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الجامعة الأردنيـــــة
كلية الدراسات العليا

التاريـــخ: 19 / 12/ 2010 م

نموذج رقم (18)
اقرار والتزام بالمعايير الأخلاقية والأمانة العلمية وقوانين الجامعة الأردنية وأنظمتها وتعليماتها لطلبة الماجستير

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عنوان الرسالة:

(Interindividual Variation in Aflatoxin B1 Exposure and The Concomitant Polymorphisms of certain CYP450s Among Jordanian Population)

اعلن بأنني قد التزمت بقوانين الجامعة الأردنية وأنظمتها وتعليماتها وقراراتها السارية المفعول المتعلقة باعداد رسائل الماجستير عندما قمت شخصيا" باعداد رسالتي وذلك بما ينسجم مع الأمانة العلمية وكافة المعايير الأخلاقية المتعارف عليها في كتابة الرسائل العلمية. كما أنني أعلن بأن رسالتي هذه غير منقولة أو مستلة من رسائل أو كتب أو أبحاث أو أي منشورات علمية تم نشرها أو تخزينها في أي وسيلة اعلامية، وتأسيسا" على ما تقدم فانني أتحمل المسؤولية بأنواعها كافة فيما لو تبين غير ذلك بما فيه حق مجلس العمداء في الجامعة الأردنية بالغاء قرار منحي الدرجة العلمية التي حصلت عليها وسحب شهادة التخرج مني بعد صدورها دون أن يكون لي أي حق في التظلم أو الاعتراض أو الطعن بأي صورة كانت في القرار الصادر عن مجلس العمداء بهذا الصدد.

توقيع الطالب: غادة سلامة ..... التاريخ: 19 / 12/ 2010 م

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التوقيع ..... التاريخ ٢٠١٠/١٢/٢٠

INTERINDIVIDUAL VARIATION IN AFLATOXIN B1 EXPOSURE AND THE CONCOMITENT POLYMORPHISMS OF CERTAIN CYP450s AMONG JORDAINAIN POPULATION

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December, 2010

COMMITTEE DECISION

This Thesis (Interindividual Variation in Aflatoxin B1 Exposure and the Concomitant Polymorphisms of Certain CYP450s Among Jordanian Population) was Successfully Defended and Approved on 6th December 2010

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تعتمد كلية الدراسات العليا
هذه النسخة من الرسالة
التوقيع.....التاريخ.....

Dedication

I dedicate this work to my precious family, friends and all the people who guided me through, all the way.

Thank you

Acknowledgment

This work wouldn't be accomplished without my supervisor, Dr Mohammed Al. Khateeb and my Co advisor Dr Kamal Al. Hadidi advice and support

My Deepest gratitude for the people in faculty of medicine in particular molecular lab staff for their generous help and support (Dr Said, Nida'a, Fatiam, Bara'a, Randa and Esmail)

Warm hearted gratitude for all the volunteers, who shared me the same enthusiasm in search of knowledge.

And for those who donated financially and helped me to complete this study
I will always appreciate what you have done

Maha, thank for your help, kindness and support

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List of Abbreviations

AFB1	Aflatoxin B1
AFBO	Aflatoxin B1 and Aflatoxin Q1
Ahr	Aryl hydrocarbon receptor
bp	Base pair
CAR	Constitutive androgen receptor
CYP	Cytochrome enzymes
Da	Dalton
dNTPs	Deoxy Nucleotides Triphosphate
DNA	Deoxyribonucleic acid
ELiza	Enzyme linked immunoassay
EDTA	Ethylene diamine tetra acetic acid
EPXH	Epoxide hydrolase
FDA	Food and drug administration
GST	Glutathione stransferases
KDa	Kilo Dalton
HPLC	High pressure liquid chromatography
ng	nanogram

HBV	Hepatitis B virus
HCC	Hepatic Carcinoma
IDMS	Isotop mass spectrometery – tandem mass spectrometery
kg	kilogram
LC/MS	Liquid chromatography / mass spectrometery
µg	Microgram
OD	Optical density
OATP2	organic anion transporting polypeptide 2
PCR	Polymerase chain reaction
PXR	Pregnane X- receptor
RNA	Ribonucleic acid
rpm	Round per minute
SRS	Substrate recognition sequence
SULT	sulfotranferases
SNP	Single nucleotide polymorphism
Taq DNA	*Thermus aquaticus* DNA
TBE	Trisbase Boric acid EDTA
UGT	UDP-glucuronosyltransferases
UTR	Untranslated region
χ^2	Chi square

Mg	magnesium
mRNA	Messenger Ribonucleic acid
MRPs	multidrug resistance associated proteins
NAT	N- acetiltransferases
Pgp	P-glycoprotein

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INTERINDIVIDUAL VARIATION IN AFLATOXIN B1 EXPOSURE AND THE CONCOMITENT POLYMORPHISMS OF CERTAIN CYP450s AMONG JORDANIN POPULATION

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ABSTRACT

Cytochromes P450s (CYP450) plays extremely vital role in the oxidation, reduction and peroxidation of numerous endogenous and exogenous compounds such as drugs and procarcinogens. They are mainly expressed in the liver in polymorphic forms, therefore they are proposed as biomarkers for susceptibility to carcinogencity and toxicity.

The mycotoxin Aflatoxin B1 (AFB1) is a food and feed contaminant that is oxidized principally by CYP1A2, 3A4, 3A5 and 3A7 and is believed to increase susceptibility to liver and lung cancer in people working in AFB1 contaminated environment.

The objective was to find allelic frequencies of (CYP3A5*2,*3,*4,*5,*6,*7, CYP3A4*1B, CYP3A7*1C and CYP1A2*1C, *1D, *1E, *1F) enzymes and compare them in people working in contaminated areas with general population.

To genotype the above mentioned alleles; polymerase chain reaction - restriction fragment length (PCR-RFLP) was applied on the DNA of 194 workers in feed and food factories who were exposed to AFB1 and 397 DNA of apparently healthy unrelated volunteers. Results were analyzed using SPSS Statistical package, and as expected, no difference in the allelic frequency between the two groups was found. Consequently we pooled both groups and calculated the frequency of each polymorphism. Hardy Weinberg equilibrium was used to calculate the frequencies

The frequency of the alleles were as follows 0.2% for CYP3A5*2, CYP3A5*3 (86.6%), CYP*3A5*4,*5*7 were not detected, CYP3A5*6 (1.7%), 11.7% for CYP3A4*1B, 1.7% for CYP3A7*1C. and 6.5%, 18.2%, 6.0% and 67.3% for CYP1A2*1C, 1D, 1E and 1F respectively.

In conclusion, the allelic frequencies of the mostly known CYP450 enzymes have been determined. The limitation of this study was the inability to determine the aflatoxin-albumin adducts levels.

1.0

Introduction

In toxicology the conventional framework for studying chemicals and xenobiotics toxicities relies on toxicity assessment which was developed by using descriptive means and finding relationships between chemicals in different doses with tissue pathology at the site of action as well as to system level toxicity and explicit mortality. However the advancement in biological research and molecular biology in particular has raised the interest to understand toxicities at a molecular level, indisputably this urged the need to correlate genotyping information at a pharmacokinetic and pharmacodynamic level. In other words, this would be shown by individual's genotype which significantly influences any chemical disposition and determines his susceptibility to chemical's and xenobiotic's toxicities, where the exposure to chemicals can result in different genes expression profiles which in turn leads to different pharmacodynamic effects.

Overall the newly emerged science; Toxicogenomic studies the individual's predisposition to carcinogenic, teratogenic and other toxic effects of drugs and other xenobiotics (Suter, et al., 2004).

To exert their toxic and or carcinogenic effect, most environmental toxic chemicals and carcinogens have to be metabolically activated and the Cytochrome enzyme (CYP) system in the human body appears to be the perfect tool for such purposes. It is the major enzyme system in xenobiotic metabolism, but not necessarily the leading factor for initiating toxicities exerted by xenobiotics, as CYP – catalyzed metabolism is one of the precursors for the detoxification of toxic chemicals (Gonzalez, 1993, Denisov, et al., 2005 and Guingerich, 2006).

Bozina, et al. (2009) have categorized the main proteins involved in xenobiotic disposition involved in the efflux mechanisms into; oxidative phase I; conjugative phase II (metabolizing enzymes) and the transporters phase III. Cytochrome 450 (CYP450) are heme thiolate proteins and form the major enzymes of phase I. They are considered the prerequisite for phase II catalyzed conjugation with positions created by some functional groups generated by CYP450s.

Phase II enzymes includes, UDP-glucuronosyltransferases (UGT), sulfotranferases (SULT), Glutathione stransferases (GST) and N- acetiltransferases (NAT). The reactions of these enzymes are important for the lipophilic compounds to be biotransformed into water soluble products to be readily excreted in urine later on.

The Phase III transporters enzymes includes the collectively called; P-glycoprotein (Pgp), multidrug resistance associated proteins (MRPs) and organic anion transporting polypeptide 2 (OATP2), they control the absorption, distribution and excretion of xenobiotics, these enzymes are expressed in many tissues such as the liver, intestine, kidney and brain.

Moreover Bozina, et al. (2009) have illustrated the presence of a considerable catalytic diversity among the CYP enzymes of phase I like CYP families 1 to 3 which exhibits an interindividual variability in catalytic activity, which may be either related to genetic polymorphisms or to variability in the expression levels.

Of the CYP enzymes discovered, CYP1A1, 1A2, 2A6, 2C9, 2D6, 3A4 and others have shown to be polymorphic at the phenotypic or the genotypic level or both and because an individual's ability to metabolize these toxicants can be altered; depending on the variant alleles, these variations which cause genetic polymorphism have been proposed to be used as biomarkers for susceptibility to environmental carcinogenesis and toxicity according to Hong and Yang, 1997.

It is well known that most of the hydrophobic substrates, like polychlorinated biphenyls and dioxin-like compounds, are oxidized by CYP1 (CYP1A1, CYP1A2, and CYP1B1). More hydrophilic compounds (nitrosoureas, aminoazo dyes, biphenyls, fluorenes, and heterocyclic amines) are metabolized by CYP1A1 and CYP1B1 mainly. Carcinogens however including environmental pollutants are oxidized by CYP2C8, CYP2C9, CYP2C19, CYP2E1, CYP3A4, CYP3A5, CYP2A6, and CYP2B6. CYP2E1 activates predominantly small molecules like benzene, vinyl chloride and others, while CYP3A4 and CYP3A5 activate larger molecules such as aflatoxins and in particular Aflatoxin B1 (Belitsky and Yakubovskaya, 2008).

Aflatoxin B1 is economically important due to its effects on human health, livestock and the marketability of agricultural products. It has been categorized as a group 1 carcinogen after several studies linked between human cancer and exposure to aflatoxin (Dvorackova, 1976). Hayes, et al., (1984), reported higher incidence of respiratory tumors such as lung cancer in workers of a linseed processing plant exposed to (0.04 to 2.5 µg AFB1 per week) of aflatoxin B1. Similar results indicating that workers exposed to airborne aflatoxin B1 is a sustained risk in potentially developing lung cancers were reported by others (Kelly, et al., 1997 and Van Vleet, et al., a, b, 2002)

1.2 Study Objectives:-

Taking into consideration that aflatoxin is a toxin found in the environment in general and in some crops and commodities in particular and that it is metabolized by CYP450 enzymes we designed this study with the following objectives:

1. To compare the gene frequency of some of the well characterized CYP450 polymorphisms in workers in food and feed manufacturing plants who are supposed to be exposed to aflatoxin B1 with that of general population.
2. To determine the gene frequency of different polymorphic forms of this enzyme in Jordanian population
3. Compare Jordanian with other ethnic groups.

In order to follow these objectives, genomic DNA of the study group and control group were screened using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) for several CYP 450 polymorphic forms (CYP3A4*1B, CYP3A7*1C, CYP3A5*2 – *7, CYP1A2*1C, *1D, *1E and *1F).

2.0

Literature review

2.1 Fungi; the origin of Aflatoxins

Fungi are heterotrophic euokaryotes that feed by adsorbing organic compounds from their environment, by secreting acids and enzymes to break down polymeric compounds into simpler components which are then absorbed back into the fungal cell. The fungi reproduce by sporulation, they are described of being ubiquitous (grow almost any where) (Speijers and Speijers, 2004 and Bennett, 2010).

2.1.1 Aspergillus Genera

According to Bennett (2009), Aspergillus which was named by Antonio Micheli (1679 – 1736) when he described 1900 plants of which 900 were fungi "sprinkles the world with spores" (Fig.1). Aspergillus colonies usually grow rapidly as white, yellow, yellow brown to black or shades of green, mostly consisting of a dense feet of straight conidiophores. The conidiophores consist of elongated stalk or stipe culminates in an expanded bulbous region called the columella or vesicle. This fertile area of the vesicle gives rise to a layer of cells called philaides that produce long chains of mitotic spores called conidia or conidiospores. The base and the origin of the conidiophore is T or L shaped (foot cell) which forms during mycelial differentiation where some cells enlarge and develope this heavy cell walled diagnostic feature of the aspergillus's conidiophores (Speijers and Speijers, 2004 and Bennett, 2010).

There are at least 200 species in aspergillus genus and decisively Aspergillus species now are classified within the ascomycota upon extensive molecular phylogentic analysis for both classification and nomenclature (Bennett, 2010).

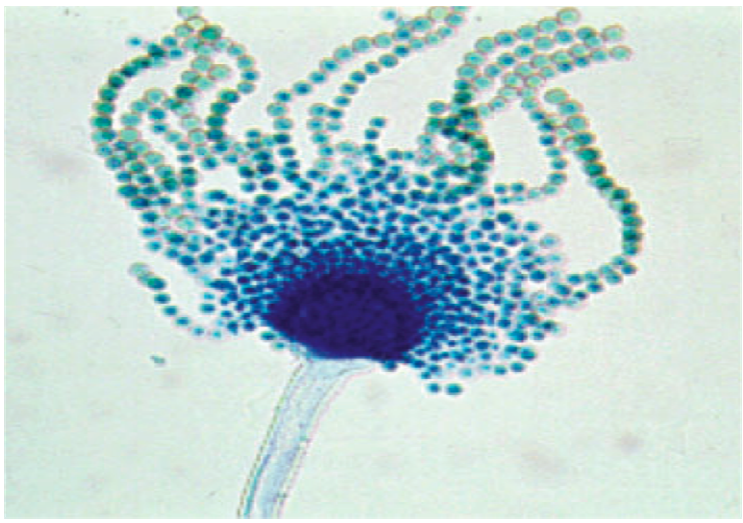

Figure 1: microscopic feature of *Aspergillus Flavus* (Hedayati et al., 2007)

2.1.2 Penicillium Genera

Comparable to aspergillus, Penicillium is a filamentous fungi, they are versatile and opportunistic, with branched conidiospores, produces conidia in a structure termed a penicillus; from Latin, little brush. A penicillus consists essentially of a well-defined cluster of phialides or similar cells bearing small, single-celled, dry conidia in chains. The phialides are either attached to a stipe directly or through one or more stages of branching. Branches are of generally similar diameter to stipes. Penicillium species tend to have a small hyphae which makes its protoplasmic movement hard to detect and leads to small peripheral growth zones, they are of hydrophobic spore surfaces but capable of getting wet, penicillium are osmotolerant and heterotrophic and produces asexually (Pitt and Hocking, 2009).

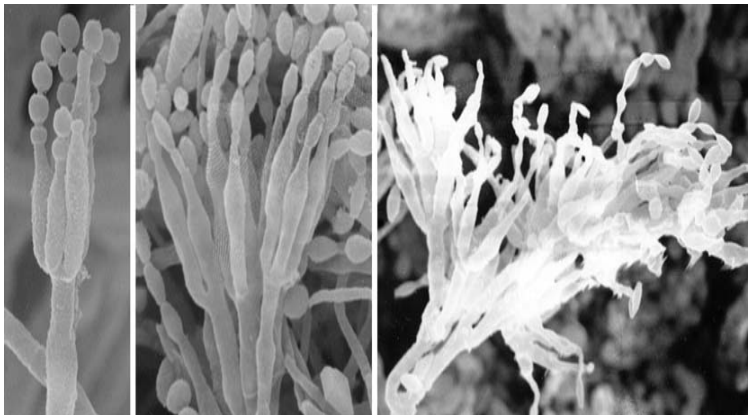

Figure 2: Scanning electron micrograph of *Penicillium spp* (Pitt and Hocking, 2009)

2.1.3 Mycotoxins

2.1.3.1 Mycotoxins definition

Human health not only can be impaired by allergy and infection resulted from fungi but through toxic metabolites they produce (Hedayati, et al., 2007). Fungi secret enzymes of different types when they colonize to digest organic matter into simpler compounds, these compounds are the primary and secondary metabolites (Mellon, et al., 2007). Mycotoxins are the natural secondary metabolites encompass a diverse and large structural group of toxins produced by the mycelial structure of the filamentous fungi or moulds known for their low molecular weight approximately 700 Daltons (Da) (Hussein and Brasel, 2001 and Mayer, et al., 2008).

Originally the term Mycotoxin as the name indicates is a combination of the Greek words, fungus (mykes) and the Latin word (toxicum), which means poisoning (Turner, et al., 2009). To date there are over 300 different mycotoxins known and classified into approximately 25 structural types with a diversity in chemical structure what makes mycotoxin toxicities and their presumed health effect on both human and animals a growing concern (Mayer, et al., 2008).

Mycotoxins are not required for growth nor for the development of the fungi and evidently the reasons for their production are not clear. Several authors proposed that the production of mycotoxins could be a defense mechanism (Fox and Howlett, 2008, Mayer, et al., 2008 and Turner, et al., 2009). Kempken and Rohlfs (2009) in their review of different approaches on why Mycotoxins are produced, they have suggested that secondary metabolism is under a tight regulatory control that allows adjustment of secondary metabolites formation to adapt diverse ecological challenges.

Since 1961, the Modern Mycotoxicology have grown progressively and dramatically after a veterinary crisis in London following the death of 100,000 turkey poults which was associated and linked afterwards to peanuts contamination with *Aspergillus flavus* secondary metabolites known as Aflatoxin B1 (Richard, 2007).

2.1.3.2 Mycotoxigenic Fungi

All mycotoxins are of fungal origin; conversely not all toxic compounds produced by fungi are called Mycotoxins. For instance fungal products that are toxic to bacteria are called antibiotics, on the other hand ethanol is a low molecular weight fungal metabolite but only toxic at higher concentration but not considered a Mycotoxin. The effect of mycotoxins and their metabolites on humans depends on their concentration and the target organ. Mushroom poisons are not considered Mycotoxins because their intake is attributed usually with a criminal intent (Bennett and Klich, 2003).

It should be pointed out that not all molds are toxigenic and mycotoxigenic fungi implicated the human food chain belong mainly to *Aspergillus, Fusarium* and *Penicillum* (Sweeney and Dobson, 1998).

Fusarium is considered as a destructive endophytic plant pathogen, that produces mycotoxin before or immediately to post harvesting crops such as cereal grains and forages, while *Aspergillus* and *Penicillum* saprophytically contaminants commodities and foods during drying and storage. Due to the fact that the toxigenic molds needs large amount of energy to produce toxins, Mycotoxins problems are more common in grains than forages (Sweeney and Dobson, 1998, Hussein and Brasel, 2001 and Meerdink, 2002).

2.1.3.3 The Effects of mycotoxins on health.

Mycotoxicosis is the reflection of the toxic effect of Mycotoxins after the dietary, respiratory or dermal exposure. Mycotoxicosis pathologies resembles those caused by the exposure to pesticides and heavy metal residues.

It should not be conflicted with Mycoses that result from the growth of fungi on animal hosts (Bennett and Klich, 2003). It is well recognized that the mycotoxin type , amount of toxin, duration of exposure, the age, general health, and the sex of the individual and other inadequately implied synergistically effects (that involves genetics, dietary status interaction with other toxic insults), affect Mycotoxicosis symptoms (Bennett and Klich, 2003). In the same time mycotoxicosis can increase the vulnerability to microbial diseases and worsen the effect of malnutrition and could synergistically interact with other toxins (Bennett and Klich, 2003 and Speijers and Speijers, 2004). Moreover the severity of mycotoxins toxic effects can be affected by some factors like vitamin deficiency, caloric deprivation, alcohol abuse and infections or disease status (Bennett and Klich, 2003)

The impact of Mycotoxins on human health and veterinary is the burden associated with the chronic exposure to Mycotoxins which is manifested by cancer induction, kidney toxicity and immunosuppresion. While the acute effects were best known for Mycotoxin exposure and proven scientifically as in the case of Turkey X disease (Bennett and Klich, 2003 and Mayer, et al., 2008). The magnitude of the toxicity may vary according to the species and subspecies and the mode of action of the mycotoxin (Hussein and Brasel, 2001 and Mayer, et al. 2008).

2.1.3.4 Mycotoxins groups

Fungal secondary metabolites (Mycotoxins) are separated into four groups: alkaloids, non ribosomal pepetides, polyketides, and terpenes (Kempken and Rohlfs, 2009). Examples of Mycotoxins of great public health concern and agroeconomic significance include: Afalatoxins, Ochratoxin, Zeraleneon, Fuminosin, Tremogen and Ergots (Hussein and Brasel, 2001). The Food and Agriculture Organization (FAO) reports that 1/4 of the world's crops are contaminated to some extent with the aforementioned Mycotoxins (Van Egmond and Jonker, 2005).

2.1.4 Aflatoxin B1

Aflatoxins B1, G1, B2, G2 are members in the Aflatoxin group, Aflatoxin B1 is the most important toxin that contaminants food and feed in many developing and developed countries. According to the Food and Drug Administration (FDA) they are considered to be an unavoidable contaminant of foods (Van Egmond and Jonker, 2005). Aflatoxins were first isolated in early 1960's after an outbreaks of disease and death in turkeys and in rainbow trout fed on rations formulated from peanut and cotton seed meals (Wild and Turner, 2002 and Williams, et al., 2004).

These contaminants can be detected in food and feed during growth, harvest and long term storage especially at inappropriate conditions. They are the secondary metabolites of *Aspergillus flavus* (shown in figure 1) and *Aspergillus parasiticus*, members of the Genera *Penicillium* are known to produce Ochratoxin, predominately and according to FAO Aflatoxins are produced in nature solely by *Aspergillus spp* (Van Egmond and Jonker, 2005).

Growth and contamination eventually aggravate within any commodity when temperatures are between 24ºC and 35ºC and if the moisture content exceeds 7% - 10% with ventilation, importantly Aflatoxin formation is exaggerated by a sum of biotic and abiotic factors in the environment of the fungus (Wogan, 1973, Williams, et al., 2004, Van Egmond and Jonker, 2005, Klich, 2007 and Hedayati, et al., 2007)

2.2 The Cytochrome 450 enzyme system (CYP450 or P450).

2.2.1 Diffinition and History

The intracellular hemeproteins CYP450 activates molecular oxygen for the oxidative metabolism of a wide spectrum of lipophilic organic chemicals. The presence of thiol group from cystein residue serves as a ligand to the heme iron and differentiates them from other heme proteins. This ligand will provide electron center for the activation of molecular oxygen by alternating the electron density porphoryn ring resonance in the heme molecule, Figure (3) (Hasler, et al., 1999).

CYP450 were first recognized by Martin Klinberg in (1958) when he was studying a chromosomal pigment, he noticed an absorbance band with a maximum density at 450 nm wave length. This property was unique among heme proteins and served as a signature for the CYP450 protein and consequently this region was given the same name. Omura and Sato in (1962 – 1964) have solved Klinberg's spectrophotometeric incident and characterized this pigment as a heme protein (Hasler, et al., 1999).

2.2.2 Nomenclature and Categorization.

The categorization of CYP450 is based on the families and subfamilies amino acids sequence, The families have more than 40% sequence resemblance while sub families have at least 55% resemblance (Nelson, 1996) and (Hasler, et al., 1999). The standard nomenclature, enzyme within a super family are designated by Roman numerals; subfamilies are designated by capital Roman letters, while isoforms are marked by Arabic numerals. For example, CYP2D6 means cytochrome P450, family 2, subfamily D, polypeptide 6, (Nelson, 1996 and Belitsky and Yakubovskaya, 2008).

2.2.3 Occurrence and natural distribution

Denisov (2005) described the CYP450 as "they catalogs the diversity of life forms, since they are found in all branches of the tree of life", (page 2253). Around 7700 CYP450's sequences and 866 families have been identified, of which 2740 sequence were found in animals and 2675 in plants (Hamdane, et al., 2008).

Upon the completion of the sequence of the human genome, 107 human CYP450 genes were found, 59 of them are active and 48 are pseudogenes (Guengerich, 2006). Remarkably the number of CYP450's in lower organisms was found to account for 239 pseudogenes in Arabidopsis (small flowering plant) and 458 pseudogenes in rice, yet CYP450 based detoxification potential appears to be higher in rodents, were mouse are proven to have 108 functional CYP genes (Guengerich, 2006).

2.2.4 Cytochrome P450 role in life and activity.

Fifty seven CYP450 enzymes involved in drug and chemical toxicity have been identified and gain considerable interest (Guengerich, 2006). Quarter of them are involved in metabolism of xenobiotics but only 5 human CYP450's are involved in 90-95% of the metabolism (Guengerich, 2008). 75% of drugs can be oxidized by three CYP450's (3A4, 2D6, 2C9). Conversely only 6 (1A1, 1B1, 2A6, 2E1, 1A2 and 3A4) of CYP450's are involved in metabolism of chemical carcinogens (Hasler, et al., 1999, Guengerich, 2005 and Guengerich, 2008).

Human CYP450 forms can be divided into three major groups; those in 5- 51 CYP families have endogenous importance since they have significantly high affinity for the substrates and are relatively well conserved during evolution. Family 1 – 3, usually have less affinity for their substrates and they are less conserved evolutionary Nevertheless this group gained reputation through exhibiting important genetic polymorphism and accounting for 70 – 80% of all phase I dependent metabolism of most clinically used drugs. The third group has a role in fatty acid and related substrate metabolism of some xenobiotics (Ingelman-Sundberg, 2004).

2.2.5 Cytochrome P450, nature and metabolic activity.

Most CYP450's are members of the class of enzymes called oxygenases, specifically monooxygenases or mixed function oxidases were they induce the incorporation of one atom of oxygen from O_2 into the substance being oxidized as indicated in Figure (4) (Degtyarenko and Archakov, 1993, Hasler, et al., 1999 and Hamdane, et al., 2008). Preferentially they are expressed in the centrilobular area of the liver; a sensitive area consequently is borne to damage by drugs and ethanol that are activated by CYP450 action (Guengerich, 2006).

CYP450 are membrane bound, hydrophobic and hard to crystallize. Contains approximately 500 amino acids (a cystein molecules located near the carboxy terminus of the protein provides the essential thiol ligand for the heme iron). The signature sequence of most P450 containing this cystein is FxxGxxxCxG. The amino terminus of the protein is rich in hydrophobic amino acids and is believed to act as a domain for binding the proteins to membranes and the P450s catalyzes the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and oxygen dependent oxidation transformation of large number of different chemical compounds (Hasler, et al., 1999 and Denisov, et al., 2005).

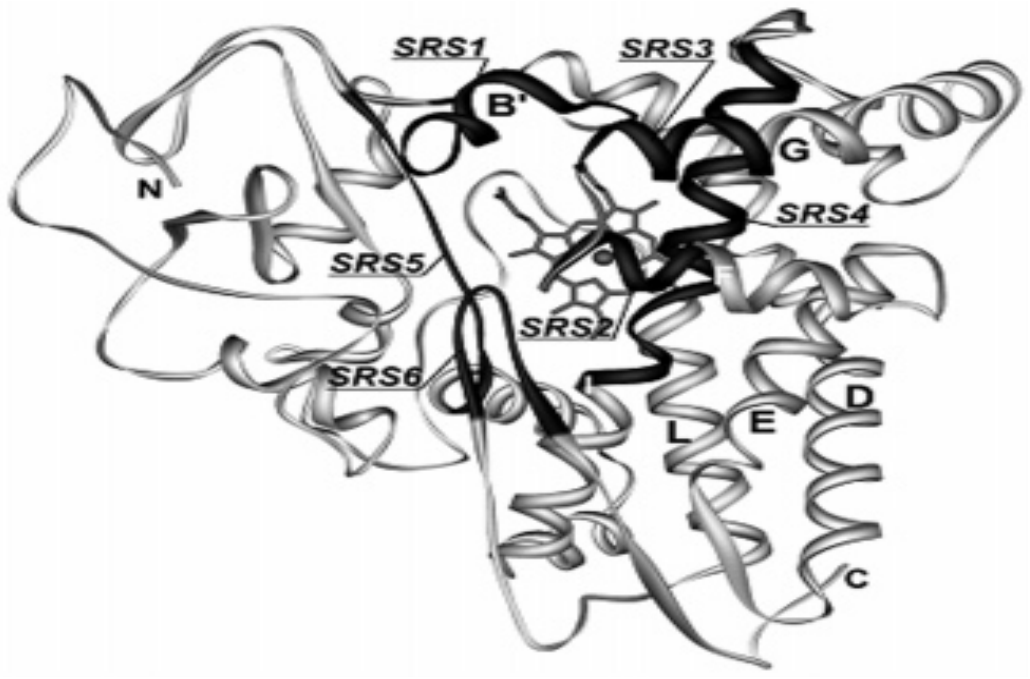

Figure 3: Distal face of cytochrome P450s folding which is highly conserved and shown in a ribbon representation Substrate recognition sequence (SRS) regions are shown in black and labeled. (Denisov, et. al 2005).

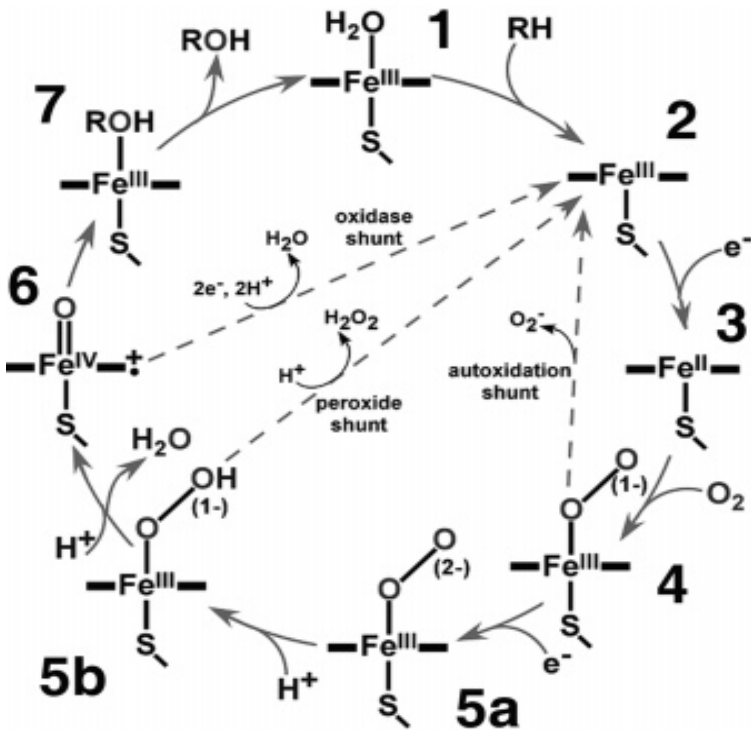

Figure 4: Catalytic cycle of the CYP450, (i) oxygen binding to the reduced heme iron and formation of an oxygenated heme Fe2+-OO or Fe3+-OO- (ii) one-electron reduction of this complex to a ferric peroxo state Fe3+-OO2-, which is easily protonated to form the hydroperoxo Fe3+-OOH-, (iii) second protonation of the latter Fe3+-OOH- complex at the distal oxygen atom to form an unstable transient Fe-OOH2, which is followed by heterolytic scission of the O-O bond and release of the water molecule (iv) the various reactions of the remaining higher valent porphyrin metal-oxo complex, often described as a ferryl-oxo *ð*-cation porphyrin radical (Denisov, et al., 2005)

2.2.6 Expression and Expression Sites

Concentration of CYP450 in the liver, intestine and the cortex of the adrenal gland exceeds the concentration of other hemeproteins (Hasler, et al., 1999 and Guengerich, 2005). Even though they are found to be distributed through out every organ in the human body; the type of the CYP450 in a tissue appears to be specific. Most of them are inducible and the control of the CYP450 expression can be exerted at the transcriptional, (messenger ribonucleic acid) mRNA, at translational, and post translational levels. Post translational regulation has been described in CYP450 1A2 and 3A4 (Hasler, et al., 1999 and Guengerich, 2006). On the other hand the 57 CYP450's that have been mostly examined; appeared to be expressed primarily in the endoplasmic reticulum and only 6 are located exclusively in the mitochondria (Guengerich, 2005).

It is obvious through browsing out literature that CYP450's play a distinctive role in the cellular metabolism and the maintenance or stability of the cellular homeostasis. Some of the reactions catalyzed by human CYP450 are required for the conversion of cholesterol to androgens, estrogens, gluco and mineral ocortiocoids. Synthesis and degradation of prostaglandins and other unsaturated fatty acids and these reactions are restricted to only 4 CYP450's. Conversion of vitamins to their active forms specifically (A and D) also catalyzed 4 CYP450s. An added role is metabolism of cholesterol to bile acids. Apart form their role in endogenous reactions, 15 CYP450s are involved in the metabolism of xenobiotics and change them to reactive metabolites through the free radicals that interacts with cellular deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and proteins or undergo detoxification by the reaction with cellular constituents such as glutathion (Hasler, et al., 1999, Denisov, et al., 2005 and Guengrich, 2005).

2.2.7 Cytochrome P450 substrates, induction and inhibition.

As a group CYP450 have more substrates than any other group of enzymes (table 1), at least among drugs and most of the reactive products (for example: epoxides and hydroquinones) (Guengerich, 2006) and (Guengerich, 2008). Higher eukaryotes have gained evolutionary an adaptive mechanisms that allowed them to detect any insulting agents and thus increase their metabolism and eventually their clearance, clearly this defense front line against such harsh insults is signified by the CYP450's monooxygenases; as they catalyze the first step in the metabolism of the lipophilic xenobiotics to a more soluble compounds which can be readily excreted. Therefore upon exposure to xenobiotic substrates, CYP450 expression rate increases, and this induction is synchronized and regulated by a controlled process at the transcriptional level (Guengerich, 2005).

The process of the catalytic cycle of CYP450 (Figure. 4) are exposed and vulnerable to inhibition at different levels: at the binding of the substrate, at the binding of molecular oxygen, and at the catalytic step in which the substrate is actually oxidized.

CYP450 inhibitors can be divided into three mechanistically diverse classes, agents that bind reversibly, agents that form quasi irreversibly complexes with the heme iron atom and agents that bind irreversibly to the protein or the heme moiety or accelerate degradation and or oxidative fragmentation of the prosthetic heme (Williams, et al., 2005 and Pelkonen, et al., 2008). Inhibition and even destruction of CYP450's do not produce toxicity, except in cases where CYP450 is involved in a critical physiological pathway such as steroidgenesis. Further more if an individual has an inherited genetic deficiency of a particular CYP450 or the enzyme it self is inhibited by another drug; toxicity may develop, especially if a drug or a xenobiotic accumulates over repeated doses or exposure (Guengerich, 2008).

Table 1: Some of the mostly known substrates, inhibitors and inducers of the CYP3A4, 3A5 and 1A2.

Cytochrome P450	substrates	Inhibitors*	Inducers**
CYP3A4 and 3A5	Midazolama, Testosterone Felodipinea Erythromycin Nifedipine Triazolam Simvastatin Atorvastatin Alprazolam Cyclosporin Buspirone Alfentanil Quinidine Lovastatin Eletriptan Sildenafil Vincristine Tacrolimus	Ketoconazole Itraconazole Troleandomycin Verapamil Indinavir Saquinavir Diltiazem Clarithromycin Gestodene Ritonavir Nelfinavir	CYP3A4 Inducer Rifampicin Rifabutin Amprenavir Aprepitant Bosentan Ritonavir St. John's wort Sulfinpyrazone Topiramate Carbamazepine Efavirenz Nevirapine Barbiturates (phenobarbital, etc.) Phenytoin Dexamethasone Methylprednisolone Prednisolone Artemisinin antimalarialsh Metamizole Modafinil CYP3A5: Rifampicin Topical clobetasol 17-propionate
CYP1A2	Caffeine Phenacetin Ethoxyresorufin Tacrine Theophylline Melatonin Riluzole Tizanidine Zolmitriptan Ropivacaine Flutamide Frovatriptan Lidocaine Ropinirole Mirtazapine Clozapine	Fluvoxamine a-Naphthoflavone Ciprofloxacin Rofecoxib Mexiletine Propafenone Enoxacin	PAHsb Indole-3-carbinolc TCDD Omeprazole Rifampicin Ritonavir Sulfinpyrazone Carbamazepine Barbiturates (phenobarbital, etc.) Phenytoin Smokers Some foods broccoli char grilled meat

* These will slow down substrate drug metabolism and increase drug effect.

** These will speed up substrate drug metabolism and decrease drug effect.

Source: (Pelkonen, et al., 2008) and (Guingerich, 2005).

2.2.8 Genetic basis of CYP metabolic polymorphism:

2.2.8.1 Polymorphism definition and type of mutations in the CYP450

Phenotyping was the first level of polymorphism studied in pharmacokinetic, defined as the observable characteristic of an individual produced by the interaction of genes and environment. In this case the phenotypic character was the unusual responses to drugs due to reduced metabolism was obvious in some of the cases studied. The second level was the studying allelic polymorphisms of the CYP450, which is defined as the occurrence in a population of two or more genetically determined forms in frequencies greater than 1% in the population. Any frequency less than 1% it becomes monogenic of rare genetic trait (mutation) such as mutations in inborn error of metabolism diseases (Guengerich, 2005).

2.2.8.2 Types of mutations in the CYP450 enzymes and their significance.

Mutations in the CYP genes are the chief mechanisms for shifting, altering enzyme expression and/or catalytic activities, where point mutations and deletions are observed in the polymorphic CYP genes. Depending on their locations in the gene sequence, polymorphic changes could have either one of the two general effects or none at all. Mutations in the coding region of a CYP gene that cause amino acid substitutions could alter the catalytic activity by causing a direct change in the protein structure. Mutations in the noncoding region, on the other hand, may modify the level of mRNA expression influencing the transcription, mRNA stabilization, or premature RNA splicing. Remarkably an individual's competence to metabolize certain drugs and environmental chemicals can be subjective to the functional significance of mutations (Hong and Yang, 1997). Mainly the genetic alterations in CYP gene family may be caused by either deletions, missense mutations, splicing defects mutations and nonsense mutations

and rarely mutations in the 5'- or 3'-untranslated regulatory regions affect the CYP phenotype (Rodregiguz and Ingelman 2006).

Genes encoding xenobiotic metabolism (CYP1-3) differ in their characteristics from genes responsible for the metabolism of principally endogenous compounds (CYP4-CYP51), 6 out of the 20 CYP450s significant in the metabolism of endogenous compounds have revealed a genetic polymorphism. The genes in families 1 - 3 are functionally polymorphic with exception of CYP1A1, 2E1, 3A4 are relatively conserved. This group includes a number of pseudogenes, such genes are also present in some CYPs involved in endogenous metabolism (CYP21P), with less variable comparing to CYP families 1 to 3 (Bozina, et al., 2009 and Guengerich, 2006). The frequency of these genes shows variability in different races and different ethnic groups which indicate the needs for genotyping the population to get the utmost benefits of treatment (Ingelman- Sundberg, 2004).

2.2.8.3 Levels of CYP450 expression.

The CYP450 expression can be controlled at the transcriptional, mRNA, translational and posttranslational levels. At the transcriptional control level, three vital cytosolic receptors are responsible for the detection the concentration of environmental xenobiotics, namely the pregnane X-receptor (PXR), constitutive androgen receptor (CAR) and aryl hydrocarbon receptor (AhR) respectively called the xenosensors. The AhR regulates CYP1A1, CYP1A2 and CYP2S1, the PXR regulates CYP2C9 and CYP3A4 and the CAR regulates CYP2B6, CYP2C9, and CYP3A4. Apparently these transcriptional factors are involved in the control of most human drug metabolizing CYP450s. Polymorphisms of receptors CAR, PXR, and AhR have also been described in literature. For instance more than 10 mutations in the AhR gene have been recognized to be able to modulate CYP activity (Kuehl, et al., 2001, Burk and Wojnowski, 2004, Pelkonen, et al., 2008 and Bozina, et al., 2009).

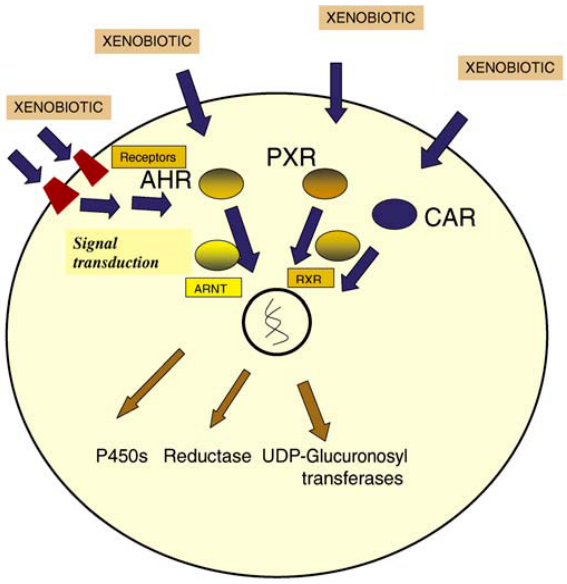

Figure 5; sensing mechanism for the regulation of expression of cytochrome P450 and other xenobiotic metabolizing enzymes, the level of the xenobiotic is sensed by receptors in the cytosol. In addition some extracellular receptors can activate some of these receptors by signal transduction pathways. (Ingelman-sundberg, 2004)

2.2.9 Cytochrome 3A sub family (CYP3A)

The Cytochrome 3A family has been studied extensively due to its clinical importance since it has a wide substrate spectrum, susceptibility to drug interaction in addition to the high inter individual variability in expression (Burk and Wojnowski, 2004).

Although the genetic factors accounts for 70 – 90 % of the variations in CYP3A the activity and expression can be affected by many other factors; including drug interactions, induction or inhibition by drugs, environment, age, race, disease status, organ function and dietary factors (Lee and Goldstein, 2005).

Single nucleotide polymorphisms (SNPs) are the most common form of genetic variation in the CYP3A genes (Roy, et al., 2005 and Kuehl, et al., 2001).

CYP3A is the most abundant subfamily of CYP450 mostly localized in the kidney, intestine and the liver and accounts for 30 – 60% of the total CYP450 (Burk and Wojnowski, 2004 and Lee and Goldstein, 2005). This family contains four isoformes CYP3A4, 3A5, 3A7 and 3A43 located adjacent to each other on chromosome 7q21 and consists of 231 - 232 (kilo base) kb in length and responsible for the metabolism of 45–60% of currently prescribed drugs (Van Schaik, et al., 2002), (Burk and Wojnowski, 2004 and Lee and Goldstein, 2005). CYP3A4 and CYP3A5 are the most expressed in the liver and intestine. 80 SNPs has been reported in this family. Both CYP3A4 and CYP3A5 contain similar sequences and that is why they have substrate overlapping specificities (Shimada, et al., 1994 and Lee and Goldstein, 2005).

2.2.9.1 Cytochrome 3A4 (CYP3A4)

This gene is encoded by a 27 kb sequence on human chromosome 7q21.3 – q22.1 and spans 13 exons. Coding for enzyme consists of 502 amino acids with a molecular weight of 57 kilo Dalton (kDa). Hepatic expression of 3A4 protein is vary and could reach up to 90 folds, and the vivo variability in clearance is much lower being less than 10 folds for several CYP3A substrates (Lee and Goldstein, 2005).

Although approximately 30% of the CYP3A4 expression is in the liver showing a unimodal distribution (Burk and Wojnowski, 2004), it is also expressed in the lungs, the small intestine, prostate and colon. It is involved mostly in the oxidative metabolism of a broad range of structurally diverse foreign compounds and the endogenous steroid hormones. Substrates bioactivated by CYP3A4 includes acetaminophen, AFB1 and many others shown in previously mentioned table (1) (Lee and Goldstein, 2005) and (Burk and Wojnowski, 2004).

CYP3A4 activity is depending on CYP3A4 mRNA concentration, indicating that transcriptional control is the primary mechanism for regulating expression of CYP3A4. The functional CYP3A4 is found in most "adults" individuals with varies expression ranging 10 – 40% (Kuehl, et al., 2001). CYP3A4 can catalyze multiple metabolic pathways including C- and N terminal dealkylation, and C terminal-hydroxylation, dehalogenation, dehydration and nitroreduction (Li, et al., 1995).

A total of 18 CYP3A4 coding variant (CYP3A4* 2 – 18) has been reported. And it was identified that CYP3A4 have 28 SNPs, five that caused a non-synonymous changes and one caused changes in the CYP3A4 coding region, and those SNPs that affect protein activity "the non-synonymous" have a low frequency and are likely to occur at different frequencies in different populations (lee and Goldesteine, 2005).

CYP3A4 variant CYP3A4*2, *4, *5, *6, *17, and *18, have been shown to alter enzyme activity, compared with the wild type. It has been estimated that 90% of the human variation in CYP3A4 activity is due to genetic origin (Lamba, et al., 2002).

Single neucleotide polymorphisms found in 5'- UTR include CYP3A4*1A – F and in the 3'-UTR includes CYP3A4*1G – J and 1N – T. (Lamba, et al., 2002). The most common variant in the 5' – untranslated region (UTR) is CYP3A4*1B with a point mutation results from nucleotide substitution (A>G) at -392. (Originally described as CYP3A4*V) had attained a specific status among CYP3A4 variants, with the postulation that this variant might be associated with the reduced CYP 3A4 expression and thereby decreasing the metabolism of steroids in the prostate which latter proven to be an associated with prostate cancer, another involvement is with the reduced risk for the treatment – related leukemia (Burk and Wojnowski, 2004). The frequency of CYP3A4*1B is highly variable in different racial populations. Kuehl, et al. (2001) reported that CYP3A4*1B don't influence CYP3A4 gene expression or enzyme activity due to the linkage disequilibrium between CYP3A4*1B and CYP3A5*1.

2.2.9.2 Cytochrome 3A5 (CYP3A5)

The CYP3A5 gene has 13 exons encoding protein of 502 amino acid, expressed at a higher level than CYP3A4 in extra hepatic tissues such as the lungs, kidney, breast, prostate and polymorphonuclear leukocytes (Lee and Goldstein, 2005 and Kuehl, et al., 2001).

Cytochrome 3A5 variants in the 5'- and 3'- UTR are CYP3A5*1B-E, where most of these variants in the 5'-UTR occur at frequencies less than 5%, However in the 3'-UTR the most dominant variant is the CYP3A5*1D and of the SNPs occur in the coding region in the exons or entrons these includes CYP3A5*1 – 10.

Many reports showed that CYP3A4 is more inducible and active than CYP3A5, while other reports suggest that CYP3A5 has greater activity towards certain substrates. (Lee and Goldstein, 2005 and Kuehl, et al., 2001).

CYP3A5 is more likely to be polymorphically expressed and accounts for at least 50% of the CYP3A content when CYP3A4 expression is lower in the specified organ and in individuals expressing CYP3A5*1 (Burk and Wojnowski, 2004 and Kuehl, et al., 2001). All CYP3A5 SNPs are located on the chromosome 7, CYP3A5*2 is to be found on exon 11 and has a C >**A** substitution at codon 389, this nucleotide substitution in the coding region is a non-synonymous mutation with an amino acid modification of the protein composition from the uncharged polar residue threonine to uncharged polar residue Aspragine, however this mutation doesn't change proteins polarity (Roy, et al., 2005 and Lee and Goldstein, 2005).

With CYP3A5*4 which is positioned on exon 7 and with A>**G** nucleotide substitution at codon 200 causes an amino acid modification from Glutamine to Arginine. CYP3A5*5 is Another SNP found at the 5' end of intron 5 or at the splicing donor site results from T >**C** substitution, consequently resulting in a splicing defect. A distinctive

defect found in another allele belonging to CYP3A5; is CYP3A5*6 at exon 7 causes a splicing defect with G>**A** at codon 208 as a deduction and deletion of this exon; a decreased Enzymatic activity would result, however CYP3A5*3 is considered the major allele that causes CYP 3A5 decreased activity, is located at intron 3 and codes for a splicing defect as a consequence of an A>**G** nucleotide change. The splicing defect is illustrated by the production of a modified (unstable and degradable) mRNA responsible in creating defective CYP3A5 enzymes (Chou, et al., 2001, Kuehl, et al., 2001 and Roy, et al., 2005). A frame shift mutation with an un truncated non functional enzyme as an outcome and a result of a single base insertion " threonine" at codon 346 at exon 7 is the tombstone of CYP3A5*7 allele (Lee and Goldstein, 2005).

Researchers considered that genotyping analysis for mutations responsible on generating splicing defects especially (CYP3A5*3 and *6) would predict the expression to great extent, what was a prediction turned to be true for CYP3A5*3; due to the apparent and attributable decreased and variable expression as a result of this allele in several populations and ethnic groups. Out of 30 variants found, the variant CYP3A5*3A (g.6986G) showed the highest specificity and selectivity as a marker of the CYP3A5 polymorphisms, The prominence of CYP3A5*3 cryptic splice is that it produces a premature stop codon and almost a significant null expression of the CYP3A5 protein as a consequence in people who are homozygous to the allele, or who are to the great extent carriers of the homozygous recessive allele in other words all livers expressing high amounts of CYP3A5 are heterozygous or homozygous for an adenine at the position g.6986 (the wild type CYP3A5*1 allele), whereas all livers expressing low amounts of CYP3A5 protein are homozygous for guanine at this position (Lee and Goldstein, 2005 and Kuehl, et al., 2001).

Although CYP3A5 induce-ability is achieved through CAR and PXR agonists the substantial induction of this enzyme is related to the proximal element PXR (Kuehl, et al., 2001, Burk, et al., 2004 and Lee and Goldstein, 2005).

The association between CYP3A5 expression and certain drugs may results from higher substrate specificity rather than the level of expression where variability in expression in the liver may be attributed to the variant allele CYP3A5*3 (Kuehl, et al., 2001 and King, et al., 2003).

In relation to drug metabolism, CYP3A5 contribution is considered to vary from 6 – 99 % of the total CYP3A activity among different populations (Kuehl, et al., 2001).

In adult white peoples as well as Asians 10 – 30 % is the only reported detectable amounts of CYP3A5 according to (Lee and Goldstein, 2005). Where's Kuehl, et al. (2001) found out that this amount is remarkably increased to 60% in the African American. These apparent variations may be attributable on one side to the wide variation each population experience when they are subjected to specific environmental factors that makes this variation acknowledgeable. And on the other hand there would be the effect of drug interaction and to some extent to the genetic variation.

Seemingly and upon these important findings CYP3A5 would have a considerable contribution to the interindiviual and interracial differences in the CYP3A dependent drug metabolism, response and clearance (Kuehl, et al., 2001 and Roy, et al., 2003).

CYP3A5 has an 85% sequence identity with CYP3A4. And on the contrast to the CYP3A4, is expressed in the fetal liver but in a polymorphic way (Guengerich, 2005).

The over extended substrate specificities and the tissue expression of CYP3A4 and CYP3A5 obstruct the establishment of associations between gene variants and phenotypic results. And individuals having defective alleles of both cytochromes would be predicted to have lower CYP3A activity than those carrying mutations in single CYP3A gene (Lee and Goldstein, 2005). Lee and Goldstein (2005) had emphasized that most CYP3A4 and CYP3A5 defective variants occur at low allelic frequencies except for CYP3A5*3,*6,*7.

2.2.9.3 Cytochrome 3A7 (CYP3A7)

This gene is part of a cluster of Cytochrome P450 genes on chromosome 7q21.1 – 22 (Lee and Goldstein, 2005). CYP3A7 have arose through the alteration of a 60bp portion of the CYP3A4 promoter in (¯188 - ¯129) as shown in figure (6), and this what explains the existence of this enzyme in some adult individuals, despite the idea that it is solely expressed during fetal stage and starts to diminish with adulthood (Kuehl, et al., 2001).

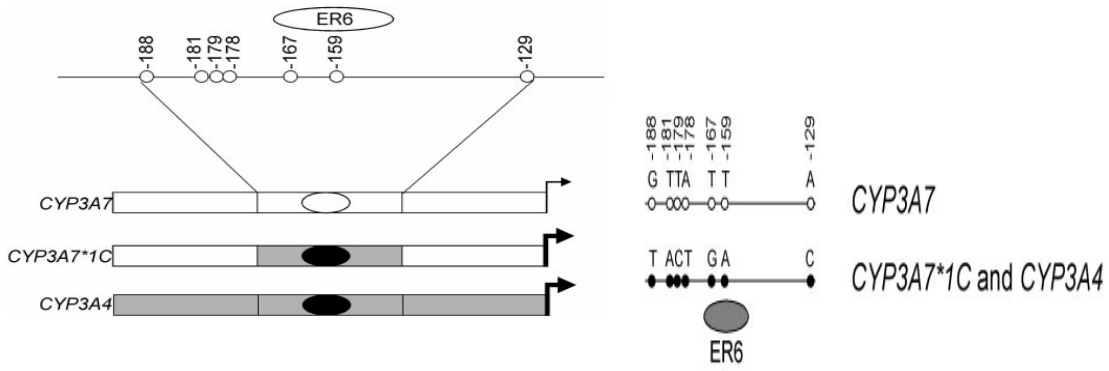

Figure 6: **Proximal promoter region mutated in the *CYP3A7*1C* allele and hypothesized consequences for gene expression.** In the *CYP3A7*1C* promoter allele, the region between positions –188 and –129 is replaced by the corresponding region of *CYP3A4*. Bases different between *CYP3A7* and *CYP3A4* in the region replaced are depicted as *open circles*. Numbering is with respect to the transcriptional start site. The size of the *arrows* indicates the hypothesized strength of gene expression in (Burk, et al., 2002)

CYP3A7 have a low expression in comparison to CYP3A4 post-natal, which suggests its limited role in drug clearance (Burk and Wojnowski, 2004).

Many reports showed that CYP3A7 is expressed mainly during fetal life, where it accounts for 50% of the total CYP protein and the expression is silenced after birth (Leeder, et al., 2005).

while others such as King, et al.,(2003) showed that both CYP3A5 and CYP3A4 in 10 - 11% of the adult livers express CYP3A7 mRNA, but it is not clear wither the protein is expressed or not, others showed that the expression of CYP3A7*1C is polymorphic (-167 T>G variant) (Burk and Wojnowski, 2004, Lee and Goldstein, 2005 and Sim, et al., 2005). CYP3A7 specific effects may arise in organs such as adrenal gland, where 3A7 transcripts constitute an eminent CYP3A mRNA species (Burk and Wojnowski, 2004 and Guengerich, 2005).

2.2.10 Cytochrome 1A subfamily (CYP1A)

CYP1 family comprises three members namely 1A1, 1A2 and 1B1, with CYP1A2 being one of the major CYP in the liver (Zhou, et al., 2009). The CYP1A subfamily, constitute approximately 15% of the total liver CYP family (Shimada, et al., 1994 and Gunes and Dahl, 2008).

2.2.10.1 Cytochrome 1A2 (CYP1A2)

The CYP1A2 gene cluster is mapped to chromosome 15 and there is a close linkage between CYP1A2 and CYP1A1 genes as they share a common 5- flanking region, they are separated by 23kb segment, the CYP1A2 it is approximately 7.8 kb long and includes seven exons and six introns and the first exons in addition to 55 base pair (bp) long non coding exon, CYP1A2 contains 20 variants (*1B to *16), where CYP1A2*1A is referred as the wild type, and more than 177 SNPs in the upstream sequence, and there are 22 non-synonymous SNPs in the CYP1A2 coding region in rare frequency < 1% (Ghobti, et al., 2007 and Zhou, et al., 2009). CYP1A2 codes for a 515 residue protein with a molecular weight of 58.294 Da (Zhou, et al., 2009).

Most of the CYP1A2 substrates are hydrophobic and the most common feature of\ CYP1A2 ligand their content of one to two hydrophobic regions, an aromatic ring and hydrogen bond acceptor. Phenactine, caffeine, theophylline have been frequently used as a model substrate for evaluating the activity of CYP1A2 in vivo (Zhou, et al., 2009).

1A2 protein expression and activity shows inter individual variation ranging from 35 – 75% and most probably due to genetic factors as indicated by the concentration of CYP 1A2 mRNA in the liver displays more than 40 fold differences in some individuals (Zhou, et al., 2009).

CYP1A2 Enzyme activity is influenced by other factors such as gender, race, smoking , dietary substances, environmental chemicals, modulation by endogenous substances, in addition to genetic factors such as polymorphisms in other genes such as CYP1A1 and GSTM1 and methylation in the 5' flanking region of the CYP1A2 gene (Gunes and Dahl, 2008).

The polymorphic 1A2 alleles are found in different regions of the gene, CYP1A2*1C, *1D, *1E and *1F which show variability in the promoter and enhancer region don't cause any change in the protein sequence (Sachse, et al., 2003).

CYP1A2*1C located on intron 1 in the 5' flanking region in the enhancer region and contains -3860 G>A nucleotide substitution, CYP1A2*1D allele located on the promoter region and causes a thiamine deletion at -2467 in the 5' flanking region,

CYP1A2*1E (and 1*G) causes a T>G substitution at -739 in intron 1 in the promoter region, CYP1A2* 1F is an SNP as a consequence of a C >A nucleotide change at -163 in intron 1 of the promoter region, an altered enzyme activity was shown to be associated with CYP1A2*1C, *1D, *1F, and *1K (Nakajima et al. 1999, Sachse, et al., 2003, Ghobti, et al., 2007 and Zhou, et al., 2009).

2.3 Aflatoxin B1 and CYP450 beyond their metabolizing relationship

2.3.1 Aflatoxin B1 worldwide importance

With no doubt, the FAO considers aflatoxin to be an important public health problem not only in regions where high heat and high humidity prevail, which is the typical character of tropical and subtropical regions, but also temperate zones like Europe and northern America and that is why limits for exposure were set in most countries of the world and according to the same organization at least 99 country world wide had Mycotoxins regulation for feed and food in the late 2003 in more commodities and products. For instance the accepted limit for aflatoxin B1 in food stuff including ground nuts and dried fruits intended for direct human consumption is 2 µg/kg (Van Egmond and Jonker, 2005). And in Jordan the maximum tolerable levels since 1981 as declared by the FAO in 1997 in food like (almonds, cereals, maize, peanuts, pistachio, pine nuts and rice) for B1 is 15 ng/g and the combination of aflatoxins (B1, B2, G1 and G1) is 30 ng/g and tested back then by TLC, which was developed to the imuunosorbent assays, the same levels where given for all feed stuff for AFB1 and the Combination (CAST, 2003).

2.3.2 Target organs

While the liver is the primary target organ after the dietary exposure, lungs are considered to be the route for the occupational exposure toxicities for those people involved in feed and food manufacturing or handling activities (Kelly, et al., 1997) and (Van Vleet, et al., a, b, 2002). Further more aflatoxin can cross the placenta, that is why aflatoxin adducts were identified in cord blood of newborn Gambian children which is considered advancement in aflatoxin transmission from mothers during pregnancy raising the risk of childhood cancer (Wild, et al., 1991 and Doi, et al., 2002).

2.3.3 Aflatoxicosis and other clinical implications

The poisoning that results from aflatoxin ingestion is called Aflatoxicosis, which is an acute illness with complete liver cirrhosis leads to death due to large doses of the toxin. While nutritional and immunological disorders are consequences of chronic sub lethal doses and eventually cumulative effect corresponds with the risk of cancer (Williams, et al., 2004), which is well established in many studies conducted all over the world like china, Africa and Europe mainly in Germany and Britain (Harrison, et al., 1993, Aguilar, et al., 1994, Sibanda, et al., 1997 and Turner et al., 1998).

Since the discovery of Aflatoxins and over the decades the effect of aflatoxin on human health have been studied and found to be involved in series of human diseases, and mainly the liver cancer, and some have linked between the incidence of liver cancer "in many countries" with the distinctive geographical distribution which was similar to that of aflatoxin contamination of food in these particular geographical areas (Sibanda, et al., 1997 and Jolly, et al., 2006).

Other human diseases believed to characterize the involvement of aflatoxin like Reys syndrome, Indian childhood cirrhosis, chronic gastritis, kwashiorkor and lung cancer (Eaton and Gallagher, 1994, Sibanda, et al., 1997 and Williams, et al., 2004).

2.3.4 Aflatoxin B1 metabolic pathway

Aflatoxin B1 is the most frequently occurring and the most toxic among the Aflatoxins group (Wild and Turner, 2002), can be oxidized by certain CYP450s and play the key role in determining the metabolic pathway that initiates the reaction with cellular macromolecules and cause aflatoxin toxic effect (Eaton, et al., 1994). Generally, the contribution of these enzymes to AFB1 metabolism in vivo depends on affinity and expression of these enzymes (Wild and Gong, 2010).

Aoyama, et al., (1990) found that at least five human liver CYP450 enzymes are capable of activating AFB1 to its mutagenic and carcinogenic metabolite (CYP1A2, 2A6, 2B7, 3A3, 3A4 and 3A5) in adults and 3A7 in prenatals (Doi, et al., 2002) and in adults (Kamdem, 2006). Cytochrome 1A2 & 3A4 were considered the most important boactivating enzymes (Shimada and Guengerich, 1989 and Forrester et al., 1990).

Aflatoxin is biotransformed by CYP450 to the reactive AFB1 -8-9 epoxide (AFBO) the endoepoxide and exoepoxide to exert the hepatocarcinogenic effect through the covalent binding with the DNA and the potency of carcinogenicity is directly related to the extent of DNA binding (Eaton and Gallagher, 1994). The exoepoxide binding to DNA exerts another mutagenic effect through the formation of 8-9-dihydrol-AFB1 (AFB1-N7-Gua), (Wild and Turner, 2002).

2.3.5 Levels of exposure:

The level of the dietary exposure to a particular toxin is important for assessing an individual risk as well as the activity of enzymes involved in the activation and detoxification of any toxic compound (Mace et al., 1997). That is why there are many conflicting evidences on the role of the specific P450 cytochromes that are responsible for the activation step in the liver, those studies concluded that when aflatoxin B1 intake is at the dietary concentrations, CYP1A2 is the dominant bioactivating enzyme (Gallagher, et al., 1994, Eaton, et al., 1995, Gallagher et al., 1996 and Wild and Turner, 2002). Yet, CYP3A4 is the catalyst of activation at higher substrate concentrations (Forrester, et al., 1990, Ramsdell, et al., 1991, Gallagher, et al., 1994 and Gallagher, et al., 1996).

Along with the aforementioned findings Hayes, et al. (1984), Kelly, et al. (1997) and Van Vleet, et al., a, b. (2002), have studied aflatoxin B1 activation in human lungs and have found that although human lungs distinguished by cytochromes scarcity especially in both CYP1A2 and CYP3A4, the pattern of activation is consistent with what have been found in liver studies but with a lower level of activation for CYP3A4 and CYP1A2 compared with liver capability of activation, consequently the risk of developing lung cancer for those who inhale contaminated dust in certain occupations evidently can occur**.**

2.3.6 Detoxification

AFB1 is also bioactivated into more polar metabolites AFM1, AFQ1 and AFP1 or the none "carcinogenic metabolites" of AFB1 to be readily excreted into urine (Eaton and Gallagher, 1994, Wild and Turner, 2002 and (Kamdem, 2006).

One metabolic pathway of detoxification is the conjugation with glutathione S-transferase (GST), researchers found that humans do not possess GST isoenzymes with high specific activity toward AFBO compared with other animal species (Forrester, et al., 1990, Kirby, et al., 1993 and Heinonen, et al., 1996).

And one of the conclusions drawn depending on this lack in GST-mediated potential against AFB1 in human liver is that the variation in hepatic CYP450 due either to genetic polymorphism or to modulation by environmental factors can be a determinant in the risk of liver cancer developed in AFB1 exposed individuals (Kirby, et al., 1993).

2.3.7 Aflatoxin other Mutational Consequences

Both CYP 3A4 and 1A2 contribute to the formation of AFB1-induced P53 mutations. And dose response relationship was set between levels of AFB1 and the prevalence of this mutation p53 (249 ser) mutation in primary Hebatic Cellular Carcinoma (HCC) (Mace, et al., 1997 and Stern, et al., 2001).

This was clearly shown when AFB1 exhibits some sequence selectivity towards guainine bases or cytosine as the 5' base, therefore inducing predominantly G-T transversions. Molecular analysis reveled a high prevalence of an AGG---AGT (arg---ser) transversion at codon 249 in tumors from areas with reported high aflatoxin exposure, this fact based on the assumption that mutational spectra in humans reflects the etiological agent (Wild and Turner, 2002).

2.3.8 Afaltoxin B1 biomarkers of exposure and Carcinogencity

A lot of researchers used biomarkers of exposure and found that AFB1-N7-guainine adducts in urine are the reliable biomarkers of exposure from urine which only reflects recent exposure to aflatoxin (Groopman, et al., 1992). While chronic exposure is more likely to be interpreted by AFB1-albumin adducts (AFB1-alb) level in peripheral blood (Wild, et al., 1992).

These biomarkers have provided information implicating aflatoxin as a considerable risk factor for hepatotoxicity and carcinogenicity to those populations exposed, (Wang, et al., 1996, Turner, et al., 1998 and Chen, et al., 2007).

Other AFB1 metabolites can be considered as useful biomarkers of human exposure to aflatoxin like AFM1, Q1, P1 have been detected in urine samples (Groopman, et al., 1985).

In addition to the use of these biomarkers in studies of HCC etiology, they have been used in assessing polymorphism of the Aflatoxin metabolizing CYP450s and wither these polymorphisms results in altered levels of metabolites in some individuals, some researchers went beyond this and used biomarkers out comes to develop chemo prevention methods for aflatoxin exposure in their preliminary prevention studies (Wild and Turner, 2002).

2.3.9 Epidemiological studies and assessment

Many Epidemiological studies have been conducted in some countries like Gambia, China, Taiwan and these studies linked between aflatoxin dietary intake and the incidence of HCC with an afterward establishment of relationship between individuals susceptibility and their consequent response findings (Wang, et al., 1996 and Chen, et al., 2007).

Moreover observer can find substantial apparent individual responsiveness variability within the same population and solidly sustained when compared with variability in other different populations.

Eaton and Gallagher (1994) therefore suggested that the prevailing factors confounded these studies could be the lack of accurate assessment of chronic aflatoxin intake and the presence of endemic hepatitis B virus in regions where the incidence of both HCC in and AFB1 contamination are high.

Shimada and Guingerich (1994), Heinonen, et al. (1996), and Doi, et al. (2002) came up with a conclusion that there is a significant individual variability in AFB1 metabolism and binding between humans which is consistent with the high level of DNA binding and protein adducts formation and they suggested the presence of genetic and or environmental factors that may confer large variability in susceptibility to AFB1.

Other researchers have studied the individual variability AFB1 metabolism and detoxification pathways from a genetic perspective and especially related to the genetic basis for susceptibility to HCC (McGlynn, et al., 2003).

Wild, et al. (2000) had studied the effect of certain variables in determining the level of aflatoxin albumin adduct, these variables were of environmental nature like the place of residence and timing of sample collection, other variables considered as host factors like

age, sex, Hepatitis B virus (HBV) status and interindividual variations in carcinogen metabolizing enzymes. Their results showed that adducts levels were higher in those living in rural than those living in peri urban areas. And twice high in dry seasons than wet seasons. Albumin adduct levels were not associated with other variables studied, but was significantly higher in those non-HBV infected subjects with GSTM1 null genotype. This study emphasized and pointed out the importance of environmental factors aforementioned on levels of aflatoxin albumin adducts.

Other studies showed that aflatoxin exposure may be associated with advanced liver disease in chronic hepatitis C patients in endemic areas in Taiwan, and this environmental exposure may enhance the hepatic carcinogenic potential of hepatitis B virus using aflatoxin albumin adduct levels as biomarker of exposure (Wang, et al., 1996 and Chen, et al., 2007).

Quite the contrary, Eaton and Gallagher, (1994) suggested that hepatitis B virus enhances the carcinogenic response of aflatoxin significantly by 30 folds which was defined by the presence of a synergetic relationship between both to cause the carcinogenicity.

2.4 CYP450 polymorphisms and Aflatoxin B1 analysis

Many studies were conducted to assess whether genetic polymorphisms in CYP1A2 are associated with HCC and exposure to Aflatoxin B1.

Chen, et al. (2006) in their study to correlate genetic polymorphisms in CYP1A2 with HCC susceptibility in a high risk region of China, have found a supporting evidence for the existence of genetic susceptibility to HCC in genes involved in the bioactivation of AFB1 in a high epidemic regions in China. But interestingly of all biomarkers tested as reflective of AFB1 exposure none were detected and therefore a conclusion regarding whether or not the association observed was only a direct effect of CYP1A2 genetic polymorphisms on AFB1 exposure was hard to be drawn. Never the less according to their findings "environmental aflatoxin exposure may contribute to shape the nucleotide diversity pattern at CYP1A2 locus during human history, and the risk genotype is deleterious and thus less favored by natural selection in HCC endemic regions" (Page 25). However, the existence of individual variability can be found not only in this enzyme but researchers should concentrate on other targets for their studies like CYP3A4, CYP3A5, aflatoxin detoxification pathway and DNA repair mechanism, because aflatoxin carcinogenicity is caused by the set of the aforementioned targets as well as to other biological processes.

In another study conducted by Wojnowski, et al., (2004), on Gambian sample population with a considered high aflatoxin exposure, they tried to link polymorphism of CYP3A5 enzyme with HCC susceptibility, using PCR for genotyping and using aflatoxin albumin adduct as the biomarker for exposure. Their study group was composed of 288 Gambians, CYP3A5 activity was assessed by using haplotypes of the three variant loci (CYP3A5*3, *6 and *7) associated with decreases in protein expression. CYP3A5 expression was then compared to aflatoxin-albumin adduct. They

reported in their results that the effect of the CYP3A5 polymorphism was strongest in individuals with low CYP3A4 activity with a 70.1% increase in AF-albumin in high, compared to low expressers. And therefore their findings can amend the risk of HCC among individuals exposed to aflatoxin B1.

When studying the impact of CYP3A5 genetic polymorphism on the biotransformation of drugs and environmental toxins, kamdem, (2006) research results indicated that most of AFBO (AFB1 and AFQ1) produced were due to CYP3A4 activity and AFB1 disposition to these metabolites, tightly correlated to CYP3A4 expression level. Further more according to his findings, when CYP3A5 content represents a significant fraction of the total hepatic CYP3A pool, the source of interindividual variability among population would be due to CYP3A5.

2.5 aflatoxin B1 albumin adduct levels among different populations using different approaches and different methods: -

Turner, et al. (1998), used immunoassay and high performance chromatography to screen and confirm aflatoxin albumin adducts in serums of 104 British individual using self questionnaires to compare dietary habits with the level of aflatoxin exposure, which found to be not related in his study, even though some levels were found in the study population and according to the researchers the source of that exposure may be a reflection of the difficulties during monitoring regulated imported food stuffs or to the lack of tight regulations.

Earlier, others have studied the exposure of workers in feed mills and feed factories using the same concept which was aflatoxin albumin adducts using enzyme linked immunosorbent assay like (Autrup, et al., 1991), who assessed the exposure to aflatoxin B1 of workers in animal feed processing plant in Denmark, the workers served as their own controls, samples therefore withdrawn after a vacation they had and after four weeks of work, he found seven samples out of forty five were positive with an average daily intake of 64ng/kg, the workers who had exposed the most those who were unloading cargos, and he concluded that this exposure might partly explain the increased risk for liver cancer in the animal feed processing industry. In a similar study conducted by Ghosh, et.al. (1997), he assayed airborne aflatoxin in rice and maize processing plants in India using indirect, competitive enzyme linked immunosorbent assay (Eliza). The same study performed in Thailand by Nuntharatanpong, et al., (2001) in animal feed processing plant and both confirmed the inhalation of aflatoxin from air dust.

Currently a wide range of methods can be used and available for scientists ranging from immunoassays to the newly advanced liquid chromatography tandem mass spectrometry (LC/MS).

In their study McCoy, et al. (2005), McCoy, et al. (2008), Scholl and Groopman (2008) and Scholl, et al. (2006) had set up a method using isotop dilution mass spectrometery – tandem mass spectrometery (IDMS) for the analysis of afaltoxin albumin adducts which is considered a major advance in the analysis of aflatoxin albumin adducts, where it improved the detection limits and the confirmation provided by on line mass spectral fragmentation patterns and the ability to filter out by mass any impurities that interfere in spectrophotometeric detectors, (Shepard, 2009). Both groups of scientists had compared this method with Eliza and High pressure liquid chromatography (HPLC); were they found that the IDMS was the most sensitive method. Added to this Scholl and Groopman, (2008) have found that aflatoxin albumin adducts in human serum stability may last for years if stored at -80C, this was proven using samples from 1989 and analyzed by liquid chromatography / masss spectrometery (LC/MS) in 2008.

2.6 Methodology

2.6.1 Polymerase Chain Reaction (PCR)

PCR is DNA synthesis in a test tube using the elementary components of the natural DNA replication. It starts and proceeds by the three steps governed by temperature repeated for 20-40 times (Figure 7). It starts by template (which carries the DNA segment or the target we wish to amplify) denaturation to separate the complementary strands, then the reaction cooled to an annealing temperature to allow the oligoneucleoutides primers to hybridize; the primer which is a short, single-stranded piece of DNA (20 – 30 nucleotides) that anneals or attach to its complementary sequence on the template and the 3' end points, a pair of primers will attach to either side of target DNA segment, providing initiation site for the DNA to synthesis. The reaction is heated to a temperature close to the optimal polymerization temperature for the polymerase, which is the enzyme used to synthesize new strands of DNA and consequently it adds bases complementary to the template strand (Saiki, et al., 1988, Andy Vierstraete, 1999 and McPherson and Moller, 2001). Since both strands are copied during PCR, there is an exponential increase of the number of copies of the gene (Figure 8) (McPherson and Moller, 2001).

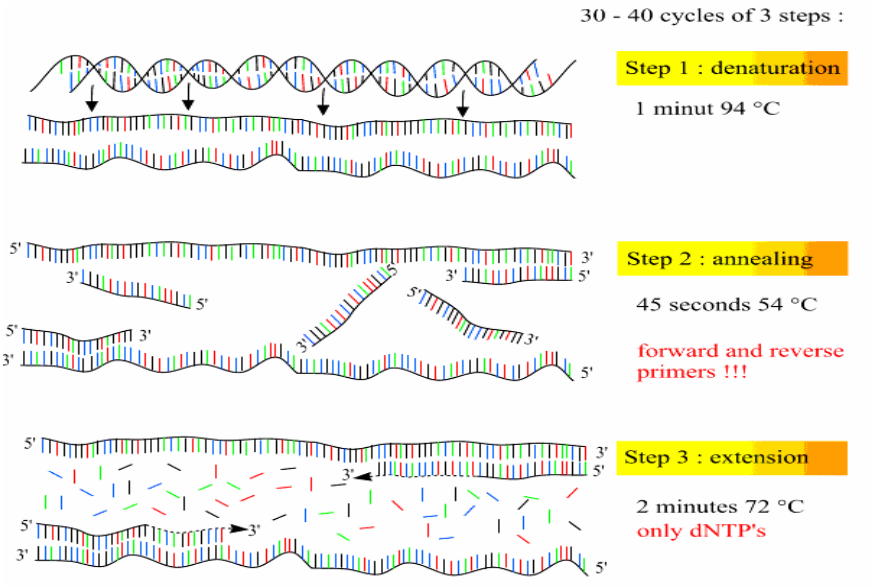

Figure 7: Polymerase Chain reaction .
This figure shows an illustration of the principle behined the PCR in amplifing DNA molecules in vitro. Each cycle consists of three steps (1) Denaturation of DNA,(2) anneling of denatured DNA to the primers ,and (3) enzymatic replication of the region of interest by Taq polymerase.
Source Vierstraete, Andy, (1999)

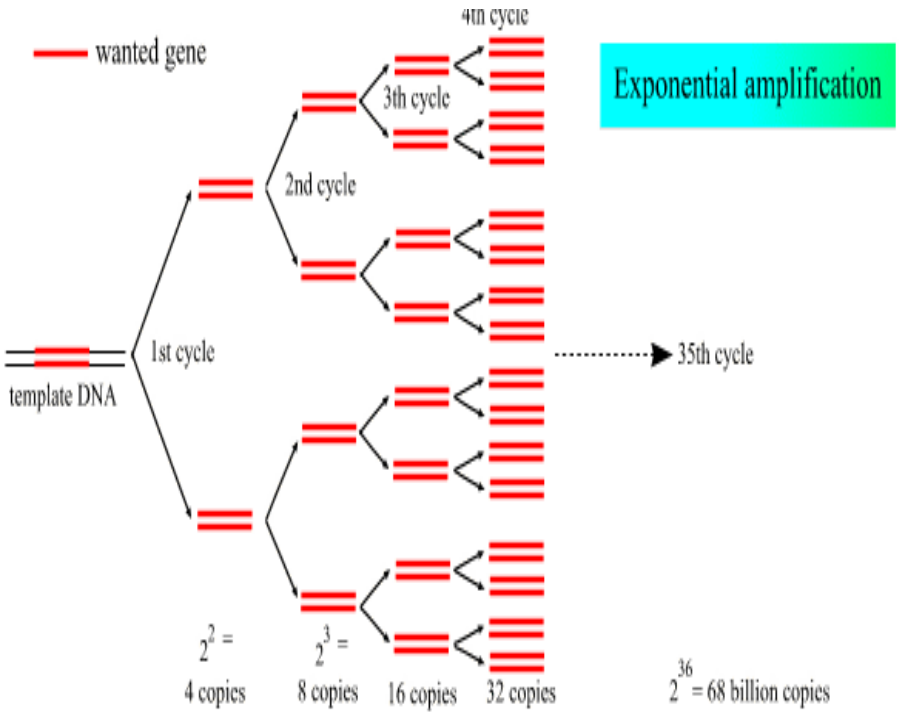

Figure 8: The exponential amplification of the gene in PCR
An illustration of the exponential amplification for the template DNA.
Source: Vierstraete, Andy (1999)

2.6.2 Restriction fragment length polymorphism (RFLP).

Restriction enzymes are molecular scissors that cut DNA into fragments at specific sites in their sequence. Many bacteria make these enzymes to protect themselves from foreign DNA brought into their cells by viruses.

A restriction enzyme functions by "scanning" the length of a DNA molecule. It is looking for a particular pattern of nucleotides, the enzyme's recognition sequence. Once it encounters its specific recognition sequence, generally 4 to 6 nucleotides long, the enzyme will bond to the DNA molecule and makes one cut in each of the two sugar-phosphate backbones of the double helix. Once the cuts have been made, the DNA molecule will break into fragments. These fragments can be visualized by electrophoresis due to their mobility in the gel according their length (Tait, Robert, 1999 and Todd, et. al., 2001).

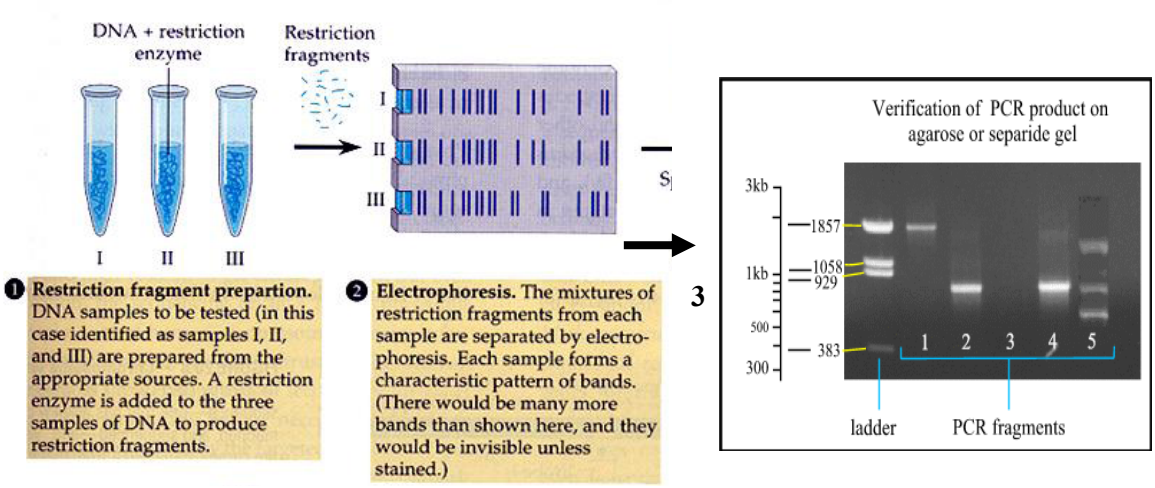

Figure 9: RFLP procedure
1: Sample preparation and
2: Electrophoresis (source 1 and 2 from source: (Pawlik, 2008)
3: visualization and interpretation on gel. Source: (Vierstraete, Andy, 1999)

2.6.3 Gel electrophoresis

Often electrophoteric or chromatographic separation techniques are used to distinguish the size or hybridization state of the PCR products and Agrose gel electrophoresis is simple to perform and when an unknown is compared to DNA size standards, the product length can be estimated (De Silva and Wittwer, 2000). The most common form of DNA electrophoresis involve molding a polymer with conductive medium like (Tris boric acid EDTA) TBE which became the favored cation for gel electrophoresis and applying a voltage so that multiple samples can migrate in parallel, producing both the current and the electrokinetic movement of the negatively charged DNA (Brody and Kern, 2004). Over the years agarose proved convenient for sizing DNA, separating, identifying and purifying 0.5 – 25 Kb DNA fragments and ethidium in gel and buffer allowed visualization of DNA bands directly upon illumination with UV light (Voytas, 2001).

3

Materials and Methods

3.1 Goal of the Method

The experimental part of this study relied on genotyping the major CYP450s involved in metabolizing environmental xenobiotics, carcinogens and drugs and these were CYP3A4 for (CYP3A4*1B), CYP3A7 (CYP3A7*1C), CYP3A5(*2 ,*3, *4, *5, *6 and *7) and CYP1A2 (*1C, *1D,*1E and *1F) the choice of alleles based and mostly known genotyped variants and the change they make which is indicated in (Table 2), This part was conducted to serve the following two goals:

1. Establish a genotyping method to be used in the laboratory and to know of the aforementioned enzyme genotypes expressed as frequencies among the Jordanian population.

2. To determine vulnerability of individuals upon exposure to certain environmental toxins depending on the genotyping information, as we highlight on Aflatoxin B1.

Table (2): Genotyping of the CYP3A4, 3A7, 3A5 and 1A2 by PCR-RFLP based upon the following variants

CYP alleles	**Variant (Nucleotide changes)**	**Amino acid change**	**References**
CYP 1A2*C	-3858G>A	None	(Chida, et al., 1999), (Nakajima,et al. 1999) and (Sache, et al., 2003)
CYP 1A2*D	-246T >delT	None	(Chida, et al., 1999), (Nakajima,et al. 1999) and (Sache, et al., 2003)
CYP 1A2*E	-740T > G	None	(Chida, et al., 1999), (Nakajima,et al. 1999) and (Sache, et al., 2003)
CYP 1A2*F	-164A>C	None	(Chida, et al., 1999), (Nakajima,et al. 1999) and (Sache, et al., 2003)
CYP 3A4*1B	-392A>G or -289A>G	None	(Ando, et al., 1999)
CYP 3A7*1C	T-167G	None	(Smit, et al., 2003)
CYP 3A5*2	27289C>A	T398N	(Van Shaik, et al., 2002)
CYP 3A5*3	6986A>G,	Splicing defect	(Van Shaik, et al., 2002)
CYP 3A5*4	14665A>G	Q200R	(Van Shaik, et al., 2002)
CYP 3A5*5	12952T>C	Splicing defect	(Van Shaik, et al., 2002)
CYP 3A5*6	14690G>A	Splicing defect	(Van Shaik, et al., 2002)
CYP 3A5*7	27131_27132 insT	Frame shift	(Van Shaik, et al., 2002)

3.2 Materials and Reagents:

- DNA Extraction kits (Promega, Madison, WI, USA)
- Ethanol Molecular grade (Sigma, St. Louise, MO, USA)
- Isopropanol Molecular grade (Sigma, St. Louise, MO, USA)
- Taq DNA (Promega, Madison, WI, USA)
- dNTP 10mM mix (Promega, Madison, WI, USA)
- 5X Green PCR buffer (Promega, Madison, WI, USA)
- Restriction Enzymes (New England Biolabs Inc., Beverly, MA.), see table (3) for the enzymes and primers
- 100 bp DNA ladder and 50 bp DNA ladder (Fermentas Inc., USA)
- Primers (Promega, Madison, WI, USA), see table (3) for the enzymes and primers.
- Nuclease Free water (Promega, Madison, WI, USA)
- 10 X Tris base EDTA buffer (TBE), for gel preparation and as an electrophoresis buffer (Promega, Madison, WI, USA).
- Ethidium Bromide staining (Promega, Madison, WI, USA).

Table (3) CYP alleles and their relevant primers (forward and reverse), per restriction enzymes used

CYP allele	Primers	Restriction enzyme
CYP3A4*1B-F[1]	5'-GGACAGCCATAGAGACAAGGGGA-3'	mboII
CYP3A4*1B-R[2]	5'-CACTCACTGACCTCCTTTGAGTTCA-3'	
*CYP3A7*1C* –F	5'-CCATAGAGACAAGAGGAGA-3'	SspI
*CYP3A7*1C* –R	5'-CTGAGTCTTTTTTTCAGCAGC-3'	
*CYP*1A2*C-F	5'-GCT ACA CAT GAT CGA GCT ATA C-3′	DdeI
CYP 1A2*C-R	5′-CAG GTC TCT TCA CTG TAA AGT TA-3′	
*CYP*1A2*D-F	5′-TGA GCC ATG ATT GTG GCA TA-3′	NdeI
*CYP*1A2*D-R	5′-AGG AGT CTT TAA TAT GGA CCC AG-3′	
*CYP*1A2*E-F	5′-AAA GAC GGG GAG CCT GGG CTA GGT GTA GGA G-3′	StuI
*CYP*1A2*E-R	5′-AGC CAG GGC CAG GGC TGC CCT TGT GCT AAG-3′	
*CYP*1A2*F-F	5′-CCC AGA AGT GGA AAC TGA GA-3′	ApaI
*CYP*1A2*F–R	5′-GGG TTG AGA TGG AGA CAT TC-3′	
*CYP3A5 *2-F*	5'-CTGTTTCTTTCCTTCCAGGC-3'	TasI
*CYP3A5 *2-R*	5'-CTCCATTTCCCTGGAGACTTG-3'	
*CYP3A5 *3-F*	5'-CATCAGTTAGTAGACAGATGA-3'	SspI
*CYP3A5 *3-R*	5'-GGTCCAAACAGGGAAGAAATA-3'	
*CYP3A5 *4-F*	5'-TCGACTCTCTCAACAATCCTC-3'	TaqI
*CYP3A5 *4-R*	5'-AAAGTGTGTGAGGGCTCTCGA-3'	
*CYP3A5 *5-F*	5'-CCATGAAGATCACCACAACT-3'	NlaIII
*CYP3A5 *5-R*	5'-CCTGTCCCCAGATTCATGC-3'	
*CYP3A5 *6-F*	5'-GTGGGGTGTTGACAGCTAAAG-3'	DdeI
*CYP3A5 *6-R*	5'-TGGAAGATGATTCAGCAGATAGT-3'	
*CYP3A5 *7-F*	5-CTTCAATAGTACTGCATGGAC-3'	DdeI
*CYP3A5 *7-R*	5'-CTGTACCACGGCATCATAGCT-3'	

1= F; Forward 2=R; reverse

3.3 Instruments:

- Centrifuge (Kendro Laboratory Product Gmbh, Hanau, Germany)
- Vortex mixer (KMC-1300V,Vision scientific Co.Ltd,Korea)
- PTC- Programable thermo cyclar (MJR Inc.) for the PCR reaction (Waltham, MA, USA).
- Horizontal electrophoresis (PS3002 Apelex, France).
- UV transillumiator (Sony-modle: SSM-125CE, Tokyo, Japan)
- DNA quantifier (documentation system): Black and white monitor Video Graphic printer UP-89MD (Sony Corporation-Tokyo, Japan)

3.4.1 Subjects:

The study consisted of randomly selected apparently healthy unrelated individuals separated into two groups:

Study group: Consisted of 194 workers (51 female and 152 male with an average age of 27 years for females and 33 for males volunteered to participate in this study**.** Another 24 Egyptians working in the conditions were included for further comparison purposes.

All the participants in the study group are workers in feed manufacturing plants including, corn and soya for animal consumption, reserving silos, flour or corn processing food, spices and nuts processing plants and other related sectors. Since all of them are working in feed and food industry we suspect their exposure to aflatoxin B1.

Control group: Sex and age matched group consisted of 397 apparently healthy unrelated volunteers (91 female and 306 male) Average age of the female controls 30.12 and 31 years for males were used as a control group.

3.4.2 Questionnaire

A special questionnaire (appendix 1) was designed to collect information such as; gender, age, smoking, coffee consumption, dietary habits, along residence and their general job. The questionnaire contained a consent that had to be signed before obtaining blood samples.

Table (4) some regular habits (smoking and coffee consumption) obtained from self-questionnaire of the study groups.

Study	**Smoking**		**Coffee consumption Based on small cups (10 – 15 ml)**	
Population	**Category /daily**	**percentage**	**category**	**Percentage**
Male	≤15 cigarette	12%	<10 daily	65%
	20 cigarette	19%	> 10 daily	4%
Average age	20 - 40 cigarette	15%	Weekly	3%
32.9	> 40 cigarette	4%	Monthly	
	Arjillah	4%	Rarely	7%
	Not a smoker	46%	No coffee	21%
Female				
Average age	≤ 15cigarette		<10	23%
27.3	20 cigarette	4%	> 10	3%
	20 - 40 cigarette		Weekly	
	> 40 cigarette		Monthly	
	Arjillah		Rarely	10%
	Not a smoker	96%	No coffee	64%

Table (5) some regular habits (smoking and coffee consumption) obtained from self-questionnaire of the control groups

Control	**Smoking**		**Coffee consumption Based on small cups**	
Population	**Category/daily**	**percentage**	**category**	**percentage**
Male	≤ 15 cigarette	11%	<10 daily	63%
	20 cigarette	29%	> 10 daily	5%
Average age	20 - 40 cigarette	12%	Weekly	2%
31	> 40 cigarette	2%	Monthly	
	Arjillah	6%	Rarely	5%
	Not a smoker	40%	No coffee	25%
Female				
Average age	≤ 15 cigarette	7%	<10 daily	49%
32	20 cigarette		> 10	
	20 - 40 cigarette	1%	Weekly	3%
	> 40 cigarette		Monthly	3%
	Arjillah	9%	Rarely	12%
	Not a smoker	83%	No coffee	33%

3.4.3 : Inclusion and exclusion criteria

Inclusion criterion was based on the residence; we selected individuals from almost all over the country and mostly concentrated on two major areas (Amman and Aqaba). Where Amman is categorized as a temperate climatic zone, conversely Aqaba is recognized for its higher temperatures and humidity through out the year.

Exclusion criteria: individuals with chronic diseases (diabetes, high blood pressure, hepatitis).

3.5 Genotyping method

Genotyping was performed blind to the identity of the controls and the study group giving the fact that each sample marked with a serial number only.

3.5.1 Samples collection and DNA Extraction

Ten ml of blood was drawn from each subject participated in this study in an ethylene diamine tetra acetic acid (EDTA) anticoagulant blood samples and stored at 2-8°C until DNA was extracted.

DNA Extraction:

Genomic DNA was extracted from peripheral blood leukocytes using (Promega) DNA Extraction as recommended by the manufacturers, reagents, quantities shown below in Table (6), briefly as follows

Table (6) Promega's standard reagents versus quantities used for DNA extraction

Sample	Cell Lysis Solution (RBC lysis)	Nucleic Lysis Solution	Protein Precipitation Solution	Isopropanol	DNA Rehydration Solution
300 µL	900 µL	300 µL	100 µL	300 µL	150 µL

1. Nine hundred µl of cell lysis solution was added to 300 µl of whole blood in an Eppendorf tube, mixed with inversion and incubated for 10 minutes at room temperature.
2. The tube was centrifuged at 15000 rpm for 30 seconds.
3. The supernatant was aspirated using a Pasteur pipette and the white pellet was left with around 20 µl of supernatant.
4. The pellet was vortex-mixed and 300 µl of nucleus lysis solution was added and mixed well. 100 µl of protein precipitation solution was added and the tube was vortex-mixed vigorously until complete mixing.
5. After the tube was centrifuged at 15000 rpm for 3 minutes the supernatant, which contains DNA, was transferred to another Eppendorf tube containing 300 µl of isopropanol and mixed gently until the DNA threads were observed.
6. The tube was centrifuged at 15000 rpm for 1 minute.
7. Supernatant was discarded and the white DNA pellet was kept in the tube.
8. The pellet was washed with 300 µl of (70%) ethanol.
9. The pellet was centrifuged at 15000 rpm for 1 minute.
10. Supernatant was discarded and the pellet was kept at room temperature for 15 minutes.
11. 100 ml of DNA rehydration solution was added to the pellet and the sample was kept at 4˚C overnight.

3.5.2 Genomic DNA Quantitation.

DNA yield was determined by measuring absorbance at 260 nm (A_{260}) using (UV) spectrophotometer (BIO-RAD). Four µl of DNA solution were added to 96 µl of distilled water as a solvent to make a 25-fold dilution, and absorbance was measured at 260 nm against distilled water as a blank. DNA concentration was automatically calculated by the instrument applying the following equation:

Concentration of DNA (ng/ µl) = 25 X absorbance at 260 nm X 50

25 is the dilution factor, and 50 is such that every optical density (OD) is 50 (ng/ µl)

Genomic DNA concentration was within the range of 100-250 ng/ µl .

Samples then were stored at -20°C prior to analysis.

3.5.3 Polymerase chain reaction procedure.

3.5.3.1: Genotyping of the alleles

The detection of both wild type and mutated alleles of CYP3A5 *2 – CYP3A5*7 was performed using PCR – RFLP as described in (Van Schaik, et al., 2002), the same method used for CYP1A2*C,*D, *E and *F (A-D) as described by (Nakajima, et al., 1999 and Chida, et al., 1999). Both CYP3A7*1C and CYP3A4*1B were also genotyped according to the methods described by Smit et al., 2008 and Ando, et al., 1999 respectively. All have shared approximately the same approach in their methods and as a result we made slight modifications and came up with a method that is applicable for genotyping all enzymes chosen in this study.

3.5.3.2 : Procedure of PCR

PCR was run on a 50µl reaction volume using approximately 47 µl of the following; 1X PCR buffer of pH 8.5 and 1.5 mM $MgCl_2$, 0.25 mM of each deoxyribonucleoside triphosphate (dntps), 2 units of DNA Taq polymerase, 40 µM each forward and reverse primers (previously mentioned in table 3). And to the reaction mixture we added 3 µl genomic DNA to a final volume of 50µl.

Amplification consisted of an initial denaturation step for 7 minutes at 94 Cº followed by 35 cycles each consisting of the following steps; Denaturation for 1 minute at 94 ^{o}C followed by Annealing for 1 minute at 55 ^{o}C (raised for 60 ^{o}C for 1A2*E) and Extension for 1 minute at 72 ^{o}C another final extension for 7 minutes at 72 ^{o}C . This program used to genotest all enzymes mentioned above.

3.5. 4 Electrophoresis:-

PCR products were visualized using 2% agarose - trisborate-EDTA gel with ethidium bromide staining.

3.5.4.1 Gel preparation:

First 2gm of the agarose powder was added to 100 ml 1X TBE buffer (which was prepared from the 10X TBE stock buffer, by adding 100 ml of this stock to 900 ml De-ionized distilled water) in a conical flask and subjected to heat to solubilize the agarose powder by boiling and shaking or swirling the solution constantly if necessary until completely dissolved (heat applied by using a microwave). Under the fume hood we added few drops of the ethidium Bromide staining (approximately 5 - 20 µl/ 100ml gel solution) and then stirred well and making sure that we are not subjected to the vapor after adding the stain, the mixture was then poured slowly into the appropriate tank.

The bubbles were pushed away to the side using a disposable tip. The combs were suitable inserted and checked if correctly positioned before pouring the gel. The gel is then left to set and hardens for 30 minutes or more.

3.5.4.2 Loading of the samples into the gel.

After the gel is hardened, the combs were removed and submerged it in the suitable PCR Horizontal electrophoresis tank containing fresh 1X TBE buffer, to forbid and reduce pH changes in the electrical field. Then we started loading 5 - 8 µl of the samples and in the same lane we added in the middle or at the beginning the marker or the ladder.

The gel tank was then closed and the power source was switched on and the gel left to run at 5V/cm with constant monitoring of the current flow. The gels were lifted from the tanks when bands and the ladder bands significantly separated, we didn't permit it to last for longer than 30 minutes or longer than it was required, because Heat generated usually causes gel melting and reduces the buffering capacity required.

3.5.4.3 Interpretation of results:

Usually the results are interpreted after DNA migration from the loading wells after applying an electrical charge, which will cause the negatively charged nucleic acid to move forward through the agarose gel to the positively charged electric field as indicated with the arrow in the Figure (10).

Moreover, the shorter the molecules; the faster they will move compared with the larger ones and the easier they will migrate through the gel pores.

Electrophoresis and interpretation of the results was performed on two stages:

Stage one: checking the PCR product, where we checked the appearance of the PCR products indicated by the Enzyme DNA band at a specific length, which was specified

by measuring its length when it intersects with the ladder bands used. (See each enzyme with each correspondent separated band at the specified length in table 7).

The second stage was after using restriction endonuclease enzymes where the procedure will be discussed afterwards. The DNA Products is cut or digested into smaller fragments through these restriction enzymes through a specific recognition site at a specific band length indicative for this inherited genotype and therefore each individual sample may exhibit the wild type genotype (WT), homozygous (HOM) or the Heterozygous (HET) as indicated in the figure below. in this figure for CYP3A5*6 the PCR product was digested with a specific restriction enzymes and individuals showed the wild type genotype, had CYP3A5*6 DNA bands at 230, 137 and 103 measured in distinction with the ladder bands lengths, individuals having the Homozygous genotype had recognized bands at 230, 137 and 128 bp. Heterozygous however showed the 4 bands and they were 230, 137, 128 and 103 collectively distinctive.

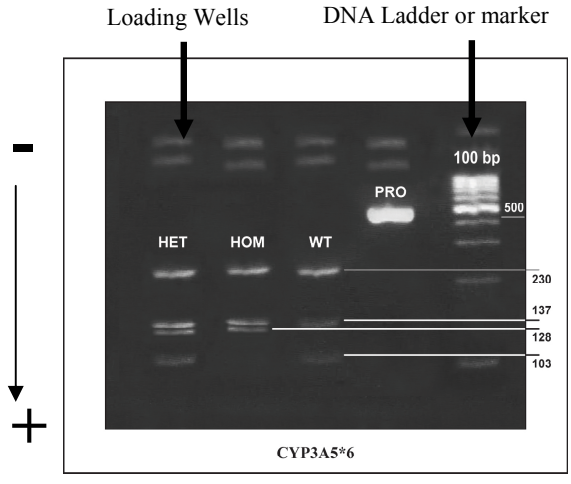

Figure 10: schematic drawing of an agarose gel and a PCR product compared with the ladder and DNA movement from negative to positive. And bands after restriction of the PCR product.

3.5.5 Analysis using Restriction Enzymes.

After the digestion of 15 µl of the PCR product with 4 units of each enzyme (Table 7), pure water and 1X restriction buffer were added to a total volume of 25 µl. Each reaction tube was incubated at required temperatures for each enzyme as shown in Table (7) and incubated for a given time, the digested product was further analyzed on 2.5 - 3% agarose - trisborate-EDTA gel with ethidium bromide staining for visualizing of band patterns. The fragments obtained for wild type and variant alleles are indicated in Table (7) and visualized images for each enzymes are indicated in Figures from (6 to 17)

Table (7): CYP alleles genotyped, with restriction enzyme used per volume and incubation time, and the resultant PCR product before restriction and after restriction

CYP alleles	**PCR product Size in bp**	**Enzymes required for restriction**	**Recommended Units per reaction volume**	**Incubation temperature**	**Fragment sizes in bp**	
					Wild type allele	**Variant allele**
CYP 1A2*C	596	DdeI	10 Units	37Cº	596	464, 132
CYP 1A2*D	167	Nde I	10 Units	37 Cº	149	167
CYP 1A2*E	169	Stu I	10 Units	37Cº	169	137
CYP 1A2*F	243	Apa	50 Units	25Cº	124, 119	243
CYP 3A5*2	269	TasI (Tsp5091)	10 Units	65 Cº	269	182, 87
CYP 3A5*3	293	SspI	5 Units	37Cº	148, 125, 20	168, 125
CYP 3A5*4	281	Taq I	20 Units	65 Cº	261, 20	241, 20
CYP 3A5*5	240	NIaIII	10 Units	37Cº	226, 14	189, 37, 14
CYP 3A5*6	495	DdeI	10 Units	37Cº	230, 137, 103, 25	230, 137, 128
CYP 3A5*7	108	DdeI	10 Units	37Cº	61, 24, 22	41, 24, 22, 20
CYP 3A4*1B	168	MboII	5 Units	37Cº	134, 34	168
CYP 3A7*1C	370	SspI	5 Units	37Cº	244, 126	370

3.6 Statistical analysis:-

Both control group and study group were genotyped for the aforementioned 12 alleles by using the Hardy Weinberg equilibrium (HWE) (Gun and Thompson, 1992); using χ^2 and both groups then compared statistically using Statistical package for social sciences program (SPSS) version 16 at Jordan University for Science and Technology, Irbid; to prove if there is a relation ship between allelic frequency in healthy individuals and those exposed to stressed environment with AFB1 contamination.

Hardy-Weinberg equilibrium equations and parameters used:

We used the following equations to estimate frequencies

- The first equation: $(p^2 + 2pq + q^2 = 1)$; where p is defined as the frequency of the dominant allele and q as the frequency of the recessive allele for a trait controlled by a pair of alleles (A and a).
- The chi-square test statistic is calculated with the following formula:

$$\chi^2 = \sum \frac{(o-e)^2}{e}$$

o: observed allelic frequency

e: Expected allelic frequency

- We used 1 degree of freedom (degrees of freedom for test for Hardy–Weinberg proportions are number of genotypes − number of alleles); (3-2=1).
- And The 5% significance level

4.0

Results

A total of 591 Jordanian participants (397 healthy individuals and 194 individuals supposed to be exposed to the effect of aflatoxin B1 in the most vulnerable working environment) and 24 Egyptian workers (exposed to the same environment) were genotyped using PCR-RFLP for CYP3A4 polymorphism -392A>G (CYP3A4*1B), CYP3A7 polymorphism -167 T>G (CYP3A7*1C), CYP3A5 polymorphisms 27289C>A (CYP3A5*2), 6986A>G (CYP3A5*3), 14665A>G (CYP3A5*4), 12952T>C (CYP3A5*5), 14690G>A (CYP3A5*6), 27131_27132 insT (CYP3A5*7) and some of known CYP1A2 polymorphisms such as -3860G>A (CYP1A2*1C), -2467delT (CYP1A2*1D), -379T>G (*1E,1G,1J,1K) and -163C>A(*1F, 1J and 1K). The results of genotyping are shown in the Figures 11 – 22. After digestion with the appropriate restriction enzyme, bands for the wild type (wt), homozygous (hom) and heteroztygous (het) produced were obvious for each polymorphism on the gel images (Figures 11 -22); percentages of wts, hom and het for each polymorphism in both groups are shown in Table (8).

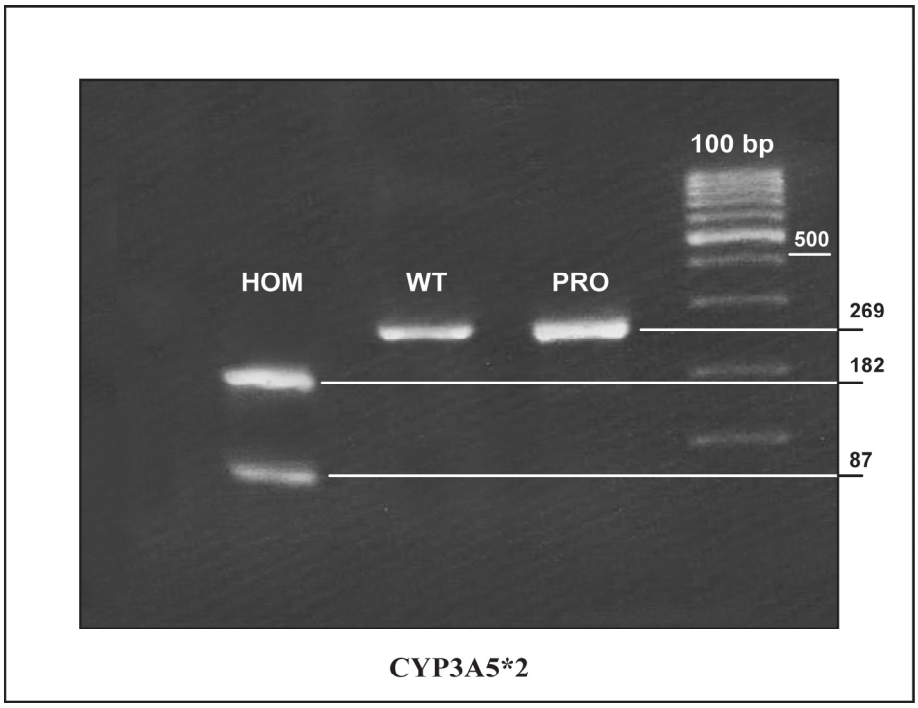

Figure 11: CYP3A5*2 on (3%) agarose gel electrophoresis using TasI restricted enzyme for the PCR product. Using 100bp ladder, PRO; product appeared at band size 269, WT; wild type appeared at band size 268.

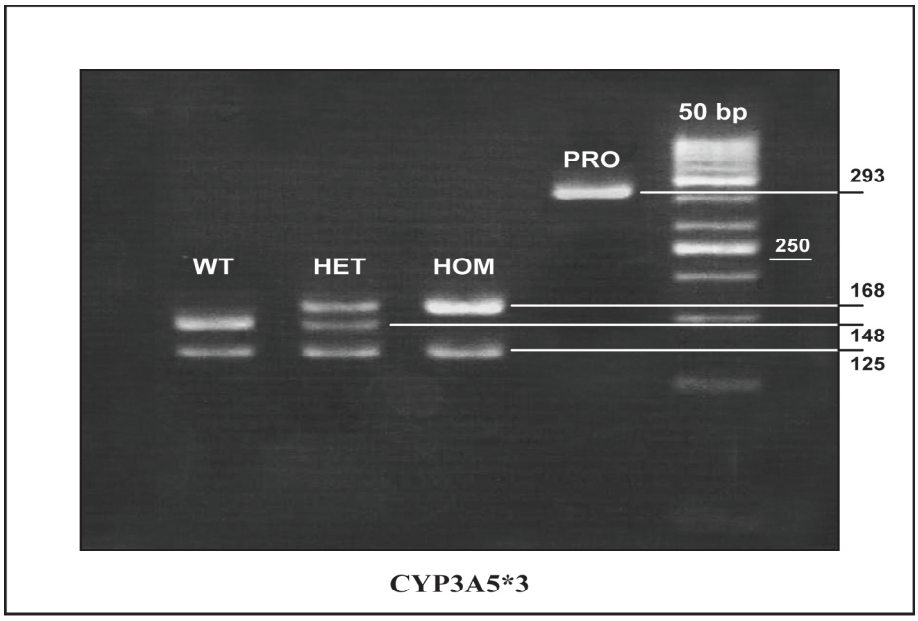

Figure 12: CYP3A5*3 on (3%) agarose gel electrophoresis using SspI as arestriction enzyme for the PCR product. Using 50bp ladder, PRO; product appeared at band size 293, WT; wild type appeared at band size 140 and 125, (HOM) homozygous gave bands at 168 and 125, (HET) heterozygous 168, 148 and 125.

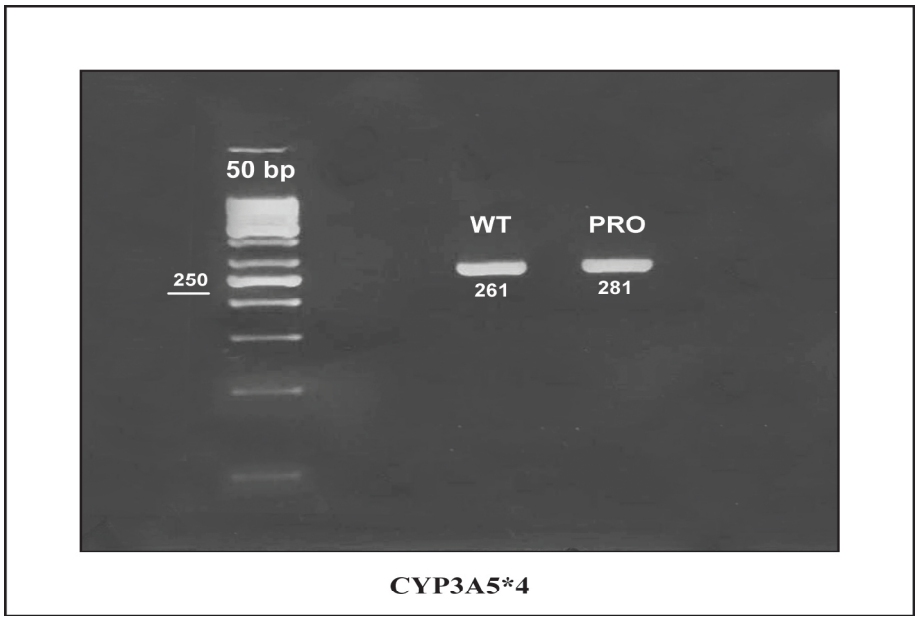

Figure 13: CYP3A5*4 on (2.5%) agarose gel electrophoresis using Taq I as a restriction enzyme for the PCR product. Using 50bp ladder, PRO; product appeared at band size 281 and only WT; wild type appeared at band size 261bp

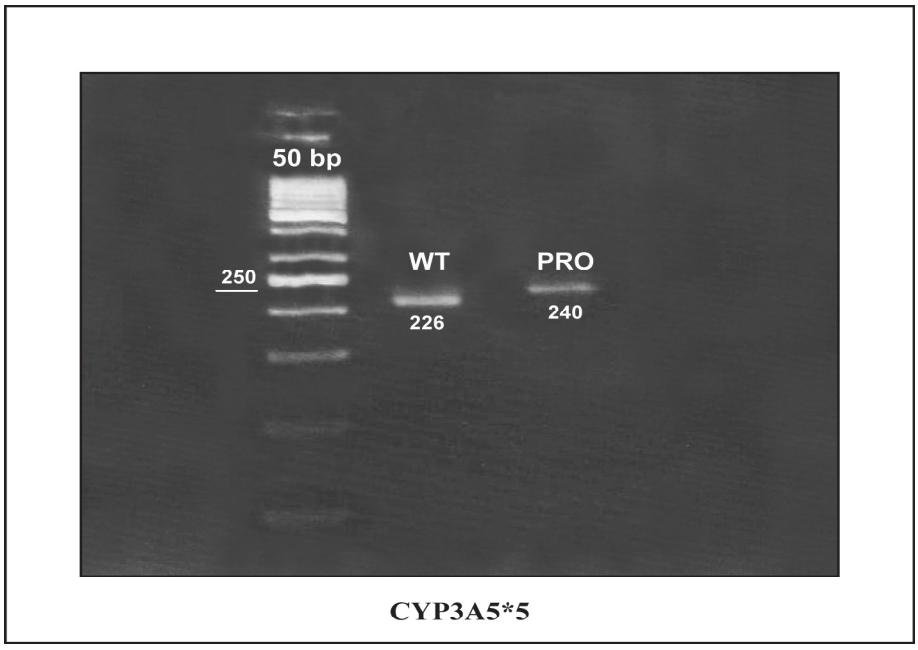

Figure 14: CYP3A5*5 on (3%) agarose gel electrophoresis using NIaIII as a restriction enzyme for the PCR product. Using 50bp ladder, PRO; product appeared at band size 240 and only WT; wild type appeared at band size 226bp

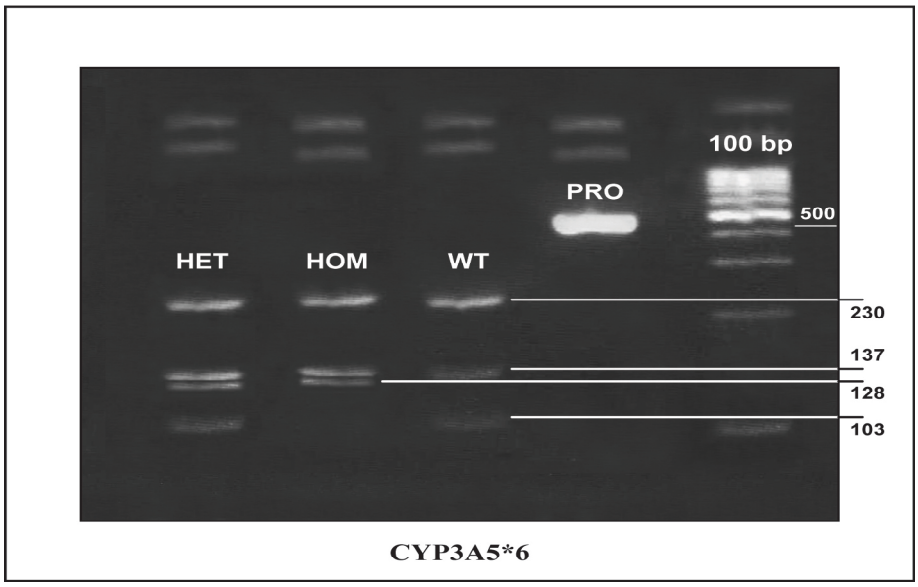

Figure 15: CYP3A5*6 on (3%) agarose gel electrophoresis using DdeI as a restriction enzyme for the PCR product. Using 100bp ladder, PRO; product appeared at band size 495 bp, WT; wild type appeared at band size 230, 137 and 103, (HOM) homozygous gave bands at 230, 137 and 128, (HET) heterozygous bands were at 230, 137, 128 and 103.

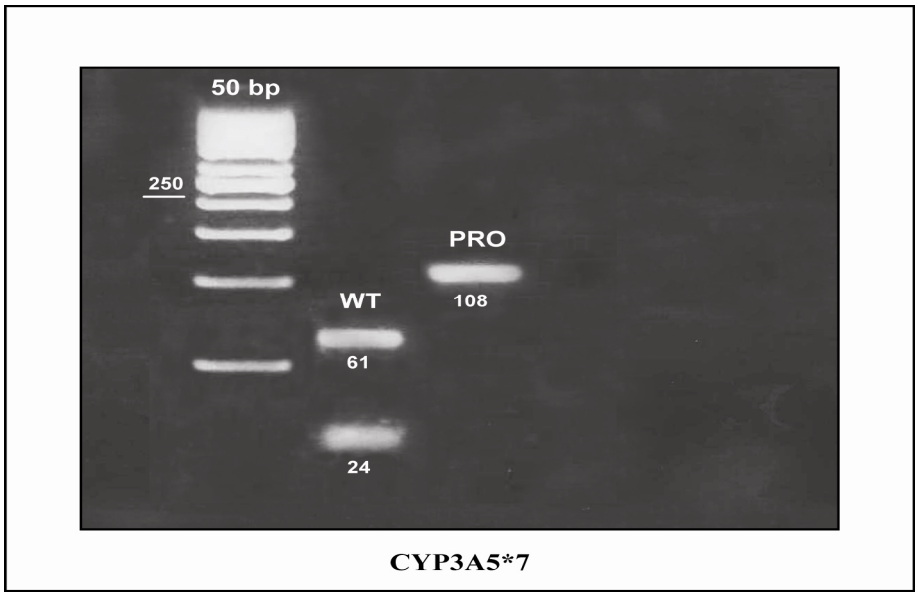

Figure 16: CYP3A5*7 on (3.5%) agarose gel electrophoresis using DdeI as a restriction enzyme for the PCR product. Using 50bp ladder, PRO; product appeared at band size 108 bp, WT; wild type appeared at band size 61 and 24, No (HOM) homozygous or (HET) heterozygous found in any of the samples.

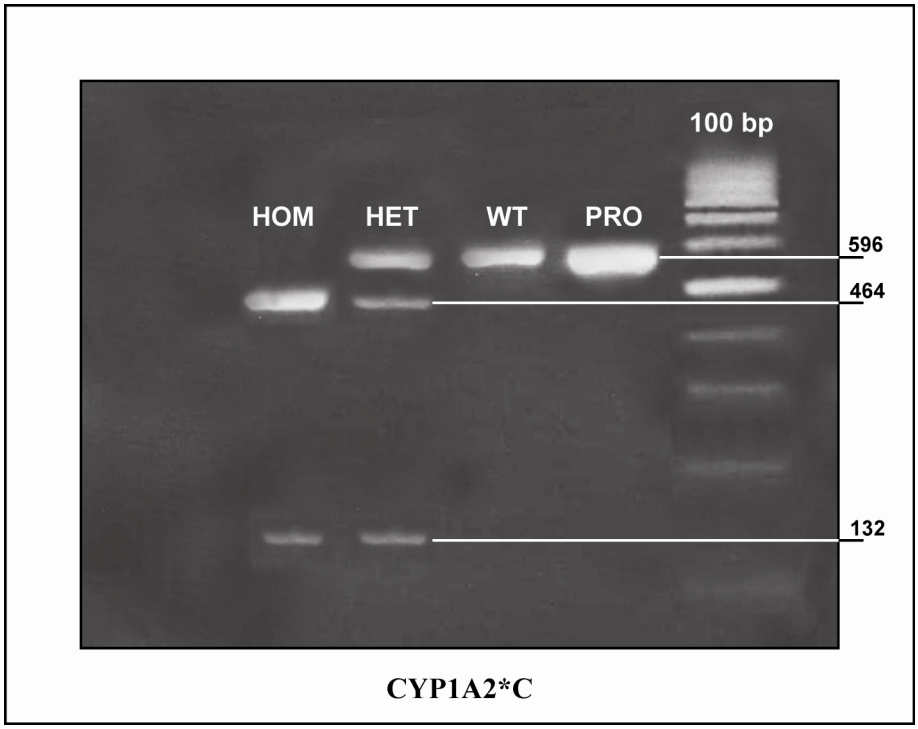

Figure 17: CYP1A2*C on (2.5%) agarose gel electrophoresis using DdeI as a restriction enzyme for the PCR product. Using 100bp ladder, PRO; product appeared at band size 596 bp, WT; wild type appeared at band size 596, (HOM) homozygous gave bands at 464 and 132, (HET) heterozygous bands were at 596, 464 and 132.

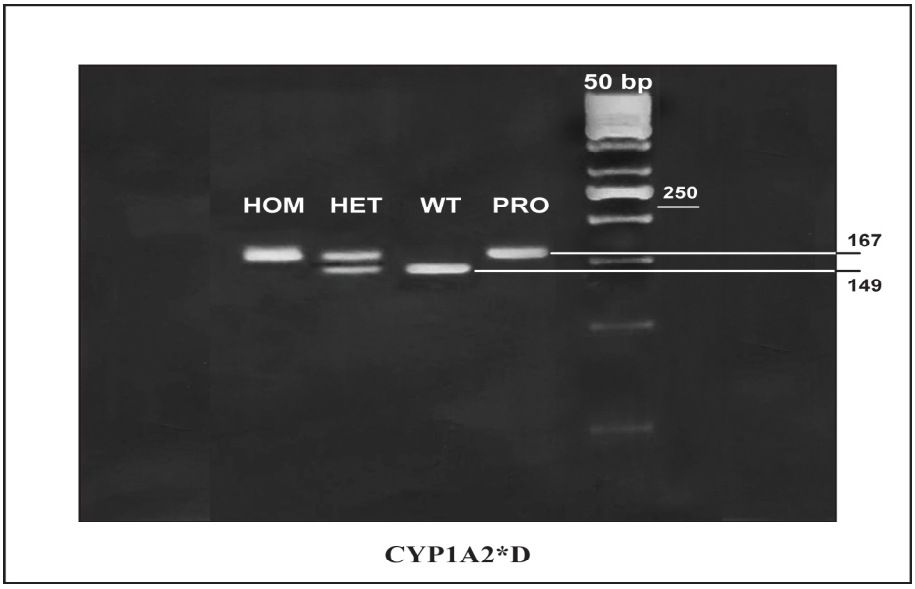

Figure 18: CYP1A2*D on (3%) agarose gel electrophoresis using NdeI as a restriction enzyme for the PCR product. Using 50bp ladder, PRO; product appeared at band size 167 bp, WT; wild type appeared at band size 149, (HOM) homozygous gave bands at 167, (HET) heterozygous bands were at 167 and 149.

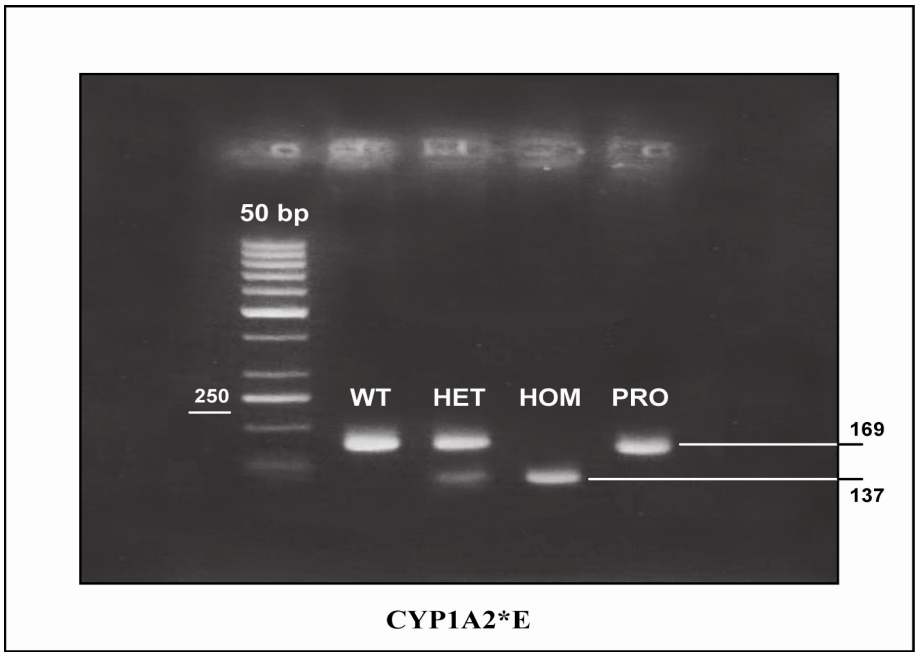

Figure 19: CYP1A2*E on (3%) agarose gel electrophoresis StuI using as a restriction enzyme for the PCR product. Using 50bp ladder, PRO; product appeared at band size 169 bp, WT; wild type appeared at band size 169, (HOM) homozygous gave bands at 137, (HET) heterozygous bands were at 169 and 137.

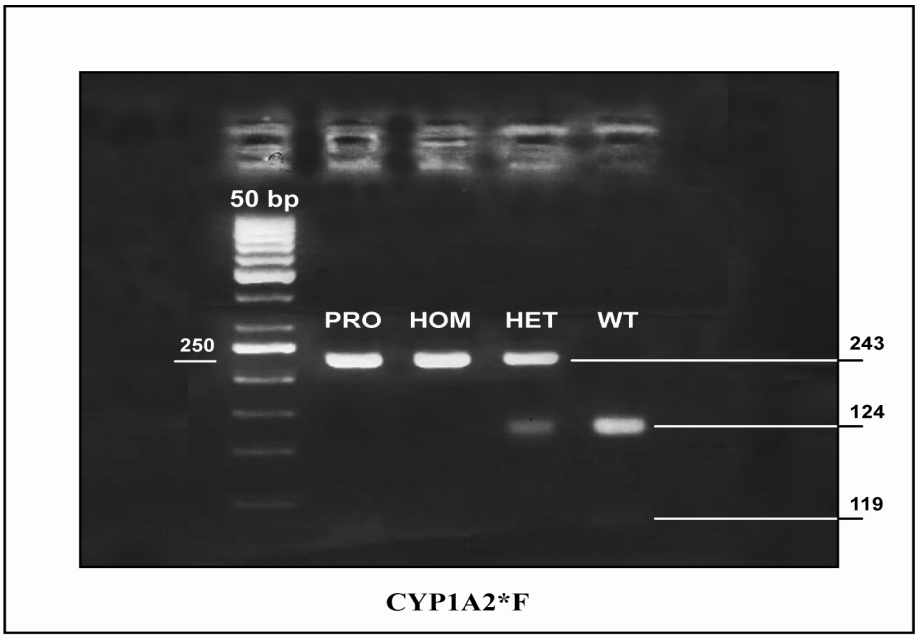

Figure 20: CYP1A2*F on (3%) agarose gel electrophoresis StuI using as a restriction enzyme for the PCR product. Using 50bp ladder, PRO; product appeared at band size 243 bp, WT; wild type appeared at band size 124 and 119 (not that clear), (HOM) homozygous gave bands at 243, (HET) heterozygous bands were at 243 and 124 and 119 (not that clear).

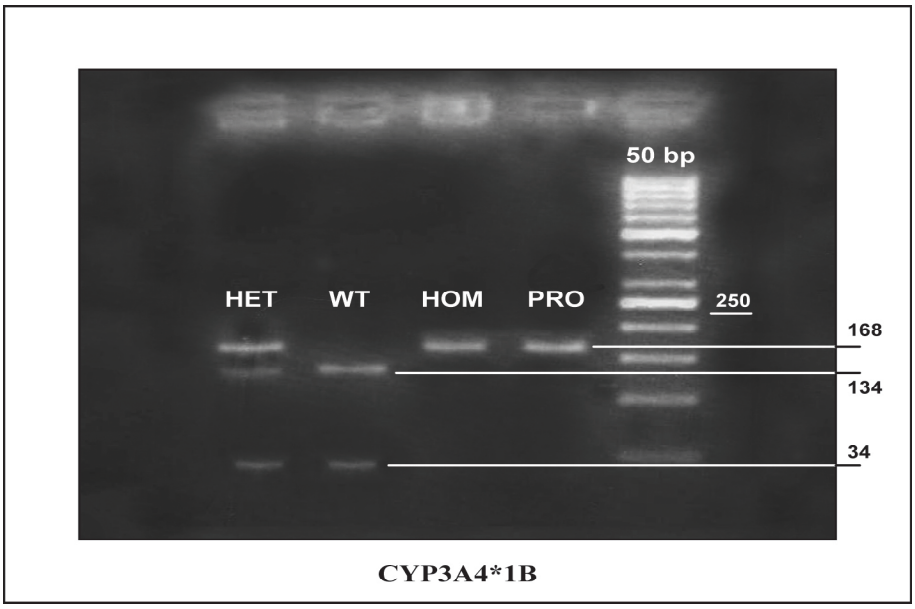

Figure 21: CY3A4*1B on (3.5%) agarose gel electrophoresis using MboII as a restriction enzyme for the PCR product. Using 50bp ladder, PRO; product appeared at band size 168 bp, WT; wild type appeared at band size 134, 34, (HOM) homozygous gave bands at 168, (HET) heterozygous bands were at 168 and 134 and a faint band at 34 bp.

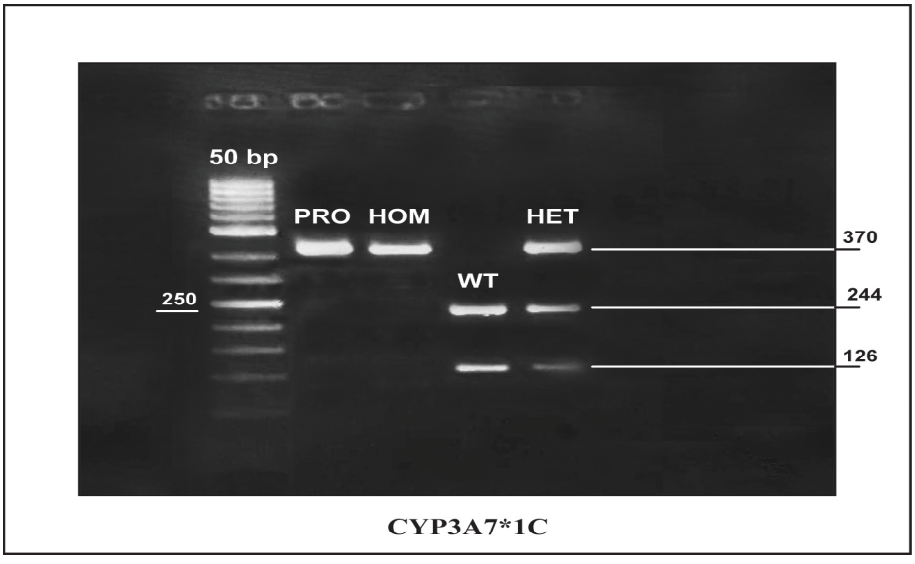

Figure 22: CY3A7*1C on (3%) agarose gel electrophoresis using SspI as a restriction enzyme for the PCR product. Using 50bp ladder, PRO; product appeared at band size 370 bp, WT; wild type appeared at band size 244 and 126, (HOM) homozygous gave bands at 370, (HET) heterozygous bands were at 370, 244 and 126.

To answer our question we raised in the introduction wither or not there is a risk that might threaten the workers in aflatoxin B1 exposed environment, and relying on the fact that these polymorphism will be present in Jordanians and then can be used as biomarkers for exposure we used SPSS program to analyze our results for the existence of differences in SNPs or allelic frequencies between the two groups assumed exposed group and healthy unexposed group to heavy levels of AFB1 contamination. with consistency to what have been speculated their were no significant difference in allelic frequencies between those in the most exposed environment to AFB1 and those who are not exposed directly to this mycotoxin, the provided chi-square value (χ^2), degress of freedom (df) and Population number (N) were for CYP3A5*2; χ^2 = 2.064 (df=1, N=557) based on P<0.05, for CYP3A5*3; χ^2= 0.034(df= 2, N= 434), P<0.05. For CYP3A5*4; no statistics are shown because of either the allele is constant or of low variant allele frequency. CYP3A5*5; no statistics are shown for the same reasons. CYP3A5*6; χ^2 = 0.118 (df= 1, N= 532), P<0.05. CYP3A5*7; no statistics are shown. CYP3A4*1B; χ^2 = 0.671(df=0.671, N= 494). CYP3A7*1C; χ^2 = 3.818 (df=2, N=521), P<0.05. As for CYP1A2*C; χ^2 = 0.724 (df=2, N=524), P<0.05. CYP1A2*D; χ^2 = 4.359 (df=2, N= 473) P<0.05, CYP1A2*E; χ^2 = 0.011 (df=2, N= 518) P<0.05 and for CYP1A2*F; χ^2 = 0.487 (df=2, N= 527) P<0.05. P values for all the comparison were higher than the alpha value of significance (P>0.05) as indicated in Table (8).

Table (8) Spss results for the comparison between both groups (study group, might exposed to aflatoxin) and control healthy group, comparison based on pvalue of the Pearson chi square.

SNP/ alleles	study group %	control group %	P values Pearson χ^2
CYP3A5*2			
wt	181 (99.5%)	375 (100%)	0.151 Fisher exact test p value = 0.327
hom	1 (0.5%)	0	
het	0	0	
CYP3A5*3			
wt	3(2.4%)	8(2.6%)	0.983
hom	95 (76.6%)	235(75.8%)	
het	26(21%)	67(21.6%)	
CYP3A5*4			
wt	180 (100%)	355(100%)	No statistics reported
hom	0	0	
het	0	0	
CYP3A5*5			
wt	182 (100%)	396 (100%)	No statistics reported
hom	0	0	
het	0	0	
CYP3A5*6			
wt	172 (97.2%)	343 (96.6%)	0.731 Fisher exact test p value = 0.801
hom	0	0	
het	5 (2.8%)	12 (3.4%)	
CYP3A5*7			
wt	174 (100%)	355(100%)	No statistics reported
hom	0	0	
het	0	0	
CYP3A4*1B			
wt	136(78.8%)	251(78.2%)	0.715
hom	2 (1.2%)	7(2.2%)	
het	35(20.2%)	63(19.6%)	
CYP3A7*1C			
wt	173(98.9%)	331(95.7%)	0.148
hom	0	1(0.3%)	
het	2(1.1%)	14 (4%)	

Table (8) continue

SNP/alleles	Exposed study group	Non exposed control group	P values Pearson χ^2
CYP1A2*1C			
Wt	156(88.6%)	302(86.8%)	0.696
Hom	1(0.6%)	1(0.3%)	
Het	19(10.8%)	45(12.9%)	
CYP1A2*1D			
Wt	106(70.2%)	220(68.3%)	0.133
Hom	12(7.9%)	13(4%)	
Het	33(21.9%)	89(27.6%)	
CYP1A2*1E			
Wt	145(88.4%)	314(88.7%)	0.955
Hom	1(0.6%)	2(0.6%)	
Het	18(11%)	38(10.7%)	
CYP1A2*1F			
Wt	25(14.3%)	43(12.2%)	0.743
Hom	81(46.3%)	170(48.3%)	
Het	69(39.4%)	139(39.5%)	

Since no correlation was found and the results opposed the theory for exposure consequences at least at the allelic frequency level, we aimed to fulfill the second objective and genotype the whole population, as a result we pooled the allele frequencies of both groups (Jordanian only) to represent the whole Jordanian population regardless to their exposure status; we found that the most frequent polymorphism of the CYP3A5 was the SNP CYP3A5*3 with an overall frequency of 86.8% in whole population (86.7% in controls and 87.1% in study group), the Egyptian studied frequency was 91.7%. Two SNPs were far less frequent; the CYP3A5*2 and CYP3A5*6 where they showed an over all 0.2% frequency (only 0.5 % in study) and overall 1.7% frequency (1.8% in controls and 1.4% in study) for the two SNPs respectively, Egyptians study group showed the same pattern but with higher frequency for CYP3A5*6 (4.5%).

CYP1A2*1F represented the highest frequency among CYP1A2 SNPs studied in pooled population with 67.3% frequency (68% in control group and 65.7% in the study group). In the Egyptian group the frequency was lower showing 59.1%. The second frequent SNP was CYP1A2*D in Jordanian showing 18.2% (17.9% in controls and 18.9% in study group), while Egyptians frequency was 16.7% for the same SNP. Both CYP1A2*C and CYP1A2*E had nearly the same frequency with an overall 6.5% (6.8% in controls and 6% in the study group) and 6% in the sum of the population (5.9% in controls and 6.1% in the study group) correspondingly. Egyptians studied showed a lower frequency by 2.2% for CYP1A2*1C and 2.6% for CYP1A2*1E.

On the other hand CYP3A4*1B frequency in the Jordanian population appeared to be 11.7% (12% in controls and 11.3% in study group) and higher in Egyptian study group with 15% frequency.

CYP3A7*1C in Jordanians had 1.7% frequency (2.3% in controls and 0.6% in the study) where as in Egyptian group this SNP was not frequent. (χ^2 values, P values, frequencies and the 95% confidence intervals (95% CI) values for overall population designated in Table 9 and sub groups presented in Table 10 for control results and Table 11 for study and Egyptian group results shown in Table 12.

Table (9) allelic frequencies for 12 SNPs in the total population (both grouped combined together), presented by the Chi square calculations.

SNP	N	Chi	Pvalue	frequency	95% CI	
3A5*2	588	556.000	0.000	0.002	-0.001	0.004
3A5*3	465	2.019	0.155	0.868	0.845	0.891
3A5*4	567	--------	--------	0.000[a]	0.000	0.000
3A5*5	583	--------	--------	0.000 [a]	0.000	0.000
3A5*6	563	4.872	0.027	0.017 [b]	0.009	0.025
3A5*7	559	--------	--------	0.000 [a]	0.000	0.000
3A4*1B	525	0.917	0.338	0.117	0.097	0.138
3A7*1C	550	4.726	0.030	0.017 [b]	0.009	0.025
1A2*C	554	0.023	0.880	0.065	0.050	0.080
1A2*D	504	8.376	0.004	0.182 [b]	0.157	0.207
1A2*E	550	0.799	0.371	0.060	0.045	0.075
1A2*F	560	6.151	0.013	0.673[b]	0.644	0.702

a: Hardy Weinberg proportions P value couldn't be estimated due to low variant allele frequency
b: Hardy Weinberg proportion P value <0.05.

Table (10) allelic frequencies for 12 SNPs in the control grouped, presented by the Chi square calculations.

SNP	N	χ^2	Pvalue	Frequency	95% CI	
CYP3A5*2	395	--------	--------	0.000[a]	0.000	0.000
CYP 3A5*3	331	1.458	0.227	0.867	0.839	0.894
CYP 3A5*4	376	--------	--------	0.000 [a]	0.000	0.000
CYP 3A5*5	390	--------	--------	0.000 [a]	0.000	0.000
CYP 3A5*6	376	6.767	0.009	0.018[b]	0.016	0.028
CYP 3A5*7	375	--------	--------	0.000 [a]	0.000	0.000
CYP 3A4*1B	341	1.610	0.205	0.120	0.094	0.145
CYP 3A7*1C	365	3.748	0.053	0.023[b]	0.012	0.035
CYP 1A2*1C	368	0.250	0.617	0.068	0.049	0.077
CYP 1A2*1D	343	1.077	0.299	0.179	0.148	0.209
CYP 1A2*1E	375	0.516	0.473	0.059	0.042	0.077
CYP 1A2*1F	374	2.982	0.084	0.680	0.645	0.716

a: Hardy Weinberg proportions P value couldn't be estimated due to low variant allele frequency
b: Hardy Weinberg proportion P value <0.05.

Table (11) allelic frequencies for12 SNPs in the exposed study grouped, presented by the Chi square calculations.

SNP	N	χ^2	Pvalue	frequency	95% CI	
CYP3A5*2	193	182	0.000	0.005[b]	-0.002	0.013
CYP 3A5*3	134	0.559	0.455	0.871	0.828	0.914
CYP 3A5*4	191	--------	--------	0.000[a]	0.000	0.000
CYP 3A5*5	193	--------	--------	0.000[a]	0.000	0.000
CYP 3A5*6	187	0.037	0.848	0.014	0.002	0.027
CYP 3A5*7	184	--------	--------	0.000[a]	0.000	0.000
CYP 3A4*1B	184	0.023	0.880	0.113	0.079	0.147
CYP 3A7*1C	185	0.006	0.939	0.006	-0.002	0.014
CYP 1A2*1C	186	0.034	0.854	0.060	0.035	0.085
CYP 1A2*1D	161	5.459	0.019	0.189[b]	0.144	0.234
CYP 1A2*1E	175	0.033	0.855	0.061	0.035	0.087
CYP 1A2*1F	186	14.276	0.000	0.657[b]	0.606	0.708

a: Hardy Weinberg proportions P value couldn't be estimated due to low variant allele frequency
b: Hardy Weinberg proportion P value <0.05.

Table (12) allelic frequencies for 12 SNPs in the Egyptian exposed study grouped, presented by the Chi square calculations.

SNP	N	χ^2	Pvalue	frequency	95% CI	
CYP3A5*2	23	--------	--------	0.000[a]	0.000	0.000
CYP 3A5*3	18	0.149	0.70	0.917	0.825	1.009
CYP 3A5*4	22	--------	--------	0.000[a]	0.000	0.000
CYP 3A5*5	23	--------	--------	0.000[a]	0.000	0.000
CYP 3A5*6	23	0.0499	0.82	0.045[a]	0.017	0.108
CYP 3A5*7	21	--------	--------	0.000[a]	0.000	0.000
CYP 3A4*1B	20	0.623	0.62	0.150	0.037	0.263
CYP 3A7*1C	21	--------	--------	0.000[a]	0.000	0.000
CYP 1A2*1C	23	0.011	0.92	0.022	0.021	0.065
CYP 1A2*1D	21	0.429	0.51	0.167	0.052	0.282
CYP 1A2*1E	19	0.014	0.91	0.026	0.026	0.078
CYP 1A2*1F	22	0.079	0.78	0.591	0.443	0.739

a: Hardy Weinberg proportions P value couldn't be estimated due to low variant allele frequency or low sample size

In general and taken as a whole, the Jordanian population were on hardy Weinberg Equilibrium (HWE) for allelic frequencies for CYP3A5*3, CYP3A4*1B, CYP1A2*C and CYP1A2*E. In the sub groups on the other hand, SNPs frequencies in the controls met hardy Weinberg equilibrium were CYP3A5*3 and CYP3A4*1B, CYP3A7*1C and CYP1A2*1C-*1F, in the study group; CYP3A5*3, *6 and CYP3A4*1B, CYP3A7*1C and CYP1A2*1C and*1E were on HWE in the control group. These discrepancies are largely because of the population size difference within each group.

We have analyzed frequencies based on two other factors, the gender and the smoking status. Based on the gender we have found that females most frequent SNP of the CYP3A5 was CYP3A5*3 with a percentage 85% which is lower than what was found in males 87.9%. Yet, the other SNPs could not be estimated by Hardy Weinberg proportions except for CYP3A5*2 in males (0.2%) and CYP3A5*6 (1.6%) and (2.3%) in females. CYP1A2 most frequent SNP in both genders was consistent with the overall population frequency 67% in males and 68% in females. CYP3A4*1B and CYP3A7*1C frequency was obviously higher in males (12.5% and 2% respectively) than females (9.1% and 0.8% respectively). In the CYP1A2 group of SNPs, CYP1A2*1F was slightly higher comparing both genders 67% in males and 68.5% in females. Other CYP1A2 frequencies in males and females were and in descending order CYP1A2*D (males 16.7% and 23.6% in females), CYP1A2*E (males 5.5% and 7.9% females), CYP1A2*C (males 6.3% and 7.4% in females) (χ^2 values, P values, frequencies and the 95% confidence intervals (95% CI) for both genders presented in Table 13 and 14.

Table (13) allelic frequencies for 12 SNPs in the Jordanian males, presented by the Chi square calculations.

SNP	N	Chi	Pvalue	frequency	95% CI	
CYP3A5*2	455	455.000	0.000	0.002	-0.001	0.005
CYP 3A5*3	348	2.192	0.139	0.879	0.855	0.904
CYP 3A5*4	438	------	------	0.000[a]	0.000	0.000
CYP 3A5*5	450	------	------	0.000[a]	0.000	0.000
CYP 3A5*6	434	7.200	0.007	0.016[b]	0.008	0.025
CYP 3A5*7	432	------	------	0.000[a]	0.000	0.000
CYP 3A4*1B	409	0.084	0.772	0.125	0.102	0.148
CYP 3A7*1C	421	4.165	0.041	0.020[b]	0.010	0.030
CYP 1A2*1C	426	0.056	0.814	0.063	0.047	0.080
CYP 1A2*1D	398	3.101	0.078	0.167	0.141	0.194
CYP 1A2*1E	429	0.072	0.789	0.055	0.039	0.070
CYP 1A2*1F	430	1.773	0.183	0.670	0.638	0.702

a: Hardy Weinberg proportions P value couldn't be estimated due to low variant allele frequency
b: Hardy Weinberg proportion P value <0.05.

Table (14) allelic frequencies for 12 SNPs in the Jordanian females, presented by the Chi square calculations.

SNP	N	Chi	Pvalue	frequency	95% CI	
CYP3A5*2	133	------	------	0.000[a]	0.000	0.000
CYP 3A5*3	117	0.077	0.781	0.850	0.804	0.897
CYP 3A5*4	129	------	------	0.000[a]	0.000	0.000
CYP 3A5*5	133	------	------	0.000[a]	0.000	0.000
CYP 3A5*6	129	0.073	0.787	0.023	0.004	0.042
CYP 3A5*7	127	------	------	0.000[a]	0.000	0.000
CYP 3A4*1B	116	1.40	0.237	0.091	0.053	0.128
CYP 3A7*1C	129	0.01	0.929	0.008	-0.003	0.019
CYP 1A2*1C	128	0.82	0.364	0.074	0.041	0.107
CYP 1A2*1D	106	7.57	0.006	0.236[b]	0.178	0.294
CYP 1A2*1E	121	2.48	0.115	0.079	0.044	0.113
CYP 1A2*1F	130	6.08	0.014	0.685[b]	0.627	0.742

a: Hardy Weinberg proportions P value couldn't be estimated due to low variant allele frequency
b: Hardy Weinberg proportion P value <0.05.

When we have gone through questionnaires we have collected in the beginning of the study, we have noticed that 50 – 60% of the volunteers enrolled in this study were smokers, and regardless to their history of smoking we analyzed allelic frequency depending on the smoking habits which were categorized in 4 groups and compared them with the allelic frequency of the non smokers, which should not differ from the smokers. The highest frequency of the CYP3A5*3 was found in the Arjillah smokers and who intakes 20 – 30 cigarette (88.2 % - 88%) respectively, this group also showed higher CYP3A5*6 frequency (3.8%) compared with 2.4% in non smokers and CYP3A4*1B was highest in groups (2 and 3) approximately 14%. CYP3A7*1C did not differ from the non smokers group (2%). Smoker having 1 – 30 cigarette had (6.7 – 6.9%) frequency in CYP1A2*C, compared with the non smokers (7.5%), however smokers of more than 40 cigarette and arjillah inhalers had lower frequency (4.5%) and (3.8%) in that order. Smokers in groups 2, 3 and 4 had lower frequencies of CYP1A2*D (average frequency 15.6%, than the non smoker groups and smokers who had 1 – 15 cigarette (19.9% - 19.6). smokers in general had lower frequency for the CYP1A2*F (65.1%, 64.5%, 67%, 66%), in groups 1 – 4, compared to non smokers 69%. (χ^2 values, P values, frequencies and the 95% confidence intervals (95% CI) for the both all groups presented in Table 15.

Another Comparison made for people who consume Nuts on daily, weekly and monthly basis, which is a basic source of aflatoxin B1 dietary exposure, and a habit increases rapidly among the Jordanian, (χ^2 values, P values, frequencies and the 95% confidence intervals (95% CI)) for each category of consumption presented in Table 16.

Table (15) allelic frequencies for 12 SNPs for the Jordanian smokers and non smokers individuals, presented by the Chi square calculations.

SNP	Allelic frequency per group of smokers				
	Group 1 smokers 1- 20 cigarette /day	**Group 2 smokers 20 – 30 cigarette /day**	**Group 3 smokers 40 – 80 cigarette /day**	**Group 4 smokers Arjillah**	**Non smokers**
CYP3A5*2	0.000	0.009	0.000	0.000	0.000
CYP 3A5*3	0.872	0.880	0.814	0.882	0.876
CYP 3A5*4	0.000	0.000	0.000	0.000	0.000
CYP 3A5*5	0.000	0.000	0.000	0.000	0.000
CYP 3A5*6	0.000	0.009	0.018	0.038	0.024
CYP 3A5*7	0.000	0.000	0.000	0.000	0.000
CYP3A4*1B	0.125	0.141	0.142	0.100	0.104
CYP3A7*1C	0.020	0.014	0.009	0.020	0.021
CYP 1A2*C	0.067	0.069	0.045	0.038	0.075
CYP 1A2*D	0.196	0.160	0.142	0.167	0.199
CYP 1A2*E	0.115	0.050	0.100	0.077	0.048
CYP 1A2*F	0.651	0.645	0.670	0.660	0.686

Table (16) allelic frequencies for 12 SNPs for the Jordanian Nut consuming individuals, presented by the Chi square calculations

SNP	Allelic frequency per group of Nuts consumers in Jordan				
	Daily consumption	**Weekly**	**Monthly**	**Rarely**	**No consumption**
CYP3A5*2	0.000	0.000	0.000	0.010	0.000
CYP 3A5*3	0.828	0.881	0.911	0.848	0.890
CYP 3A5*4	0.000	0.000	0.000	0.000	0.000
CYP 3A5*5	0.000	0.000	0.000	0.000	0.000
CYP 3A5*6	0.006	0.026	0.008	0.020	0.022
CYP 3A5*7	0.000	0.000	0.000	0.000	0.000
CYP3A4*1B	0.127	0.119	0.086	0.114	0.137
CYP3A7*1C	0.031	0.012	0.025	0.005	0.022
CYP 1A2*C	0.056	0.051	0.136	0.047	0.069
CYP 1A2*D	0.145	0.179	0.233	0.171	0.188
CYP 1A2*E	0.059	0.064	0.047	0.069	0.037
CYP 1A2*F	0.675	0.656	0.731	0.633	0.701

5.0

Discussion

It is evident that the metabolism of certain drugs and toxicants is controlled by certain enzymes, among which are the CYP450 enzymes. The knowledge about population frequencies usually elucidates the clinical relevance of CYP450 enzymes in optimizing individualized pharmacotherapy and susceptibility to toxicants that are metabolized by the CYP450 enzymes. Therefore variation in expression and activity, of these enzymes could be genetically originated with certain CYP450 enzymes or governed by environmental stresses of all types. Mutations in the CYP genes moreover may cause the absence of the enzyme, reduced or increased enzymatic expression and altered enzymatic substrate specificity (Rodriguez and Ingelman, 2006).

According to Kuehl, et al. (2001), heterozygous expression of the three CYP3A family members: 3A4, 3A5, 3A7 at least, is responsible for revealing and explaining human CYP3A activities and expression. Therefore genetic polymorphism in these enzymes may alter the mutagenic and carcinogenic effects of a given level of aflatoxin exposure and contribute to the variable susceptibility to carcinogenesis initiated by this toxin and many other environmental toxins (Hao and Xiang-Me, 2000).

One of the objectives of this study was to compare the prevalence of the frequency of the studied alleles between the exposed population and non exposed population and since there was no difference found between the two groups, we compiled them in one study group.

5.1 CYP3A5

In this study I have investigated the occurrence of six variants belonging to the CYP3A5 in the current study and compared their variability in different populations and ethnic groups. Although CYP3A5*6 is not present in Asians and Dutch caucasians, it is present in 13% - 16% of African Americans (Kuehl, et al., 2001, Van Schaik, et al., 2002 and Lee and Goldstein, 2005). CYP3A5*7 frequency in African Americans is 10% (Kuehl, et al., 2001). It is reported that the frequency of the CYP3A5*3 variant, which allow the normal splicing of CYP3A5 transcripts, were 5% in caucasians, 73% in African Americans according to (Hustert, et al., 2001). And 94% among British population (king, et al., 2003). Similar result was reported (91%) by Van Schaik, et al. (2002), in Dutch population for the CYP3A5*3, while he found CYP3A5*2 in 1% for and 0.1% for CYP3A5*6 while the rest of other alleles CYP3A5*4,*5,*7 were not pronounced to be present in his population study.

In the current study the frequency of CYP3A5 alleles were <0.1% (CYP3A5*4, *5, * and *7), which suggests that the screening for such alleles for estimating many toxins and aflatoxins risks in particular is irrelevant. This is consistent with the findings in Dutch caucasians (Van Schaik, et al. (2002). Heterozygots and those which have the wild type of CYP3A5*3 may show enzymatic activity (Kuehl, et al., 2001 and Van Schaik, et al., 2002), in our study 87.1 % of the overall population do not have any enzymatic activity and those would not be able to metabolize AFB1 through CYP3A5 if exposed.

In some ethnic groups like the Dutch and despite being heterozygous for (CYP3A5*2) and (CYP3A5*3) the effect and expression of CYP3A5 most probably in these individuals was diminished, this was supported by (Kuehl, et al., 2001 and Van Schaik, et al., 2002). As indicated in Table 8, no heterozygotes for the CYP3A5*2 variant have been detected.

On the contrary of this decreased expression caused by these alleles, CYP3A5*1 is the predominant allele responsible for CYP3A5 increased expression. Wojnowski, et al. (2004) found that CYP3A5*1 predominate in the Gambian population and therefore might endure a high risk of exposing to the mutagenic metabolite AFB1, similar findings are reported by (Kuehl, et al., 2001).

Table 17: CYP3A5 allelic frequencies in different populations.

Ethnic group	**Number of subjects**	**Frequencies of CYP 3A5 SNPs in different Ethnic groups compared with the Jordanian control and study groups based on P-value**						**References**
		27289 C>A CYP 3A5*2 %	**6986A>G CYP3A5*3 %**	**14665A>G CYP3A5*4 %**	**12952T>C CYP3A5*5 %**	**14690G>A CYP3A5*6 %**	**27131_ 27132 insT CYP3A5*7**	
White Canadian	160	0.7	92.9		0.0	0.0	0.0	(Hustert, et al., 2001)
White European	160	2.0	70.0			0.0	0.0	(Hustert, et al., 2001)
Dutch Caucasian	500	1.0	91.7	0.0	0.0	0.1	0.0	(Van Schaik, et al., 2002)
British	100	----	94					(King, et al., 2003)
Asian	48, 220	----	75		0.9	0.0	0.0	(Chou, et al., 2001)
Zimbabwean	200	0.0	77.6		0.0	22	10	(Roy, et al., 2005)
African-American	30, 90, 15	0.0	27 – 50			13 - 16	10	(Kuehl, et al., 2001)
Egyptian	18 - 23	0.0	91.7	0.0	0.0	4.5	0.0	Results of this study
Jordanian overall population	400 - 590	0.2	87	0.0	0.0	1.7	0.0	

Genetic variants of CYP3A5 shows demographic distribution particularly in industrialized countries due to the exertion of selection pressure on specific alleles by different environmental factors in that geographical area. This fact as well as to the boosted 2 – 3 fold increase in enzymatic activity that can be attained due to these factors, would indicate along with the allelic frequency, the tangled role of environment stress factors beside the genetics on CYP3A5 rate of expression (Chou, et al., 2001, Kuehl, et al., 2001, Roy, et al. 2005 and Lee and Goldestin, 2005).

In our study we could not establish any significant relationship between aflatoxin exposure as a stress factor and CYP3A5 allelic frequency, although there is a slight difference in the allelic frequency between both groups in the study group (87.1%) and (86.7%) in the control. Nonetheless, the higher frequency (91.7%) of CYP3A5*3 in the Egyptian study group, higher than the only African ethnic group we found in literature (the Zimbabwean 77%) (Roy, et al., 2005) may indicate the presence of such factor.

5.2 CYP3A4

Although it is estimated that 90% of the human variation in CYP3A4 activity is genetically determined (Lamba, et al., 2002 and Burk and Wojnowski, 2004), there is a wide interindividual variability in expression and activity directly related to some physiological, pathological and environmental factors; such as age, food, Hepatic diseases, smoking, concomitant drug intake of steroids, anticonvulsants and antifungal agents (Ando, et al., 1999).

The frequency of CYP3A4*1B is highly variable in different populations, ranging from 0% in Chinese (Lee and Goldstein, 2005), Taiwanese and Japanese (Ando, et al., 1999) and (Lee and Goldstein, 2005), to 4 – 10% in Caucasians (Rebbeck, et al., 1998, Van Schaik et al., 2002 and Lamba, et al., 2002), to 19% in Scottish Caucasians (Tayeb, et al., 2002), to (9 – 10%) in Mexican, to 15% in middle eastern (Lamba, et al., 2002) and with higher frequency reaching (48 – 80%) in African American (Lamba, et al., 2002) and to 53% in Africans (Rebbeck, et al., 1998 and Ando, et al., 1999). The frequency of this variant was 11.7 % in our group, which is similar the Middle Eastern group as shown in Table 18. We were unable to demonstrate any relationship between the allelic frequencies and the exposure to aflatoxin (11.3 % in study group versus 12 % in control group).

Some investigators reported that CYP3A4*1B is associated with increased CYP3A5 expression due to its linkage with CYP3A5*1A, but don't affect gene expression or activity of CYP3A4 (Kuehl, et al., 2001 and Wojonwski, et al., 2002). While others suggested, regardless to the many conflicting data about CYP3A4 expression, that CYP3A4*1B is the only allele found to affect this expression (Rodriguez and Ingelman, 2006).

Such expression found to be increased in most liver biopsies of individuals heterozygous for CYP3A4*1B which exclusively indicates that this allele is the marker for CYP3A5 polymorphism (Kuehl, et al., 2001 and Wojonwski, et al., 2002). As indicated in Table 8, although heterozygosity for CYP3A5*3 was 21% in overall population and 20% - 19.6% in study and control, only 7.5% of the control sample group were heterozygous for CYP3A4*1B and CYP3A5*3 and 9.7% of the study group and (11.1%) of Egyptian group shared this heterozygosity, therefore the individuals carrying both SNPs, seems to be more vulnerable.

Table 18: CYP3A4 allelic frequencies in different populations.

Frequencies of CYP 3A4 SNP in different Ethnic groups compared with the Jordanian population			
Ethnic Group	Study population Number	CYP3A4*1B Allelic frequency %	References
Caucasians	94	9.6	(Rebbeck, et al., 1998)
Caucasians	56	19.6	(Tayeb, etal., 2002)
Caucasians	53	6.5	(Lamba, et al., 2002)
Mexican	20	10	(Lamba, et al., 2002)
South east Asian	20	0.0	(Lamba, et al., 2002) (Lee and Goldsestine, 2005)
Chinese	20	0.0	(Lamba, et al., 2002)
Japanese	20 128	0.0 0.0	(Lamba, et al., 2002) (Ando, et al.,1999)
African		53	(Rebbeck, et al., 1998)
African-American	21	48	(Lamba, et al., 2002) (Ando et al., 1999)
Middle eastern	40	15	(Lamba, et al., 2002)
Egyptian	20	15	Results of this study
Jordanian	525	11.7	

5.3 CYP3A7

Burk, et al. (2002) had specified a polymorphic expression of CYP3A7 mRNA in 11% of the Caucasian livers with a frequency of 3.5% and CYP3A7*1C was the consistent marker for increased expression of CYP3A7 in both the liver and intestine.

Even so Sim, et al. (2005) Had came up with a conclusion that CYP3A7 protein expression is significant as the CYP3A5 in adult human liver and that it may influence the metabolism of certain CYP3A substrates.

This conclusion was consistent with Smit, et al. (2005) when he found that heterozygozity of CYP3A7*1C polymorphisms which causes the persistence of the enzyme and subsequently related with the reduced serum steroid hormone levels during adult life.

Table 19 shows the frequency of this variant in different populations, being 3 % in caucasians (Kuehl, et al., 2002, Burk, et al., 2002 and Smit, et al., 2008), 6% in African Americans to 15% in French Caucasians. Conversely to the Jordanian where we found 1.7% lower frequency in comparison to the previously mentioned ethnic populations, Table 19, (higher in the control (2.3%) compared with 0.6% in study group). this difference may be due to the sample number in the control group compared with study group. or due to the effect of some stress factors exerted on allelic frequency and the environment which may signify the genetic difference causing inconsistency in regulation, expression or activity of the CYP3A enzymes which could be relevant factors that alter cancer vulnerability or clinical out come (Gervasini, et al., 2007). Egyptian group alternatively in our study showed no CYP3A7*1C SNP frequency.

Table19: CYP3A7 allelic frequencies in different populations.

Frequencies of CYP 3A7 SNP in different Ethnic groups compared with the Jordanian population			
Ethnic Group	Study population Number	CYP3A7*1C Allelic frequency	References
Caucasians	79 500	3 3.2 3.5	(Kuehl, et al., 2001) (Smit, et al., 2008) (Burk, et al., 2002)
French Caucasians	10	15	(Kuehl, et al., 2001)
African American	40	6	(Kuehl, et al., 2001)
Egyptian	21	0.0	Results of this study
Jordanian	550	1.7	

5.4 CYP1A2

The individual variation in the CYP1A2 gene expression ranging from 10 – 200 fold, which may be affected the pharmacotherapy by the sum of genetic, epigenetic and environmental elements that adjust or regulates CYP1A2 expression and activity,(Zhou, et al. (2009), with an estimated 35 – 75% variability in 1A2 activity (Gunes and Dahl, 2008).

Smoking is considered a very important inducer of CYP1A2 activity, through polycyclic aromatic carbons present in the smoke, dietary habits and heavy exercise known to induce its activity, while drugs like flouroquinolones and oral contraceptive may inhibit enzyme activity (Gunes and Dahl, 2008).

Another important possible contributor of the interindividual variability in activity is the existence of polymorphisms in the CYP1A2 gene and over the few past years several SNPs have been discovered and found to be important in reflecting enzymatic activity (Nakajimma, et al., 1999, Chida, et al., 1999, Sachse, et al., 2003 and Gunes and Dahl, 2008).

The frequency of CYP1A2*1C varies from one population to another 23.3% (Nakajima et al., 1999) and 21.1 % (Chida, et al., 1999) in Japanese, 4% in Turks (Bilgen, et al., 2008) , 0.07 in Egyptians (Hamdy, et al. (2003), 6.5% of Jordanians in the current study. The frequency of this allele in the Egyptian study group found was 2.2%, higher than that reported by (Hamdy, et al., 2003)

In regard to enzymatic activity, CYP1A2*C have no effect on this (Zhou, et al., 2009), for that reason drawing conclusions about its relation with toxin metabolism at this level of study would be hard to do.

Only a humble conclusion may be drawn if we compared the Egyptian groups, those found in literature with a CYP1A2*1C frequency (0.07%), matched up with what we have observed 2.2% a difference that can be due to the limited sample number we have studied or to the effect of environmental factors surrounding workers.

CYP1A2*1D showed lower frequencies in caucasians (4 – 19.3%) (Ghobti, et al., 2007) in comparison to Asians (44 – 70%) (Chida, et al, 1999 and Ghobti, et al., 2007), in Turkish 92% (Bilgen, et al., 2008) and African "Egyptian in particular" (40%) (Hamdy, et al., 2003). Egyptians CYP1A2*1D frequency again in our study was not consistent with what have been mentioned previously, with a frequency of 16.7% lower than the Jordanian (18.2%) in both study and control.

CYP1A2*1E (and 1*G) which was shown to be frequent in Asians (8.2%) in Japanese specifically, but less frequent in the European caucasians 0.44% - 2.3% (Ghobti, et al., 2007) and Africans (3%)and In Turks (1%) in (Bilgen, et al., 2008) 6% in Jordanian (current study).

The frequency of CYP1A2*F allele among the studied population was the highest among studied alleles, being with 27% - 33% among European (Sachse, et al., 2003) and (Ghobti, et al., 2007). 27% in Turks (Bilgen et al., 2008), 61% - 68% in Japanese (Chida, et al., 1999) and Egyptians (Hamdy, et al., 2003) respectively. The frequency in our Jordanian group studied was (67.3%) and 59.1% in Egyptians studied. The inducibility of this allele known to be influenced by smoking, and since 64% of males and 17% of the females in our studied group are smokers, they would mostly vulnerable to a higher enzymatic induction among Jordanians.

As signified in Table 20 the CYP1A2 selected SNPs frequencies were comparison with other ethnic groups, we suggest that CYP1A2*1C, *1D, *1E and *1F, may be suitable for screening in the Jordanian population.

Table 20: CYP1A2 frequency in different populations:

Ethnic group	Study population number	Frequencies of CYP 1A2 SNPs in different Ethnic groups compared with the Jordanian control and study groups based on P-value				References
		-3860G>A CYP1A21*C	-2467delT CYP1A2*1D	-379T>G *1E,1G,1J,1K	-163C>A *F, 1J and 1K	
Caucasian						
British	114	0.9	4.8	4	33.3	(Sachse, et al., 2003)
Swedish	194	0.8	19.3	2.3	28.6	(Ghobti, et al., 2007)
Switzerland	100	2			68	(Todesco, et al., 2003)
Turkish	110	4	92	1	27	(Bilgen, et al., 2003) (Zhou, et al., 2009)
Asian						
Chinese	27	22	50	9	30	(Zhou, et al., 2009)
Japanese						
Japanese	159	21	42	8	61	(Chida, et al., 1999)
Saudi Arabian				10		(Gunes and Dahl, 2008) (Zhou, et al., 2009)
Korean	150	26.7	70.7	2.7	37.3	(Ghobti, et al., 2007)
African						
Egyptian	212	7	40	3	68	(Hamdy, et al., 2003)
Egyptian	19 - 23	2.2	16.7	2.6	59.1	Results of this study
Jordanian	500 – 560	6.5	18.2	6	67.3	

The common CYP1A2 polymorphisms (-163C>A, -2467delT, -3860G>A, -379T>G and other SNPs) were found to be in linkage disequilibrium, which generated several CYP1A2 haplotypes most of them have no impact on the enzymatic activity (Sachse, et al., 2003, Ghobti, et al., 2007 and Gunes and Dahl, 2008), this linkage disequilibrium however explains the discrepancies of the influence of these polymorphisms on the enzyme activity in smokers, which was characterized of being inconsistent and fluctuant (Gunes and Dahl, 2008).

The distribution pattern and the frequencies of CYP1A2 varies in different studies and populations, this may attributed to the manipulation and endurance of environmental factors on the activity of CYP1A2 through induction and /or inhibition which may concealed possible genetic variation in enzyme activity (Gunes and Dahl, 2008).

Single nucleotide polymorphisms Per se, cannot completely explain the interindividual differences in CYP1A2 related metabolic capacity. Therefore it had been suggested that chronic exposure to AFB1 might contribute to distinct pattern diversity of the nucleotide at CYP1A2 locus. Such diversity may be resulted through evolutionary process and generate risky genotype which may be detrimental and adverse and thus less favored by natural selection especially in HCC endemic regions (Chen, et al., 2006).

As to the gender differences in allelic frequencies reported in literature we found that only among the CYP3A5, CYP3A5*6 allelic frequency was higher in females as well as CYP1A2*1C – 1F higher allelic frequency compared to men, which supports the gender differences in the CYP1A2 frequencies, still men had higher 3A5*3 and 3A4*1B frequencies.

5.5 Deviation from Hardy Weinberg Equilibrium.

Another subject of debate is the deviation from the Hardy Weinberg equilibrium which assert that; the frequency of alleles or genotypes in any population remains unvarying and in equilibrium between generations, unless specific interruptions where commenced.

And when P value calculated is less than 0.05 the population will not be consistent with HWE. In our study SNPs in the overall all population (CYP1A2*1D and *1F, CYP3A5*2 and *6, CYP3A7*1C) were not consistent with the HWE compared with the controls (which showed only deviation in CYP3A5*6) and the study (CYP1A2*1D and *1F, CYP3A5*2).

The deviations from HWE can be caused by sum of reasons; of these are the population sizes (the small population size: where controls had higher population size than study group). And to what is called the random genetic drift (which is the change in frequency of the gene variant in a population due to random sampling). Another reason would be the population structure, where it may include inbreeding (which causes increase in the Homozygozity in all genes), assortative mating, stratification or admixture of different ethnic groups. Therefore testing for HWE should be performed among controls only, to eliminate special exposure or risk groups, assuming that aflatoxin exposure or any risk disease is rare in the general population (Schaid and Jacobsen, 1999).

5.6 Interpretations related to Aflatoxin

As mentioned previously the exposure to aflatoxin happened mainly either by food consumption or inhalation. Table 21 shows the consumption of certain stored none cocked food is widely varies in the population we studied. The most consumed food was the nuts range from daily consumption 14.3 % - 40.2 % weekly consumption, followed by cereals. People usually dependent on vegetables and fruits, which can be added as a source of contamination, see Table 21 for comparison.

Table 21: dietary habits among Jordanian population taken by questionnaire

dietary habits/period	Nuts %	Cereals %	Figs %	Dried fruits %	Raisins %
Daily	14.3	7.0	4.4	0.7	1.0
Weekly	40.2	11.0	4.3	0.5	1.3
Monthly	12.0	7.9	1.6	1.0	1.0
Rarely	17.9	34.4	32.5	21.1	13.1
Not at all	15.7	39.8	57.1	76.8	83.6
Total population questioned	**610**	**611**	**609**	**607**	**611**

Although the studies dealing with feed and food contaminants and their subsequent toxicities or even the level of exposure by inhalation or dietary intake are rare in Jordan. Herzallah, (2009) had studied aflatoxin and other Mycotoxins contaminations in different commodities when he tested a sum of 220 samples of different types of meats, milk, eggs and feed samples collected during January – May 2007 and measured the contamination level of Mycotoxins like the aflatoxins and their metabolites in feed from different sources in the country. Levels of contamination tested are shown in Table 22.

Table 22: Occurrence of aflatoxins in meat, milk, egg and feed samples from different location from Jordan

Table Occurrence of Aflatoxins in meat , milk, eggs and feed samples collected from different locations and seasons in Jordan.

Sample name	Collection time	No of samples	No of positive samples	Mean µg/kg	Range µg/kg	Incidence (%)	Average Aflatoxin concentration µg/kg					
							AFB1	AFB2	AFG1	AFG2	M1	M2
Feed	W S	20 20	12 5	9.92±0.03 5.32±0.02	1.02– 30.67 1.12-28.73	60 25	7.10^{a} 4.02^{b}	1.23^{a} 0.36^{b}	1.08^{a} 0.73^{a}	0.51^{a} 0.21^{b}	<0.05 <0.05	<0.05 <0.05
Milk (cows, sheep,goats)	W S	30 30	5 0	2.79±0.02 <0.05	0.16-5.23 <0.05	16.7 0	2.27^{cd} <0.05	0.21^{b} <0.05	0.08^{c} <0.05	0.09^{c} <0.05	0.14^{c} <0.05	<0.05 <0.05
Meat locally produced Beef, sheep's and goat	W S	30 30	4 2	6.36±0.03 3.01±0.02	0.13-5.10 0.15-3.28	13.3 6.7	3.25^{c} 2.53	1.27^{a} 0.27^{b}	0.87^{a} 0.13^{b}	0.31^{ab} <0.05	0.56^{b} 0.08	0.10^{a} <0.05
Meat Imported	W S	10 10	3 3	4.84±0.05 3.77±0.04	2.15-8.32 1.10-8.20	30 30	3.46^{c} 2.85^{cd}	0.18^{b} 0.60^{ab}	0.10^{b} 0.14^{b}	<0.05 0.10^{c}	1.1^{a} 0.08	<0.05 <0.05
Eggs (Hubbard)	W S	20 20	4 1	6.15±0.01 1.77±0.03	0.31-5.80 0.20-5.15	20 5	4.50^{b} 1.45^{d}	1.03^{a} 0.12^{c}	0.32^{ab} 0.08^{c}	0.15^{bc} <0.05	0.15^{c} 0.12^{c}	<0.05 <0.05

[A] detection limits of aflatoxin (B1 , B2, G1, G2) and Metabolites (M1,M2) were 0.05 µg/kg

[B] S; spring while W; winter

[C] results are represented as means (n=5) +/- SD. Means within a column with different subscripts differ statistically (P<0.05).

Salem and Ahmad, (2010) scanned 108 different food samples for the presence of mycotoxins and found that 24% of these samples were contaminated with mycotoxins, Table 23, Ochratoxins being the most common followed by Aflatoxins, Table 24.

Table 23: Occurrence of ochratoxin in foods from Jordan (regular foods contaminated)

Ochratoxin A levels in different analyzed samples of food from Jordan, as detected by ELISA.

Food commodity	No. analyzed samples	No. positive samples (%)[a]	Mean of contamination[b] (μg kg^{-1})	Range of contamination (μg kg^{-1})
Cereals	48	7 (14.6%)	2.8	2.04–5.86
Wheat	17	5 (29%)	2.26	2.04–2.56
Barely	8	1 (13%)	5.86	
Rice	23	1 (4%)	2.17	
Nuts	15	7 (47%)	5.64	2.75–7.42
Green coffee	11	7 (63%)	4.09	2.19–6.57
Legumes	10	0		
Others				
Sesame seeds	2	0		
Sunflower seeds	3	1 (33%)	4.34	

[a] Percentage of contamination.
[b] Mean contamination of positive samples.

Table 24: Occurrence of Mycotoxins in foods from Jordan

Occurrence of mycotoxins in foods from Jordan, as detected by ELISA.

Mycotoxins	No. analyzed samples	No. positive samples (%)[a]	Mean of contamination[b] (μg kg^{-1})	Range of contamination (μg kg^{-1})
Ochratoxin A	89	22 (25%)	4.17	2.04–7.42
Aflatoxins	67	2 (3%)	9.62	5.16–14.08
Deoxynivalenol	51	2 (4%)	600	250–950
Fumonisins	55	1 (2%)	250	
Zearalenone	50	0		
T-2 toxin	61	0		

[a] Percentage of contamination.
[b] Mean contamination of positive samples.

The exposure through inhalation will increase risk of lung cancer depending on the contamination levels in addition to swallowing the dusts, food, or water in heavily contaminated areas.

The lung CYP pool concentration is not dominantly reliant on CYP3A4 and CYP1A2, where He, et al. (2006) revealed a newly identified CYP2A13 enzyme in the lungs in high concentrations and contributes to AFB1 metabolism and activates lung cancer. The study of the persons with proven aflatoxin contamination for CYP450 (CYP3A4, 3A5, 3A7 and 1A2) and compare them with control group will make the study more powerful. But unfortunately there is limitation due to the resources constrains.

6.0

Conclusion

In our pursue to compare the gene frequency of some of the mostly known CYP450 polymorphisms in the workers of some of Jordanian food and feed manufacturing plants who are supposed to be exposed to aflatoxin B1 with general population we could not establish a relationship depending on the allelic frequencies of both groups. In spite of this we determined the gene frequency of the selected polymorphic forms of the CYP450s and calculated their prevalence among Jordanian population, we have succeeded in scanning 9 SNPs out of 12: CYP3A5*2, *3 and *6, CYP3A4*1B, CYP3A7*1C and CYP1A2*1C -1F). The remarkable point of strength is that they will serve good for general screening purposes in the Jordanian population elucidated by their clinical relevance in individualized Pharmacotherapy. This comprehensive study introduced some of the major SNPs that play a role in altering the metabolizing activity of CYP450 enzymes, either by deduction or the increase of its activity, and we have concluded that these common polymorphisms of the CYP3A4, 3A5 3A7 and 1A2 genes do not modify the risk of exposure to AFB1 and the concomitant cancer initiation without the true measurement of aflatoxin levels per occupation or exposure, where comparison will be of more scientific value which was a constrains that forbidden us from comparing the results of allelic frequencies with possible exposure levels. Another limitation was the lack of possibility to draw medical history for the workers or to restrain respiratory problems that may have resulted from exposure.

What worth noting is that the variation in Hepatic CYP450 could be either to polymorphism or to the inflection of environmental factors and these two can be detrimental in the risk of liver or lung cancer in AFB1 exposed individuals, and studying one factor is not enough to judge properly and to sort out this conflict.

Dietary exposures of AFB1 should be further estimated; through AF- albumin adduct measurement in general population and in occupationally exposed people and continue genotyping other polymorphisms like CYP3A5*1 and CYP2A31 isoenzyme then concentrating on the elimination of the effect of inducers and inhibitors on the CYP450s metabolizing process of AFB1.

Collectively epidemiological studies have reported a significant association between the studied CYP450 enzymes and carcinogenesis, and most of these studies have indicated that AFB1 is a major etiological agent of human liver and lung cancer, an added constraint in our study where we could not identify any possible cancer cases, never the less allelic frequencies would serve as strong start point for cancer future studies especially cancers initiated by CYP450 SNPs.

Finally we hope that this revision we have introduced on AFB1 and its health risks would open the eyes more widely on many toxicants and xenobiotics, among them Mycotoxins which obscure our lives, to be considered as candidates for further and continued study where health risks illustrated more fairly and expansively with the focus on analysis of the biomarker it self.

7.0

Recommendation

Upon the finding of this study, the recommendations would be as follows.

1. Proceed with AFB1 albumin adducts analysis to set the significance of the CYP450 polymorphisms which determines AFB1 metabolic pathway.

2. Other polymorphisms for CYP2A1 and CYP3A5*1 discovered to define susceptibility to lung and liver cancer respectively.

3. Make Aflatoxin B1 exposure level testing as a routine test in occupations and commodities that show possible risk.

4. analyze aflatoxin albumin adducts by other approaches rather than Eliza; instead use HPLC or LC/MS

5. Concentrate on liver and lung cancers in such occupations and other etiological factors affect the most, like AFB1 and Hepatitis in the case of liver cancer.

6. To use the allelic frequencies we have found in cancer studies especially cancers initiated by CYP450 polymorphisms.

7. To use the allelic frequencies we have found in Pharmacotherapy studies and other related studies.

7.0

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Appendix 1
Thesis Questionnaire

إسم التجربة	الإختلاف بين الأفراد في التعرض للأفلاتوكسين من نوع B1 و التعدد الجيني في السيتوكرومات من نوع P450 المصاحبة لهذا التعرض بين عينة من الأردنيين.		
إسم الطالب	غادة عبد المجيد سلامة	التخصص	علم السموم المخبري
الجهة المبعوثة	كلية الطب / الجامعة الأردنية	طبيعة البحث	بحث لدرجة ماجستير
رقم العينة المتسلسل		هذا الإستبيان خاص بـــ	Study group: ---- Control group:---
نوع العينة المطلوبة	دم	الكمية	5 + 10 مل (عدد 2)
تاريخ جمع العينه		طريقة حفظ العينه	

إسم المتبرع كاملا		الجنس	
العمر		مكان السكن	
هاتف المتبرع		مكان العمل/الشركة	
طبيعة العمل		عدد ساعات العمل	
عنوان العمل			
طبيعة المواد التي يتم التعامل معها (تحديدا)	----------		
هل تعاني من أمراض معينه مع ذكر للمرض.(ذكر أمراض سبق الإصابة بهاخصوصاالتهاب الكبد الوبائي)	----------	التدخين/ عدد السجائر في اليوم	----------

النمط الغذائي مع ذكر تقريبي لكمية الإستهلاك اليومي لهذه المواد	تركيز على المواد التالية (الحبوب الجافة و/أو المخزنة مثل الذرة ومنتجاتها، القمح (الفريكة، البرغل)، الشوفان أو أي منتج من مشتقاتها. الخضار و الفواكه غير المطبوخة والمخزنة أو الفواكه المجففة مثل الزبيب.أنواع المكسرات المختلفة (فستق، لوز، جوز، كاجو، أنواع البزر المختلفة).البهارات بكافة أنواعها، (يمكن للمتبرع إختيار الماده الأكثر إستهلاكامن المواد السابقة و ذكرها)	
المادة	درجة الإستخدام	الكمية المستهلكة تقريبا
1. ----------	يوميا---- أسبوعيا---- شهريا----أكثر من ذلك----------	------
2. ----------	يوميا---- أسبوعيا---- شهريا----أكثر من ذلك----------	------
3. ----------	يوميا---- أسبوعيا---- شهريا----أكثر من ذلك----------	------
4. ----------	يوميا---- أسبوعيا---- شهريا----أكثر من ذلك----------	------
5. ----------	يوميا---- أسبوعيا---- شهريا----أكثر من ذلك----------	
6. ----------	يوميا---- أسبوعيا---- شهريا----أكثر من ذلك----------	
7. ----------	يوميا---- أسبوعيا---- شهريا----أكثر من ذلك----------	
8. ----------	يوميا---- أسبوعيا---- شهريا----أكثر من ذلك----------	

أنا المتبرع---------- أقر بأن المعلومات التي تم ذكرهاسابقا صحيحة و أنني أتبرع بالعينة بكامل إرادتي. وفي حال تم طلب عينة أخرى فإنه لا مانع لدي. و لا يحق لي السؤال عن نتيجة البحث لأغراض خاصة بجهة البحث التي تعلن عن النتائج بطريقتها الخاصة.

التوقيع:----------

إسم وتوقيع جامع العينة: ----------	إسم و توقيع الجهة المسؤولة عن المتبرع: ----------

الاختلاف في التعرض للأفلاتوكسين B1 بين الأفراد و الطفرات الجينية في بعض أنزيمات ال CYP450 المصاحبة لهذا التعرض بين عينة من الأردنيين

إعداد
غادة عبد المجيد سلامة

المشرف
الأستاذ الدكتور محمد سعيد الخطيب

المشرف المشارك
الأستاذ الدكتور كمال عبد الحميد الحديدي

الملخص

تلعب الأنزيمات CYP450 دورا حيويا للغاية في أكسدة و اختزال وتفاعلات البيروكسيد للعديد من المركبات التي ينتجها الجسم أو التي يتعرض لها من أدوية و مواد مسرطنة. و تعتبر من الانزيمات الهامة ذات تعددية الطفرات و الأشكال والتي يتم تمثيلها بشكل أساسي في الكبد و عليه فانه من الممكن استخدام هذه الأنزيمات كأحد العلامات الحيوية لدراسة قابلية التسمم أو الإصابة بالسرطان.
إن السم الفطري الأفلاتوكسين ب 1 من الملوثات الشائعة للغذاء و المواد العلفية وتتمم أكسدته بواسطة الأنزيمات CYP1A2, 3A4, 3A5, 3A7 و التي تحدد مساره الأيضي ويعتقد أنه يقوم بزيادة قابلية الإصابة بسرطان الكبد و الرئتين عند الأشخاص الذين يعملون في بيئة عمل ملوثة بهذا السم الفطري.
الهدف العام من هذه الدراسة هو القيام بمقارنة النسق الجيني و الترددات الأليلية لطفرات بعض أنزيمات CYP450 والمذكورة انفا و هي (CYP*3A5*2*3*4,*5*6*7) (CYP3A7*1C), (CYP3A4*1B.) و(CYP1A2*1C, *1E,*1D,*1F) لعينة من الأردنيين ومقارنتهم بعينة من العاملين ببيئات ملوثة بالأفلاتوكسين.
و لدراسة التركيب الوراثي لطفرات الأنزيمات سابقة الذكر في كلا العينتين فقد قمنا باستخدام تفاعل البلمرة (PCR-RFLP) لتحليل الحمض النووي لدى 194 عامل في الصناعات الغذائية و العلفية من المرجح تعرضهم للأفلاتوكسين ب1 و تحليل الحمض النووي لدى 397 فرد من الأصحاء ظاهريا غير الأقرباء. و باستخدام معادلة هاردي وينبيرغ الإحصائية فقد قمنا بحساب تردد الأليلات ونسقها الجيني لدى الأفراد.
أما بالنسبة للنتائج فقد تم تحليلها إحصائيا باستخدام برنامج الحزمة الإحصائية (SPSS) ولم نجد أي فرق في وتيرة النسق الجيني أو تردد الأليلات بين المجموعتين ولهذا السبب فقد تم جمع المجوعتين في مجموعة واحدة لحساب. تردد الأليلات فكانت النتيجة كما يلي:
0.2% ل CYP3A5*2 (86.6%), CYP3A5*3، أما CYP*3A5*4,*5*7 فلم تظهر أي تردد جيني ، CYP3A4*1B (11.7%) ، CYP3A7*1C (1.7%) ، ولكل من CYP1A2*1C، 1D و 1E و1F فقد كانت نسبة التردد 6.5% ، 18.2%، 6.0%، 67.3% على التوالي.

و في الختام فقد تم تحديد التردد الأليلي لمجموعة من أنزيمات CYP450 مع وجود أحد المعوقات التي واجهت هذه الدراسة هو عدم إمكانية فحص مستوى الأفلاتوكسين في الدم لكلا المجوعتين وذلك لاستدراج استنتاجات أكثر فاعلية لدراسة العلاقة بين الأنزيمات السابقة و الأفلاتوكسن.