1, AFM_1, AFP_1 and AFB_1-N^7-Gua adduct in urine were statistically analyzed. For these variables, analysis of contrasts showed differences between healthy control and groups at risk and between viral diseases and alcoholic cirrhosis.

In addition, a univariate analysis of variance, contrasts, and ANOVA tests were applied to consumption of food as a dependent variable.

Results

FB_1-N^7-Gua adducts *in vitro* synthesis

The carcinogen AFB_1-N^7-Gua adduct was synthesized *in vitro* to be used as a standard in the CII ELISA test; the chromatographic peak appeared at a retention time of 22.4 min, and a concentration of 2.1 mg/mL was obtained (Figure 1).

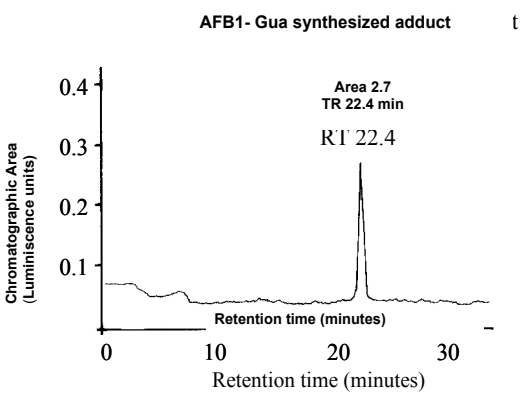

Figure 1: AFB_1-N^7-Gua adduct synthesized and concentrated by immunoaffinity column. The chromatographic peak at a retention time (RT) of 22.4 min with a concentration of 2.1 µg/mL.

Comparison of HPLC and ELISA methods

The best inhibition of the AFB_1-BSA monoclonal antibody was obtained with a 1:300 concentration from the tissue culture, and the chosen dilution was 1:1000.

An optimal 94% of inhibition was obtained. ELISA was more sensitive and was able to detect lower concentrations (0.1 ng AF/mg Cr) with better recovery of total AFs (AFt) (38/210) than the HPLC method (24/210), which could not detect low AF levels in agreement with previous rat and human urine studies [19,34,35].

Analytical quality assurance

The Costar 3590 plate with a high adherence was chosen for the entire study, and the optimal AFB_1-Ov coating dilution for ELISA plates was 10 ng; the HPLC and ELISA methods showed a 0.95 correlation coefficient and a > 0.90 with AF concentrations of >10 ng/kg.

The retention times (RT) in minutes of standards in isocratic HPLC analysis were as follows: AFB_1-N^7-Gua adduct (3.9), AFM_1 (4.8), AFP_1 (6.1) and AFB_1 (7.4), which are all in agreement with previous reports [29,34]. Internal HPLC standards confirmed the chromatographic interpretations. The correlation coefficient (R^2) values for the calibration curve for HPLC isocratic analysis were as follows: AFB_1 (R^2=0.999); AFM_1 (R^2= 0.998); AFP_1 (R^2=0.996); and AFB_1-N^7-Gua adduct (R^2=0.996).

Food questionnaire

Alcoholics experienced behavior problems and memory failures, and did not answer the questionnaire reliably. The standard deviations (SD) for maize, rice, and dairy product consumption were higher for the alcoholics than the other groups, and because these answers were skewed, the same analyses were performed after omitting these answers (Table 1).

The healthy control population ate the same foods as the risk groups, but they excreted fewer carcinogens; the difference was primarily the ingested amount of each type of food, as shown in Table 2.

Excretion of different AF types in hepatic diseases

Table 1 shows the frequency of AF positive urine samples, individual percentage frequency, AF types, sex, ranges of positive samples from total AF concentrations, and P values. AFM_1 is a mutagenic and carcinogenic [49,50] detoxification metabolite of AFB_1 and appears with high frequency in all of the risk groups, although its excretion was different ($P \leq 0.029$). The VC group had the highest amount of AFM_1 (14-101 ng/mL), AFB_1 (423-502 ng/mL) and AFt (0–4106 ng/cr mg). The HBV group exhibited the second highest of AFM_1 (2-90 ng/mL), AFB_1 (170-324 ng/mL) and AFt (0.1–1543 ng/cr mg). The HCV group had the third highest amount of AFM1 (1-44 ng/mL) and AFt (3-60 ng/cr mg) and exhibited no AFB_1. In contrast, the healthy control groups had traces of AFM_1 (1 to 4 ng/mL), no AFB_1 and low AFt (9-42 ng/cr mg). The amounts of AF required to kill dogs and humans via hepatitis have been reported in India [12] to be from 2 to 6 mg of AF daily over a period of a month.

Alcoholics have poor metabolism and thus did not excrete AFM_1. Patients with HCV and alcoholics did not excrete AFP_1 in urine which was probably eliminated as glucuronide by bile [51]. The frequency and concentration of AFP_1 in urine have no relation to dosage-exposition [49] but have been correlated with the development of primary HCC [52]. AFP_1 is excreted as a free compound in urine [49].

Groups	Age range	%AF+ig/ %AF+ tc	Sex fem/masc	T				HPLC ngAF/mg cr (N° + cases)	ELISA ngAF/mg cr (N°+ cases)
				AFB_1	AFM_1	AFP_1	AFB-Gua		
Risk groups									
HBV	26-68	50/7	11/19	170 - 324 (3)	2 - 90 (4)	20 - 36 (3)	1 - 43 (5)	1 - 590 (6)	0.1 - 1543 (15)
HCV	34-64	17/2	19/11	0	1 - 44 (4)	0	1 - 4 (4)	2 - 63 (7)	3 - 60 8)
VC	30-72	27/4	18/12	423 - 502 (2)	14 - 101 (5)	48 - 127 (2)	0.4 - 323 (6)	0.3 - 1693 (11)	0 – 4106 (15)
Range	26-72	10-50%	48/42	170 - 502	1 - 101	20 - 127	0.4 – 323	0.3 -1693	0.1 - 4562
N° positive cases				5/210 (2.4%)	13/210 (6.2%)	5/210 (2.4%)	15/210 (7.2%)	24/210 (11.4%)	38/210 (18.1%)
Control groups									
AC	41-74	10/1	10/20	0	0	0	1 - 2 (3)	0.3 - 3 (2)	1 - 10 (3)
KF	35-71	3/1	17/13	0	0	0	0	0	2
CNHD	34-69	0/0	19/11	0	0	0	0	0	0
H	26-72	10/1	15/15	0	1 - 4 (3)	2 - 3 (2)	1 (1)	1 - 10 (4)	9 - 42 (6)
Total	26-74		109/101						
N° positive cases				0	3/210 (1.4%)	2/210 (1.0%)	4/210 (1.9%)	6/210 (2.9%)	9/210 (4.3%)
Statistical analyses									
P value in F test*				0.036	0.0532	0.0013	0.277		
Total number of 47 positive cases in 210 total number of samples				5/210 (2.4%)	16/210 (76.2%)	7/210 (3.3%)	19/210 (9.1%)		
P values in contrasts[a]				VC vs HBV : 0.036	VC vs H: 0.019 VC+HBV+ HCV vs H : 0.029	VC vs H : 0.002 HBV vs H :0.023 VC+HBV vs H: 0.0005	Non-significant at 0.05%		

HBV : Viral B Hepatitis; HCV: Viral C Hepatitis; VC:Viral cirrhosis; AC:Alcoholic cirrhosis; KF:Kidney failure. CNHD: Chronic non hepatic diseases; % AF + ig/ % AF + tc total cases: Percentage of aflatoxin positive cases inside the group of 30, between aflatoxin positive cases inside total 210 cases. *With data previously transformed using Box & Cox method. a: Always lower averages in Healthy (H). (N° + cases): Number of positive cases

Table 1: Excretion of AF types in hepatic diseases, by HPLC and ELISA methods, and statistical analysis.

Disease / control	Average of food rations reported ingested								
	24 and 48 hours reported consumption		Annual reported consumption						
	Animal products (meats,dairy and eggs)	Cereals, oil seeds and spices	Maize	Wheat	Rice	Oil seeds	Spices	Animal products	
								Dairies	Meats
HepatitisB virus	3	9	1824	591	186	57	306	748	388
Hepatitis C virus	4	6	1505	584	187	169	354	928	615
Viral cirrhosis	4	5	1466	609	213	108	293	869	402
Healthy cases	3	6	1035	542	145	52	211	582	400

Table 2: Average of food rations reported ingested.

The adduct frequency in sick individuals was higher than in the healthy group. This results suggests that AFB_1-N^7-Gua adducts are a carcinogenic biomarker in comparison to the other AFs. The correlation of AF content in diseased patients was statistically significant: $P \leq 0.05$ including alcoholics, and $P \leq 0.033$ without alcoholics. The differences between viral disease and control groups were highly significant ($P \leq 0.0002$), and the presence of virus and AF were correlated. CNHD patients were AF negative, as shown in Table 1. No significant differences were found between AF concentrations in healthy and alcoholic groups compared with the viral disease groups, the differences were significant in the frequency of positive cases.

Relation between diseases and food diets

The contrasts between groups, the reported food consumption and the AF logarithm are reported in Table 3. The clusters according the reported food consumption are presented in Table 4.

Cluster 1, associated with the healthy control group, showed low ingestion of all foods. Cluster 3, associated with HBV, showed high consumption of maize, rice, dairy food and low ingestion of wheat, oil seeds and spices. Cluster 7, associated with HCV, showed high consumption of wheat, spices, dairy, an oil seeds, and exhibited an average consumption of other foods. The remaining clusters were not associated with diagnostics.

Cereal, oil seeds and spices (c.os.s.) ingested within the 24 to 48 hours (c.os.s. 24-48) prior to urine sample collection were highly significant ($P \leq 0.0005$) as foods with AF contamination risk in relation to liver diseases. The consumption of maize and derivatives was highly significant ($P \leq 0.011$); consumption of oil seeds was also significant ($P \leq 0.019$). Patients with hepatic viral diseases exhibited a significant difference in the consumption of dairy products ($P \leq 0.014$) and of maize ($P \leq 0.0005$) and condiments ($P \leq P\ 0.028$); they also showed a significant difference in AF levels ($\ln \log_{10}$ transformed) ($P \leq 0.0009$) (Table 4). Foods that were most highly associated with liver diseases were maize, oil seeds, and spices, as summarized in Figure 2.

Healthy controls consumed fewer dairy products than the remaining groups ($P \leq 0.012$). They also consumed fewer oil seeds, but this was marginally significant ($P \leq 0.065$) in relation to the

Variance analysis followed by contrast test	P values per variable in contrast (with alcoholic cirrhosis)											AF log
	24 and 48 hours consumption			Annual consumption								
	Ani	c.os.s.	OH	Maize	Wheat	Rice	Oil	Spices	Dairy	Meats	[OH]	
VC,AC, HBV,HCV vs KF,CNHD & H	.014	.524	155	.032	.390	.810	.139	.105	.288	.651	0	.0002
H vs the rest	.130	.257	365	.032	.625	.09	.025	.104	.029	.350	0	.0002
	P values per variable in contrast (without alcoholic cirrhosis)											
VC,HBV&HCV vs KF,NHD & H	.014	.211	.159	.0005	.343	.279	.263	.028	.146	.617	.363	.0009
H vs the rest	.106	.368	.528	.109	.620	.184	.065	.139	.012	.368	.689	.500
VC vs HBV & HCV	.463	.005	.159	.400	.773	.477	.874	.528	.785	.225	.787	.558

AF log: Aflatoxin logarithm; VC: Viral cirrhosis; HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; C: Controls (AC: Alcoholic cirrhosis; KF: Kidney failure; CNHD: Chronic non hepatic diseases and H: Healthy groups); Ani: animal derivates; [OH]:Annual alcohol ingestion; c.os.s.: cereals, oil seeds and spices

Table 3: Contrasts between groups, consumption of food and AF logarithm.

Clusters/groups**			Average reported ingested rations									
N°	Groups*	N° of cases	24-48 hours reported consumption		Annual reported consumption							
			Animal derivates	Cereals, oilseeds and spices	Maize	Wheat	Rice	Oil seeds	Spices	Dairy	Meats	[OH]
1	H	69 45	2 5	6 5	1,106 – 1,206	626 381 -	162 - 127 -	47 - 52	188 - 177 -	614 - 610 -	354 - 460	0 1
2	Not associated with diagnostics											
3	HBV	11 8	4 2	13 8	2,929 + 2,888 +	413 498	317+ 184	70 92	169 - 410	1,310 + 664 -	388 317 -	0 92
4-6	Not associated with diagnostics											
7	HCV	15 22	3 5	6 6	1,247 1,283	875 + 704	191 164	486 + 114	328 748	1,135 1,324 +	604 + 714 +	2 2

There was no 24-48 hours reported consumption of alcohol; [OH]: Annual reported ingested alcohol; Meats: Meat products and derivates (dairy products and eggs). + Maximum value or near it. -Minimum value or near it. *Diseased/ healthy groups most associated with each cluster, in a correspondence analysis.
** Clusters 4 and 6 were not associated with diagnostics

Table 4: Clusters according food reported consumption.

rest. Compared with the HCV and HBV groups, the VC group was significantly different in the consumption of c.os.s. 24-48 hours prior to urine sample analysis ($P \leq 0.005$). A discriminant analysis of three liver diseases and of the healthy control groups showed a correlation between the disease and the ratio of the food consumed (Figure 3).

Discussion

Humans with chronic HBV and those that are exposed to AFs are more at risk to develop liver cancer, which might be prevented by decreased consumption of AF-contaminated foods [10].

Studies of liver cirrhosis in Mexico have shown the effects rather than the causes of liver disease. AFB_1-N^7-Gua in urine and AFB_1-FAPY in tumor adducts are useful molecular biomarkers to determine the etiology of liver diseases, in contrast to agents, such as alcohol, that cause damage and then disappear.

Our results showed a positive association of AF in the urine of patients with hepatic diseases such as HBV, HCV and VC in agreement with previous reports [53,54]. AF analysis should be included in epidemiological studies [19]. The urine samples from Mexican risk groups had higher levels of AF (0.12- 4106.2 ng/cr mg) than cases from Africa (0.100-1.0 AF ng/urine mL) [55] after measuring units were equalized. Healthy Mexicans had levels of AF five times higher (6.9-33.6 ng/cr mg) than those in the healthy Danish human urine samples (6.5 AFB_1 ng/cr mg), with an increase of AF excretion when beer was consumed [56]: The amount of maize, hot peppers, spices and eggs consumed in both countries may account for the difference.

AFB_1 toxicity is higher than that of its metabolites, but only 10% was recovered as free AFB_1, whereas 90% was metabolized. Free AFB_1, which is not activated, enters and leaves the body without causing major changes and has little impact on diseases, but when it is oxidized, it forms the highly reactive 8,9 AF exo-epoxide, which can bind to DNA, forming the adduct that is the active carcinogen. Metabolic detoxifying processes are glucuronation, sulfonation and acetylation, which produce compounds soluble enough to be excreted and have hydroxylated metabolites as primary substrates of AFB_1 (i.e., AFM_1, AFP_1 and AFQ_1), with less toxic and carcinogenic activities [57,58]. Free AFB_1 represents recent exposure (24-48 hours). Ingested amounts of AFB_1 (0.2%) and AFB_2 (0.6%) can be calculated when excreted as AFB-N^7-Gua in urine [37].

AFB_1-N^7-Gua adducts, with an average of 31 ng, were found to be a biomarker metabolite that was detected in 9% of the 210 Mexican urine samples, a level inferior to 12.6% adduct in urine samples reported from Kenya [59] or the excretion of 200 ng adducts with 51% primary HCC mortality in China [49]. One explanation for this discrepancy is that the use of lime to prepare Mexican tortillas may lower the levels of AF and primary HCC. Excreted AFB-N^7-Gua adducts and AFB_1 ingestion had a significant correlation of 0.80 and $P<0.000001$, indicating a close association between exposure and molecular dosimetry [49,53].

The concentration of AF in alcoholics was very low (0.21 ng/mL) because they are an undernourished group; however, in the questionnaire, they said that they ate very well. Unexpectedly, only a single healthy control excreted the adduct (1.38 ng/mL), but no differences were found between the amounts of adducts from alcoholics and healthy controls.

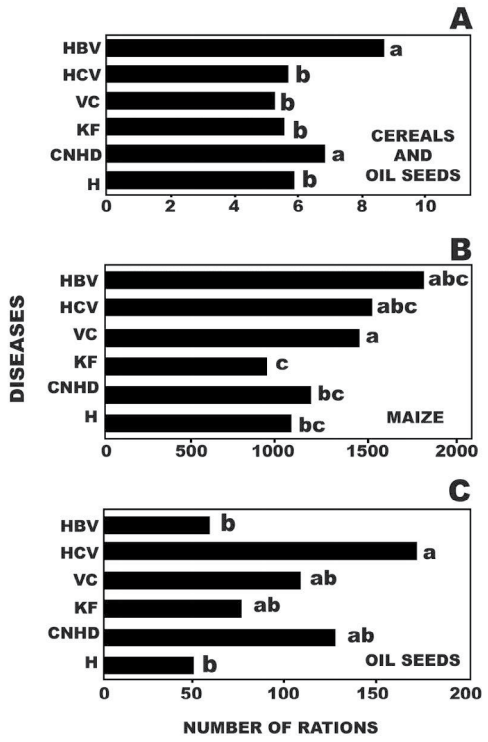

Figure 2: Tukey test from food survey (relation between diseases and number of reported rations). Number of rations: A in 24 to 48 hours; B and C in a year. Groups with a letter (a, b, c) in common are not statistically different, with no common letter are statistically different ($p<0.05$). Diseases: HBV= Hepatitis B Virus, HCV=Hepatitis C Virus, VC=Viral cirrhosis, KF= Kidney failure, CNHD=Chronic non hepatic diseases, H=Healthy.

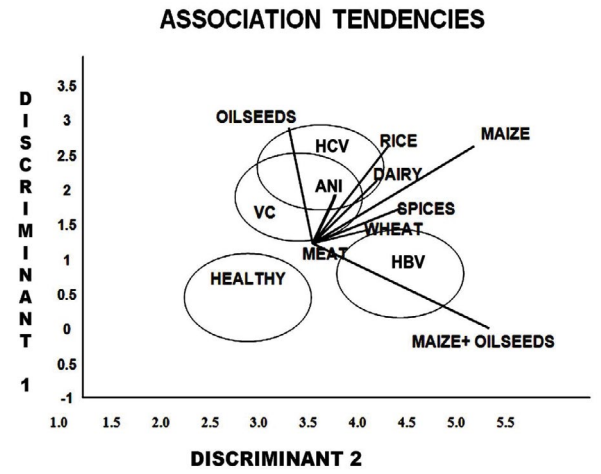

Figure 3: The association shows a circle for each group and axis for each reported food. If a circle is in the direction of an axis, that group has more reported consumption of the food of the axis. The Healthy group was moderated in the reported consumption of all foods; Hepatitis C Virus (HCV) reported to consume more dairies, meats and oil seeds; Viral cirrhosis (VC) more oilseeds; Hepatitis B Virus (HBV) reported to eat more cereals and oil seeds. The food reported consumption of the HCV and VC groups were very similar. Ani=Animal derivates, meat and eggs, were not so significant.

AFB_1-N^7-Gua adducts are rapidly formed DNA repair products excreted by the urine, which indirectly indicate the risk of a person developing liver damage or cancer, and are useful biomarkers to determine the role of AF in chronic liver viral diseases [60,61]. The presence of AFB_1-N^7-Gua adducts in patient's shows a direct connection with viral infection and suggests an etiopathogenic role of AF toward HCC tumors in countries with high exposure to AF [62-64].

Foods with significant association ($P \leq 0.0004$) with liver diseases are maize, oil seeds, spices and dairy derivatives. Patients in the healthy group ingested the same types but a lower amount of these foods and metabolized AFB_1 to AFM_1 and AFP_1; one patient did exhibit adducts but no free AFB_1. Risk groups excreted more AF of the following types in diminishing order: AFB-N^7-Gua adducts, AFM_1 and AFP_1 metabolites, free AFB_1. AFB-N^7-Gua adducts in human urine were confirmed as a molecular biomarker of the risk of developing hepatic diseases by AF, in agreement with other reports [64]. If the food data from 210 patients would have been obtained from chemical analysis, they would have included 42,000 samples from many types of foods and derivatives, and analysis would have been expensive. Thus, we chose to use epidemiological methods to measure human exposure to AFs by dietary questionnaires, and the statistical analysis was accurate, and provided new data in this respect. Aflatoxin exposure biomarkers provide great potential for accurate assessment [65], and food data can explain the origin of the carcinogens; thus, both aspects of this study are complementary.

HCC is associated with HBV chronicity and dietary exposure to aflatoxin, a mutagen targeting codon 249 of the tumor suppressor *TP53* (*R249S* mutation). Complete sequencing of the *HBX* gene indicated that *R249S* mutations were associated with HCC with no documented prior cirrhosis but not with HCC developing in a context of cirrhosis or in non-cancer chronic liver diseases. Thus, the *R249S* mutation may specifically cooperate within the *HBX* gene in a pathway that leads to HCC but bypasses cirrhosis [66].

Exposure to AFM_1 can account for a substantial part of the risk of HCC in men with chronic HBV hepatitis and adds to the evidence that HCV and family history of HCC increase the risk of HCC in men with chronic HBV hepatitis [67].

HCC is related to various etiologies including HBV, HCB, high alcohol intake, AFB_1, dietary exposure to AF and metabolic syndrome related to genetic and environmental factors. Most of the cases of HCC developed from cirrhosis. Thus, the mechanisms of carcinogenesis and the risk factors cannot be separated from the initiation events leading to cirrhosis. AFB_1 and HBV have a clear direct oncogenic role through point mutations in the *TP53* tumor suppressor gene and insertional mutagenesis, respectively. The sequencing and transcriptome analysis will clarify the etiology and genetic events [68].

AFB_1 causes HCC, and exposure begins in utero and is life-long. AFB_1 is converted by proteins in the cytochrome p450 (CYT 450) family into mutagenic and carcinogenic intermediates. AFB_1 is converted into AFB_1-8,9 exo-epoxide, which is in turn converted into AFB_1-N^7-Gua adducts. This adduct is metabolized into AFB_1-FAPY adducts, and these two adducts are mutagenic and carcinogenic. In addition, an arginine to serine mutation at codon 249 of the p53 tumor suppressor gene is produced, abrogating the function of the tumor suppressor gene and contributing to hepatocarcinogenesis. AFB_1 acts synergistically with HBV to cause HCC by integrating into the gene and interfering with nucleotide excision repair, activating p21waf1/cip1, generating DNA mutations, and altering gene methylation. The

precise pathogenetic mechanisms responsible for AFB_1-induced HCC as well as the interaction between AFB_1 and HVB in causing the tumor are unknown [69].

The AF regulatory standards around the world (4 to 20 ng/g total AF) are not adequately protective for human health, showing a HCC risk of >1 case in 100,000 persons. In contrast, when large amounts of maize and peanuts are consumed and HBV prevalence is high, the protection level is 1 in 10,000 lifetime HCC cases in the population. However, almost all aflatoxin regulations worldwide are adequately protective, with the exception of several nations in Africa and Latin America [70].

In conclusion, studies have reported the link between HBV and AF, but the present work also shows a link with AFB_1-N^7-Gua as a novel biomarker for HBV, HCV and VC and shows that the levels correlate with the type and amount of food ingested. This is one of the most thorough studies of the relationship between AF in foods and hepatic disease; it summarizes 42,000 data points of food rations, detects different AF and adduct levels in the urine of risk and control groups, and shows their relation to viral hepatic diseases and the importance of AF adducts as biomarkers in the risk of HCC.

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