The effect of early life aflatoxin exposure on growth, immune function and cancer predisposition

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I confirm that the work submitted is my own and that appropriate credit has been given where reference has been made to the work of others

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Abstract

Aflatoxin exposure through contamination of dietary staples is prevalent in developing regions such as sub-Saharan Africa. Aflatoxin exposure has been linked to a G→T transversion in *p53*, which has been suggested to be the main mechanism of aflatoxin-associated liver carcinogenesis. In young children, *in utero* and weaning exposure has been linked to growth and immune function impairment through unknown mechanisms. The role of the insulin-like growth factor (IGF) axis was examined in aflatoxin-exposed Kenyan school-children. Through analysis of plasma samples, it was identified that 16% of the effect of aflatoxin on growth impairment was through disrupted IGF1 levels. It was also observed that mRNA and secreted protein levels of IGF growth-axis components were significantly reduced in aflatoxin-treated cultured liver cells. In another study using a Gambian mother-child cohort, it was determined that in most women aflatoxin exposure was consistently high or low during gestation and that this exposure was influenced by season of exposure. In infants born to highly aflatoxin-exposed women, significant changes to DNA methylation and gene expression patterns in white blood cells were observed in a number of genes following whole genome analysis. Among the observed changes, hypomethylation of *HORMAD2* at the promoter-level and *MIR24-2* at the gene-level as well as hypermethylation of *CD3D* at the promoter-level were identified and successfully validated by pyrosequencing. Additionally, *USP4* and *STAT3*, associated with cancers and growth impairment, were both upregulated in the high AFB_1 exposure group and were also successfully validated by qPCR. Changes in methylation and expression in aflatoxin-treated liver cells were assessed using a

whole genome approach. Hypomethylation of *IL2* and *MIR24-2* at the gene level and upregulation of *NFKB1A* and *IL6* were observed in AFB_1-treated liver cells compared to controls. Overall, the study provides evidence that the IGF axis may be involved in aflatoxin-induced growth impairment and the STAT3 and NF-κB signalling pathways, associated with hepatocarcinogenesis and growth impairment, were found to be activated in both Gambian infants exposed *in utero* and in treated liver cells. These pathways may play important roles in the mechanisms of aflatoxin exposure-associated health effects and need to be explored further.

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Abbreviations

AD	Atopic disorder
AF-alb	Aflatoxin-albumin adduct
AFB_1	Aflatoxin B_1
AFB_1-FAPY	AFB_1-formamidopyrimidine
AFB_1-N^7-Gua	Aflatoxin exo-epoxide adduct
AFB2a	Aflatoxin B2a
AFG_1	Aflatoxin G_1
AFM_1	Aflatoxin M_1
AFP_1	Aflatoxin P_1
AFQ_1	Aflatoxin Q_1
AMN	Amnion associated transmembrane protein
AS3MT	Arsenic methyltransferase
B[a]P	Benzo[a]pyrene
BCG	Bromocresol green
BMX	Bone marrow non-receptor tyrosine kinase
bp	Basepair
BSA	Bovine serum albumin
ChIP	Chromatin immunoprecipitation
CI	Confidence intervals
ComBat	Combining Batches
CRC	Colorectal cancer
CV	Coefficient of variation
CYP	Cytochrome
DAVID	Database for Annotation, Visualization and Integrated Discovery
DMR	Differentially methylated region
DMSO	Dimethyl sulfoxide
DNMT1	DNA methyltransferase-1
DOHaD	Developmental origins of health and disease

DON	Deoxynivalenol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENID	Early Nutrition and Immune Development
ER-α	Estrogen receptor-α
FDR	False Discovery Rate
GH	Growth hormone
GM	Geometric mean
GO	Gene Ontology
GOI	Gene of interest
GPR56	G-protein coupled receptor 56
GSH	Glutathione
GST	Glutathione-*S*-transferases
GSTM1	Glutathione-*S*-transferase M1
GSTP1	Glutathione *S*-transferase P1
HAZ	Height-for-age Z score
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCG	Human chorionic gonadotropin
HCV	hepatitis C virus
HIF-2α	Hypoxia-inducible factor 2α
HPLC	High-pressure liquid chromatography
HPLC-f	HPLC with fluorescence detection
HSA	Human serum albumin
IAP	Intracisternal A particle
IARC	International Agency for Research on Cancer
IDMS	Isotope dilution mass spectrometry
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor receptor 1
IGFALS	Insulin-like growth factor acid labile subunit
IGFBP	Insulin-like growth factor binding protein
IKK	IκB kinase

LD50	Lethal dose, 50%
MBL2	Mannose-binding lectin-2
MDEG	Methyl Donors and Epigenetics
MDFS	Methylation-dependent fragment separation
MGMT	O^6-methylguanine-DNA methyltransferase
MIR24-2	MicroRNA 24-2
MSM	Multi-Sample Amplification Master Mix
MWCO	Molecular weight cut-off
NA	Non-annotated gene
NES	Nuclear export signal
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B
NLS	Nuclear localisation sequence
nuID	Nucleotide Universal Identifier
OR	Odds ratio
PAH	Polycyclic aromatic hydrocarbon
PBCs	Peripheral blood cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG10	Paternally expressed gene 10
PES	Polyethersulfone
Pfs-IgG3	Plasmodium falciparum schizont antigen IgG3
PHC	Primary hepatocellular carcinoma
PHLDA2	Pleckstrin homology like domain, family A, member 2
QC	Quality control
qPCR	Quantitative PCR
QTA	Quantitative Trait Analysis
RHD	Rel homology
RIA	Radioimmunoassay
RIN	RNA integrity number
RLGS	Restriction Landmark Genome Scanning
ROS	Reactive oxygen species
RS1	Repeat sequence 1
RSN	Robust Spline Normalisation
SDS	Sequence detection system

SHP1	Src homology region 2 domain-containing phosphatase-1
SNCG	Synuclein-gamma
SNP	Single nucleotide polymorphism
SOCS-3	Suppressor of cytokine signalling-3
SSRI	Selective serotonin reuptake inhibitors
STAT3	Signal transducer and activator of transcription-3
TAD	Transcriptional activation domain
TF	Transcription factor
TLC	Thin-layer chromatography
TMB	3,3′,5,5′-Tetramethylbenzidine
TSLP	Thymic stromal gene
USP4	Ubiquitin-specific protease-4
VEGF-R	Vascular endothelial growth factor receptor
VST	Variance Stabilization Transformation
WAZ	Weight-for-age Z score
WHZ	Weight-for-height Z score

1 Introduction

1.1 Literature Review

1.1.1 Discovery of Aflatoxins

In May 1960, several thousand turkeys in England showed symptoms of loss of appetite, lethargy and a high mortality rate leading to a diagnosis of Turkey 'X' disease, so called as the cause was undetermined (Blount 1961). The series of experiments that followed upon characterisation of the disease revealed no involvement of infectious agents (Blount 1961). However, very high mortality rates and symptoms indicative of Turkey 'X' disease were observed in poults that were given suspect feed during feeding trials (Blount 1961). As the common factor in all cases was Brazilian imported groundnut meal, it was hypothesised that contaminated feed was responsible for manifestation of the disease, directing research towards the discovery of aflatoxin-producing *Aspergillus* fungal contamination of the groundnut meal (Wogan 1966).

Aflatoxins are secondary metabolites produced primarily by *A. paraciticus* and *A. flavus* but also by other less commonly occurring fungal species belonging to the *Aspergillus* genus (Pitt 2000). Four main structurally-related types of aflatoxins have been characterised and extensively studied: aflatoxin B_1, B_2, G_1 and G_2 (Wogan 1966) so named due to the blue (B_1 and B_2) and yellow-green (G_1 and G_2) light emitted respectively when exposed to ultraviolet light, with aflatoxin B_1 being the most commonly occurring in nature (Wogan 1966). When ingested, aflatoxin B_1 and B_2 can be hydroxylated to form subtypes M_1 and M_2 respectively (Wogan and Pong 1970). The chemical structures of these main types of aflatoxins are depicted in Figure 1.

Aflatoxins have been the subject of a wide range of studies aimed to determine the effects of exposure on animal and human health. The ubiquitous nature of these toxins makes it imperative to fully comprehend sources of exposure, metabolism once ingested and the effects on health, the latter of which is the focus of this thesis.

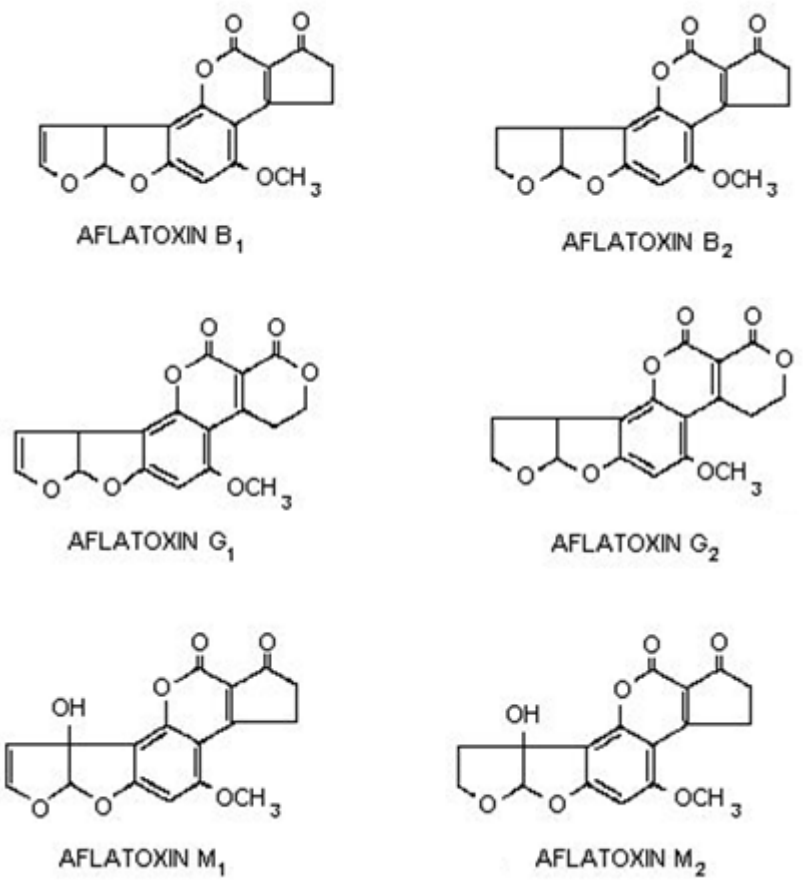

Figure 1: Chemical structures of the four naturally occuring aflatoxins and their hydroxylated metabolites: B_1, B_2, G_1, G_2, M_1 and M_2. Adapted from http://www.food-info.net/uk/tox/afla.htm (accessed 12th January 2013)

1.1.2 Contamination of crops with aflatoxin

Contamination of crops by aflatoxin-producing *Aspergillus spp.* occurs at temperatures between 24˚C and 35˚C with 7-10% relative humidity- conditions usually observed in countries situated in the tropics (Williams *et al.* 2004). Contamination is therefore usually observed between approximately 40˚N and 40˚S of the equator, particularly in developing countries within this region (Cardwell and Cotty 2002). It has been estimated that mycotoxins contaminate approximately a quarter of the world's food supply including several dietary staples such as maize and groundnuts consumed in developing countries (Strosnider *et al.* 2006) with approximately 4.5 billion people exposed to aflatoxin contamination worldwide (Williams *et al.* 2004).

Most aflatoxin is produced by *A. flavus* of which there are two main types based on differences in morphology: strains L and S (Cotty 1994). The L strain produces larger and fewer sclerotia and differs in its ability to produce aflatoxin with several L strain isolates known to be atoxigenic. On the other hand, the S strain produces many small sclerotia and tends to produce more aflatoxin. Furthermore, atoxigenic S strain isolates are rarely found (Cotty 1994). The soil is the main medium through which contamination of crops occurs (Horn 2003). Variability in the distribution of these strains can affect the extent to which crops are contaminated with aflatoxin. In a study based in Benin, the S strain was found in higher numbers in the soil in the dry agroecological zone in the north compared to higher numbers of the L strain observed in the soil in the humid and tropical agroecological zone in the south (Cardwell and Cotty 2002). Further variation in the production of aflatoxin by the L strain can result in irregular contamination in different regions.

Contamination of crops with aflatoxin-producing fungi can occur in two distinct phases. The first contamination phase may initiate during growth due to mechanical insect damage as well as dispersal of aflatoxin-producing fungi by insect activity (Cotty 1994). Additionally, the hot and humid climate can increase crop susceptibility to contamination and promote fungal growth (Cotty and Jaime-Garcia 2007). Post-harvest contamination usually constitutes the second phase of contamination through storage practices, which can introduce or propagate fungal growth and consequently aflatoxin production in grains (Vincelli 1995). Storage practices including the type of material used and length of storage can differ by the agroecological zone and ethnicity. In Benin, less contamination with aflatoxins was detected in maize stored in the 'Ago', (a giant basket made from woven raffia palms, tree branches or bamboo) when made of bamboo as opposed to woven palms, compared to other materials (Bandyopadhyay *et al.* 2007). Additionally, it was observed that aflatoxin production was higher after approximately 6 months of storage compared to levels at the beginning of storage. However, when maize was stored for longer periods, a lot of care was taken when drying the grains and use of insecticides was more common. For this reason, aflatoxin levels were in fact lower in grains stored for longer than 6 months (Bandyopadhyay *et al.* 2007).

Contamination of crops with aflatoxins is highly dependent on environmental factors such as temperature and moisture. Any change to these conditions can alter contamination levels and distribution. Indeed, climate is suggested to be the driving force of fungal growth and mycotoxin production (Magan *et al.* 2003). Increased heat and draught conditions can lead to increased physical damage to crops such as 'hull cracking' resulting in elevated aflatoxin contamination (Paterson and Lima 2010). Additionally, increased CO_2 levels can result in increased host susceptibility

due to a change in physiology and morphology. Warm temperatures also allow fungal species to compete well than at cooler temperatures. Therefore, global warming may accelerate contamination in regions where temperatures become optimal for fungal growth (Paterson and Lima 2010). Indeed, it is expected that as global climate changes, developing countries, including those in Africa, will experience warmer temperatures with increased risk of draught stress. These changes are predicted to increase contamination of crops with aflatoxin in these regions (Magan *et al.* 2011). Therefore, the risk of contamination and of consumption of aflatoxin-contaminated foods needs to be assessed in order for effective strategies to be put in place for the prevention of acute and chronic dietary exposure.

1.1.3 Impact of aflatoxins on animal and human health

1.1.3.1 Structure of AFB_1 related to health effects

The discovery of aflatoxins as the causal agent in sickness and deaths in poultry marked a spate of studies dedicated to understanding the effects of exposure and the mechanism by which aflatoxin affects animal health. Animals and humans are exposed to aflatoxins through consumption of contaminated grains. Following the detection of aflatoxins in poultry and cattle feed, studies were focussed on understanding the effects and dose response of aflatoxin contamination on animals. Early research on laboratory rats and guinea pigs discovered the formation of lesions in the liver as well as the development of hepatocarcinomas (Brown *et al.* 1999). Initially, continual exposure to aflatoxin through feed was considered a prerequisite for liver lesion formation. However, further studies showed that hepatic necrosis and tumours in rats could be induced following a single sub-lethal dose of aflatoxins

(Butler 1964; Carnaghan 1967). It was soon discovered that periportal necrosis occurred slowly over 48-72 hours following exposure with no observed rapid recovery (Butler 1964) making aflatoxin B_1 the most potent hepatocarcinogen known at the time (Carnaghan 1967).

Hepatic tumours and slowed growth were symptoms common to different species of animals exposed to varying doses of aflatoxin in feed (Butler 1969) with cattle and sheep being more resistant to the effects of exposure compared to poultry (Robens and Richard 1992). Table 1 presents the LD_{50} concentrations of aflatoxin B_1 for some animal species, which shows the variation in concentrations that are lethal in different species.

Table 1: LD_{50} doses of AFB_1 in different animal species.

Species	LD_{50} dose (mg/Kg)	Reference(s)
Duckling	0.4	(Carnaghan *et al.* 1963)
Rabbit	0.3	(Butler 1969)
Pig	0.6	(Butler 1969)
Guinea Pig	1.4	(Butler 1966)
Rainbow Trout	0.8	(Bauer *et al.* 1969)
Rat (neonate)	1.0	(Wogan 1966)
Rat (weanling)	5.5-7.2	(Butler 1964; Wogan 1966)
Mouse	9.0	(Butler 1969)
Hamster	10.2	(Butler 1969)

The basic structure of aflatoxin was first described by Asao et al in 1963 and followed up in 1965 (Asao *et al.* 1963; Asao *et al.* 1965). Aflatoxin was found to

consist of a fused coumarin-cyclopentene system attached to a dihydrofurofuran moiety. Additionally, a double bond exists at the 2,3 (8,9 by IUPAC nomenclarture) position of the terminal furan ring. In AFB_1 and AFB_2, a cyclopentenone ring is fused to a lactone ring in the coumarin moiety while in AFG_1 and AFG_2, the coumarin moiety consists of a fused lactone ring (Lamplugh *et al.* 1988; Partanen *et al.* 2010). In two key studies designed to determine the structure-related activity of aflatoxins, both naturally occurring aflatoxins (B_1, G_1, B_2 and G_2) and synthetic compounds were tested in Male Fischer rats at low doses (Josse *et al.* 2012; Randhawa 2008). The potency of the four naturally occurring aflatoxins was confirmed to be in the order $B_1 > G_1 > B_2 > G_2$. The synthetic compounds tested lacked the dihydrofurofuran ring and contained the fused coumarin-cyclopentene ring in varying configurations. While toxicity and development of hepatocellular carcinoma (HCC) in the rats was observed with exposure to AFB_1 and AFG_1, neither was detected when rats were fed meals containing the synthetic compounds revealing the importance of the dihyrdofurofuran ring in potency and carcinogenicity (Josse *et al.* 2012). Additionally, these compounds were found to be non-toxic in ducklings, which are much more sensitive to AFB_1 and AFG_1 than rats (Josse *et al.* 2012). In the second related study, it was also discovered that AFB_1 and AFG_1 inhibited hepatocyte RNA polymerase activity and reduced nuclear RNA synthesis. Moreover, one of the synthetic compounds studied, 5,7-Dimethoxycyclopentenone [2,3-*c*] coumarin, which was closest in configuration to AFB_1, had the highest affinity to bind DNA followed by AFB_1 (Randhawa 2008). AFB_1 binds purine bases in single-stranded DNA weakly and the presence of an amino group plays a role in the interaction between AFB_1 and DNA (Maxwell *et al.* 1989).

1.1.3.2 Metabolism of AFB_1

It was observed that when one-day old ducklings were exposed to the milk obtained from cows exposed to AFB_1, they produced bile duct hyperplasia similar to that seen with direct exposure to AFB_1 (Campbell and Hayes 1976). This suggested the presence of a toxic metabolite in milk, which was identified as aflatoxin M_1 (AFM_1), a hydroxylated form of AFB_1 (De Iongh *et al.* 1964) and was also excreted in sheep milk and urine (Allcroft *et al.* 1966). AFM_1 was revealed to have carcinogenic potential in rainbow trout and rats albeit with lower potency than AFB_1 (Wogan and Paglialunga 1974; Sinnhuber *et al.* 1974).

Further inquiry into the metabolism of AFB_1 facilitated the identification of another metabolite called aflatoxin Q_1, which is an isomer of AFM_1. AFQ_1 was one of two metabolites produced when monkey liver microsomes were incubated with an NADPH regenerating system, the other metabolite being AFM_1 (Masri *et al.* 1974). Using a bacterial *Salmonella typhimurium* strain TA 98 test, AFQ_1 was found to be mutagenic although at least 50 times less potent than AFB_1 (Wong and Hsieh 1976). It was also found to be 100 times less carcinogenic than AFB_1 in rainbow trout (Hendricks *et al.* 1980).

Another metabolite that in the form of a conjugate is the main component of urine of AFB_1-exposed rhesus monkeys and rats is the AFP_1, which is a phenol product of demethylated AFB_1 (Dalezios *et al.* 1971; Wogan *et al.* 1967). This metabolite was found to be slightly toxic in new-born mice at a 150 mg/kg dose causing approximately 13% mortality (Buchi *et al.* 1973) but was mostly non-toxic and non-teratogenic in both new-born mice and fertile chicken eggs (Buchi *et al.* 1973; Stoloff *et al.* 1972).

AFB_1 is also biologically reduced to a metabolite called aflatoxicol by liver enzymes (Pawlowski *et al.* 1977), which is almost as carcinogenic as AFB_1 in rainbow trout (Schoenhard *et al.* 1981). Metabolism of AFB_1 to aflatoxicol at the human placental interface *in vitro* provides evidence for the extension of the effects of AFB_1 from mother to the foetus (Partanen *et al.* 2010).

When water reacts with the 8,9 double bond in the terminal furan ring of AFB_1, aflatoxin B2a is formed and is therefore the monohydroxylated derivative of AFB_1. As with the other identified metabolites, AFB2a is also much less toxic than AFB_1 and has a lower capacity to bind DNA (Ciegler and Peterson 1968; Lillehoj and Ciegler 1969).

It was first suggested by Schoental in 1970 that aflatoxin may be metabolised to an epoxide that has elevated toxicity, binds to DNA and to which animals may be more vulnerable (Randhawa and Cohen 2005; Schoental 1970). Subsequently, this metabolite was discovered in an assay subjecting *Salmonella typhimurium* TA 1530 to liver microsomes derived from rats exposed to AFB_1 (Agag 2004). The unknown metabolite was found to bind tightly to liver microsomes (Gurtoo and Campbell 1974). This epoxide was proposed to be a 2,3-oxide (8,9-oxide by IUPAC nomenclature) of aflatoxin B_1 and found to be important in the *in vitro* binding of AFB_1 to DNA (Essigmann *et al.* 1977). This was further supported by the fact that most of the binding of AFB_1 to liver DNA and ribosomal RNA (rRNA) in rats was by the AFB_1-2,3-oxide and that this oxide was also produced *in vitro* by human liver microsomes (Swenson *et al.* 1974). The formation of the epoxide-DNA adduct was confirmed in an *in vivo* system in rats and the epoxide was found to mainly bind the N^7 atom of guanine via an S_N2 reaction forming the 2**,**3-dihydro-2-**(**guan-7-yl**)**-3-hydroxy-aflatoxin B_1 DNA adduct (Lin *et al.* 1977; Croy *et al.* 1978; Johnson and

Guengerich 1997). Further analysis of the epoxide revealed the potent toxin to be a highly unstable exo-epoxide while an endo-epoxide, which being more stable did not interact with DNA and was classed as non-mutagenic, was also identified (Iyer *et al.* 1994). The instability of the exo-epoxide is due to the presence of a positively charged imidazole ring. The adduct can then either undergo depurination resulting in the formation of an apurinic site or under alkaline conditions, it can be converted to the relatively more stable imidazole ring-opened AFB_1-formamidopyrimidine (AFB_1-FAPY) (Marzilli *et al.* 1998). A significant proportion of adducts formed *in vivo* comprises AFB_1-FAPY in rats (Croy and Wogan 1981) as well as in human liver tissue (Hsieh *et al.* 1988). The formation of AFB_1-FAPY involves considerably less alteration to the structure of DNA compared to the AFB_1-N^7-Gua adduct, which makes it easier for it to bypass most DNA repair mechanisms. It has therefore been suggested that the susceptibility to AFB_1-induced carcinogenesis is a result of the variation in DNA repair systems among mammals (Bedard and Massey 2006).

It was soon established that the metabolic activation to the more toxic epoxide was catalysed by the cytochrome (CYP) P450 system in the liver (Shimada and Guengerich 1989) particularly the CYP 1A2, 2A3, 2A7, 3A3 and 3A4 forms (Aoyama *et al.* 1990). The fact that the bioactivation of AFB_1 had been observed to occur in human livers (Shimada and Guengerich 1989; Shimada *et al.* 1989) warranted research on the potential effects of the epoxide on human health. CYP 3A4 has been identified as the main enzyme involved in the bioactivation of AFB_1 in humans and is largely present in the liver as well as the small intestine (Guengerich *et al.* 1998). It has also been found to only form the mutagenic exo-epoxide and AFQ_1 (Ueng *et al.* 1995). CYP 1A2 is also considered very important in the oxidation of low concentrations of AFB_1 (Gallagher *et al.* 1994) and can form the

endo- and the exo-expoxide as well as metabolic products AFM_1 and small amounts of AFQ_1 (Ueng *et al.* 1995).

Liver enzymes not only activate ingested AFB_1 but also engage in a detoxification pathway. In fact, formation of an 8,9-exo-AFB_1 epoxide-glutathione conjugate made up about 10% of the dose given to female Wistar rats (Degen and Neumann 1978). Glutathione-*S*-transferases (GST), produced by liver microsomes, inhibits the binding of the 8,9-exo-AFB_1 epoxide to DNA and has provided further evidence of a detoxification pathway via glutathione (GSH) conjugation (Lotlikar *et al.* 1980). It has also been suggested that differences in rate of conjugation with GSH contributes towards species variation in susceptibility to AFB_1 (Degen and Neumann 1981) with the mouse liver cytosol conjugating the exo-epoxide almost exclusively, the rat liver cytosol conjugating mostly the endo-epoxide and human liver cytosols conjugating both epoxides but at very low levels (Raney *et al.* 1992). While the half-life of the exo-epoxide has been determined to be approximately 1 second in an aqueous buffer of neutral pH at 23°C, it reacts almost immediately with DNA and GSTs (Guengerich *et al.* 1996). It has been suggested that variations including genetic polymorphisms in the P450 enzymes can alter the rate of activation and detoxification of AFB_1 and therefore susceptibility to the carcinogenic effects of AFB_1 (Peter Guengerich and Shimada 1998). The metabolism of AFB_1 is depicted in Figure 2.

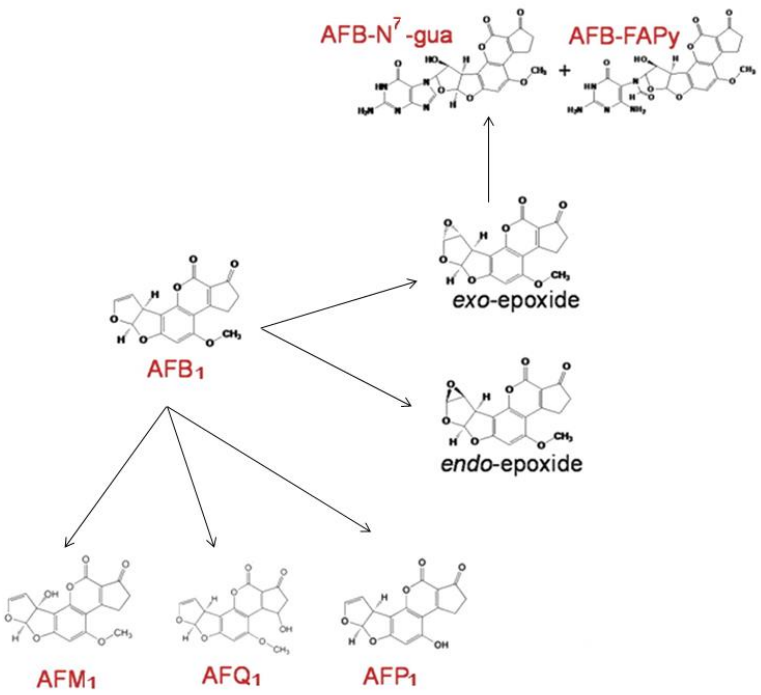

Figure 2: Metabolism of AFB_1- activation to the endo- and exo-epoxides and detoxification to AFM_1, AFQ_1 and AFP_1 metabolites. Adapted from (Kensler *et al.* 2011).

1.1.4 A brief history of the effects of AFB_1 on the human liver

As more research on AFB_1 revealed it to be a potent hepatocarcinogen in animals, it became apparent that exposure to aflatoxins also potentially contributes to certain health effects in humans. An early study on the effects of AFB_1 on human cell cultures reported increased toxicity and inhibited growth in HeLa and Chang liver cells (Gabliks *et al.* 1965). In an embryonic lung cell line, L-132, AFB_1 was found to reduce cell numbers by suppressing mitosis. It was also observed to reduce protein and DNA content and alter morphology of the cells with increasing dose (Legator 1966).

Human liver microsomes were known to activate AFB_1 to its toxic epoxide (Shimada and Guengerich 1989; Shimada *et al.* 1989) but it was also observed that AFB_1 bound DNA in human bronchus and colon cell lines forming a DNA adduct similar in structure to those seen in animals studies previously (Autrup *et al.* 1979). It was first proposed by Oettlé in 1965 that aflatoxin exposure through diet may be a contributing factor in the relatively high liver cancer incidence in African countries compared to Western countries (Oettle 1964). An epidemiological study by Shank et al, found that aflatoxin levels in ingested food in the Ratburi region in the north was about 9-10 times higher than that consumed in the Songkhla region in the south (Shank *et al.* 1972b). They also discovered that liver cancer incidence in the Ratburi area was about 3 times greater than that in the Songkhla area (Shank *et al.* 1972a) providing preliminary clues as to the causal effect of aflatoxins on human liver carcinogenesis. In two studies by Peers et al, aflatoxin levels in food measured by thin-layer chromatography (TLC) were significantly associated with liver cancer incidence in the Murang'a district, Kenya and in Swaziland (Peers and Linsell 1973; Peers *et al.* 1976). In a study based in The Philippines, aflatoxin levels in different food items were first determined using TLC. The diet of 90 liver cancer patients and 90 controls was then assessed using a questionnaire and the aflatoxin load calculated using the results from the aflatoxin analyses. As association was found between aflatoxin load and liver cancer (Bulatao-Jaym *et al.* 1982).

1.1.4.1 Evidence of a potential mechanism of AFB_1-associated cancer

A key characteristic of many carcinogens is their ability to form covalent bonds with DNA in somatic and/or reproductive cells, with the resulting interaction product called a DNA adduct (Harris 1989). The formation of a DNA adduct is usually the precursor lesion to a mutation, which can occur through replication errors during DNA synthesis (Marnett and Burcham 1993). Following exposure, a carcinogen is usually metabolically activated to form a reactive metabolite, which is then either inactivated through detoxification mechanisms or forms an adduct with DNA. Repair mechanisms aimed at the resulting carcinogen-DNA adduct are attempted. If these fail, the adduct can lead to mutations and eventually carcinogenesis (Strickland *et al.* 1993). In the past, ^{32}P post labelling and immunoassays laid the groundwork for the detection and determination of DNA adducts in humans (Strickland *et al.* 1993). A number of interesting factors were noted about DNA adducts. It has been discovered that adduct formation can occur in target as well as in non-target tissues. Also, specific sites of carcinogen reaction can have different efficacies in tumour formation. For instance, potent carcinogens that were large were found to mostly form adducts at the N^7 position of guanine while *O*-alkylation was more effective for smaller alkylating agents. Interestingly, the stability of the adducts was related to the probability of tumour formation and in some cases, more DNA adducts were formed in tissues of animals sensitive to carcinogenic potential than in resistant species (Hemminki 1993).

Mutation spectra have enabled researchers to determine common sites of mutations in tumours in specific cancers. With this information, it has been possible to compare the site of DNA adduct formation and potentially the site of mutations to the

mutation spectra of tumour suppressor genes or oncogenes in specific cancers in order to determine if the mutational signature of a particular carcinogen corresponds to the mutations most commonly observed in tumours. This provides evidence towards the mechanism of carcinogenesis induced by covalent DNA adduct formation (Pfeifer *et al.* 2002). Indeed, specific G->T transversions in *p53* in lung cancer patients who are smokers have been associated with the site of polycyclic aromatic hydrocarbon (PAH) adduct formation in the *p53* gene (Smith *et al.* 2000). Additionally, G->T transversions have been found to be significantly higher in lung cancer patients who are smokers than in patients who are non-smokers (Hainaut and Pfeifer 2001).

The carcinogenic mechanism of aflatoxin is typical of this DNA adduct/mutation paradigm. In an *in vitro* study using *Escherichia coli* (*E. coli*), the aflatoxin 8,9-exo-epoxide was found to induce a G:C -> T:A transversion in the *lacI* gene (Foster *et al.* 1983). When phage M13AB28 DNA modified with AFB_1-2,3-dichloride was transfected into *E. coli*, this transversion was detected in the *lacZ* M13 phage gene in the *E. coli* host together with a G-to-A transition (Sambamurti *et al.* 1988). In rats, the G:C rich regions flanking N-*ras* and c-Ki-*ras* oncogenes were found to be sites of aflatoxin-induced damage (Mcmahon *et al.* 1990) suggesting that alterations in this region may be specific to AFB_1 mutagenesis. The tumour suppressor gene, *p53*, has been observed to be frequently mutated in several human cancers (Hollstein *et al.* 1991). In HCC patients from Qidong, China, G -> T and G -> C transversions were seen in the third-base of codon 249 in the *p53* gene (Hsu *et al.* 1991). This specific mutation was confirmed in HCC patients from south African and some Asian countries but was not detected in patients from North America, Europe and the Middle East. Also, the codon 249 mutation was detected in about 50% of patients

from Mozambique, where aflatoxin intake was observed to be higher compared to only 8% of patients from Transkei, a district in South Africa where aflatoxin intake was lower (Bressac *et al.* 1991).

1.1.4.2 Hepatitis B virus, aflatoxins and liver cancer

As research into the health impact of aflatoxins on animals and potentially in humans was being conducted, primary liver cancer was already being thoroughly investigated particularly due to the fact that it is one of the most common cancers afflicting African people (Berman 1959). Hepatitis B virus (HBV) infection, which is highly prevalent in Africa and Asia, was suggested as the stimulus leading to development of HCC in Africa (Higginson 1963). Indeed, the PLC/PRF/5 hepatoma cell line was shown to produce the hepatitis B surface antigen (HBsAg) without any artificial infection providing an *in vitro* clue to the association between HBV and HCC (Macnab *et al.* 1976) and these cells were also found to contain at least six viral integration sites in the host DNA (Edman *et al.* 1980). The association between HBV infection and HCC was further demonstrated by the presence of HBV DNA integrated into HCC tumour tissue and the PLC/PRF/5 hepatoma cell line (Brechot *et al.* 1980). When introduced into transgenic mice, the HBV viral regulatory gene, *HBx*, alone was observed to induce morphological changes, which developed into liver cancer (Kim *et al.* 1991). However, it has been suggested that infection with HBV is the first insult, which precedes HCC development possibly by several years. This could be followed by a carcinogenic event in the form of aflatoxin exposure which ultimately leads to HCC development (Enwonwu 1984). In a cohort of 7917 men based in Southern Guangxi, China, it was found that 91% of men who died of

primary hepatocellular carcinoma (PHC) were positive for blood HBsAg. Additionally, estimates of aflatoxin intake in this region had a near linear relationship with PHC mortality rates (Yeh *et al.* 1989).

While signifying important associations between aflatoxin exposure and development of HCC, these studies are reliant on potentially inaccurate data on aflatoxin load in intake and therefore lack a well-validated and comprehensive marker to determine aflatoxin exposure. A consistent biomarker of exposure would provide assurance into observations of associations between AFB_1 and liver cancer with and without the presence of HBV infection but would also represent a biologically effective dose (Groopman *et al.* 1994).

Several of the described studies using either food consumption data, measurement of toxin levels in food samples or validated biological markers of exposure have consistently observed high exposures to aflatoxins in developing regions and its associations with adverse health effects, particularly liver cancer. This work led to the upgrade of naturally occurring mixtures of aflatoxins from 'possibly carcinogenic to humans' (Group 2a) to 'carcinogenic to humans' (Group 1) by the International Agency for Research on Cancer (IARC) in 1993 (International Agency for Research On 1993), an assertion which was maintained in a following monograph in 2002 (Iarc 2002).

1.1.5 Advantages of biomarkers

In epidemiological studies assessing the impact and influence of exposures, precise measurement of these exposures is necessary. Identification and rigorous validation of biomarkers of exposures or effects is therefore paramount to make accurate

inferences providing confidence to any findings. There are two main types of biomarkers: biomarkers of exposure, which are useful in risk prediction, and biomarkers of disease, which are used to diagnose and monitor disease progression (Mayeux 2004). These markers can allow for the identification of disease risk at an early point following exposure and can reflect changes as a result of exposures (Gil and Pla 2001).

In some cases, the metabolites of a compound can be measured in body fluids and can be biomarkers for the internal dose. Some biomarkers of the 'biologically effective dose' can be measured in the target organ or a surrogate source. For example, exposure to lead could be determined by air pollution statistics. However, measurement of lead or its metabolites in tissues including blood can provide a more accurate framework of exposure (Mayeux 2004). In addition to potential inaccuracies of questionnaire measurements and workplace exposure monitoring, individual variations in absorption and metabolism of external exposures can further confound any results from epidemiological studies relying on these measures to determine exposure (Timbrell 1998).

1.1.6 Molecular markers of AFB_1 exposure

It was first identified that the AFB_1-N^7-Gua DNA adduct found in liver tissues of rats could also be detected in urine in a dose-dependent manner using high-pressure liquid chromatography (HPLC) (Bennett *et al.* 1981). Using affinity chromatography, these adducts were also detected in urine from people living in Guangxi, China where dietary aflatoxin exposure was estimated by measuring peanut oil and corn consumed by the people in the study (Groopman *et al.* 1985). In a small study

consisting of 20 subjects from The Gambia, AFB_1 was measured using antibody-affinity HPLC in food items collected over a period of one week and AFB_1 metabolites- AFB_1-N^7-Gua DNA adduct, AFQ_1 and AFP_1 in urine were measured using a competitive enzyme-linked immunosorbent assay (ELISA). Dietary AFB_1 load was strongly correlated to AFB_1-N^7-Gua DNA adduct levels in urine but no association between HBsAg surface antigen status and urine adduct levels was observed (Groopman *et al.* 1992a). Dietary AFB_1 intake was also strongly correlated with urine AFB_1-N^7-Gua adduct levels in 42 subjects from Guangxi, China, which leant further credence to the use of the AFB_1-DNA adduct as a biomarker of dietary AFB_1 exposure (Groopman *et al.* 1992b).

Ross et al. performed a comprehensive case-control study nestled within a prospective cohort study of 18,244 middle-aged men from Shanghai, China. Importantly, this study used biomarkers of aflatoxin exposure to determine the association between exposure and liver cancer. A total of 22 liver cancer patients and 140 controls matched for age, time of sample collection and neighbouring residence were recruited and urine samples were used to measure AFB_1, AFM_1, AFP_1 and AFB_1-N^7-Gua adduct levels. Higher levels of aflatoxin metabolites were observed in liver cancer patients than in controls. Furthermore, there was a strong interaction between aflatoxin exposure and HBsAg status associated with liver cancer incidence than with either factor alone (Ross *et al.* 1992). However, as the sample size was relatively small, an absolute inference cannot be made. This study was taken further to include a total of 50 HCC cases and 267 matched controls (Qian *et al.* 1994). Measured urinary AFB_1 and its metabolites were once again found to be significantly higher in HCC cases than in controls. Also, the individual HCC risk for patients with HBsAg antigen positivity and for urinary aflatoxin presence was 7.3- and 3.4-fold

respectively. However, when subjects were positive for both factors, there was a 59.4-fold increase in HCC risk (Qian *et al.* 1994). However, only a few other studies have found similar impressive risks of HCC development with either HBV or AFB_1 exposure alone or with both (Chen *et al.* 1996b; Wang *et al.* 1998).

AFB_1-N^7-Gua and other metabolites measured in urine only reflect short-term dietary exposure (Groopman *et al.* 1993). Therefore, the discovery markers that reflect longer-term exposure was undertaken. AFB_1 has also been observed to bind plasma albumin paralleling DNA binding and after 7-14 days following exposure, has been seen to accumulate to a level 3-fold higher than the dose administered in rats (Wild *et al.* 1986). This relatively new adduct was subsequently isolated in AFB_1-treated rats and it was found that the AFB_1 predominantly bound lysine in albumin. It was also observed to have a half-life of 2.5 days, which is similar to the half-life of albumin in rats. This would translate to a half-life of approximately 20 days in man and could therefore reflect exposure over a period of weeks or months (Sabbioni *et al.* 1987). Additionally, this adduct is produced through the binding of the 8,9-exo-AFB_1 epoxide to albumin and is proportional to the degree of DNA damage in the liver (Wild *et al.* 1986; Sabbioni *et al.* 1987). One of the first human studies to confirm the validity of aflatoxin-albumin (AF-alb) adduct as a biomarker was done by Gan et al in 1988 (Gan *et al.* 1988). Blood from 42 residents from Guangxi, China was analysed for AF-alb levels by a competitive radioimmunoassay and aflatoxin intake and urinary AFM_1 levels were also measured. AF-alb levels were found to correlate significantly with both food intake as well as urinary AFM_1 levels (Gan *et al.* 1988). These blood samples were also analysed using fluorescence measurements to quantify AF-alb adduct levels and on comparison with food intake

data, it was determined that 4% of ingested aflatoxin was bound to serum albumin (Gan *et al.* 1989).

1.1.6.1 Methods of AF-alb measurement

The discovery and characterisation of AF-alb adducts warranted the development of techniques for the accurate, quick and simple measurement of adduct levels in blood. Previously used techniques involving radioimmunoassays (RIA) and fluorescence measurements quantified total aflatoxin-albumin adduct levels in the serum. However, the RIA technique assumed similar affinity of the antibody to both AF-alb adducts as well as AFB_1, which was used as a standard (Gan *et al.* 1988). However, it has since been shown that the specific antibody used has a 10 times greater affinity to AF-alb than to AFB_1, resulting in an overestimation of the amount of adduct in the serum (Sabbioni *et al.* 1990). Also, the fluorescence method used intact protein without the separation of lysine residues bound to aflatoxin (Gan *et al.* 1989). Subsequently, an HPLC method was developed to overcome these issues and to accurately quantify AF-lysine residues (Sabbioni *et al.* 1990). In this method, sera from 42 Guangxi residents were first digested with pronase followed by filtration using a monoclonal antibody immunoaffinity column and were finally analysed by HPLC. Comparison of the results from this technique with those of the RIA by Gan et al. confirmed that RIA detected 3.6 times more AF-alb than the HPLC method, probably due to the inappropriate standards used in the former (Sabbioni *et al.* 1990). The HPLC technique with an added purification step involving the use of C18 cartridges, an ELISA on unmodified albumin and a competitive ELISA on digested and hydrolysed albumin were compared by Wild et al. using serum samples from

The Gambia, Kenya, Thailand and France (Wild *et al.* 1990b). Of the three methods tested, the competitive hydrolysis ELISA and hydrolysis HPLC with fluorescence measurement had much lower detection limits compared to the direct ELISA, although the recovery, determined by measurement of [^{14}C]-AFB_1 bound to albumin in rats, was less than 30% in both assays. However, the hydrolysis ELISA was decided to be the better technique due to the overall low cost, ease of performance and the large numbers of samples that could be quantified at once (Wild *et al.* 1990b). This ELISA was developed and tested further by Chapot and Wild and included an albumin extraction and hydrolysis stage followed by purification with C18 cartridges. The purified albumin was incubated with specific antibodies and this was then analysed on plates coated with an AFB_1-ovalbumin antigen (Chapot and Wild 1991). This competitive ELISA has been used in a number of studies to understand the effects of AFB_1 exposure on human health (Wild *et al.* 2000; Gong *et al.* 2002; Gong *et al.* 2004; Turner *et al.* 2007).

The measurement of AF-alb adducts in 102 Kenyan serum samples collected during the 2004 aflatoxicosis outbreak (Azziz-Baumgartner *et al.* 2005) by hydrolysis ELISA, HPLC with fluorescence detection (HPLC-f) and HPLC with isotope dilution mass spectrometry (IDMS) revealed that all three methods performed at different laboratories correlated well across all samples tested and were predictive of outcome when samples were classed as cases or controls. Additionally, it was noted that the IDMS method was the most sensitive with HPLC-f being the least sensitive (Mccoy *et al.* 2008).

The development of these non-laborious, accurate and quick methods has allowed for easier quantification of AF-alb adducts in sera. Importantly, these techniques have enabled a number of studies to link exposure levels with health outcomes,

thereby contributing greatly to our understanding of the consequences of dietary exposure.

1.1.7 Aflatoxin exposure levels and associated health effects

1.1.7.1 AFB_1 exposure and liver cancer risk

Liver cancer is the fifth most prevalent cancer in the world and is the fourth most leading cause of cancer-related mortality resulting in 8.2% of total cancer-related mortality. Eighty per cent of liver cancer cases occur in developing countries particularly in West and Central Africa (Parkin *et al.* 2008).

The identification and validation of AFB_1-N^7-Gua DNA and AF-alb adducts have enabled discovery of stronger associations between exposure and health effects including liver cancer and have improved our understanding of the mechanistic action of aflatoxins on animal and human health. Using an immunoassay based approach together with HPLC fluorescence for positive samples, AF-alb adducts were detected in nearly all of Gambian child and adult sera with levels up to 350 picogram AFB_1-lysine equivalent per milligram albumin (350 pg/mg). On the other hand, no adducts were detected in sera from Poland and France. AF-alb adducts were also detected in sera samples from Senegal, Kenya, Uganda and Thailand although at lower levels than in The Gambia (Wild *et al.* 1990a). These results indicated that AFB_1 exposure is higher in regions where liver cancer incidence is reported to be higher but is also suggestive of the varied dietary staples in these different geographical locations (Wild *et al.* 1990a).

1.1.7.1.1 Cohort studies on AFB_1 exposure

HBsAg surface markers and urinary AFB_1-N^7-Gua adduct levels were compared with liver cancer incidence in 983 subjects from districts in the Central, Eastern and Western provinces in Kenya and found to vary over region (Autrup *et al.* 1987). Interestingly, in this study, multiplicative and additive analyses did not reveal any synergistic association between aflatoxin exposure and HBV infection status. Also, while aflatoxin exposure was not significantly associated with liver cancer incidence, there was an association between them only when considering the Bantu ethnic group. This suggested potential differences in diet or an existing genetic predisposition in these people (Autrup *et al.* 1987).

Wang et al. conducted a cohort study based in seven townships of Taiwan with a total of 12,024 males and 13,594 females being recruited. Serum samples were assayed for HBsAg antigen and HCV infection and participants were monitored for HCC development. Fifty-six HCC cases were identified and 220 controls were matched for age, sex and township. Aflatoxin metabolites including AF-alb, B_1, B_2, G_1, G_2 and P_1 were measured in blood and urine samples. It was found that the odds ratio for liver cancer corresponding to AF-alb adducts was 4.6 (95% CI: 2.0-10) before adjustment for HBsAg and 1.6 (95% CI: 0.4-5.5) after adjustment. Additionally, for the high aflatoxin metabolite levels, the odds ratio was 3.3 (95% CI: 1.4-7.7) before adjustment for HBsAg and 3.8 (95% CI: 1.1-13) after adjustment. Interestingly, among HBsAg-negative subjects, there was little or no effect of aflatoxin biomarkers on HCC. However, in HBsAg-positive subjects, there was quite a strong effect.

In another cohort study on 357 adults based in The Gambia, plasma AF-alb levels analysed by a competitive ELISA, were found to vary by region and season (Wild *et*

al. 2000). People residing in rural regions had 1.5-fold higher AF-alb levels than those living in urban regions. Also, AF-alb levels were approximately twice as high in the dry season compared to the rainy season potentially due to consumption of stored grains with accumulated aflatoxin contamination during the dry season. However, this study also failed to find any association between HBV infection status and aflatoxin exposure. Additionally, different genotypes of GSTs were not found to influence AF-alb exposure (Wild *et al.* 2000).

In contrast, when Turner et al. analysed samples from 444 Gambian children, a strong association between plasma AF-alb levels and HBV infection status was observed with levels averaging among uninfected children at 31.6 pg/mg, chronic carriers at 44.9 pg/mg and acutely infected children at 96.9 pg/mg. A seasonal variation in plasma AF-alb levels did exist with exposure being twice as high during the dry season compared to the rainy season. Also, there were no differences in aflatoxin exposure within the ethnic groups involved in the study (Turner *et al.* 2000).

1.1.7.1.2 Case-control studies associating AFB_1 exposure with liver cancer risk

In a small case-control study based in different regions in Thailand, 65 HCC cases and 65 controls matched for age, sex and residence were recruited. Aflatoxin metabolite levels in urine and AF-alb adduct levels in blood were measured and were not found to vary by region and were not different between cases and controls (Srivatanakul *et al.* 1991).

Mandishona et al. (Mandishona *et al.* 1998) carried out a small case-control study in South Africa with the aim to determine the role of dietary iron in the aetiology of

HCC. However, they also measured AF-alb adducts in 24 cases with two control series. One set of 48 controls were matched for age, sex and ethnicity while the second set of 75 controls consisted of relatives and family members. In addition, HBsAg antigen levels and HCV infection status were determined. It was found that the median of AF-alb levels was lower in cases than in both sets of controls while HBsAg levels were higher in cases than in controls (Mandishona *et al.* 1998). The discrepancy in these results may have been due to the comparison of medians in groups, which may not reflect the numbers of samples with high exposure values per group.

Besides plasma biomarkers, urinary AFM_1 levels, analysed in 43 HCC cases compared to 86 matched controls in a study based in Taiwan, were found to have a dose-dependent association to the risk of HCC development in chronic HBV carriers (Yu *et al.* 1997) providing more evidence for an association between AFB_1 and HBV infection in the development of HCC. Additionally, urinary AFB_1-N^7-Gua adduct levels and HBV infection status were strongly associated with higher risk of HCC development. With an odds ratio (OR) of 10.0 (95% confidence intervals (95% CI) = 1.6, 60.9) people with both detectable AFB_1-N^7-Gua adducts and plasma AF-alb levels were at a very high risk of HCC development (Yu *et al.* 1997).

However, these case-control studies have relatively small sample sizes and may have other confounding factors including HCV infections status, ethnicity and village. Therefore, the association between AFB_1 exposure, HBV infection and liver cancer risk would need to be reanalysed in larger studies.

1.1.7.1.3 p53 mutation as a mechanism of AFB_1-associated liver carcinogenesis

An insight into the potential mechanisms of these interactions was provided by Chen et al, in a study analysing AF-alb adduct levels in newly diagnosed HCC patients and matched controls all of whom were HBsAg carriers. A significant association between AFB_1 exposure and HCC in HBsAg carriers was only observed if the patients with null GST M1 and T1 genotypes, while this was not observed in patients with non-null genotypes. The study concluded that HBsAg surface antigen positive people with high AFB_1 exposure and no GST M1 and T1 detoxification enzymes were at 10 times increased risk of developing HCC (Chen *et al.* 1996a).

The *p53* gene is mutated in more than half of all human cancers and over 70% of mutations are missense resulting in mutant proteins. Due to the large number of missense mutations in *p53* than in other tumour suppressor genes, it has been suggested that the *p53* mutations confer a selective advantage in carcinogenesis (Hussain and Harris 1998). Indeed, *p53* mutations are thought to encourage the clonal expansion of pre-neoplastic and neoplastic cells particularly in livers chronically infected with HBV (Harris 1996). It has been observed that when *p53*-deficient Hep3B cells were transfected with mutant p53-249ser, the survival and mitosis rates increased significantly compared to non-transfected cells. However, the tumourigenic potential of the mutant cell line was not unequivocally determined due to conflicting results (Ponchel *et al.* 1994).

A specific G -> T transversion in codon 249 of the *p53* gene has been suggested to be the mechanism of aflatoxin-associated HCC. However, the vast majority of the studies showing a link between the *p53* mutation and aflatoxin exposure have not directly measured exposure instead relying on aflatoxin levels in food, food intake frequency and geographic residence information (Lasky and Magder 1997).

Therefore, the majority of the evidence does not strongly support the specific *p53* mutation as the mechanism of AFB_1-induced carcinogenesis.

A transgenic mouse model with a p53ser246 mutation, equivalent to the human p53ser249, was generated and used to determine the mechanism of AFB_1-induced HCC. In AFB_1 treated mice, the lowest incidence of tumours were observed in wild type mice negative for HBsAg. A higher percentage of tumours was found in heterozygous mice negative for HBsAg and the highest percentage of tumours were detected in HBsAg-positive mice homozygous for the *p53* mutation. These results were suggestive of an important role for the mutant *p53* protein in the co-carcinogenesis of AFB_1 and HBV infection (Ghebranious and Sell 1998).

Another animal model to further explore the mechanism of AFB_1-associated HCC made use of the Hupki mouse, which is a human *p53* gene knock-in mouse. These mice were treated with AFB_1 and followed for 80 weeks after which it was observed that HCC developed in 44% of the Hupki mice compared to only 19% of control mice. Interestingly, examination of the liver tumours and normal surrounding tissue did not find any mutations in the *p53* gene (Tong *et al.* 2006). However, it is possible that the mutation was not detected due to the lack of expression of HBV antigens, which may play an important role in AFB_1-induced carcinogenesis (Wild and Montesano 2009).

A potential role for the *p53* mutation in the interaction between AFB_1 and HBV infection in HCC development has been disputed by Stern et al., who besides determining *p53* mutation incidence in HCC patients from southern Guangxi, China, also conducted a meta-analysis of 48 studies. While their study found that 36% of patients carried the *p53* 249^{ser} mutation, the meta-analysis observed a strong

correlation between the *p53* 249^{ser} mutation and AFB_1 exposure but no association with HBV status (Stern *et al.* 2001).

Interestingly, significant adduct formation was observed in HepG2 cells exposed to AFB_1 that were metabolically activated by rat liver microsomes. While the single base mutation in codon 249 was detected, mutations in other codons including 244, 245 and 248 were also observed, in some cases at a higher frequency (Denissenko *et al.* 1998). This suggests that a mutation in codon 249 in *p53* may not be specific with potentially other mutations and mechanisms at play in hepatocarcinogenesis.

In a retrospective study based in Qidong, China, only 2 of 234 plasma samples from HBsAg carriers were positive for the *p53* mutation in codon 249. However, neither patient went on to develop HCC. Also, while 67% of the subjects were positive for AF-alb biomarkers, the levels were low and equally distributed among cases and controls. The *p53* mutation was detected in 61% of HCC tumours suggesting that tumours containing the mutation can occur in the absence of recent exposure to high levels of AFB_1 (Szymañska *et al.* 2009). The fact that the *p53* mutation was detected in tumours despite the overall low AFB_1 exposure in this population suggests either that previous higher exposure may have initiated the carcinogenesis process leading to the mutation and tumour development, or that the complete picture of the mechanistic action of AFB_1 in the development of HCC is still lacking.

1.1.7.2 Epigenetics: Role in cancer prediction, diagnosis and prognosis

In 1939, Conrad Waddington introduced the concept of epigenetics to explain the relation between genetics and development biology (Holliday 2006). He described

the ‘epigenotype’ as being a complex of developmental processes, linked in a network, which exists between the genotype and the phenotype. He also suggested that early disruptions to these processes could slowly lead to greater changes in different tissues (Waddington 2012). Eventually, epigenetic alterations were defined as heritable changes in gene expression that do not alter the actual DNA sequence. These changes result in patterns, which can be inherited mitotically and/or meiotically and include DNA methylation and histone methylation and acetylation (Holliday 2006).

One of the most studied epigenetic changes is the methylation of cytosine in CpG islands, which usually overlap with the promoter regions of several genes (Esteller 2008). Hypermethylation of a gene promoter region typically results in transcriptional inactivation while the opposite is true of hypomethylation. In normal cells, repetitive sequences are hypermethylated to avoid chromosomal instability and reactivation of transposable genomic sequences (Esteller 2008). DNA methyltransferases (DNMTs) are responsible for de novo methylation in previously unmethylated regions but also for maintaining pre-existing methylation during DNA replication (Klose and Bird 2006).

In addition to DNA methylation, changes to histone profiles can also affect gene expression. Acetylation, methylation and phosphorylation at specific positions in histone residues either activate or inactivate gene transcription depending on the residue where the change occurs (Esteller 2008). For instance, methylation of H3 lysine 4 is associated with transcriptional activation while methylation of H3 lysine 9 is associated with repression (Bernstein *et al.* 2007). As lysine methylation can be monomeric, dimeric or trimeric and as histones can also undergo other posttranslational modifications, there are numerous combinations of histone

modifications potentially constituting a 'histone code', which can be read into biological functions (Jenuwein and Allis 2001). Additionally, histone modifications are now known to interact with DNA methylation (Egger *et al.* 2004) adding another layer of complexity to the epigenetic regulation of gene expression.

Genomic imprinting is another epigenetic mechanism to distinguish between maternal and paternal alleles of a gene (Feinberg *et al.* 2002). It is the preferential expression of a specific parental allele in somatic cells through hypermethylation of one parental allele. It is also important in the reduction of gene dosage in X chromosome inactivation in female mammals (Feinberg *et al.* 2002).

Environmental carcinogens not only alter gene expression through mutations but also through reversible epigenetic events (Jones and Baylin 2007). As a consequence, a number of studies are now focussing on identifying markers of gene expression and epigenetic changes due to carcinogen exposure leading to eventual cancer development (Andrew *et al.* 2008; Liu *et al.* 2009; Lu *et al.* 2006; Chen *et al.* 2003; Couvert *et al.* 2008). The specific gene expression and epigenetic profiles generated as a result can be used to predict cancer development and prognosis thus enabling certain preventive and protective measures to be put into place. These profiles are especially significant since epigenetic changes are potentially reversible.

Some characteristic epigenetic changes have been discovered in aflatoxin-related HCC, which are potential markers of cancer development. In a series of studies, Zhang et al. attempted to identify methylation changes in specific tumour suppressor genes in tumour tissues from HCC patients. Hypermethylation of the promoter regions of *RASSF1A*, *p16* and O^6-methylguanine-DNA methyltransferase (*MGMT*) were detected in 85%, 47% and 39% of tumour tissues, respectively. The

methylation status of all three genes was also significantly linked to AFB_1-DNA adducts in the tumour tissues (Zhang *et al.* 2002; Zhang *et al.* 2003). They also correlated AF-alb adducts in plasma with AFB_1-DNA adduct levels in tumours before finding an association between *p16* hypermethylation and AF-alb adduct levels in the plasma of HCC patients, which upon further validation could possibly be used as a marker for development of HCC (Zhang *et al.* 2006). Hypermethylation in the promoter region of *p16* is a commonly observed epigenetic change in cancer patients detectable both in tumour tissues as well as in the plasma (Nakahara *et al.* 2006; Wong *et al.* 1999). However, one study failed to find a direct biological influence with relation to cell cycle regulation in multiple myeloma. This was because it was determined that methylation in the promoter region mostly occurred in non-crucial regions and thus did not affect gene expression. For this purpose, *p16* methylation has been suggested as a marker of overall epigenetic changes during myeloma disease progression (Gonzalez-Paz *et al.* 2007).

Another epigenetic change in HCC patients as a result of AFB_1 exposure is the hypermethylation of the promoter region of the glutathione *S*-transferase P1 (*GSTP1*) gene that codes for the detoxifying GSTs. This hypermethylation has also been significantly associated with AFB_1-DNA adducts in HCC tumour tissue (Zhang *et al.* 2005). Furthermore, reduced expression of the *GSTP1* gene was observed in aflatoxin-exposed tree shrews that developed HCC (Li *et al.* 2008).

Zhao et al. demonstrated that AFB_1 induced demethylation of synuclein-gamma (*SNCG*) in the HCC cell line, HepG2, which was treated with 1 µM (~0.3 ng/ml) AFB_1 for 1 or 3 days. It was also shown that *SNCG* was demethylated in the early stages of HCC and the SNCG protein was highly expressed in the later stages of the cancer (Zhao *et al.* 2006). Altered gene expression of *SNCG* through demethylation

has also been observed in the advanced stages of gastric cancer and has been suggested to be a proto-oncogene (Yanagawa *et al.* 2004).

Human populations chronically exposed to aflatoxins may exhibit similar alterations to gene expression and methylation patterns affecting genes linked to cancer development. When discovered and validated, these changes could serve as predictive, diagnostic and prognostic markers of aflatoxin exposure-induced liver carcinogenesis. A model designed by Farazi and DePinho shows the different factors thought to contribute to hepatocarcinogenesis and has been modified in Figure 3.

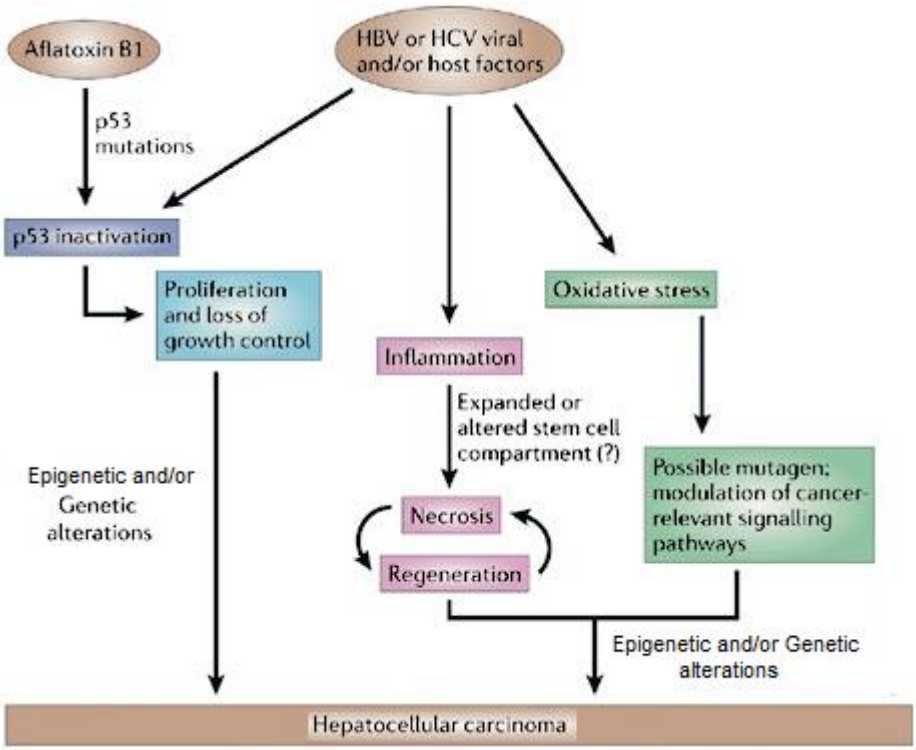

Figure 3: Model depicting the mechanisms of HCC development through exposures to AFB_1 and HBV/HCV. Exposures can induce epigenetic and genetic changes that can eventually lead to HCC development. Modified from (Farazi and Depinho 2006).

1.1.7.3 Identifying mechanistic markers of gene expression and epigenetic alterations

Gene expression and epigenetic markers can be utilised for three main applications: prediction, diagnosis and prognosis of cancer (Simon 2003). These markers are important for elucidating the effects of childhood carcinogenic exposure due to the prospect of reversing or modulating the effects of the carcinogen.

The advent of microarrays has enabled us to identify differences in gene expression in tumours versus normal tissue and within tumour subtypes. In fact, it is possible to use gene expression profiles generated using microarray technology to determine the mechanism of action of various toxins. Indeed, by developing such profiles, Waring et al were able to group together different hepatotoxins based on their mode of toxicity (Waring *et al.* 2001). This approach was also used by Thomas et al. enabling them to classify 24 different treatments in mice based on the mRNA expression profiles examined using custom cDNA microarrays (Thomas *et al.* 2001).

A number of concerns have been put forward about the reliability of microarrays in discovering robust and stable disease markers owing to dissimilarities in gene expression profiles generated for the same tumours in different studies. However, it has been shown that even though there are few overlapping altered genes in similar microarray experiments, the different gene expression profiles generated reveal the same information about disease diagnosis and prognosis (Sotiriou and Piccart 2007). This was observed by Fan et al. who attempted to compare different gene expression models for breast cancer prediction and prognosis on a single data set of 295 patients. Four of the five sets of gene expression profiles identified in different studies were significantly similar in the prediction of the outcome in this data set

proving that concordance exists among gene expression profiles generated by different studies (Fan *et al.* 2006).

A number of methods including bisulfite sequencing, pyrosequencing, chromatin immunoprecipitation (ChIP) and genome-wide methylation arrays have been developed for the identification of epigenetic changes (Ho and Tang 2007). Due to the relatively limited information obtained from methylation studies focussing on single genes of interest (GOI), the spotlight is slowly shifting to genome-wide studies. A few methods have been developed to determine the methylation status of the entire genome through the use of enzymes and antibodies that detect methylated portions of the genome (Esteller 2007). One such method is Restriction Landmark Genome Scanning (RLGS), through which DNA is first cut by restriction enzymes that are methylation-sensitive and is then labelled with a radioactive isotope. These DNA fragments are then run on a 2-dimensional gel and differences in spots and patterns are compared between different treatment groups on an autoradiogram (Kawai *et al.* 1993). Using RLGS with NotI, which selectively recognises unmethylated DNA, Yaoi et al were able to identify 48 spots showing altered methylation following bisphenol A exposure in murine forebrains (Yaoi *et al.* 2008).

Illumina's Infinium technology has made it possible to investigate DNA methylation levels at a single nucleotide resolution. The Illumina Infinium 27K bead chip array, which studied methylation in 27, 578 CpG sites, has been used to determine methylation patterns in several cancers (Teschendorff *et al.* 2010; Fackler *et al.* 2011; Rodriguez *et al.* 2012). This array was improved upon to produce the Infinium 450K bead chip array, which interrogates more than 485, 000 CpG loci. These sites cover about 99% of the genome with an average 17 CpG sites per gene. Besides CpG islands, the array also covers island shores and surrounding regions (Dedeurwaerder

et al. 2011a; Zhang *et al.* 2012). These assays enable a comprehensive examination of DNA methylation patterns as a result of environmental exposures.

For instance, using the Illumina Infinium 27K methylation bead chip array, Wang et al. were able to identify an association between hypomethylation of thymic stromal lymphopoietin (*TSLP*) with high cord blood cotinine levels, representing prenatal smoke exposure, in 14 Taiwanese children. Methylation status of *TSLP* was further associated with prenatal smoke exposure as well as atopic disorder (AD) in 150 Taiwanese children who were followed up for 2 years. Altered methylation was successfully validated using methylation-dependent fragment separation (MDFS) (Wang *et al.* 2013).

Differences in environmental exposures due to location can also affect DNA methylation patterns as has been observed in a study based in the Czech Republic. Benzo[a]pyrene (B[a]P) concentrations measured by the Czech Hydrometeorological Institute were compared with whole genome DNA methylation using the Infinium 27K array in Ostrava, which is highly polluted and in Prachatice, which was treated as a control. Hypomethylation of more than 10% was observed in 58 individual CpG sites in genes involved in the immune response, signalling and metabolism in people living in Ostrava than in Prachatice (Rossnerova *et al.* 2013).

Investigating the mechanism of *in utero* exposure to smoking, Joubert et al. used the Infinium 450K array to examine changes to the methylome following birth. Maternal plasma cotinine levels were significantly associated with differential methylation in 26 CpG sites in 10 genes. One of the genes identified, *AHRR*, has also been observed to be differentially methylated in adult smokers versus non-smokers. Another gene, *GFI1*, which is involved in developmental processes, had not been previously

implicated in response to smoking and therefore was a novel finding of the study (Joubert *et al.* 2012).

The Illumina Infinium 450K array together with the Illumina Human HT12 v.4 were used to examine the influence of arsenic exposure in adult Argentinian Andean women and in new born Bangladeshi infants. Eight single nucleotide polymorphisms (SNPs) in arsenic methyltransferase (*AS3MT*), seen specifically in people living in the Argentinian Andean region, increase arsenic metabolism. While this haplotype does exist in the Bangladeshi population, it is present at a much lower frequency. The study determined that the *AS3MT* haplotype status strongly predicted DNA methylation and gene expression of *AS3MT* as well as a number of neighbouring genes, thus potentially novel genes involved in arsenic metabolism (Engström *et al.* 2013).

Advances in technology have produced myriad techniques available for the identification and validation of unique markers of environmental exposures on health effects. These techniques can be utilised effectively to contribute to our understanding of the mechanisms involved in AFB_1-assocaited health effects. Careful investigation into the results of whole genome examinations as a result of early life exposures can potentially be used to prevent certain adverse health outcomes in later life.

1.1.8 Child growth and development

It has been estimated that in 2008, the number of children under 5 years of age that died was 8.8 million. Of these, nearly half of the deaths occurred in India, Pakistan, China, Nigeria and the Democratic Republic of Congo (Black *et al.* 2010). The

number of child deaths in Africa was more than 20-fold the number of deaths recorded in Europe and USA. Diarrhoea and infectious diseases were the major contributors to child mortality in Africa and Asia while non-communicable diseases were the main concern in developed regions (Black *et al.* 2010). Additionally, stunting, severe wasting, under nutrition, vitamin A and zinc deficiencies have been recognised as the underlying causes of a third of the deaths of children under 5 years (Black *et al.* 2008).

While death is usually the final outcome of consistent insults to child development, it has also been suggested that more than 200 million children under 5 years from low income regions do not attain their full developmental potential as a result of undernutrition and nutritional deficiencies, poverty, infectious diseases and environmental exposures usually occurring concurrently (Walker *et al.* 2007). Nearly 37% children in developing countries are estimated to live in poverty, which has been identified as the main contributor to poor nutrition and sanitation and can make a child prone to stunting and infections (Grantham-Mcgregor *et al.* 2007). Consequently, nearly 30-40% of children under 5 years are stunted in sub-Saharan African, South-East and South-Central Asian countries (De Onis and Blössner 2003).

Several intervention trials, using either single or multi nutritional supplementation, have reported a lower incidence of stunting in children (Chhagan *et al.* 2009; Remans *et al.* 2011; Umeta *et al.* 2000). However, these trials only recover a fraction of the growth potential in children suggesting that other factors besides nutritional deficiencies play an important role in child stunting.

1.1.9 AFB_1 exposure and effects on growth

One of the first observations of the effect of AFB_1 on growth in animals was reported by Butler and Barnes in 1963 (Butler and Barnes 1963). In this study, rats were fed groundnut meal that had been shown to be either toxic or non-toxic to ducklings. One group of rats was given 50% toxic meal continuously while another was given 50% non-toxic meal and a third group was given 50% toxic meal for 16 weeks followed by normal diet for a total of 36 weeks. Rats in the first group had slower growth rates compared to the rats in the second group. Also, growth rates improved in the third group of rats after around 4 weeks following a change in diet although the growth never reached the same level as the rats in the non-toxic meal group (Butler and Barnes 1963).

In another early study using known concentrations of AFB_1, 10-day old ducklings and weanling rats were fed doses of 60 and 600 µg/Kg/day respectively for 5 consecutive days. Following the treatment, which represented 10-12% of the LD_{50} dose, it was observed that weight gain and liver size were drastically reduced in treated animals compared to controls (Shank and Wogan 1966).

Specific doses of AFB_1 were found to either have no effect or cause reduced weight gain as was observed in weanling pigs and cattle (Keyl *et al.* 1970). When meals containing up to 233 ppb AFB_1 for pigs and 300 ppb AFB_1 for cattle for 120-130 days were fed to animals, no change in weight gain was seen compared to controls. However, when given meal containing more than 465 ppb AFB_1 for pigs and 700 ppb AFB_1 for cattle, weight gain was significantly reduced in treated animals (Keyl *et al.* 1970). Significant effects on growth were observed in broiler breeder hens exposed to dietary AFB_1 at 5 µg/g and 10 µg/g for four weeks. Egg hatchability and

weights were significantly lower in hens treated with the higher dose of AFB_1 and chicks born of these hens were considerably lighter (Howarth and Wyatt 1976).

Interestingly, it was observed that when pregnant rats were administered AFB_1 at a dose that was ¼ of the LD_{50}, the effects on the rats and their foetuses depended on time of treatment. When given on day 6 of pregnancy, the effects on the mothers were similar to non-pregnant rats and there were no significant effects on the foetuses in terms of growth. However, when AFB_1 was given on Day 16 of pregnancy, more severe effects on the livers were observed in the mothers and the foetuses were significantly stunted compared to controls. It was also suggested that the observed stunting was secondary to the effects of AFB_1 on the maternal livers as at certain AFB_1 doses, stunting was not seen in foetuses when liver lesions were not present in mothers (Butler and Wigglesworth 1966).

Growth retardation in foetuses was observed in hamsters given either 4 mg/kg or 6 mg/kg on days 8 or 9 of gestation. The foetuses were examined on days 11 or 15 following gestation and the observed stunting was most striking on day 15 in hamsters given either dose of AFB_1 on day 9 of gestation compared to those observed on day 11, suggesting that a single dose of AFB_1 at a time point in pregnancy can affect foetal growth and can continue to have an effect as pregnancy progresses (Schmidt and Panciera 1980). These initial studies suggest that AFB_1 plays a key role in growth and development of foetuses throughout pregnancy but is potentially more significant towards the later stages.

Early work studying humans have also observed similar effects on growth associated with AFB_1 exposure. Examination of 125 Kenyan pregnant women found that 53% of the women and 37% of cord bloods were positive for aflatoxins as ascertained by

analysis using fluorescence HPLC, which can detect AFB_1, B_2, M_1, M_2, G_1, G_2 and aflatoxicol. Among females, infants born to aflatoxin-positive mothers were significantly lighter than infants born to aflatoxin-negative mothers. However, male infants born to aflatoxin-positive mothers showed an increase in weight compared to those born to aflatoxin-negative mothers, although this was not found to be significant. This discrepancy could be attributed to the much smaller sample size for male infants from aflatoxin-positive mothers compared to all other groups (Vries *et al.* 1989).

Gong et al (Gong *et al.* 2002; Gong *et al.* 2003) confirmed the potentially adverse effect of dietary AFB_1 exposure on growth in children from Benin and Togo. The study included 480 children aged 9 months to 5 years belonging to different geographical zones. AF-alb levels were measured using the competitive ELISA described by Chapot et al (Chapot and Wild 1991) and were detected in 99% of the samples. Children with stunting and who were underweight had 30-40% higher AF-alb adducts than those in the control group. Additionally, maize consumption as ascertained by detailed dietary questionnaires was associated with AFB_1 exposure in these children (Gong *et al.* 2003; Gong *et al.* 2002). The higher AF-alb levels were also associated with increased *A. flavus* infestation in maize, higher levels of aflatoxins in maize and greater consumption of maize (Egal *et al.* 2005). However, micronutrient deficiency and HBV infection status were not determined and could potentially be confounders to the observed association between AFB_1 exposure and growth impairment.

These findings were analysed further with a longitudinal study over 8 months in Benin involving 200 children aged 16-37 months recruited from villages of either high or low aflatoxin exposure (Gong *et al.* 2004). AF-alb levels measured using

ELISA were detected in nearly all samples studied at three time points and were higher in fully weaned children compared to partially breast-fed children. The main weaning food was a maize-based porridge, which was the potential source of the exposure. Also, children belonging to the highest quartile of AFB_1 exposure had a mean 1.7 cm reduction in height compared to children in the lowest AFB_1 quartile. Moreover, no association was observed between AF-alb levels and vitamin A and zinc levels in plasma suggesting that these nutrients do not have a confounding effect on the association between AFB_1 exposure and growth (Gong *et al.* 2004). While some micronutrient levels were measured, total energy intake and levels of other important micronutrients were not measured and could be confounding factors in the study. Additionally, HBV or HCV infection status were not determined.

Higher maternal AFB_1 exposure determined through measurement of AF-alb levels was significantly associated with infant birth and height gain providing evidence for the *in utero* effects of AFB_1 on foetal development (Turner *et al.* 2007). In this study, maternal blood was collected at two time points during pregnancy and 138 infants were followed for 1 year following birth. AF-alb levels were detected in all maternal samples, approximately half of cord blood samples and in a few infant samples. This is consistent with the *in utero* exposure through the maternal diet and also with the lower exposure in infants during breast-feeding. Interestingly, in this cohort, it has been predicted that for a 6 month old female infant with medium height-for-age Z score (HAZ), if the maternal exposure was reduced from 110 to 10 pg/mg, the corresponding height would increase by 0.8 cm (Turner *et al.* 2007). However, socio-economic status, which can have a strong impact on diet and micronutrient levels were not assessed for this population and therefore could be confounders.

Although aflatoxin biomarkers were not analysed by Okoth and Ohingo, a significant association between wasting in Kenyan children aged 3-36 months and aflatoxin-contaminated flour was observed. However, wasting was only observed in 6% of the 242 children studied making it a very small sample size of about 14 children (Okoth and Ohingo 2004).

1.1.9.1 Mechanisms of growth impairment

Growth faltering in children in developing regions is a common phenomenon with malnutrition being frequently implicated as the major cause (Goulet 2010). However, strategies to supplement children with essential nutrients have failed to allow normal expected growth in some children (Chhagan *et al.* 2009). This has led to other theories being put forward including a role for infections in child stunting, which can result in micronutrient deficiency or nutrient malabsorption (Stephensen 1999). Diarrhoea has also been implicated in growth faltering with the odds of stunting increasing by 1.13 (95% CI = 1.07, 1.19) for every diarrhoeal episode (Checkley *et al.* 2008). Enteropathy leading to an inefficient intestinal nutrient transport could also consequently cause growth impairment (Campbell *et al.* 2003).

Other mycotoxins have been found to have an effect on nutrient transport and intestinal function. Ochratoxin A, which is a mycotoxin also produced by *Aspergillus* species, was observed to disrupt glucose transport in a human colon carcinoma caco-2 cell line. The results of the study further suggested that ochratoxin A may enhance its own absorption through a paracellular route by destabilising the tight junctions of the monolayer (Maresca *et al.* 2001). In another study, the effects on nutrient transport of deoxynivalenol (DON) were studied using another human

intestinal cell line, HT-29-D4 and it was found that DON inhibited the uptake of glucose, fructose and L-serine in these cells (Maresca *et al.* 2002). Treatment with AFB_1 significantly compromised integrity of the intestinal barrier, which is an important defence against pathogens and toxins (Gratz *et al.* 2007). This effect was observed to be time-dependent. Co-incubation with *Lactobacillus rhamnosus* GG strain, present in probiotic yoghurt, appeared to alleviate some of this disruption (Gratz *et al.* 2007). Therefore, lack of access to certain food items together with chronic AFB_1 exposure may explain a part of the mechanism of AFB_1-associated growth impairment.

However, although a strong association between dietary AFB_1 exposure and child growth impairment has been observed in several studies based in Africa, the complete mechanisms remain unclear.

Infants with intrauterine growth restriction, described as having a birth weight below the 5-10^{th} percentile following gestation, are known to have a substantially increased risk of morbidity and mortality compared with infants having higher birth weights (Aucott *et al.* 2004). Incidence of low birth weight is high in developing countries and has been attributed to intrauterine growth restriction rather than preterm births (Kramer 2003).

Infections and environmental exposures during gestation have been identified as being the potential causative agents of intrauterine growth restriction. For instance, exposure to organophosphorous insecticides either *in utero* or after birth was significantly associated with intrauterine growth restriction with an adjusted odds ratio of 2.3 (95% CI = 1.0, 5.3) (Levario-Carrillo *et al.* 2004). Also, in a prospective cohort study examining 1,578 mother-child pairs, gestational arsenic exposure up to

100 µg/L measured in urine was significantly associated with lower birth weight and head and chest circumferences such that a 1 µg/L increase in arsenic levels in urine was associated with 1.68 g reduction in birth weight (Rahman *et al.* 2009). Intrauterine growth restriction has also been attributed to maternal smoking during pregnancy in Brazilian mother-child pairs with an odds ratio of 2.07 (95% CI = 1.69, 2.53) (Horta *et al.* 1997)

A number of studies have attempted the identification of mechanistic associations between exposures and a growth restricted outcome. It has been observed that the expression of *CYP1A1* and *CYP2E1* genes was significantly higher in maternal liver microsomes and placenta samples of rats exposed to nicotine during pregnancy. Also, the nicotine exposure was strongly linked to lower birth weights of the foetuses. The study suggests that increased expression of the CYP genes leads to increased oxidative stress and lipid peroxidation (Wang *et al.* 2009), which in turn have been associated with intrauterine growth restriction (Elsayed 2001). In women homozygous for the A1 allele in the *CYP17* gene, increased foetal restriction was observed compared to women who had an A2 allele. Additionally, increased foetal retardation was seen in women with the *GSTM1* (glutathione-*S*-transferase M1) null genotype compared to women who carried the *GSTM1* gene (Yamada *et al.* 2004). While the presence of the A2 allele in *CYP17* had been associated with increased oestrogen, which in turn has been linked to placental growth (Bukovsky *et al.* 2003), *GSTM1* is involved in the detoxification of xenobiotic molecules including aflatoxin (Mcglynn *et al.* 1995).

A similar mechanism may be at play in aflatoxin-associated growth impairment although polymorphisms in important carcinogen-activating and -deactivating genes may be the result of natural individual genetic variability. However, these

differences would alter susceptibility to growth impairing agents such as aflatoxin and thus set the stage for the actual mechanism of aflatoxin-induced growth retardation.

One hypothesis for the mechanism of aflatoxin-associated growth restriction suggests that AFB_1 alters the insulin-like growth factor (IGF) pathway thus affecting growth and development. IGF1 is a mediator of the growth hormone (GH) and is also involved in embryonic development although the latter function is independent of GH activity while IGF2 is the main hormone involved in prenatal growth (Baker *et al.* 1993). The effects of the IGFs are mediated by activation via the IGF1 receptor (IGF1R), which is a transmembrane tyrosine kinase and plays a major role in foetal growth (Adams *et al.* 2000). The bioactivity and availability of the IGFs are regulated by a family of six binding proteins (IGFBPs), which prolong the half-life of IGFs in the system but can also work independently of IGFs as growth factors (Mohan and Baylink 2002).

It has been observed that serum IGF1 levels are markedly lower in infants with intrauterine growth restriction compared to controls. Although IGF1 levels increase significantly during pregnancy, the actual values at both the second as well as the third trimesters were reduced in growth restricted children compared to controls. However, no difference was seen in IGF2, IGFBP3 and GH levels between the two groups (Leger *et al.* 1996). In another study, elevated levels of serum IGFBP2 and IGFBP4 were observed in guinea pig foetuses with growth restriction while IGFBP3 levels were much lower compared to controls and there were no changes in IGF1 and IGF2 mRNA expression in growth restricted guinea pigs (Carter *et al.* 2005).

Exposures during pregnancy can alter foetal development including growth, which can manifest through an altered IGF growth axis (Figure 4). In fact, elevated serum IGFBP1 and IGFBP2 levels were observed in sheep exposed to testosterone during pregnancy and were linked to growth restriction in the foetuses (Manikkam *et al.* 2004), which may be part of the mechanism through which testosterone can affect foetal growth. In another study, while all selective serotonin reuptake inhibitors (SSRIs) administered during pregnancy were associated with lower birth weight and length, only infants born to mothers exposed to citalopram had significantly lower cord blood IGF1 levels compared to the other SSRI groups and controls (Davidson *et al.* 2009).

DON is another mycotoxin that has been identified as a causative agent in reduced weight gain in several animal species (Pestka 2007). It has been suggested that DON induces this growth suppression through a disrupted IGF axis. Reduced mRNA expression of the IGF-acid labile subunit (IGFALS), which forms a part of the IGF1-IGFBP3-IGFALS binding complex, was observed in mice chronically exposed to DON over a period of 8 weeks. The mice also showed reduced weight gain and had lower circulating plasma IGF 1 levels compared to controls (Amuzie and Pestka 2010). These effects were confirmed in another study where mice fed on high fat diets had lower weight gain and significantly reduced plasma IGF1 and IGFALS levels compared to controls (Kobayashi‐Hattori *et al.* 2011).

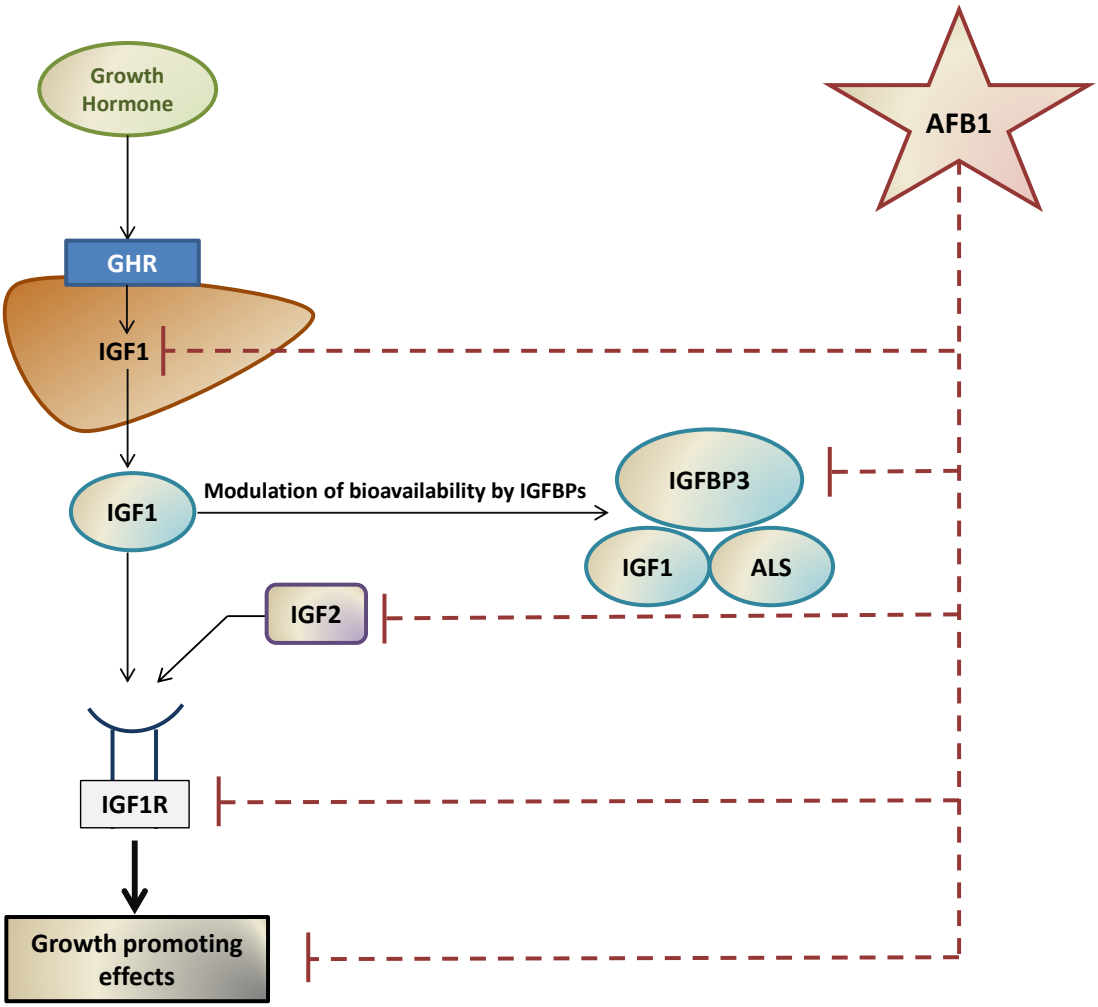

Figure 4: Model depicting the hypothesis that AFB_1 exposure alters growth through disruption of the IGF growth axis. Growth hormone, produced by the pituitary gland, signals the production of IGF1 in the liver as well as other tissues, through the growth hormone receptor (GHR). IGF1 bioavailability is modulated through binding factors including IGFBP3, forming a complex with an acid labile subunit (ALS). Both IGF1 and IGF2 exert their growth promoting potential through binding with the IGF1-receptor (IGF1R). Dietary AFB_1 may inhibit the growth promoting process at several stages (represented by the red, dashed lines).

Microarray experiments comparing placentas of intra-uterine growth restricted pregnancies and normal placentas have revealed differential expression in growth suppression. Increased expression of *leptin*, vascular endothelial growth factor receptor (*VEGF*-R), human chorionic gonadotropin (*HCG*), *follistatin-like 3* and hypoxia-inducible factor 2α (*HIF-2α*) was observed in growth restricted placentas suggesting that hypoxia may have a role to play in placental growth (Mccarthy *et al.*

2007). Elevated expression of *follistatin-like 3* as well as *IGFBP1* have been reported in another study on growth restricted placentas (Okamoto *et al.* 2006).

1.1.9.2 Role of epigenetic modifications in growth

Certain epigenetic mechanisms have also been associated with growth suppression. In further support of role that the disrupted IGF axis plays in growth impairment, epigenetic modifications were identified in growth restricted rats (Fu *et al.* 2009). There was differential CpG methylation in the promoters P1 and P2 of *IGF1* and hepatic histone H3 methylation and acetylation was altered in the growth restricted rats compared to controls. mRNA expression and serum levels of IGF1 were also significantly reduced in these rats and could be due to the observed epigenetic changes (Fu *et al.* 2009).

Intra-uterine growth restriction has been associated with a number of changes in DNA methylation at loci in rat pancreatic islet tissue affecting genes involved in cell proliferation, insulin secretion and cell death (Thompson *et al.* 2010). A whole genome approach using Illumina's Infinium HumanMethylation 27K bead chip array was used to analyse DNA methylation patterns in cord blood obtained from infants of women supplemented with folate during pregnancy. The analysis revealed significant associations between DNA methylation predominantly in CpG islands and birth weight centiles. Methylation levels of two such genes were described in this study: *BMX* (bone marrow non-receptor tyrosine kinase) methylation, which was positively correlated and *AMN* (amnion associated transmembrane protein), which was inversely correlated with birth weight centiles (Fryer *et al.* 2011).

Analysis of umbilical cord blood samples from Chinese infants showed that hypermethylation of the paternally expressed gene 10 (*PEG10*) differentially methylated region (DMR), determined by pyrosequencing, was significantly associated with lower birth weight and this was consistent with lower expression of *PEG10*. Also, increased mRNA expression of pleckstrin homology like domain, family A, member 2 (*PHLDA2*) was correlated with lower birth weight (Lim *et al.* 2012). Indeed, the presence of a 15 basepair (bp) repeat sequence (RS1) within the *PHLDA2* promoter reduces its efficiency and when the maternally inherited RS1 sequence was homozygous, the corresponding infant birth weight was significantly higher (Ishida *et al.* 2012).

Methylation levels of *APOE, MSX1, GRB10, PGRMC1, RGS14* and *SHMT2* analysed by using Illumina GoldenGate Methylation array on umbilical cord blood and placental samples, accounted for 78% of the birth weight variance. Also, methylation in *MSX1, CDK2* and *GRB10* were positively correlated with transcript levels of genes suspected to be involved in foetal and placental growth including *APOE* and *PSG4*. The authors suggest that despite the relative stability in methylation, inter-individual differences in methylation patterns at birth may predispose infants to development of disease in later life (Turan *et al.* 2012).

1.1.10 The foetal origins of adult disease

'Programming' has been described as a general process by which insults including environmental exposures during early development can alter the development of the foetus in order to prepare them for a similar environment in adulthood. These insults only have long-term effects on outcome when they occur at a critical period during

foetal development (Lucas 1991). Early studies by Barker et al. suggested that undernutrition during early life strongly influenced ischaemic heart disease risk in adulthood (Barker and Osmond 1986; Barker *et al.* 1989). This led to the 'foetal origins of adult disease' hypothesis, which states that ischaemic heart disease, stroke, hypertension and diabetes are the result of adaptations made by the foetus during early development to poor nutrition (Barker 2001). Several epidemiological studies have discovered links between low birth weight (in some cases together with environmental exposures) and adult disease providing some evidence for the foetal origins hypothesis (Barker *et al.* 1991; Rich-Edwards *et al.* 1997; Curhan *et al.* 1996; Frankel *et al.* 1996; Leon *et al.* 1998), and is therefore referred to as the 'developmental origins of health and disease' (DOHaD) (Hochberg *et al.* 2011). However, the validity of these findings has been questioned due to the inappropriate statistical methodologies used. In some cases, the inverse association between birth weight and adult disease has been suggested to be chiefly due to random error (Tu *et al.* 2005). Additionally, a systematic review of epidemiological studies based in low-to-middle income countries revealed only a weak association between size at birth and outcomes related to chronic adult disease (Victora *et al.* 2008). Also, it was determined that there was insufficient evidence to link poor nutrition with long-term immune function changes but an association with cancer incidence was better supported. However, overall, it was found that undernutrition was strongly linked to long-term impairment including stunted growth and lower offspring birth weight (Victora *et al.* 2008).

It has been argued that exposure resulting in low birth weight and impaired growth could also be contributing factors to adult disease development, especially if the exposure is constant through life (Byrne and Phillips 2000). Chronic exposure to

aflatoxins from *in utero* through childhood and into adulthood could likely be the cause of growth impairment as well as susceptibility to adult disease including cancer in high exposure regions.

The 'plastic' nature of human development allows the genotype to give rise to different morphological and physiological outcomes depending on the different developmental environmental conditions (Barker 2004). Developmental plasticity relies in part on epigenetic mechanisms including DNA methylation and histone modifications (Gluckman *et al.* 2008), which are heritable and can potentially influence disease risk in future generations (Jirtle and Skinner 2007). Alterations in the promoter regions of key genes are one of the main ways in which the epigenome plays a role in increased susceptibility to disease.

The Dutch Hunger Winter cohort produced important evidence of an association between potential adult disease risk through epigenetic changes and adverse environmental conditions during early development (Heijmans *et al.* 2008; Tobi *et al.* 2009). Methylation of the imprinted *IGF2* differentially methylated region was examined in 60 individuals prenatally exposed to a famine during 1944-1945 and in their same-sex unexposed siblings six decades following exposure. It was discovered that methylation levels were significantly low in exposed individuals than in their unexposed siblings (Heijmans *et al.* 2008). This was expanded to include the examination of methylation levels in 15 loci involved in growth and metabolic disease including *IL10* and *GNASAS (Tobi et al. 2009)*. Methylation of *INSIG* was found to be significantly lower in exposed individuals than in their unexposed siblings, while methylation levels of *IL10*, *LEP*, *GNASAS*, *ABCA1* and *MEG3* were reported to be higher in exposed versus unexposed individuals (Tobi *et al.* 2009).

Changes to genes with metastable epialleles are also important targets of environmental exposure-modulated adult disease risk (Jirtle and Skinner 2007). Metastable epialleles are epigenetically modified alleles that have variable expression in genetically identical individuals. The epigenetic modifications are established during early foetal development and are mostly influenced by environmental factors (Rakyan *et al.* 2002). For example, the degree of methylation of the intracisternal A particle (IAP) gene, which is a transposable element 100 Kb upstream of the agouti mouse gene, affects the expression of the agouti gene and therefore alters the colour of the mouse fur coat as well as diabetes, obesity and tumourigenesis risk, resulting in either obese yellow (unmethylated) or normal brown (methylated) mice. If the methylation of the IAP occurs later during development affecting only some embryonic cells, the fur coat is mottled (Wolff *et al.* 1998).

A number of enzymes are responsible for the maintenance and development of methylation patterns during development and cell proliferation. DNA methyltransferase-1 (DNMT1) is responsible for maintaining the methylation patterns of DNA by methylating nascent DNA immediately after replication (Bestor 2000). While existing DNA methylation patterns need to be maintained and replicated, *de novo* methylation is necessary during early development to establish new patterns, sometimes in relation to environmental cues (Dolinoy *et al.* 2007). *DNMT1* is known to be involved in certain types of *de novo* methylation, including most methylation occurring in embryo lysates (Bestor 2000). *DNMT3a* and *DNMT3b* also play an important role in *de novo* methylation during early embryonic development (Okano *et al.* 1999). Early embryos undergo genome-wide demethylation separately for paternal and maternal genomes after which both are

remethylated by *de novo* methylation at the same time (Reik *et al.* 2001). At these points in development, the embryo is vulnerable to environmental influences resulting in changes to the epigenome and the genome thereby potentially predisposing the child to adverse health effects in later life. Investigation of the genome-wide effects of early life AFB_1 exposure on gene expression and DNA methylation can contribute toward our understanding of the mechanisms involved in exposure-related health effects.

1.2 Project Aims

The main aims of this project are:

1. To test the hypothesis that AFB_1 exposure affects IGF1 and/or IGFBP3 protein levels in children and *in vitro* and to contribute to our understanding of AFB_1-associated growth impairment. This has been investigated using a cohort of Kenyan schoolchildren as well as non-tumourigenic liver cells (HHL-16) exposed *in vitro* to AFB_1
2. To determine exposure levels and seasonality of the AF-alb biomarker in pregnant women and to ascertain if *in utero* AFB_1 exposure affects growth in infants. A Gambian mother-child cohort was used to examine these questions
3. To develop gene expression and epigenetic profiles of AFB_1 exposure in children by dichotomising infants into AFB_1 exposure groups and comparing infant DNA methylation status and gene expression patterns between groups. These profiles could provide valuable markers of AFB_1 exposure in relation to growth and immune function impairment and eventual cancer development.
4. To develop gene expression and epigenetic profiles of *in vitro* AFB_1 exposure by studying whole genome changes in HHL-16 cells exposed to three doses of AFB_1 at two time points, to support the findings of the human study.

2 Materials and Methods

2.1 AF-alb measurement ELISA

Serum samples were analysed for AF-alb levels by a competitive ELISA described previously (Chapot and Wild 1991). Albumin was extracted using a two step approach taking advantage of the properties of albumin. In the first step, 375 µl cold ammonium sulphate (Sigma, Dorset, UK, dissolved in distilled water) was added to 250 µl sample serum in a drop-wise manner. This was done to increase the surface area of contact of the ammonium sulphate with the serum. At low salt concentrations, the solubility of a protein increases with increasing ionic strength, an effect termed salting in. As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out). Therefore, by the addition of ammonium sulphate, unwanted globulins are precipitated out while albumin is still in solution. The mixture of ammonium sulphate and plasma was then vortexed and centrifuged at 9000 g at 0°C for 15 minutes. The supernatant was transferred to a fresh microcentrifuge tube and 50 µl of 1 M glacial acetic acid (Sigma, Dorset, UK) was added. The addition of acid lowers the pH of the mixture allowing the remaining albumin to precipitate out of the solution. The mixture was vortexed and centrifuged at 9000 g at 0°C for 15 minutes. The supernatant was discarded and the pellet was reconstituted in 500 µl phosphate buffered saline (PBS) (8g NaCl, 0.2g KCl, 0.2g KH_2PO_4 and 1.15g Na_2HPO_4 dissolved in distilled water, adjusted to pH 7.4, all chemicals from Sigma, Dorset, UK). The albumin was then quantified using Biorad reagent (Bio-Rad laboratories, Hemel Hempstead, Hertfordshire, UK) in a coloured protein assay. The objective of this assay was to determine the concentration of solubilised albumin protein in solution by using

Coomassie blue dye, which binds to basic and aromatic amino acid residues of protein. The albumin was first diluted 500-fold with distilled water and assayed against human serum albumin (HSA; Sigma, Dorset, UK) standards. 40 µl of Biorad reagent was added to each well containing 160 µl of sample or standard and the plate was read at 620 nm wavelength. Based on the quantification results, 2 mg albumin was then digested with 1 mg/ml pronase (Roche, West Sussex, UK) and PBS to make up the final volume to 800 µl. The samples were incubated at 37°C overnight. The albumin was then hydrolysed with 1.8 ml 100% ethanol (Sigma, Dorset, UK) and 100 mg/ml bovine serum albumin (BSA; Sigma, Dorset, UK) for at least 2 hours. Hydrolysed albumin was centrifuged at 1000 *g* for 15 minutes at 4°C and the supernatant transferred to separate falcon tubes. The albumin was diluted with PBS to make the final volume to 30 ml. This was then purified using Sep Pak C18 cartridges (Waters, Hertfordshire, UK). The cartridges were first activated using 5 ml of 80% methanol (Rathburn, Walkerburn, Scotland, UK) in distilled water, followed by equilibrating the cartridge surface with 10 ml distilled water. The samples were then run through the cartridges at a slow rate. The cartridges were washed with 5 ml distilled water followed by 5 ml of 5% methanol in distilled water. Finally, the aflatoxin-lysine residues were eluted with 5 ml of 80% methanol. Eluted samples were dried overnight in a speed vac (SPD1010 speed vac, Thermo Scientific, Langenselbold, Germany) and reconstituted in 500 µl PBS.

The samples were then analysed by a competitive ELISA. Greiner high-binding plates (Greiner, Stoenhouse, Gloucestershire, UK) were coated with 0.4 µg/ml of AFB_1-ovalbumin (ovalbumin, Sigma, Dorset, UK) per well overnight and soaked and washed in PBS/Tween 20 (Tween 20, Sigma, Dorset, UK). The wells were blocked with 5% milk (skimmed milk powder, Sainsbury's own brand, dissolved in

distilled water) for 1 hour. A set of 8 AFB_1-lysine standards, samples and PBS blank were incubated with 1:500,000 primary anti-aflatoxin antibody produced in rabbits with 0.75% fetal calf serum (FCS, Sigma, Dorset, UK), at a 1:1 ratio for 30 minutes. Following blocking, plates were washed in PBS/Tween 20 and drained and 50 µl each of standards and samples were added to the wells. The plates were incubated for 90 minutes at room temperature with shaking following which the plates were washed and a 2500-fold dilution of a goat anti-rabbit IgG peroxidase-labelled antibody (Sigma, Dorset, UK) was added to each well. The plates were incubated for 90 minutes at room temperature with shaking and washed 5 times in PBS/Tween 20 with an additional final wash in distilled water. Substrate mix prepared by combining 20 ml of 14.7 mg/ml citrate buffer (Sigma, Dorset, UK), 200 µl of 10 mg/ml 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma, Dorset, UK) and 4 µl of 30% hydrogen peroxide (Sigma, Dorset, UK) was then added to each sample and the plates were incubated at 37°C for 20 minutes. The plates were then read at 450 nm on a Labsystems iEMS plate reader (Thermo Scientific, Langenselbold, Germany). Each sample was read against the aflatoxin-lysine standard curve and the percentage inhibition was calculated by Ascent software linked to the plate reader. Final AF-alb concentrations were presented as picogram (pg) of aflatoxin per milligram (mg) of albumin. The detection limit for this assay was 3 pg/mg albumin. Each sample was analysed in triplicate on at least two days. One negative and three positive controls with known AF-alb levels were run with each batch of the samples. Intra-assay coefficient of variation (CV) was < 15% and inter-assay CV was ≤ 25%. This CV is much higher than typically acceptable values for sensitive assays. There are a few sources of error throughout the assay, mostly due to the fact that this is a multi-step process. Not all albumin is extracted in the initial stages and this albumin may be

diluted through the presence of other proteins that were not removed during the process. However, to eliminate a major source of error at this stage, only 2 mg albumin is purified and analysed. Some variation is introduced at this point due to the potential presence of other proteins besides albumin, especially as the protein measurement method used is not exclusive to the measurement of albumin. Furthermore, digested albumin may be lost during the Sep Pak purification stage as some may pass through the cartridge during a wash step or alternatively, some albumin may remain on the cartridge during the elution step. Variation during the ELISA may also introduce further sources of error due to differences in actual antibody, coating antigen or substrate levels between samples or wells.

2.1.1 Modified AF-alb measurement ELISA

Serum samples were analysed for aflatoxin-albumin adduct (AF-alb) levels by a modified competitive ELISA described previously (Chapot and Wild 1991) and in Chapter 2, section 1. The only alteration to the methodology was in the initial stages of sample preparation. In this modified method, albumin extraction was by-passed and 150-250 µl plasma was directly digested with 250 µl 10 mg/ml pronase at 37°C overnight. A 20 µl aliquot of each sample was not digested and instead stored for albumin measurement using a bromocresol green (BCG) reagent (San Diego, California, USA). Ten µl of the plasma aliquot was added to each well of a Sarstedt 96-well plate (Sarstedt, Leicester, UK) and 200 µl of BCG reagent was added. The plate was incubated at room temperature for 5 minutes and read at 650 nm. Concentrations of albumin were calculated against human albumin standards.

Following overnight digestion with pronase, the rest of the protocol was followed as described in Section 1. Due to the modifications eliminating any potential loss of albumin, the detection limit for this assay was 0.6 pg/mg albumin. The final calculation was adjusted to account for the missing albumin extraction step. Each sample was analysed in triplicate on at least two days. One negative and three positive controls with known AF-alb levels were run with each batch of the samples. Intra-assay CV was < 15% and inter-assay CV was ≤ 25%.

2.2 IGF1 and IGFBP3 ELISA measurement

2.2.1 Plasma samples

IGF1 and IGFBP3 levels in plasma samples were measured using separate IGF1 and IGFBP3 Quantikine ELISA kits (R&D Systems, Abingdon, UK) following the manufacturer's instructions. Plates, provided in the kits, were pre-coated with a mouse monoclonal antibody against IGF1 or IGFBP3.

For IGF1, 20 µl of each sample was first treated with Pre-treatment Buffers A and B. This was done to dissociate IGF1 from its binding proteins including IGFBP3. Samples on plates were incubated with Assay Diluent while shaking for 2 hours at 4˚C followed by the addition of a polyclonal antibody against IGF1 conjugated to horseradish peroxidase and further incubation for 1 hour at 4˚C. The assay was then developed by a substrate mix containing hydrogen peroxide and TMB and incubated for 30 minutes at room temperature protected from light. One positive quality control (Human Serum, Sigma, Dorset, UK) was included in each assay and the samples were read against an IGF1 standard curve.

For IGFBP3, 10 µl plasma was diluted 100-fold with Calibrator Diluent. Samples on pre-coated plates were incubated with Assay Diluent for 2 hours at 4˚C. A polyclonal antibody against IGFBP3 conjugated to horseradish peroxidase was then added and the plates incubated at the same conditions. The assay was developed with a mixture of hydrogen peroxide and TMB for 30 minutes at room temperature protected from light. Human Serum was run as a positive control with the samples and these were compared against an IGFBP-3 standard curve.

Twenty-four randomly-selected samples for both IGF1 and IGFBP3 assays were re-measured to determine inter-assay variation. The inter-assay CV% for these repeated samples and the quality controls (QCs) was below 10% and intra-assay CV% was below 3% for both assays.

2.2.2 Cell media samples

Following AFB_1 treatment of cells for 24 and 48 hours, conditioned cell media from each treatment were collected and centrifuged at 1000 *g* for 5 minutes to separate cell debris. The supernatant was enriched using Vivaspin 2 ultrafiltration columns with 3000 Molecular Weight Cut Off (MWCO) polyethersulfone (PES) membrane (Sigma, Dorset, UK). The columns were centrifuged at 4000 *g* for 50 minutes at room temperature to obtain approximately 30-times concentrated media, which were analysed immediately.

IGF1 and IGFBP3 levels in concentrated conditioned cell media supernatants were measured using separate IGF1 and IGFBP3 Quantikine ELISA kits (R&D Systems, Abingdon, UK) following the manufacturer's instructions as described in section 2.1. For IGF1, cell media samples were not pre-treated and for IGFBP3, samples were

diluted 2-fold with Calibrator Diluent. For both assays, samples were analysed on two separate days. The intra-assay CV% was below 3% for both assays.

2.3 Non-tumourigenic cell work

2.3.1 Cell maintenance

HHL-16, a non-tumourigenic human liver cell line, was kindly provided by Dr. Arvind H. Patel (MRC Virology Unit, Glasgow). Cells were maintained in Advanced Modified Eagle's Medium (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin and 1% non-essential amino acids (all chemicals from Sigma, Dorset, UK). The cells were maintained at 37˚C in a humidified atmosphere with 5% CO_2 and were sub-cultured with 0.05% Trypsin-EDTA (ethylenediaminetetraacetic acid) (both chemicals from Sigma, Dorset, UK) in sterile PBS every 3-4 days or when cells reached ~80% confluence.

2.3.2 Cell cytotoxicity assay

AFB_1 used to treat HHL-16 cells was purchased from Sigma (Dorset, UK) and dissolved to a 20 mg/ml stock concentration in dimethyl sulfoxide (DMSO, Sigma, Dorset, UK). Cells at passage 9 were seeded at $2x10^4$ cells/well onto 96-well plates and treated with AFB_1 concentrations: 0.2 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml and 100 µg/ml. Cells were incubated with AFB_1 for 24, 48 and 72 hours with 8 repeats per treatment within the same plate.

Non-treated cells and cells treated with 0.1% and 0.5% DMSO were used as controls, as these were the DMSO concentrations present in the 20 µg/ml and 100 µg/ml AFB_1, respectively. Following treatment, 20 µl of CellTiter 96® AQueous One Solution Reagent (Promega, Southampton, UK) was added to each well and the plate was incubated at 37˚C in a humidified incubator with 5% CO_2 for 4 hours before reading it at 490 nm on an iEMS plate reader (Thermo Scientific, Langenselbold, Germany). Percentage viability was calculated as follows:

$$\left(\frac{Absorbance\ of\ sample - blank\ absorbance}{Average\ of\ (absorbance\ of\ control - blank\ absorbance)}\right) * 100$$

Untreated cells were used as the baseline for DMSO controls and the DMSO controls were used as a baseline for all AFB_1 treatments.

2.3.3 Cell treatment with AFB_1

HHL-16 cells grown in 25 cm^2 flasks (Corning, New York, USA) to approximately 80% confluence were treated with 0.5 µg/ml, 5 µg/ml and 20 µg/ml AFB_1 dissolved in DMSO for either 24 or 48 hours in triplicate. Each dose was selected due to low observed cell toxicity. DMSO levels for cells treated with 0.5 µg/ml and 5 µg/ml were at 0.0025% and those for cells treated with 20 µg/ml were at 0.1% DMSO. Cells treated with DMSO levels corresponding to treatment DMSO levels served as controls to exclude potential toxicity from DMSO alone.

2.3.4 DNA extraction

DNA extraction for HHL-16 cells (both controls and treated cells) was carried out using the QIAamp DNA Mini Kit (Qiagen Ltd, West Sussex, UK) according to manufacturer's instructions. Cells from each flask were trypsinised and washed once in PBS before lysis with 600 mAU/ml protease enzyme and Buffer AL. This mixture was then incubated at 56˚C for 10 minutes followed by DNA precipitation with equal volume of 96-100% ethanol. This was added to the QIAamp DNA column and centrifuged for 13000 rpm for 1 minute. The columns were washed in Buffers AW1 and AW2 and incubated for 10 minutes at 37˚C to facilitate complete ethanol evaporation. Columns were then incubated with 200µl DNase/RNase-free water for 5 minutes at room temperature and DNA eluted by centrifuging the column at 13000 rpm for 1 minute. Concentrations and DNA purity were determined by measurement on a NanoDrop ND-1000 Spectrophotometer V3.0.1 (Thermo Scientific, Langenselbold, Germany).

2.3.5 RNA extraction

DNA extraction for HHL-16 cells (both controls and treated cells) was done using the RNeasy Mini Kit (Qiagen, Ltd, West Sussex, UK) according to the manufacturer's instructions. Cells from each flask were trypsinised and washed once in PBS. They were lysed with Buffer RLT followed by homogenisation with a QIAshredder by centrifuging at 20,000g for 2 minutes. 70% ethanol was mixed with the homogenised lysate and this was added to an RNeasy column and centrifuged at 13000 rpm for 1 minute. The column was then washed with Buffers RW1 and RPE and RNA was eluted with 50 µl RNase-free water by centrifuging at 13000 rpm for 1

minute. Concentrations and RNA purity were determined by measurement on a NanoDrop ND-1000 Spectrophotometer V3.0.1 (Thermo Scientific, Langenselbold, Germany).

2.3.6 Quantitative PCR (qPCR)

Primers for all genes analysed by qPCR were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/).

One microgram of total RNA was reverse-transcribed to cDNA using SuperScript II First-Strand Synthesis Mix (Invitrogen, Paisley, UK). A master mix containing 24 µl random nonamers (50 µM, Sigma, Dorset, UK), 48 µl reaction buffer (5-times concentrated), 12 µl deoxyribonucleotide triphosphate (10 µM dNTP, Promega, Southampton, UK) mix, 12 µl dithiothreitol (0.1M DTT) and 12 µl SuperScript II reverse transcriptase (Invitrogen, Paisley, UK). To this, 14 µl total RNA was added, mixed well and incubated at 42°C for 2 hours. Samples were stored at -20°C until qPCR.

The PCR mix was prepared with 5 µl magnesium chloride buffer (Invitrogen, Pailsey, UK), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM) (both primers from Invitrogen, Paisley, UK) and 1 µl distilled water and 2 µl DNA per sample. The cDNA was amplified using SYBR Green (Applied Biosystems, California, USA). The qPCR reactions were run on an ABI 7900HT real-time PCR analyser (Applied Biosystems, California, USA) using absolute quantification and results were determined using the Sequence Detection System (SDS) software. Each reaction was duplicated and a negative control routinely included. Gene expression was normalised against endogenous *GAPDH* or *HPRT* gene expression. Each PCR

reaction was run for 40 cycles and included a denaturation step at 95°C for 15 seconds. After 40 cycles, a dissociation curve was produced for each PCR product at 95°C for 15 seconds and 60°C for 15 seconds.

2.4 DNA methylation and gene expression analyses

2.4.1 DNA methylation analysis

2.4.1.1 DNA extraction

Three millilitres of whole blood was diluted four-fold in 9 ml ice-cold Tris-EDTA (20mM Tris-5mM EDTA, pH 8.0; Sigma, Dorset, UK) solution and mixed well by inverting the tube. This was then incubated on ice for 20 minutes and centrifuged it at 3500 rpm at 4˚C for 20 minutes. The supernatant containing lysed red blood cells was discarded and the cell pellet resuspended in 4 ml Tris-EDTA buffer. The mixture was mixed well by vortexing and the volume adjusted to 40 ml with Tris-EDTA buffer (Sigma, Dorset, UK). This was then mixed by inverting and centrifuged at 3500 rpm at 4˚C for 20 minutes. These washing steps with Tris-EDTA buffer were repeated till the cell pellet appeared pink-white in colour and when the supernatant was no longer red. The washed cell pellet was resuspended in 1.5 ml Tris-EDTA buffer. Seventy-five µl of 20% Sarcosyl (Sigma, Dorset, UK) was added to the cell suspension and mixed well by inverting several times. Twenty µl of proteinase K (10 mg/ml solution, Sigma, Dorset, UK) was added and the tube was gently mixed by inverting and incubated overnight in a shaking water bath at 42˚C-55˚C. Samples were brought to room temperature and mixed with 1 ml 7.5 M

ammonium acetate (Sigma, Dorset, UK). Five µl of ice-cold absolute ethanol (Sigma, Dorset, UK) was then added and this was mixed well by inverting the tube. The resulting precipitated DNA was removed using a plastic inoculation loop and transferred to a tube containing 4 ml Tris-EDTA-NaCl (20 mM EDTA, 5 mM EDTA and 0.2 M NaCl, all chemicals from Sigma, Dorset, UK). The DNA was incubated in a shaking water bath at 42˚C-55˚C until the DNA dissolved. This was then centrifuged at 4000 rpm for 30 minutes at 40˚C. The supernatant was discarded and the pellet was air-dried for 20 minutes. The pellet was resuspended in 500 µl Tris-EDTA (20mM Tris and 1mM EDTA) buffer. The DNA was incubated in a shaking water bath at 37˚C-42˚C overnight. The DNA was then transferred to labelled tubes, quantified by using a Nanodrop ND-1000 Spectrophotometer V3.0.1 (Thermo Scientific, Langenselbold, Germany) and stored at -20˚C.

2.4.1.2 Bisulfite conversion and pyrosequencing

The extracted DNA was bisulfite modified using the EZ DNA Methylation kit (Zymo Research, California, USA) following the manufacturer's instructions for Illumina Infinium Methylation Assay. Five µl of M-Dilution Buffer was added to 500 ng of DNA and the total volume was adjusted to 50 µl with distilled water. The samples was mixed by repeated pipetting and then incubated at 37˚C for 15 minutes. One hundred µl of the CT conversion reagent was then added to each sample and mixed followed by an overnight incubation in a thermocycler with the following conditions: 95˚C for 30 seconds, 50˚C for 60 minutes repeated for 16 cycles and then held at 4˚C. The samples were loaded onto Zymo-Spin IC columns containing 400 µl of M-Binding buffer and mixed well by inverting the column several times. The

columns were then centrifuged at 10000 *g* for 30 seconds and the flow through discarded. One hundred µl of M-wash buffer was then added and the columns were centrifuged at 10000 *g* for 30 seconds. M-Desulphonation buffer (200 µl) was added to the columns, which were incubated at room temperature for 20 minutes and then centrifuged at 10000 *g* for 30 seconds. The M-wash buffer (200 µl) was added twice to the columns with a centrifuge step at 10000 *g* for 30 seconds after each addition. Finally, the columns were placed in fresh 1.5 ml microcentrifuge tubes and the bisulfite treated DNA was eluted with the addition of 10 µl of M-Elution buffer followed by a centrifuge step at 10000 *g* for 30 seconds. DNA was stored at -20˚C until needed for PCR and pyrosequencing.

The methylation status in *LINE1* was assessed by pyrosequencing, which is a highly quantitative method for the analysis of DNA methylation at multiple CpG sites (Tost and Gut 2007). The pyrosequencer program also has built-in internal controls to check for the completion of bisulfite conversion. DNA amplifications were carried out on bisulfite-treated DNA using a biotinylated 'TAGGGAGTGTTAGATAGTGG' as the forward primer and 'AACTCCCTAACCCCTTAC' as the reverse primer with an annealing temperature of 58°C. About 20-25 ng of modified DNA was amplified by PCR in a final volume of 50.25 µl. The mix for PCR included 5 µl Qiagen buffer (10x), 1 µl dNTP mix (10 mM), 1 µl Forward primer (10 mM), 1 µl Reverse primer (10 mM), 0.5 µl Qiagen Taq polymerase, 40.75 µl distilled water and 1 µl DNA. All chemicals were from Qiagen Ltd, West Sussex, UK except for primers, which were purchased from Sigma, Dorset, UK. PCRs were run using the conditions presented in Table 2.

Table 2: PCR conditions for pyrosequencing.

	Temperature (°C)	Time	Number of cycles
Denaturation	95	10 minutes	
Annealing	95	30 seconds	50 cycles
	(varies per gene analysed)	30 seconds	
	72	30 seconds	
Elongation	72	10 minutes	
	18	∞	

Using the PCR product, 10 µl was analysed on a 1 % agarose gel and the remaining 40 µl was used in a pyrosequencing assay using sequencing primers per gene. The sequencing primer for *LINE1* was 5' 'CAAATAAAACAATACCTC'.

Sequencing primers were diluted 250-fold in annealing buffer (20mM Tris and 2mM $MgCl_2$ from Sigma, Dorset, UK in distilled water, adjusted to pH 7.6 with 4M acetic acid) to prepare 40 µl per sample. Streptavidine Sepharose High Performance beads (GE Healthcare, Hatfield, Hertfordshire, UK) were diluted 13-fold in binding buffer (10 mM Tris, 2 M NaCl, 1 mM EDTA and 0.1% Tween 20 in distilled water, adjusted to pH 7.6 with HCl, all chemicals from Sigma, Dorset, UK) to prepare 40 µl per sample. Beads in binding buffer were mixed with 40 µl of the PCR product and agitated for 10 minutes. Sequencing primer dilutions were added to a 96-well PSQ plate (Biotage, Sweden) at 40 µl/well.

The beads mixed with DNA were first transferred onto the 96 filter probes on a hand-held vacuum pump and then denatured by aspirating 70% Ethanol (Sigma, Dorset, UK) followed by 0.2 M NaOH (Sigma, Dorset, UK) for 30 seconds each.

Finally, the DNA was washed by aspirating washing buffer (10 mM Tris in distilled water, adjusted to pH 7.6 with 4 M acetic acid) for 30 seconds. Aspiration was then stopped and the beads released into the plate containing the sequencing primers. The contents of each well were mixed and then the plate was heated at 80°C for 2 minutes. The plate was transferred into the pyrosequencer and allowed to cool down for 20 minutes.

The experiment details were input into the software program PSQ96MA 2.1.1, which proved the calculations for the dNTPs, enzyme and substrate mix provided by the PyroMark Gold Reagent kit (Qiagen Ltd, West Sussex, UK). Based on the calculated amounts, they were added to the cartridge and fixed into the pyrosequencer. Following the 20-minute incubation, the program was run.

The methylation levels at the target CpGs were evaluated by converting the resulting pyrograms to numerical values for peak heights and expressed as the percentage of methylation of individual CpG sites.

2.4.1.3 Whole genome DNA methylation assay

One of the major aims of this project was to identify unique DNA methylation signatures of *in utero* dietary aflatoxin exposure. Infant DNA was therefore analysed using the Illumina Infinium HD 450K DNA Methylation array (Illumina, Freddy van Riemsdijkweg, The Netherlands), which investigates DNA methylation in 485 764 CpG sites covering 99% of RefSeq genes distributed over the promoter, body, 5' UTR and 3' UTR regions (Sandoval *et al.* 2011). These arrays employ two types of Infinium probe designs: Infinium I and Infinium II technology. Infinium I utilises two bead types per DNA template, one for an unmethylated locus and another for a

methylated locus. Therefore, if the input DNA template is unmethylated, it would successfully bind and get extended on the 'unmethylated' probe, while there would be no binding to the 'methylated' probe (Bibikova *et al.* 2011).

The Infinium II probe design incorporates one bead type for each locus. The methylation state in this case is determined by single-base extension. For instance, if the input DNA template is unmethylated and therefore the 'C' nucleotide is converted to 'T' during bisulfite conversion, 'A' nucleotide will be incorporated during extension. However, if the template is methylated, the methylated 'C' will remain unchanged and 'G' will be incorporated instead. Each nucleotide during single-base extension is labelled with a different dye and so methylation state can be determined by the intensity of the signal (Bibikova *et al.* 2011).

The method in brief involves denaturing and neutralising bisulfite-converted DNA samples followed by whole genome amplification. The samples then went through enzymatic fragmentation and were hybridised onto the bead chips. DNA bound to the chips underwent single-base extension and staining and finally imaging to read the signals.

Initially, 20 µl of MA1 was added to each well in the MSA4 plate. Four µl of the bisulfite-converted samples was added to each well followed by 4 µl of 0.1N NaOH to denature the DNA. The plate was then sealed, vortexed at 1600 rpm for 1 minute and pulse centrifuged to 280 g. The plate was incubated at room temperature for 10 minutes and 68 µl of RPM was added to each well to neutralise the sample. Finally, 75 µl of the Multi-Sample Amplification Master Mix (MSM) was also added. The plate was re-sealed, vortexed and pulse centrifuged under the same conditions and incubated in the Illumina Hybridization Oven at 37˚C for 24 hours. The plate was

pulse centrifuged and 50 µl of FMS was added to each well to enzymatically fragment the DNA. The plate was vortexed and centrifuged as before and placed on a heat block set at 37˚C for 1 hour. In order to precipitate the DNA, 100 µl PM1 was added to each well of the MSA4 plate. The plate was vortexed at 1600 rpm for 1 minute and then incubated at 37˚C for 5 minutes. The plate was pulse centrifuged and 300 µl of 100% 2-propanol was added to each well. The plate was sealed and the contents of the wells were mixed by inverting the plate 10 times. The plate was then incubated at 4˚C for 30 minutes and centrifuged to 3000 *g* at 4˚C for 20 minutes. The supernatant was immediately decanted and remaining liquid was drained onto absorbent paper by repeated tapping for at least 1 minute. The plate was left inverted onto a tube rack for 1 hour at room temperature to air dry the pellets. The precipitated DNA pellets were then resuspended in 46 µl of fresh RA1. The plate was heat sealed with foil and then placed in the Illumina Hybridization Oven at 48˚C for 1 hour. The plate was vortexed at 1800 rpm for 1 minute, pulse centrifuged and then placed on the heat block at 95˚C for 20 minutes to denature the samples. The plate was then placed on the benchtop at room temperature for 30 minutes to cool and pulse centrifuged. Fifteen Multi-Sample Amplification Master Mix of each sample was then added onto the BeadChips, which were placed into the Hyb chamber inserts. These inserts were then loaded into the Illumina Hyb chamber and the lid was then securely fastened. The Hyb chamber was then incubated in the Illumina Hybridization Oven at 48˚C for 20 hours. The BeadChip arrays were washed in PB1 buffer by repeated submersion of the arrays using wash racks into a wash dish containing 200 ml PB1 buffer.

For each bead chip, a black frame was placed into a multi-sample bead chip alignment fixture pre-filled with PB1 buffer. The bead chips were transferred onto

the black frames and a clear spacer was added on top of each bead chip. A clean glass plate was added on top of the bead chips and bound together with metal clamps. The Hyb Chamber reservoirs were then washed with distilled water. The bead chips were removed from the alignment fixture and the spacer and back plates were removed. The chips were added into a staining rack and washed in PB1 buffer by repeated submerging and finally soaked in the buffer for 5 minutes. The bead chips were moved into a dish containing the XC4 stain. The slides were stained by repeated submerging and finally soaking for 5 minutes. The bead chip slides were moved onto a tube rack and the whole set up was placed in a vacuum desiccator set at 675 mm Hg for 55 minutes. The assay was conducted at the Epigenetic Group laboratory at IARC, Lyon, France. The chips were finally analysed at the Faculty of de Medicine and Pharmacy of Lyon - ISPBL, ProfileXpert-LCMT, Lyon, France.

A series of controls were also included in each array to account for variation due to sample preparation, hybridisation and signal generation. Additionally, each array had a number of technical replicates, which were randomly distributed across the array. Table 3 lists the different controls included in the assay.

Table 3: Description of controls included in the Illumina Infinium 450K bead array.

Control Category	Role	Number of probes
Staining	Contains dinitrophenyl (DNP) or biotin attached to beads	4
Extension	Test the extension efficiency of A, T, C and G nucleotides	2
Hybridisation	Synthetic targets complementary to array sequence. They are present in the Hyb buffer at high (5 pM), medium 1 pM) and low concentrations (0.2 pM)	3
Target removal	Tests the efficiency of the stripping step following extension	1
Bisulfite conversion I	Utilises two probes, one for converted DNA and another for unconverted DNA, to confirm successful bisulfite conversion	4
Bisulfite conversion II	Uses single base extension. If conversion was successful, incorporates 'A' and if not incorporates 'G'	1
Specificity I	Monitors allele-specific extension for Infinium I probes	2
Specificity II	Monitors allele-specific extension for Infinium II probes	1
Non-polymorphic	Tests the overall performance of the assay by querying a non-polymorphic base with DNP or biotin-labelled nucleotides	2
Negative	Random sequences that do not hybridise to the DNA template	2

2.4.2 Gene expression analysis

2.4.2.1 RNA extraction

Three ml blood was collected from infants between 3-6 months of age and centrifuged at 1000 *g* for 15 minutes at room temperature to isolate the whole blood cells. Plasma was removed from the top of each tube using a Pasteur pipette and 1.3 ml RNALater per 500 µl whole blood cell fraction was added, mixed well and stored at -20˚C for shipment to the Epigenetics Group at the International Agency for Research on Cancer (IARC), Lyon, France.

RNA was extracted from the whole blood fractions preserved in RNALater using the RiboPure Blood RNA extraction kit (Ambion, Paisley, UK) following the manufacturer's instructions. The samples were centrifuged for 1 minute at 13,200 rpm in a microcentrifuge and the supernatant discarded. Eight hundred microliters of lysis solution and 50 µl sodium acetate solution were added to the pellet and vortexed thoroughly. This was followed by inverting the tubes to homogenise the lysate. Five hundred microliters of acid-phenol:chloroform was taken from below the aqueous layer and added to the cell lysate followed by 30 seconds of vortexing. The mixture was then incubated at room temperature for 5 minutes and centrifuged for 1 minute at 13,200 rpm. The upper aqueous phase containing RNA was transferred to a fresh 2 ml tube and 600 µl 100% ethanol (Sigma, Dorset, UK) added and the mixture vortexed well. The tubes were then centrifuged briefly to collect any droplets in the lid of the tube to the bottom. Seven hundred microliters of this mixture was then added to a filter cartridge assembly and centrifuged for about 10 seconds at 13,200 rpm. The flow through was discarded and 700 µl of sample was added to the filter cartridge and centrifuged again. The cartridge was washed with

700 µl wash buffer 1 and centrifuged for 10 seconds at 13,200 rpm and the flow-through discarded. This was followed by a second wash with 700 µl wash buffer 2/3 and centrifuged for 10 seconds at 13,200 rpm. The second wash step was repeated again and the filter cartridge centrifuged for 1 minute at 13,200 rpm to remove any residual fluid. Finally, the RNA was eluted using 50 µl elution buffer, which had been pre-heated to 75˚C. The cartridge was centrifuged for 30 seconds and the elution step was repeated with a further 50µl elution buffer. The eluted RNA was quantified using the Nanodrop 1000 (Thermo Scientific, France) and stored at -20˚C.

2.4.2.2 Whole genome gene expression direct hybridization assay

In order to explore infant gene expression variation associated with maternal aflatoxin exposure during gestation, the Illumina Human HT12-v4 Expression BeadChip Kit (Illumina, Freddy van Riemsdijkweg, The Netherlands) which is based on the Illumina Whole-Genome Gene Expression Direct Hybridization Assay system, was used at IARC, Lyon, France.

The system makes use of the BeadArray technology, in which beads, with several copies of a single oligonucleotide attached to each bead, are included in each sample slot on the BeadChip.

Extracted total RNA was first quantified using the Agilent 2100 Bioanalyser (Agilent Technologies, Berkshire, UK). The RNA 6000 Nano kit was used for this purpose following the manufacturer's instructions. First the RNA ladder was heat denatured for 2 minutes at 70°C, cooled on ice and stored at - 70°C till needed. The provided RNA gel matrix was filtered using a spin filter and centrifuging at 1500 g for 10 minutes at room temperature. Next the gel-dye mix was prepared by adding 1

µl of dye concentrate to 65 µl of filtered gel matrix and mixed well by vortexing followed by centrifuging at 13000 *g* for 10 minutes at room temperature. Nine microliters of the gel-dye mix was then loaded onto the wells on the RNA 6000 Nano chip on the chip priming station. Five microliters of the RNA marker was loaded onto all wells. One µl of the RNA ladder was added to the well marked for the ladder and 1 µl of each sample was added into individual wells. The chip was vortexed for 1 minute at 2400 rpm and analysed on the Bioanalyser within 5 minutes.

The quantified total RNA was then reverse transcribed to single stranded cDNA followed by second strand synthesis resulting in double stranded cDNA. Any residual RNA was removed and then in vitro transcription which amplifies and labels several copies of biotinylated cRNA was carried out. These were achieved by using the Illumina TotalPrep RNA Amplification Kit (Illumina, Freddy van Riemsdijkweg, The Netherlands) following the manufacturer's instructions. Approximately 500 ng total RNA was brought to 11 µl with nuclease-free water. The Reverse transcription matser mix was prepared by mixing 1 µl T7 Oligo(dT) primer, 2 µl 10x First Strand Buffer, 4 µl dNTP mix, 1 µl RNase inhibitor and 1 µl ArrayScript per reaction. Nine µl of this master mix was added to each RNA sample, mixed well and incubated in a thermocycler at 42°C for 2 hours. The samples were then placed on ice while the second strand master mix was prepared on ice. For a single reaction, 63 µl nuclease-free water, 10 µl 10x Second Strand Buffer, 4 µl dNTP mix, 2 µl DNA Polymerase and 1 µl RNase H were mixed together and 80 µl of this was transferred to each cDNA sample. The samples were incubated at 16°C for 2 hours. Following the incubation, 250 µl of cDNA binding buffer was added to each sample, mixed well and added to the cDNA filter cartridges. The cartridges were centrifuged for 1 minute at 10000 *g*, the flow through discarded and 500 µl wash buffer was added to

each cartridge. The cartridges were centrifuged as before and transferred to the cDNA elution tubes. Twenty µl of preheated nuclease-free water was added to the cartridges and these were then incubated at room temperature for 2 minutes. The tubes were centrifuged for 1 minute at 10000 *g* to elute the double stranded cDNA. The IVT master mix was then prepared at room temperature by mixing 2.5 µl T7 10x Reaction Buffer, 2.5 µl T7 Enzyme Mix and 2.5 µl Biotin-NTP Mix for a single reaction and 7.5 µl of this mix was added to each cDNA sample. The samples were incubated at 37°C for 14 hours. Finally, 75 µl of nuclease-free water was added to each sample to stop the reaction. To purify the cRNA, 350 µl cRNA binding buffer and 250 µl 100% ethanol was added to each sample and mixed by pipetting. The samples were then passed through cRNA filter cartridges, centrifuged for 1 minute at 10000 *g* and the flow through discarded. The samples were washed with 650 µl Wash Buffer and centrifuged for 1 minute at 10000 *g*. The cartridges were spun again for 1 minute to remove any residual buffer, transferred to cRNA collection tubes and 200 µl preheated nuclease-free water was added to the filter cartridges. The samples were incubated at 55°C for 10 minutes and centrifuged for 1.5 minutes at 10000 *g* to elute the purified and biotin-labelled cRNA. Finally the cRNA was quantified using RNA 600 Nano chips on the Bioanalyser prior to hybridization onto the BeadChips.

The cRNA was incubated at 65˚C for 5 minutes, vortexed and pulse centrifuged at 250 *g*. After cooling the cRNA to room temperature, the amount of cRNA is normalised according to the size of BeadChip being used. In this case, 750 ng of each cRNA sample was dispensed into each hybridization tube. Five µl of RNase-free water and 10 µl HYB was then added to each hybridization tube. Two hundred µl of HCB buffer was added to humidifying buffer reservoirs on the hybridization

chamber and gasket. Fifteen µl of each sample was loaded onto the bead chips, which were placed in hybridization chamber inserts. The chamber inserts were then placed into the hybridization chamber and gasket and the whole apparatus was sealed well and placed in the hybridization oven at 58˚C for 14 hours. The BeadChips were removed and placed in a beaker containing E1BC buffer. The cover-seal covering the BeadChips were then taken off carefully and the BeadChips transferred to a dish containing E1BC buffer. The BeadChips were transferred to a waterbath containing High-Temp Wash buffer and incubated at 55˚C for 10 minutes. The BeadChips were transferred again to a dish containing fresh E1BC buffer and repeatedly plunged in and out of the dish 10 times. The dish containing the BeadChips was shaken at medium speed for 5 minutes at room temperature. The BeadChips were then washed in 100% ethanol by plunging in and out of the dish 10 times followed by shaking at room temperature for 10 minutes. The BeadChips were once again washed in E1BC following the same steps as before. Each BeadChip slide was moved to a wash tray containing Block E1 buffer and washed while shaking at medium speed for 10 minutes. The BeadChips were transferred to fresh wash tray containing Cy3-Streptavidin at a 1:1000 dilution in block E1 buffer and incubated covered for 10 minutes while shaking at medium speed. The BeadChips were then washed for a third time in Wash E1BC buffer by plunging the slides in and out of the dish 5 times and with a final wash in this buffer for 10 minutes while shaking at medium speed. The slides were centrifuged at 1,400 rpm at room temperature for 4 minutes after which they were ready to be analysed. The scanning was done using an iScan reader at IARC, Lyon.

A series of controls were also included in each array to account for variation due to sample preparation, hybridisation and signal generation. Additionally, each array had a number of technical replicates, which were randomly distributed across the array. Table 4 lists the different controls included in the assay.

Table 4: Description of controls included in the Illumina Human HT12 bead array.

Control Category	Role	Number of probes
Housekeeping	Measures intactness of biological sample; these genes should be expressed in any cellular sample	14
Cy3 Hybridisation	Cy3 labelled oligonucleotides present in the HYB buffer in low, medium and high concentrations are used to check for efficient hybridisation	6
Low stringency hybridisation	These controls are matched with the medium and high concentration-oligonucleotides in the Cy3 hybridisation category to test for stringency. If stringency is high, these oligonucleotides produce a low signal. If stringency is low, a higher signal matching the signal of their counterparts in the Cy3 hybridisation control is produced	4
Biotin	Complementary biotin-tagged oligonucleotides are present in the HYB buffer to check for signal generation	2
Negative	These probes have oligonucleotides with sequences that have been designed such that they do not correspond to any target within the genome	2

3 Insulin-like growth factor axis: a possible mechanism for aflatoxin-related child growth impairment?

3.1 Introduction

Staple diets in many parts of Kenya mainly constitute locally grown maize, which is frequently contaminated with aflatoxins. Growth-promoting conditions for aflatoxin-producing *Aspergillus Spp.* on grains, including warm temperatures and high humidity, are prevalent in Kenya (Williams *et al.* 2004). Indeed, an acute outbreak of aflatoxin poisoning with over 100 deaths was reported in Kenya in a toxicosis incident in 2004. This outbreak was attributed to aflatoxin exposure with doses up to 220-times greater than the accepted food limit of 20 ppb (Probst *et al.* 2007).

Child malnutrition is a key underlying cause of child death, which accounts for more than one third of childhood mortality under the age of 5 in the developing world (Black *et al.* 2003). Aflatoxin exposure as indicated by blood AF-alb biomarker levels, has been associated with growth retardation in children in Benin and Togo over an 8-month observation, with children belonging to the higher aflatoxin exposure quartile being 1.7 cm shorter than those belonging to the lower quartile (Gong *et al.* 2002; Gong *et al.* 2004). In another study based in Kenya, weaning flour samples were analysed for the presence of aflatoxin and the height and weight of 242 children aged 3-36 months consuming the maize foods were recorded. It was observed that significantly more children with wasting were fed aflatoxin-contaminated flour compared to children without wasting (Okoth and Ohingo 2004). Moreover, research based in The Gambia and Ghana has shown that exposure to aflatoxin *in utero* is associated with reduced body weight and height in infants at birth (Turner *et al.* 2007; Shuaib *et al.* 2010). Whilst these studies consistently indicate an association between dietary aflatoxin exposure and reduced growth velocity, evidence to demonstrate the causal association is needed as the aetiological

mechanism through which aflatoxin-induced growth impairment occurs is still unknown. Understanding the mechanistic link between aflatoxin and child stunting will contribute to establishing the causative association, and help in developing effective intervention strategies to minimize the aflatoxin attributed economic and public health burden.

As well as being the main site for aflatoxin metabolism in the body, the liver is also a major production site for IGF, which facilitates the growth promoting effects of GH, and hence is a key factor influencing child growth (Woods *et al.* 1996). Liver-derived IGF1 is primarily found in the serum bound to any member of a group of binding proteins, most notably to IGFBP3 (Ohlsson *et al.* 2009). The bound complex of IGF1 with binding proteins serves as an IGF1 reserve in the circulation, thus extending the short half-life of the growth factor (Ohlsson *et al.* 2009). The influence of IGF1 on body growth is substantiated by the significant association between low child birth weight and short stature, and polymorphisms in the *IGF1* promoter region, which lower IGF1 levels in circulation (Arends *et al.* 2002; Vaessen *et al.* 2002).

The growth promoting effects of both IGF1 and IGF2 are manifested through a signalling pathway that utilises the IGF1-receptor (IGF1R). Additionally, IGF2 exerts its effects through IGF2R (Grimberg and Cohen 2000). Indeed, mutations in the *IGF1R* gene have been associated with postnatal growth retardation in a few small scale studies (Walenkamp *et al.* 2006; Abuzzahab *et al.* 2003).

A study on male broiler chicks has shown that aflatoxin exposure was significantly linked to the down regulation of the *IGF1* gene and the subsequent reduction in body weight (Yarru *et al.* 2009). These findings may support the hypothesis that the

mechanistic action of aflatoxin on child growth occurs through a disruption of the IGF system, either by directly lowering IGF1 levels or affecting other components in the IGF axis. Although, there has been no evidence of changes to the IGF axis in relation to aflatoxin exposure-associated child growth impairment, reduced serum IGF1 levels have been linked to HCC and liver cirrhosis in hepatitis C patients (Su *et al.* 2010; Elsammak *et al.* 2006; Lorenzo-Zúñiga *et al.* 2007).

A series of experiments were used to assess the mechanistic association between child aflatoxin exposure and impaired growth via reduced IGF1/IGFBP3 levels using available serum samples collected from school-aged children for a previous study based in the Makueni District in Kenya, a high aflatoxin risk region. Additionally, a non-tumourigenic human liver cell line was used to ascertain the *in vitro* effects of AFB_1 on expression of genes and levels of proteins that play a key role in the IGF system.

3.2 Materials and Methods

3.2.1 Human study

3.2.1.1 The subjects

The study site (Wilson *et al.* 2007b) was based in the Makueni District in Kenya, which is characterised by biannual seasonal rains, falling in November-December and April-May. The study population was based in two neighbouring village schools: Matangini Primary in Lower Mangelete in the East and Yumbuni Primary in the West. Lower Mangelete has several permanent streams and irrigational canals while the surface water in Yumbuni is mainly seasonal. Maize based dishes, githeri and ugali, constitute the main dietary staple in this area (Wilson *et al.* 2007b) and thus are a potential source of aflatoxin exposure in human populations.

Recruited school-aged children were a sub-set from a cross-sectional study in which 56.4% of children were stunted with a significant association with presentation with hepatomegaly (Wilson *et al.* 2007b; Gong *et al.* 2012). About 70% of the children were negative for schistosomiasis and all recruited children were positive for malarial infection. Available serum samples collected in May/June 2002, 119 from Yumbuni and 61 from Matangini, were used in the current study. Height was measured to the nearest eighth of an inch and converted into centimetres while weight was measured to the nearest half kilogram (Wilson *et al.* 2010). The male to female ratio for these children was 1:1 and the age range was 6-17 years. Informed consent was obtained from the parents or guardians of the recruited children. Ethical

approval was obtained from the Kenya Medical Research Institute Ethical Review Committee.

3.2.1.2 Serology

Serum samples were analysed for aflatoxin-albumin adduct (AF-alb) levels by a competitive ELISA (Chapot and Wild 1991) described in Chapter 2, Section 2.1.1. IGF1 and IGFBP3 levels in the serum samples were measured using the IGF1 and IGFBP3 Quantikine ELISA kits (R&D Systems, Abingdon, UK) as described in Chapter 2, Section 2.2.1.

3.2.1.3 Infection and disease status

Malarial infection status was determined by ELISA measurement of *Plasmodium falciparum* schizont antigen IgG3 (Pfs-IgG3) levels, which is a marker of chronic exposure to Plasmodium infections (Wilson *et al.* 2007a). Schistosomiasis was diagnosed based on the *S. mansoni* egg count from five separate stool samples with two Kato Katz slides being prepared from each sample (Wilson *et al.* 2007b).

Each participant was clinically examined by palpation for enlarged livers and spleens in the supine position. An organ was considered enlarged if it was palpable more than 2 cm below the costal line. Children were grouped into four categories: no organomegaly, firm/hard splenomegaly, firm/hard hepatomegaly or firm/hard hepatosplenomegaly (Wilson *et al.* 2007b).

3.2.2 HHL-16 cell-based study

HHL-16, a non-tumourigenic human liver cell line (Clayton *et al.* 2005), was kindly provided by Dr. Arvind H. Patel (MRC Virology Unit, Glasgow) and utilised in this model.

3.2.2.1 Cytotoxicity assay

HHL-16 cells were treated with 0.2 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml and 100 µg/ml AFB_1 and cytotoxicity was assessed as described in Chapter 2, Section 2.3.2.

3.2.2.2 AFB_1 treatment for cell gene expression and cell media protein analysis

HHL-16 cells were treated with 0.5 µg/ml, 5 µg/ml and 20 µg/ml AFB_1 dissolved in DMSO for either 24 or 48 hours in triplicate. The former two doses were selected due to limited cytotoxicity. The higher dose was selected to assess effects on cells in extreme cases.

DMSO levels for cells treated with 0.5 µg/ml and 5 µg/ml were at 0.0025% and those for cells treated with 20 µg/ml were at 0.1% DMSO. Cells treated with DMSO levels corresponding to treatment DMSO levels served as controls to exclude potential toxicity from DMSO alone. Cells were treated for 1-2 days before RNA was harvested using an RNeasy Mini kit (Qiagen Ltd, West Sussex, UK) described in Chapter 2, Section 2.3.5 Quality and quantity of extracted RNA was determined using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, DE, USA).

3.2.2.3 Reverse transcription real-time qPCR

Total RNA was reverse transcribed and amplified following the method described in Chapter 2, Section 2.3.6. Primers for *GAPDH* and *IGF1* and *IGFBP3* are shown in Table 5.

Table 5: Primer sequences for *GAPDH, IGF1, IGF2, IGFBP3* and *IGF1R*.

Gene	Forward primer (5' to 3')	Reverse primer (3' to 5')	Product Size
GAPDH	TTGTCAAGCTCATTTCCTGGTAT	TCTCTCTTCCTCTTGTGCTCTTG	150 bp
IGF1	CCCAACCCAGCCCTTATTAT	CCCCATCTCACAAAAAGGAA	146 bp
IGF2	GAAGGACCCCAGAAATCACA	TGATGGAAAAGGGAGTGAGG	132 bp
IGFBP3	GTGGATCCCTCAACCAAGAA	TAGGTTCCCAGAGTGCCCTA	139 bp
IGF1R	CCATTCTCATGCCTTGGTCT	TGCAAGTTCTGGTTGTCGAG	114 bp

3.2.2.4 Cell media protein analysis

Following AFB_1 treatment of cells for 24 and 48 hours, conditioned cell media were collected and centrifuged at 1000 *g* for 5 minutes to separate cell debris. The supernatant was enriched using Vivaspin 2 ultrafiltration columns with 3000 Molecular Weight Cut Off (MWCO) polyethersulfone (PES) membrane (Sigma-Aldrich, Dorset, England, UK). The columns were centrifuged at 4000 *g* for 50 minutes at room temperature to obtain approximately 30-times concentrated media, which were analysed immediately.

IGF1 and IGFBP3 protein levels in concentrated cell media were measured using the IGF1 and IGFBP3 Quantikine ELISA kits (R&D Systems, Abingdon, UK) as described in Chapter 2, Section 2.2.2.

3.2.3 Statistical Analyses

As AF-alb, IGF1 and IGFBP3 levels were not normally distributed all three parameters were natural-log transformed before statistical analysis. Geometric means and 95% confidence intervals (CI) for AF-alb, IGF1 and IGFBP3 are presented unless otherwise stated. Multivariate and univariate regression analyses were carried out to determine if AF-alb was inversely associated with child growth parameters. Stata IC software was used for these analyses (version 10, StataCorp, College Station, TX, USA). Regression and structural equation modelling for path model analyses were constructed to determine if AF-alb was associated with impaired child height through lower IGF1/IGFBP3 levels using Amos Graphics (version 5, Amos Development Corporation, IBM Corp, NY, USA). Standardised and non-standardised coefficients were calculated based on the path model involving AF-alb,

IGF1, height and age. Confidence intervals and *P* values were calculated employing a bootstrap approach. For the *in vitro* normal HHL-16 liver cell model, student's *t*-test was used to compare each treatment with their respective controls and *P* values < 0.05 were considered statistically significant.

3.3 Results

3.3.1 Aflatoxin-albumin adduct, IGF1 and IGFBP3 protein levels in Kenyan school children

A description of the study population, including measured AF-alb, IGF1 and IGFBP3 levels is summarised in Table 6. There were no significant differences in the mean age, height and weight between children from the two schools, although boys were taller and heavier than girls. There were no significant differences in IGF1 and IGFBP3 levels in children from the two schools. Both parameters were significantly higher in boys compared to girls [geometric mean: 123.1 ng/ml (95% CI: 110.3, 138.4) vs 87.2 ng/ml (79.3, 96.1) for IGF1 and 2065.4 ng/ml (1913.2, 2229.1) vs 1776.6 ng/ml (1654.3, 1908.3) for IGFBP3)] and increased with age (regression coefficient: 0.07, $P < 0.001$ for IGF1 and 0.02, $P = 0.02$ for IGFBP3).

Table 6: Characteristics of the children in the Kenyan study.

	Mean ± standard deviation or Geometric mean (95% CI)[a]		
Variable	**Yumbuni**	**Matangini**	**Total**
N	119	80	199
Age (y)	11.7±3.0	12.6±2.8	12.0±3.0
Height (cm)	133.0±13.5	134.4±13.0	133.5±13.3
Weight (Kg)	27.3±8.1	29.2±8.9	28.1±8.4
AF-alb (pg/mg)[a]	72.2 (60.7-86.0)	207.7 (172.3-250.4)*	110.5 (95.4-127.9)
IGF1 (ng/ml)[a]	105.0 (94.8-116.2)	98.1 (86.5-111.3)	102.2 (94.4-110.5)
IGFBP3 (ng/ml)[a]	1959.8 (1825.4-2104.2)	1819.8 (1685.9-1964.4)	1902.3 (1674.4-1875.8)

[a] geometric mean (95% CI) presented for these parameters

* $P < 0.001$

AF-alb levels were significantly higher in children from Matangini compared to those from Yumbuni (207.7 pg/mg vs. 72.2 pg/mg; $P < 0.001$) (Gong *et al.* 2012). Differences in the AF-alb mean levels were not significant ($P > 0.05$) between sexes, and there was no significant association ($P > 0.05$) between AF-alb and child age. There was no significant difference in malarial infection status between Yumbuni and Matangini. However, schistosomiasis infection egg counts were significantly higher in children belonging to Matangini than in those belonging to Yumbuni (Gong *et al.* 2012). Both malaria and schistosomiasis infection status were not significantly associated ($P > 0.05$) with either IGF1, IGFBP3 or AF-alb adduct levels.

3.3.2 AF-alb, IGF1 and IGFBP3 and growth

There was no significant correlation ($P > 0.05$) between AF-alb and child height or weight. The schoolchildren were dichotomised using an AF-alb cut-off at 400 pg/mg as this was the point at which the relationship between AF-alb levels and height became linear. Using this criterion, a significant 3.8 cm decrease in body height was observed in children belonging to the higher exposure group compared to those in the lower exposure group after adjusting for age, sex, school, disease state and infection status (overall adjusted R^2 of model = 0.636, $P = 0.018$). Linear regression analysis revealed that AF-alb was inversely associated with IGF1 (regression coefficient: -0.27 (95% CI: -0.524, -0.014), $P = 0.039$) and with IGFBP3 (-0.39 (95% CI: -0.778, -0.007), $P = 0.046$).

Path analysis using structural equation modelling was introduced to establish the path and directionality of the relationship between AF-alb, height and IGF1 or

IGFBP3 levels with age as an effect mediator, as both IGF1 levels and height are influenced by age. This work was done with input and advice from Dr. Yu-Kang Tu, University of Leeds. The model shown in Figure 5 had an acceptable fit (χ^2 = 0.343, degrees of freedom = 1; *P* = 0.558). The non-standardised and standardised regression coefficients as well as the *P* values were obtained using the statistical software Amos Graphics with these results being summarised in Figure 5. Approximately 16% of the effect of AF-alb on child height can be attributed to lower IGF1 levels (estimated using standardised coefficients of the direct and indirect effects) (*P* = 0.052). A similar path diagram was tested for IGFBP3 but an indirect effect of AF-alb on height through IGFBP3 was not significant (*P* = 0.26).

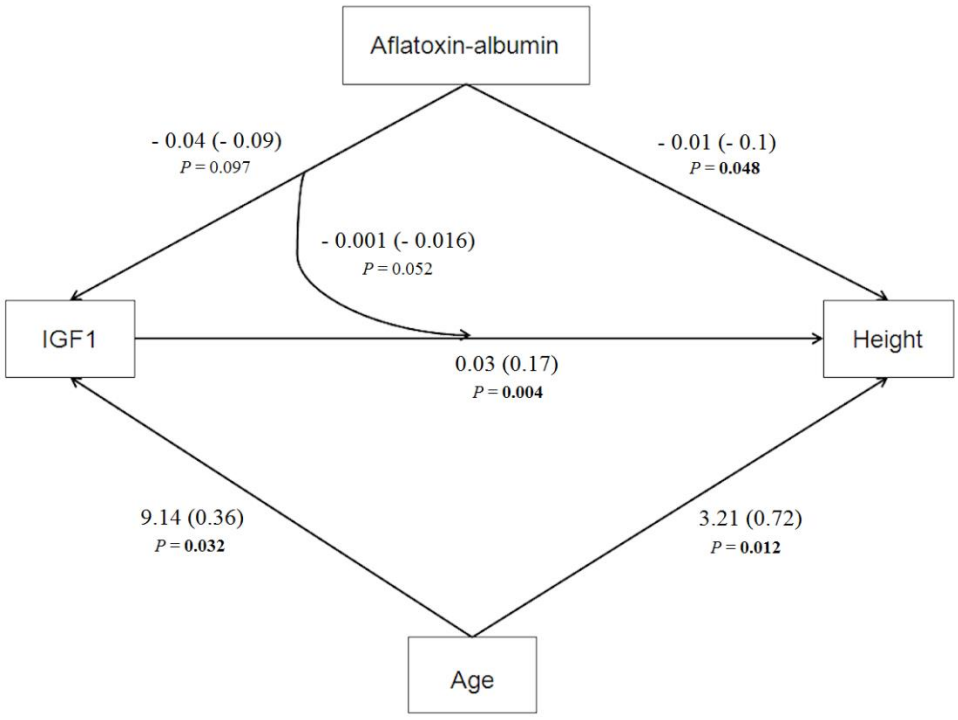

Figure 5: Diagram of the path analysis result for the effects of aflatoxin on height depicting both direct and indirect effects. Age was used as an effect mediator. Non-standardised regression coefficients (with standardised displayed in parentheses) are displayed for each path (represented by an arrow). *P* values are also shown with those considered to be statistically significant displayed in bold. AF-alb is inversely associated with child height through a direct pathway. An association between AF-alb and child height through altered IGF1 levels can be observed (*P* = 0.052). IGF1 and age significantly influence child height while age has a strong effect on plasma IGF1 levels.

3.3.3 Cytotoxicity of AFB_1 on HHL-16 cells

Cell viability following 24 hour, 48 hour and 72 hour AFB_1 treatment was determined relative to the DMSO controls (Figure 6). There was a significant reduction in viability at 20 µg/ml AFB_1 and higher doses at 24 hours of treatment ($P < 0.05$) and at 10 µg/ml AFB_1 and above at 48 and 72 hours of treatment ($P < 0.05$). At 48 hours treatment, cell viability was less than 50% in all doses above 10 µg/ml. After 72 hour treatment, viability was as low as 6% in cells treated with 100 µg/ml AFB_1.

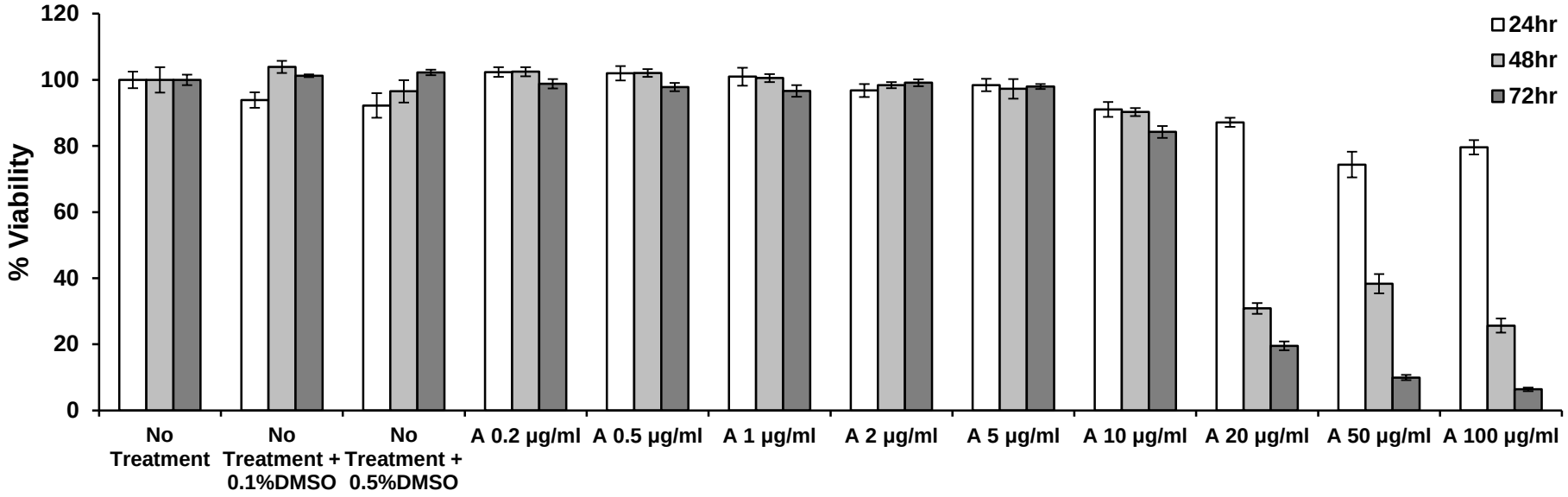

Figure 6: Cytotoxicity test at 24, 48 and 72 hours of treatment with 9 AFB_1 doses along with DMSO controls for HHL-16 liver cells. Acute toxicity is observed in cells treated with 20 µg/ml AFB_1 or above following 48 and 72 hours of exposure while no significant toxicity can be seen in untreated and DMSO controls.

3.3.4 IGF gene expression in AFB_1 treated HHL-16

IGF gene expression, measured using reverse transcription qPCR, across control and treatment groups is shown in Figure 7. After 24 hours of treatment with AFB_1, very little change was observed in IGF gene expression levels, although at the highest dose, significantly lower expression was observed for *IGFBP3* (2.4 fold change compared to control, $P = 0.005$). Expression of both IGF genes was significantly reduced following a 48-hour treatment with 5 and 20 µg/ml AFB_1 with an average 2 fold change in expression compared to controls ($P < 0.05$).

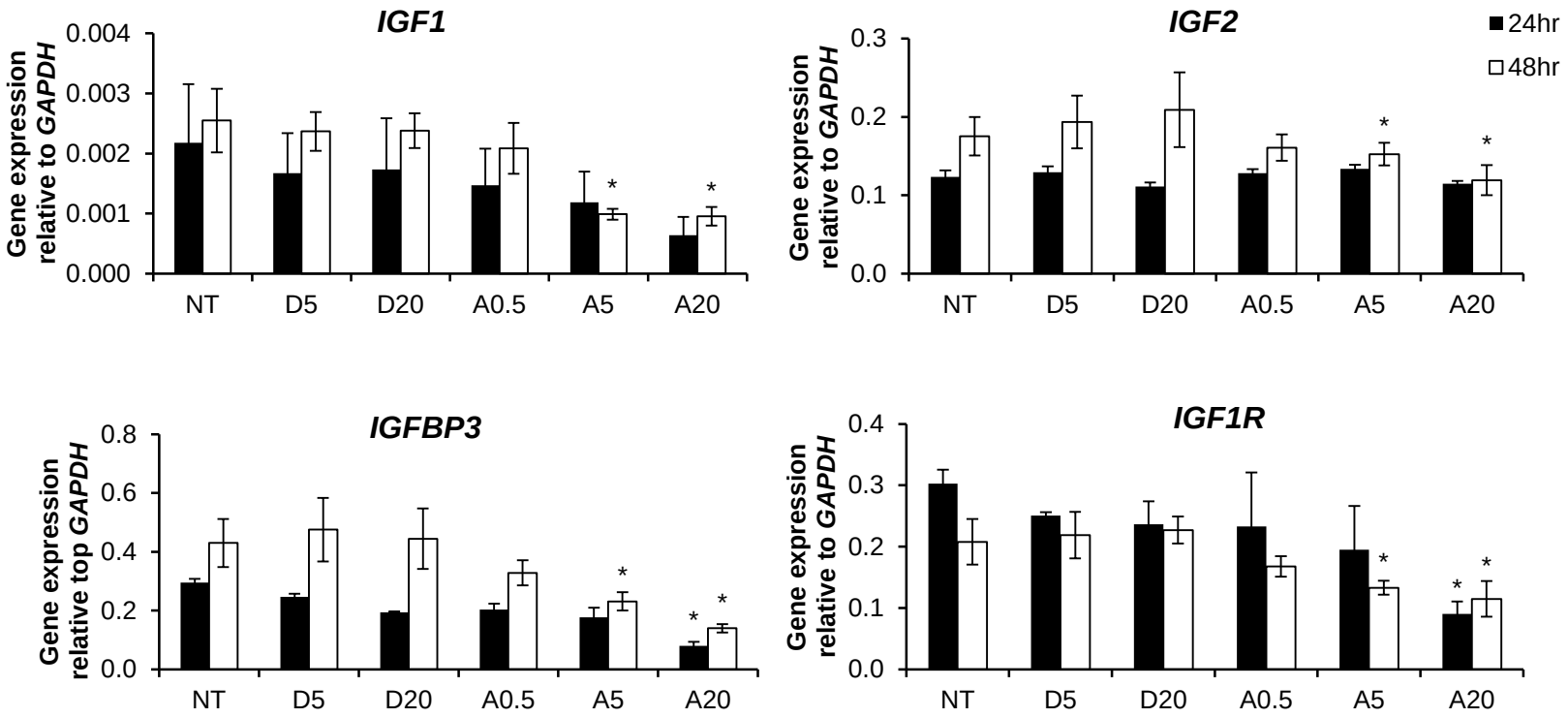

Figure 7: Graphs depicting gene expression relative to untreated cells (NT) in HHL-16 cells for *IGF1*, *IGF2*, *IGFBP3* and *IGF1R* across treatment groups- Untreated (NT), 0.0025% DMSO (D5), 0.1% DMSO (D20), 0.5 µg/ml AFB1 (A0.5), 5 µg/ml AFB1 (A5) and 20 µg/ml AFB1 (A20). * $P < 0.05$.

3.3.5 IGF1 and IGFBP3 protein levels in AFB_1 treated HHL-16

IGF protein levels in cell culture media were measured by ELISA, following 24 and 48 hour AFB_1 treatment. As with the trend observed in expression, protein levels were also reduced with increasing AFB_1 dose (see Figure 8). Overall, IGF protein expression increased with incubation time, although this was more obvious for IGFBP3 levels. However, the effects of AFB_1 on protein levels were still apparent. IGF1 levels were significantly lower in cells treated with 20 µg/ml AFB_1 after 24 hours ($P < 0.05$) and at 5 µg/ml and 20 µg/ml AFB_1 after 48 hours ($P < 0.001$). IGFBP3 protein levels were significantly lower at 5 µg/ml and 20 µg/ml AFB_1 following 24 hours and at all three doses after 48 hours of treatment. Protein levels were drastically reduced at the highest AFB_1 dose with a 2-fold average decrease ($P < 0.001$) (Figure 8).

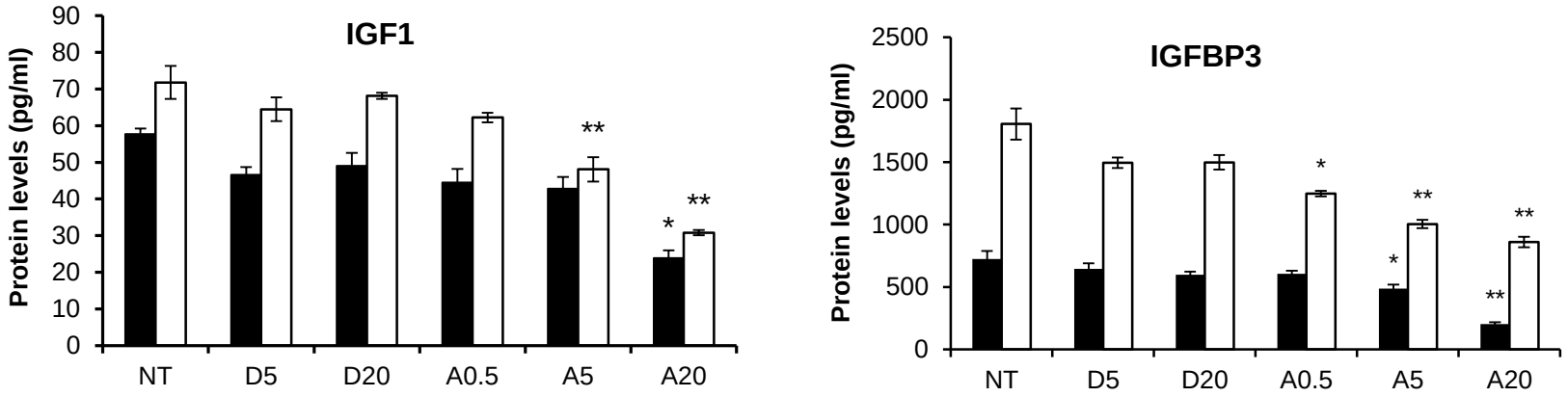

Figure 8: Graphs depicting cell media protein levels relative to untreated cells (NT) in HHL-16 cells for IGF1 and IGFBP3 across treatment groups- Untreated (NT), 0.0025% DMSO (D5), 0.1% DMSO (D20), 0.5 µg/ml AFB1 (A0.5), 5 µg/ml AFB1 (A5) and 20 µg/ml AFB1 (A20). * $P < 0.05$; ** $P < 0.01$.

3.4 Discussion

This work aimed to determine if the IGF growth axis plays a role in the mechanism of aflatoxin-associated child growth impairment in humans. The current study extends previous research to provide some evidence of the mechanistic action of aflatoxin on child growth. Aflatoxin exposure through contaminated feed has been implicated in severe growth retardation in ducks, pigs and broiler chicks (Han *et al.* 2008; Dersjant-Li *et al.* 2003). This association has also been seen in West African children exposed to aflatoxins through consumption of maize and groundnut-based food (Gong *et al.* 2002; Gong *et al.* 2004). Observations in the current study corroborate this finding as exposure to 400 pg/mg or more AFB_1 was associated with a significant 3.8 cm decrease in child height compared to children with lower AFB_1 exposure, after adjusting for confounding factors including age, sex, schistosomiasis, malarial infection status and school.

The role of the major components of the IGF axis in growth is well established (Grimberg and Cohen 2000). IGF2 is a very important factor in human foetal growth (Randhawa and Cohen 2005), and following birth, IGF1 plays a prominent role in child growth (Grimberg and Cohen 2000). The presence of binding proteins, particularly IGFBP3, regulate the growth axis by controlling the levels of free IGF1 in circulation and prolonging the half-life of IGF1 (Grimberg and Cohen 2000). The growth promoting effects of IGF2 and IGF1 during the different phases of child growth are mostly manifested through IGF1R (Grimberg and Cohen 2000). Therefore, the growth retardation associated with AFB_1 exposure may be caused by changes to the expression of key members of the IGF growth axis.

Through the use of a population-based study and an *in vitro* liver cell-based model, this study provides evidence that aflatoxin exposure-induced disruption of the IGF growth axis contributes to growth impairment. Serum IGF1 and IGFBP3 levels in school-age children were lower when children were exposed to higher levels of aflatoxin. Results from the path analysis approach revealed that ~16% of the effect on child height by aflatoxin exposure was attributed to reduced IGF1 levels (P = 0.07), indicating a potential pathway between aflatoxin and child stunting. While schistosomiasis and malarial infection status may have been confounders due to their potential impact on child growth, it has already been shown that in the current cohort of school children, neither infection was associated with growth stunting (Wilson *et al.* 2010). Other factors contributing to aflatoxin exposure-associated child stunting may include immune function changes and infections and possible damage to gut function through aflatoxin exposure leading to poor nutrient absorption (Wild and Gong 2010). A multi-component path effect may be responsible for the total effect of aflatoxin exposure on reduced child height gain.

To examine the effects of AFB_1 on IGF axis components, an *in vitro* model was set up using non-tumourigenic liver cells, HHL-16. More commonly studied liver cell lines such as HepG2 were not utilised in this study to avoid any bias towards gene expression and protein analysis due to cancer-related changes. Derived from a healthy human liver, HHL-16 cells have been immortalised with Moloney's mouse leukaemia virus and retain primary hepatocyte characteristics, expressing hepatocyte-specific markers as well as cytochrome P450 enzymes (Clayton *et al.* 2005). In the present study, these cells were treated with three AFB_1 doses for one to two days- conditions chosen based on cytotoxicity data. Reverse transcription qPCR as well as ELISA analyses correlated well showing that AFB_1 inhibited gene and

protein expression of IGFs in liver cells, particularly following 48 hours treatment with the higher AFB_1 doses. These observations provide substantial evidence that AFB_1 exposure alters expression and production of IGF axis components that play a key role in growth.

Previously, it was reported that primary human hepatocytes exposed to 0.02 µg/ml and 0.12 µg/ml of AFB_1 had repressed *IGFBP3* and *IGF1* expression (Josse *et al.* 2012). Interestingly, exposure to other mycotoxins has been associated with a decrease in IGF gene expression or IGF1 protein levels. For instance, ochratoxin A was found to down regulate *IGFBP1* and *IGF2* gene expression in the human hepatocellular cell line, HepG2 (Hundhausen *et al.* 2009), while it has been proposed that another mycotoxin, DON causes growth retardation in mice by lowering plasma IGF1 and IGF-ALS levels (Amuzie and Pestka 2010). These changes suggest that mycotoxin-associated growth impairment may follow a similar pathway involving the disruption of the IGF growth axis.

It has been shown that maternally ingested aflatoxins can cross the placental barrier and are metabolised to produce aflatoxicol, which is carcinogenic (Partanen *et al.* 2010; Maxwell *et al.* 1989). As it has been observed that aflatoxin exposure can occur during weaning as well as later in life (Gong *et al.* 2003; Wild and Gong 2010), the effects it may have on the IGF growth axis may occur during critical early development altering some or all of the major components necessary for the normal functioning of the growth axis.

Results from this study provide evidence for the hypothesis that reduced IGF1 levels contribute to aflatoxin-associated growth impairment. Other factors involved in growth and development may also be involved in the mechanism. Additionally,

aflatoxin exposure causes a significant reduction in IGF axis gene and protein expression in a non-tumourigenic liver cell line providing further support to its effects on the IGF axis.

4 Seasonal and gestation-stage associated differences in dietary aflatoxin exposure in pregnant Gambian women

4.1 Introduction

The development of aflatoxin exposure biomarkers has facilitated advances in aflatoxin research and has contributed to our understanding of causal relationships between dietary exposure and health effects. Measurement of the blood AF-alb biomarker revealed that exposure to aflatoxin is prevalent in many people living in sub-Saharan Africa and other low- to middle-income regions (Wild and Gong 2010). The AF-alb biomarker has contributed to the discovery that chronic dietary exposure to aflatoxin is not only associated with development of hepatocellular carcinoma (Gomaa *et al.* 2008; Wild and Montesano 2009) but also with child growth faltering (Gong *et al.* 2002; Gong *et al.* 2004) and immune function impairment (Turner *et al.* 2003; Jiang *et al.* 2005).

Aflatoxin contaminates a large proportion of the world's staple foods, including maize and groundnuts (Wild and Gong 2010). Seasonality has been identified as an important factor in determining aflatoxin exposure in West Africa (Hell *et al.* 2000; Wild *et al.* 2000; Cotty and Jaime-Garcia 2007). Contamination levels are usually higher following harvest and a period of storage during the annual dry season from November to May, when compared to levels during the rainy season from June to October (Wild *et al.* 2000). The latter has also been called the 'hungry' (as compared to the dry 'harvest') season because it is a period of intense physical activity but with depleted stores of staple foods (Thomson *et al.* 1966).

Exposure to aflatoxin has been consistently reported to be high in The Gambia (Allen *et al.* 1992; Wild *et al.* 2000; Turner *et al.* 2000). Maternal exposure to aflatoxin has been significantly associated with lower height and weight gain in

infants during the earlier years of life (Turner *et al.* 2007). Additionally, it is known that aflatoxin can cross the placental barrier leading to fetal exposure during pregnancy (Partanen *et al.* 2010). This is supported by the fact that AF-alb can be detected in cord blood samples (Abdulrazzaq *et al.* 2002; Turner *et al.* 2007). However, it is unclear whether aflatoxin exposure changes during pregnancy, and how such change may adversely affect the health of the mother and her child. The implications of exposure to aflatoxin *in utero* are significant, as exposures at this crucial stage can have long term-effects, including epigenetic modifications that can alter gene expression, potentially affecting the immune system, growth and development. This part of the study aimed to understand aflatoxin exposure status at the early and later pregnancy in rural Gambian women and explored possible interactions with seasonal impact on the aflatoxin exposure.

4.2 Methods

4.2.1 Study subjects

The current study utilised samples collected from two parallel studies in rural Gambia. Both studies were designed and executed by Dominguez-Salas et al., MRC International Nutrition Group, London School of Hygiene and Tropical Medicine, London, UK. The first study was a trial of pre-natal and infant nutritional supplementation on infant immune development: the 'ENID' Trial (Early Nutrition and Immune Development; ISRCTN49285450) (Moore *et al.* 2012). The second study, 'Methyl Donors and Epigenetics' (MDEG), was embedded within the ENID Trial, and followed a sub-cohort of women who conceived during the peak 3 months

of the rainy or dry seasons to study the impact of nutrition at the time of conception on DNA methylation patterns in offspring (Dominguez-Salas *et al.* 2013). The ENID and MDEG studies were performed in the West Kiang region of The Gambia, a rural subsistence farming community of savannah and farmland. All women, aged 18-45 years, were invited to participate in both studies, and informed consent was obtained. Once enrolled, women were visited monthly by a field assistant to record the date of last menstrual period. Where a menses had been missed, pregnancy and date were confirmed by a urine pregnancy test and for those testing positive, a subsequent ultrasound test was arranged in the MRC Keneba clinic. Also, a blood sample was collected before 16 weeks of pregnancy. At 16-33 weeks gestation, further blood samples were collected as part of the ENID study. Data on height, weight, village and self-reported ethnicity was also collected for all women.

Blood samples from 134 women recruited for the MDEG study were available for analysis. These blood samples, collected before 16 weeks of pregnancy as part of the MDEG study, were defined as early pregnancy samples, with the sampling falling either in the rainy (June to October) or dry (November to May) seasons. For 99 of these women, blood samples were also available for a second AF-alb test from a collection at a later time during pregnancy (between 16-33 weeks), which was collected as part of the ENID study. These samples were defined as later pregnancy samples in this study. The 99 paired blood samples collected from both the early and later pregnancy time points were subsequently used to investigate aflatoxin exposure changes between pregnancy stages. For individual women, there was at least six weeks between the collection of the early and later blood samples.

Blood collected into EDTA vacutainer tubes was centrifuged at 2750 *g* for 10 minutes at 4ºC. They were then stored at -80°C before being shipped to the

University of Leeds, UK for blood albumin and AF-alb biomarker analysis. Ethical approval was obtained from the Gambia Government/MRC Gambia Joint Ethics Committee and written informed consent was obtained from each participating woman.

4.2.2 Blood albumin and AF-alb biomarker analysis

Plasma samples were analysed for AF-alb using a modified competitive ELISA, as previously described in Chapter 2, Section 2.1.1 (Chapot and Wild 1991). Plasma albumin levels were measured according to the method described in Chapter 2, Section 2.1.1.

4.2.3 Gambian diet

Dietary information was unavailable for the pregnant Gambian women included in the study. Therefore, data on intake of groundnut and maize-containing dishes for non-pregnant women of reproductive age were analysed to gauge dietary trends in Gambian women. This data was collected by Dominguez-Salas et al., MRC International Nutrition Group, London School of Hygiene and Tropical Medicine, London, UK.

A total of 61 Gambian non-pregnant women aged 18-45 years belonging to Jiffragong, Janneh Kunda and Keneba villages in the rural area of West Kiang were recruited and followed from July 2009 to June 2010. Women were excluded from the study if pregnant, severely anaemic, at menopause, using contraceptives or planning to move away during the course of the study. Ethical approval was obtained from the

joint Gambian Government/MRC Ethics Committee (SCC/EC 1151) and the London School of Hygiene and Tropical Medicine Ethics Committee (EC 5525). Informed written consent was obtained from each woman prior to participation.

The main dietary composition and food weights were determined per day for each woman. Food intake was determined by a 24-hour weighed dietary record on two or more days per month. All meals including snacks were weighed and recorded. In the current work, intake of groundnuts and maize were important as potential sources of aflatoxin contamination. For this reason, only dishes clearly containing groundnuts or maize were included. Some dishes utilised groundnuts as part of a recipe where the dish constituted another main ingredient. However, the exact amount of groundnuts per dish was difficult to extract from each recipe. Therefore, cases where groundnuts were noted clearly to be part of a sauce or snack or eaten raw were included.

To determine groundnut intake, the number of times groundnuts were consumed per day and per meal for each woman during each season was calculated. Increase and decrease were defined by a difference of at least 5 g/day or 5 g/meal between seasons. Groundnut intake frequency was also calculated per woman per season. Increase and decrease in frequency were defined by any difference in frequency above 0.0 between seasons.

4.2.4 Statistical analysis

AF-alb was not normally distributed and was natural-log transformed prior to statistical analyses. Geometric means (GM) with 95% CI are presented for AF-alb levels. Differences in albumin and AF-alb levels by stage of pregnancy and by

season of blood collection were tested using the Student's t-test. ANOVA analysis was used to determine associations between AF-alb levels and stage of pregnancy, and season of blood collection. An interaction model was constructed to test the interaction relationship between the stage of pregnancy and season of blood collection. For the 99 women with paired AF-alb data Spearman rank correlation analysis was used to establish correlation between the AF-alb at the two stages of pregnancy. Student's t-test was used to test differences in groundnut intake and frequency between seasons.

A $P < 0.05$ was considered statistically significant. All data analyses were done on Stata IC software (version 11, StataCorp, College Station, TX, USA).

4.3 Results

At the time the early pregnancy samples were collected, the average age of the women (mean ± SD) was 28.9 ± 6.5 years (range, 18-45 years). Eighty-six % of the women were from Mandinka ethnicity background, while the remaining belonged to other ethnic backgrounds.

Early pregnancy samples were obtained from 47 women during the dry season and from 87 women during the rainy season. The mean gestation week of these women (mean ± SD) was 8 ± 4 weeks, (range 0-15 weeks). Of the 99 women who were followed-up for AF-alb measurement during later pregnancy, 47 provided samples during the dry season and 52 during the rainy season. The mean gestation week at the second sampling was 27 ± 3 weeks (range 16-33 weeks). There was an average 19 ± 5 weeks (range 6-33 weeks) gap between the two collections.

4.3.1 Blood albumin and AF-alb biomarker analysis

The blood albumin and AF-alb levels by season of blood collection and stage of pregnancy are summarized in Table 7. The blood albumin levels were significantly higher during the early stages of pregnancy compared to the later stage (37.2 ± 9.1 vs 48.1 ± 14.9 mg/ml, $P < 0.001$), and there was no significant difference in albumin levels between blood collected during the dry or rainy season ($P = 0.649$).

All samples tested had detectable AF-alb levels. AF-alb levels ranged from 4.8 to 521.6 pg/mg during early pregnancy and from 4.4 pg/mg to 556.5 pg/mg during later pregnancy. AF-alb levels were not compared between villages due to the very small number of subjects per village. Age and ethnicity background were not correlated with AF-alb levels.

We assessed aflatoxin exposure by season of blood collection. The blood AF-alb was higher during the dry season when compared to the rainy season, in both early and later pregnancy stage (68.7 vs 26.6 pg/mg, $P < 0.001$ for later pregnancy), although the difference did not reach a statistical significance at the early pregnancy stage (see Table 7 and Figure 9).

Table 7: Blood albumin (mg/ml) and AF-alb (pg/mg albumin) levels by season and stage of pregnancy.

	Albumin		
	Dry Season	**Rainy Season**	**Total**
	Mean ± SD	**Mean ± SD**	**Mean ± SD**
Early pregnancy	50.2 ± 14.6 (N = 47)	47.0 ± 15.0 (N = 87)	48.1 ± 14.9 (N = 134)
Later pregnancy	37.8 ± 8.7***** (N = 47)	36.6 ± 9.7* (N = 52)	37.2 ± 9.1* (N = 99)
	AF-alb		
	Dry Season	**Rainy Season**	**Total**
	Geometric mean (95% CI)	**Geometric mean (95% CI)**	**Geometric mean (95% CI)**
Early pregnancy	40.6 (29.5, 55.8) (N = 47)	31.6 (26.2, 38.1) (N = 87)	34.5 (29.3, 40.7) (N = 134)
Later pregnancy	68.7 (52.6, 89.7)***** (N = 47)	26.6 (21.9, 32.4)[a] (N = 52)	41.8 (34.7, 50.3)[b] (N = 99)

* Significant difference between data at early and later stages of pregnancy, $P < 0.05$

[a] Significant difference between AF-alb levels in dry and rainy seasons at the later stage of pregnancy, $P < 0.001$

[b] Significant difference between total AF-alb levels between the early and later stages of pregnancy, $P < 0.028$

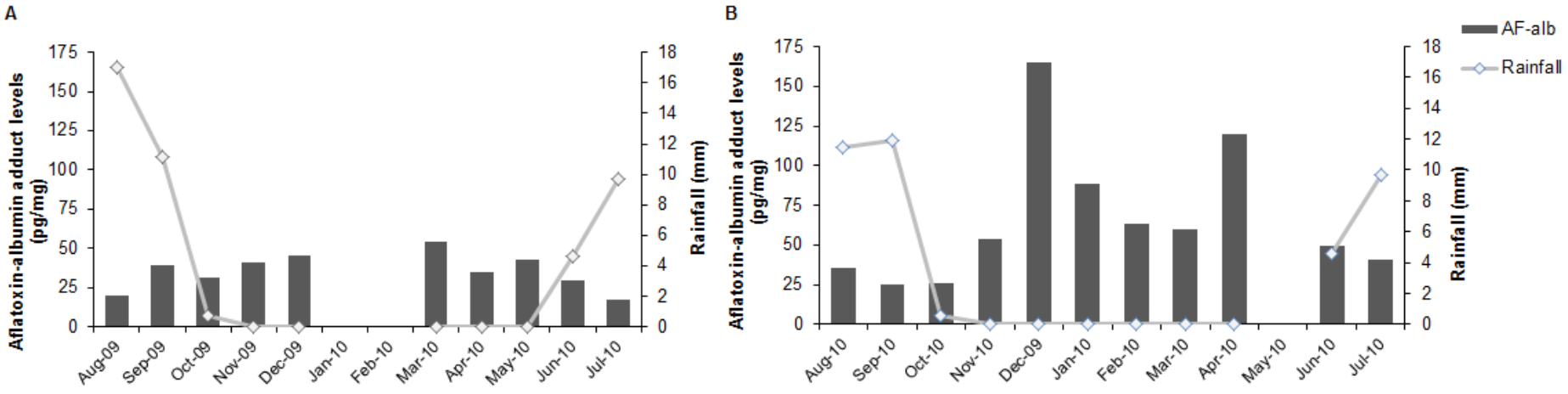

Figure 9: Geometric means of aflatoxin-albumin adduct levels presented by month of bleed with amount of rainfall per month collected during 2009-2010. A- Early Pregnancy (134 women at 0-15 weeks); B- Later Pregnancy (99 women followed up at 16-33 weeks).

AF-alb levels were significantly higher during later pregnancy compared to early pregnancy (41.8 pg/mg vs 34.5 pg/mg, $P = 0.028$). The AF-alb showed a marked difference between the two pregnancy stages when samples were collected in the dry season (68.7 pg/mg at the later stage vs 40.8 pg/mg at the early stage, $P = 0.012$), but no difference was found between the two pregnancy stages when samples were collected during the rainy season (26.6 pg/mg at the later stage vs 31.6 pg/mg at early stage, $P = 0.234$).

The interaction of season of blood collection and stage of pregnancy was further investigated using an ANOVA model (Table 8). The overall model was statistically significant ($F = 10.87$, $P < 0.001$), A significant interaction effect on AF-alb between pregnancy stage and season was found in these women ($P = 0.004$), with a 2-fold difference between the exposure in the later pregnancy at dry season as compared to early or later pregnancy at rainy season although using a similar ANOVA approach no interaction on blood albumin was found between pregnancy stage and season ($P = 0.56$).

Table 8: ANOVA analysis indicating the interaction of stage of pregnancy and season of blood collection on AF-alb but not on albumin.

Variables	Blood AF-alb F, *P*	Blood Albumin F, *P*
Pregnancy stage (early vs later pregnancy)	2.14, 0.145	45.5, <0.001
Season of blood collection (dry vs rainy season)	24.41, < 0.001	1.55, 0.21
Pregnancy stage * season of blood collection	8.29, 0.004	0.33, 0.56

F – F statistic

Correlation between AF-alb at early and later pregnancy was tested using Spearman's correlation analysis. The rank order of AF-alb for each woman at early and later stage of pregnancy was positively correlated (Spearman's coefficient: 0.231, $P = 0.022$). The women were then dichotomised into high and low aflatoxin exposure groups using the median AF-alb as cut-off values (31.5 pg/mg and 41.2 pg/mg for early and later pregnancy, respectively). When progressing from early to later pregnancy 55% of the women remained in the same exposure group, whilst others shifted their exposure groups but in agreement with the expected changes due to a shift in blood collection season, i.e. a shift from low to high exposure happened when blood collection for early and later pregnancy shifted from rainy to dry season, respectively (Table 9).

Table 9: Status of aflatoxin exposure (high or low category) of pregnant women by stage of pregnancy (early or later).

Category of AF-alb at early vs later stage of pregnancy	N.	Potential reason for change of exposure group
Low to Low	28	55% of the 99 women were in the same exposure group
High to High	26	
Low to High	24	79% of the 24 women shifted from rainy to dry season, 8% shifted from dry to rainy season,13% remained in the same season
High to Low	21	48% of the 21 women shifted from dry to rainy season, 19% shifted from rainy to dry season, 33% remained in the same season

4.3.2 Gambian diet

Only 18% of the 61 non-pregnant women consumed maize either in the form of flour, roasted or as part of a dish over an average of 2 days per month consuming 1-3 portions per day. Therefore, no further analysis was done on maize consumption in these women.

The increase and decrease in groundnut intake by season was determined and is displayed in Table 10. A majority of the women had significantly higher groundnut intake during the dry season than the rainy season when considering intake per day or intake per meal ($P < 0.001$). This is expected as the rainy season is a period of intense activity and poor food intake due to low availability. However, 10-11 women did significantly increase their groundnut intake in the rainy season compared to the dry season ($P < 0.005$). This may be attributed to higher availability through purchase from the market or other means for some women during the rainy season.

Table 10: Change in dietary groundnut intake per day or per meal for each woman by season.

Change from dry to rainy season	Intake/day			Intake/meal		
	Number of women (%)	Dry Season Mean ± SD (g/day)	Rainy Season Mean ± SD (g/day)	Number of women (%)	Dry Season Mean ± SD (g/meal)	Rainy Season Mean ± SD (g/meal)
Increase	11 (32.4%)	300.2 ± 140.6	424.0 ± 163.3 *	10 (29.4%)	178.6 ± 88.5	299.4 ± 156.8 *
Similar	1 (2.9%)	158.5	162.0	1 (2.9%)	76.7	79.5
Decrease	22 (64.7%)	350.5 ± 237.1	183.1 ± 128.5 *	23 (67.6%)	218.0 ± 112.4	124.5 ± 74.9 *

* $P < 0.005$ when comparing dry with rainy season

Groundnut intake frequency calculated per woman per season is displayed in Table 11.

Table 11: Change in dietary groundnut intake frequency per day for each woman by season.

Change from dry to rainy season	**Frequency of groundnut intake**		
	Number of women (%)	**Dry Season Mean±SD frequency**	**Rainy Season Mean±SD frequency**
Increase	15 (44.1%)	1.39 ± 0.39	1.95 ± 0.37 *
Similar	5 (14.7%)	1.3 ± 0.27	1.3 ± 0.27
Decrease	14 (41.2%)	1.79 ± 0.47	1.25 ± 0.27 *

* $P < 0.001$ when comparing dry with rainy season

A similar number of women either significantly increased or decreased their frequency of groundnut intake from the dry season to the rainy season ($P < 0.001$).

4.4 Discussion

The present work investigated changes in aflatoxin exposure during pregnancy, confirmed the effect of seasonality on aflatoxin exposure in Gambian women and also revealed differences in exposure between the early and later stages of pregnancy during the dry season. Additionally, we found that aflatoxin exposure was likely to stay high or low during the early and later pregnancy in the majority of the Gambian women studied. A change from low to high aflatoxin exposure status was most likely associated with a change in season of blood collection from rainy to dry or *vice versa*. These findings have important implications for *in utero* child health effects as it has been established that aflatoxin B_1 can cross the placenta (Partanen *et al.* 2010) and exposure to aflatoxin *in utero* has been previously linked to impaired child growth (Turner *et al.* 2007).

In the present study, during the early and later stages of pregnancy, all Gambian women had detectable AF-alb levels, which were consistent with exposure data from previous studies based in this region (Wild and Turner 2002; Turner *et al.* 2007). Previous analysis has shown that mean levels of AF-alb adducts in Gambian adults is at least 5-fold higher than exposure levels observed in adults in the USA and Europe (Montesano *et al.* 1997). Our data shows that the mean exposure levels in the Gambian women (34.1 pg/mg during early pregnancy and 41.9 pg/mg during later pregnancy) were similar to levels previously reported in Gambian adults (Wild *et al.* 1990a; Wild *et al.* 1992; Wild *et al.* 2000).

The diet in West Kiang typically consists of a staple (refined white rice, millet or maize) with a groundnut based sauce often mixed with other ingredients such as

green leaves, fish or vegetables (Prentice *et al.* 1993). The most likely sources of aflatoxin exposure are contaminated groundnuts and, to a lesser extent, maize, which form key components of the diet in The Gambia (Hudson *et al.* 1992). Harvest of groundnuts usually occurs between September and November (Roberts *et al.* 1982; Turner *et al.* 2000), following which they are either sold or stored in large open heaps making them susceptible to insect damage and fungal growth. The higher levels of AF-alb observed during the dry season as compared to the rainy season reflect the elevated production of aflatoxin in groundnuts and maize during this period. In addition, the rainy season is a period of heavy farming activity and food shortages (Paul *et al.* 1979; Roberts *et al.* 1982), which could result in lower consumption of contaminated grains and, therefore, lower exposure.

Analysis of the diet in non-pregnant Gambian women revealed significant differences in groundnut intake between the dry and rainy seasons with over 60% of the women consuming less groundnut-containing dishes in the rainy season. Intake amounts and frequency would depend on availability, harvest times and trade patterns where some families may be able to afford to buy more food than poorer families and so would affect the outcome of any analysis. This dietary analysis was not conducted in Gambian women recruited for aflatoxin exposure during pregnancy. Therefore, the dietary analysis done in this work may have missed any differences in diet due to pregnancy, which may influence aflatoxin exposure. However, this analysis does reflect the general patterns in diet in Gambian women during 2009-2010.

In the dry season we also observed higher levels of aflatoxin exposure in women during later pregnancy compared to early pregnancy. A statistically significant interaction exists between the stage of pregnancy and season of blood collection such

that the mean AF-alb level was more than doubled in blood collected during the dry season at later pregnancy as compared to that in blood collected during the rainy season at later pregnancy. It is possible that severe food shortage in the rainy/hungry season has restricted the women from consuming more food to meet the increasing demand in later pregnancy, whilst in the dry season when food supply is better; the increased consumption needed to meet the demand in later pregnancy can be satisfied. Thus, higher consumption during later pregnancy in the dry season is responsible for the increased aflatoxin exposure, even when the increased contamination during the dry season is taken into account. Although it is possible that other factors not investigated here could contribute to differences in exposure patterns (for example geographical location and socioeconomic status) the finding that later pregnancy may be associated with higher exposure may reveal a high risk period for *in utero* exposure.

It is worth noting that albumin concentrations were lower in later pregnancy versus early pregnancy. This is consistent with previous observations in French and Canadian women whereby albumin levels dropped as pregnancy progressed (Bacq *et al.* 1996; Walker *et al.* 1999), possibly due to the observed increase in blood volume in later pregnancy (Pirani *et al.* 1973; Gallery *et al.* 1979). However, as the AF-alb level is presented following adjustment for albumin concentration, a discrepancy in albumin concentrations should not make a significant influence to the aflatoxin exposure assessment. The changes in albumin levels are one of several physiological alterations occurring during pregnancy (Mattison *et al.* 1991). These changes include an increase in the activity of the CYP3A enzyme (Tracy *et al.* 2005), of which type CYP3A4 plays an important role in the metabolism and conversion of AFB_1 to the toxic AFB_1-8,9-epoxide, which then binds DNA or albumin (Guengerich *et al.*

1998). Such modifications to metabolism of AFB_1 during later pregnancy may explain the higher exposure observed in the present study.

Blood albumin concentration did not change significantly between the two seasons of collection suggesting that the hungry period during the rainy season does not adversely affect blood albumin levels. It has been reported that there is no significant difference in energy supply for pregnant Gambian women between the dry and rainy seasons (Dominguez-Salas *et al.* 2013) an unexpected finding that was explained by potential financial support from extended family permitting purchase of staple foods in the rainy/hungry season.

A critical 'window of vulnerability' exists in the early stages of fetal development, during which dietary exposures to aflatoxin, as in the case of other environment toxins, may affect the genomic programming of the foetus, including epigenetic changes that can subsequently modify gene expression (Gluckman *et al.* 2008). The changes introduced at this stage can be maintained throughout life and can adversely affect health in adulthood (De Boo and Harding 2006). However, aflatoxin exposure throughout pregnancy may also affect foetal development and growth as demonstrated in the Gambian study (Turner *et al.* 2007). The significantly higher aflatoxin exposure observed during later pregnancy in the dry season could also contribute to adverse effects on foetal development including growth.

Our data shows that aflatoxin exposure for individual women was significantly correlated between early and later stages of pregnancy, suggesting that despite the effect of seasonality dietary exposure through contaminated staple food from home stores are the key contributor to aflatoxin exposure in these women. Variation in storage practices, proximity to water bodies and genetic susceptibility have also been

reported as important determinants of aflatoxin exposure (Hell *et al.* 2000; Gong *et al.* 2012) but cannot be determined from the data collected in this study.

In summary, this work confirmed the effect of seasonality on aflatoxin exposure in pregnant Gambian women and identified reduced blood albumin as pregnancy progressed. It demonstrated an interaction between stage of pregnancy and seasonality on aflatoxin exposure in these women. Women in later pregnancy during the dry season have the highest exposure risk; high aflatoxin exposure usually persists throughout pregnancy, with far reaching implications to health of the women and their offspring.

5 Genome-wide effects of *in utero* AFB_1 exposure in Gambian infants

5.1 Introduction

Our knowledge of the mechanisms of aflatoxin-associated health effects is still incomplete. Advances in scientific technology including whole genome expression and DNA methylation analyses are paving the way towards the discovery of potential gene targets involved in aflatoxin-related effects. Identification of genes involved in different pathways may add to our understanding of the mechanistic action of aflatoxins in humans and may also contribute to the discovery of aflatoxin specific expression and methylation profiles that can serve as markers of toxin effects.

According to the foetal origins of adult disease, environmental exposures during the critical initial stages of foetal development can alter epigenetic patterns, which can then influence the risk of adverse health effects in later life (Lucas 1991). Additionally, early life environmental factors significantly affect gene expression patterns (Gibson 2008). Specific gene expression patterns have been identified in infants exposed to arsenic *in utero*. These expression signatures were highly predictive of arsenic exposure and included differential expression of genes involved in stress response, inflammation and apoptosis (Fry *et al.* 2007).

With the evidence that environmental exposures can alter gene expression in infants exposed *in utero* the possibility exists that aflatoxin exposure may act in a similar way. It is therefore important to determine the effects of early life AFB_1 exposure on genome-wide DNA methylation and gene expression patterns that can potentially affect infant development.

For this reason, we have selected a mother-child cohort based in The Gambia to analyse the effects of *in utero* AFB_1 exposure on whole genome DNA methylation and gene expression patterns. The Gambia was selected due to high dietary exposures to aflatoxins consistently observed in this region (Turner *et al.* 2000; Wild *et al.* 2000; Turner *et al.* 2007). Exposure during pregnancy has been confirmed in the selected population in Chapter 4.

Whilst the liver, as the target organ for AFB_1 exposure-related changes, would be the ideal source of tissue for the comprehensive study of exposure-associated whole genome DNA methylation and gene expression alterations, ethical considerations and difficulty of obtaining such samples preclude their use. In such cases, identifying an easily accessible source of cells to discover unique DNA methylation and/or gene expression markers of exposure that also provide some insight into a potential mechanism of exposure-associated health effects is necessary. One of the most accessible sources of cells is the blood, which can be easily obtained, processed and analysed for DNA methylation and gene expression changes using a number of efficient techniques. There are a few studies in the literature supporting the use of blood cells as a surrogate for DNA methylation and gene expression changes in tissues following environmental exposures.

In a large epigenotyping study looking at breast cancer risk and DNA methylation changes in peripheral blood cells, it was found that specific patterns in the blood cells gave a good prediction of breast cancer risk (Widschwendter *et al.* 2008). Also, two different types of breast cancer were associated with different sets of genes that were differentially methylated in blood cells. Interestingly, *ZNF217*, which is hypomethylated in several breast cancer cell lines and tissues, was also hypomethylated in blood cells even after adjusting for family history and age and

was associated significantly with estrogen receptor-α (ER-α) activity in serum (Widschwendter *et al.* 2008).

Another group was able to successfully predict breast cancer outcome in 82% of the samples collected prior to a diagnosis using a gene expression signature in peripheral blood cells (PBCs) (Sharma *et al.* 2005). This was repeated in a larger sample size and it was found that the gene expression profiles predicted outcome successfully in about 80% of the samples. Additionally, the differentially expressed genes were known to be involved in defence response and metabolic processes. These results indicate that changes in the gene expression profile of PBCs can be used for the early detection of breast cancer and can reflect changes to the target tissue (Aaroe *et al.* 2010). In another study, it was found that toxins that affect either the kidney or the liver also produce unique gene expression patterns in PBCs (Dadarkar *et al.* 2010).

Interestingly, Cui et al were able to detect loss of imprinting of *IGF-2* in peripheral blood lymphocyte DNA as an epigenetic change of colorectal cancer (CRC) (Cui *et al.* 2003). This change has also been detected in colon tissue of CRC patients (Cui *et al.* 1998) providing support to the possibility of discovering important mechanistic and predictive markers in the blood that reflect tissue-specific events.

Investigations into the effects of early life exposure to AFB_1 are crucial to our understanding of the health outcomes associated with it. A whole genome approach using DNA and RNA samples from infants exposed to AFB_1 *in utero* will provide a glimpse into the mechanistic interactions of AFB_1 on foetal development.

5.2 Gambian DNA methylation analysis

5.2.1 Methods

5.2.1.1 Sample selection and preparation

A total of 97 infant DNA samples collected at 3-6 months of age were available for analysis. These infants were born to a subset of Gambian women whose AF-alb adduct levels were analysed and described in Chapter 4. A description of the samples is presented in Table 12, showing the mean aflatoxin exposure as determined in analyses described in Chapter 4. DNA was extracted from whole blood cells following the method described in Chapter 2, Section 2.4.1.1.

Table 12: Geometric means (GM) and 95% confidence intervals (in parentheses) of AF-alb adducts in the dry and rainy seasons of conception within high and low AFB_1 exposure groups.

Season	High AFB_1		Low AFB_1		Total	
	N	**GM (pg/mg)**	**N**	**GM (pg/mg)**	**N**	**GM (pg/mg)**
Dry	19	104.4 (76.0, 143.3)	15	13.8 (10.5, 18.1)**	34	42.7 (28.4, 64.4)
Rainy	28	77.7 (60.1, 100.6)	35	14.6 (12.4, 17.1)**	63	30.6 (23.8, 39.5)
Total	47	87.6 (71.9, 106.6)	50	14.3 (12.5, 16.3)**		

N number of samples per group

** $P < 0.001$ between high and low AFB_1 exposure groups

Samples were assigned a randomised code ID and added to the bead chips, ordered by ID. Samples were analysed over four batches and three samples were replicated to measure accuracy of the whole genome DNA methylation analysis. Samples were bisulfite treated and analysed by pyrosequencing for *LINE1* methylation, to ascertain he success of bisulfite treatment, as described in Chapter 2, Section 2.4.1.2. This was followed by analysis using the Illumina Infinium HD 450K DNA Methylation array (Illumina, Freddy van Riemsdijkweg, The Netherlands), described in Chapter 2, Section 2.4.1.3. This whole genome sample analysis was done by Geoffroy Durand, IARC, Lyon, France.

5.2.1.2 Data analysis of genome wide DNA methylation

Illumina's GenomeStudio Methylation module version 1.9 (Illumina, Freddy van Riemsdijkweg, The Netherlands) was used to calculate the methylation level at each CpG site as the beta (β) value. All data analysis was done with input and advice from Dr. Hector Hernandez-Vargas, IARC, Lyon, France.

The detection *P* value for each β value, which represents a statistical test for the difference between the signal for a given probe and background (the average for all negative controls) was also calculated.

β values were then log transformed to obtain the log ratio called the M value, defined as:

$$\mathrm{M} = \log\left[\frac{\beta}{(1-\beta)}\right]$$

M values perform better in detecting true positives in both highly methylated and unmethylated DNA. Additionally, due to the transformation, M values are more suited to several statistical tests including the t test (Du *et al.* 2010). The M values were analysed using RnBeads, which is an R package specific for single CpG resolution analysis and is used extensively in Infinium data processing (Bock 2012). Additionally, the methylumi package sourced from R Studio was used in conjunction to normalise the data (Davis S 2010). Data were normalised by scaling the methylated and unmethylated channel intensities to a reference array (Davis S 2010). Finally, the Combining Batches (ComBat) package was used to remove any batch effect through an empirical Bayes framework (Bock 2012). The complete analysis produced six modules, which are described in Table 13.

Table 13: Description of RnBeads analysis modules.

Module	Description
Loading and normalisation	Summary of sample loading. Data was normalised using the methylumi package
Quality control	Box plots of all quality controls included in the 450K bead chips
Filtering	Utilises a GreedyCut algorithm which filters out probes and samples of low purity. Also removes unreliable measurements with *P* value above 0.01
Batch effects	Determines if any batch effects exist
Methylation profiles	Displays the distribution of methylation values over Infinium I and II probes
Differential methylation	Determines differentially methylated probes either at the individual level or at the region level between sample groups

5.2.1.3 Season of conception as a confounder in DNA methylation analysis

The season at the time of conception has been previously significantly associated with nutritional differences in the Gambia, which in turn can affect foetal development, particularly DNA methylation at this crucial time (Waterland *et al.* 2010). For this reason, the current data was adjusted for season of conception. However, AF-alb levels were measured a few months following conception. Therefore, a separate analysis was conducted only using samples in which the season of conception matched the season of blood collection measurement. For example, if the season of conception of a sample was dry and the season of blood collection measurement for AF-alb analysis was also dry, the sample was included in the analysis. A total of 72 samples out of 97 were classed as matched in this way. These samples are referred to as 'matched samples' in the following sections.

5.2.1.4 Analysis of differential methylation in Gambian infants

Due to the large amount of data generated from the whole genome analysis, the data were corrected for multiple comparisons. CpG sites that were differentially methylated between high and low AFB_1 exposure groups were identified by using a multivariate permutation test (Korn *et al.* 2007) providing 95% confidence that the false discovery rate (FDR) or q value was less than 5%. The FDR is the expected proportion of false discoveries that are present among all significant tests (Benjamini and Hochberg 1995). Random variance t-statistics were used for each CpG site (Wright and Simon 2003). Although t-statistics were used, the multivariate permutation test is non-parametric and does not require the assumption of normality.

Besides analysing changes at the individual probe level, alterations to DNA methylation was also assessed at the region (promoter and gene) level. A change occurring to a probe within the promoter or the gene body may be reflected in surrounding probes within the region. Additionally, when the same change occurs in several sites within a region, there is a greater chance of activation or repression of the expression of that particular gene.

Lists of probes, genes and promoter-level differential methylation were produced through RnBeads after correcting for multiple testing, either adjusted for season of conception or unadjusted in all samples. A list of promoter-level differential methylation was produced for all or matched samples either adjusted or not adjusted for season of conception. Lists were filtered to remove all probes/genes with a q value above 0.05. A shorter list was produced only including genes with at least a 3% difference in methylation levels and with a combined rank of 1000. The combined rank takes the fold change, the difference in methylation values between groups and the corrected *P* value or q value into account such that the higher the rank, the lower the significance, difference or fold change between the two groups. Heatmaps were also produced for gene lists derived at the promoter and gene region level.

5.2.1.5 Pathway analysis of differentially methylated genes

Genes identified as being differentially methylated between the two AFB_1 classes, were examined to determine if they belonged to any pathways involved in varying biological processes. The significant gene lists were used in the pathway analysis. The pathway list was determined using the Database for Annotation, Visualization

and Integrated Discovery (DAVID) v.6.7. DAVID uses sources including GenBank, UniGene, KEGG, and Gene Ontology (GO) to compare input gene lists with large databases. The functional annotation tool makes use of a batch annotation and GO term enrichment analysis to display the most relevant GO terms associated with the inputted gene list (Dennis Jr *et al.* 2003).

5.2.1.6 Validation of differentially methylated genes by pyrosequencing

A total of 30 CpG sites in twelve genes (*BRWD1/AS*, *HOXB2*, *HORMAD2*, *CREB3L3*, *USP17L1*, *PSMA8*, *SLC17A9*, *MIR24-2* and *CD3D*) were selected for validation by pyrosequencing in all 97 Gambian infant DNA samples. These CpG sites were selected both at the promoter and the gene region-level and only if the combined rank was below 1000 and the number of CpG sites within the gene was more than 30. The method used for validation is described in Chapter 2, Section 2.4.1.2. The primer sequences and PCR conditions are summarised in Table 14.

Table 14: Pyrosequencing primer sequences and annealing temperatures for genes selected for validation.

Gene symbol	Forward primer (5' to 3')	Reverse primer (5' to 3')	Sequencing primer(s) (5' to 3')	Annealing temperature
BRWD1/AS	GAGGAAGTATGGTTTAAGGG	TCCTCCTACCTTAATCTTCC	ATTTTTTTTTGGTTA	54.4
CD3D	GTTTTGGGATTATTGGTGTGAG	AAACATCTTCTAATTCCTCCCC	AGTAGTTTTATTTTG (1) ATGTTTTATTTTTTAG (2) GTTGATTTATAGGTAT (3)	53.0
CREB3L3	AGGTGGGTTGGGAGTTGTTT	CTTTCCCAACCTAAACTCCC	AGGGTTGTGAGTTTGT (1) TGGAGTAGAGATTTT (2)	56.0
HORMAD	GTATAGGTGGTTTAGGTTGG	AACAACCCTCACCTCAACT	GGTTGTTGAGGGGT (1) AAGGTYGTTGATTT (2)	56.0
HOXB2 body	AGGAGGAAATAGGTTAGGGAA	AAACCTCTCCCCTAACCTACA	Same as Forward Primer	53.0
HOXB2/AS1	GATTTTGGGAGGGGGAGATTT	CTCTCTATTTTCCACCAAACCC	GTTGATAAATATAA (1) AAATTTAGGATTTTA (2) AAATTATAGGGAATT(3)	56.0
MIR24-2	GGAAGGTAGGGGTTGTAGGT	AACCCTCACCTCCTCTAACC	TTAGTTTAGTAGGT (1) TGGTAGGTAGATAG (2)	54.4
PSMA8	GTTTGGGTGATAAAGGTTGGTAA	CCNATAATAATTACTTCCTCACC	GGAGGTAGTTTGAGG (1) TTTTGTAGTTTTGTG (2) GAATATGTTTAGGAA (3)	53.8

SLC17A9	TTGTTGGGGTTGAGTTAGGG	TCCCAACCCAAACTAACCCA	GATTTAGTTAGGG (1) GTTAGTAGGGTGGGG (2)	58.0
USP17L1P	ATTTGGAGGAGGAGGAGG	CTACCCTCCCAAAACCC	GTTTTAATTAGGTTAG	
USP17L1P (2)	ATAGGGAGTTTTTTAGAATG	CATCTCCATACTAAAATTAC	GTATTTTATATATTG	56.0
USP17L1P (3)	GGTAATTGTGYGGTTATTGT	CCCACATCATAACTTCTAAA	Same as Forward Primer	

For some genes, there were more than one sequencing, forward and/or reverse primers if more than one CpG site was selected.

5.2.2 Results

5.2.2.1 Distribution of differential methylation

5.2.2.1.1 Probe-level analysis

A comparison of high versus low AFB_1 exposure was done at the probe level. While, some probes were identified as being differentially methylated, none had a q value (equivalent to FDR) below 0.05 and were therefore not considered to be significantly different.

5.2.2.1.2 Promoter-level analysis

The comparison of high versus low AFB_1 exposure was repeated at the promoter level in all samples and matched samples either adjusted or not adjusted for season of conception. This was done to determine if different methylation patterns would be observed if adjusted for season of conception. Matched samples were also analysed to test if the fact that AF-alb measurement was done a few months following birth had any bearing on methylation patterns. The numbers of significant probes in these comparisons are displayed in Table 15.

Table 15: Summary of promoter-level differential methylation analysis comparing high versus low AFB_1 exposure groups in all or matched samples either adjusted or not adjusted for season of conception. Results were corrected for multiple comparisons and loci with q values below 0.05 were considered.

Conditions	All Samples (n = 97)		Matched Samples (n = 72)	
	Non-adjusted	Adjusted*	Non-adjusted	Adjusted*
q < 0.05	512	316	213	210
q < 0.05 and combined rank < 1000	180	120	62	23
q < 0.05 and mean difference between groups of at least 3% in either direction	38	32	27	24

* adjusted for season of conception

Gene lists from the four different comparisons revealed similar genes in all lists. In the matched samples gene lists, there was less power due to the smaller sample size. There was a reduced number of significantly different regions compared to gene lists produced when analysing all 97 samples. For this reason, the rest of the analysis was performed on all 97 samples. Additionally, due to a biological effect of season of conception on DNA methylation, all further analysis was done on adjusted data. In the final adjusted gene list at the analysing all 97 samples, the number of hypomethylated genes in the high AFB_1 exposure group compared to the low exposure group with at least a 3% difference in mean methylation levels was 19 and the number of hypermethylated genes was 13.

Some of the significant differentially methylated genes in the adjusted gene list derived from analysing all samples included *CREB3L3*, *AREG*, *SLC17A9*, *IL8*, *IL18*, *CD3D* and *USP17L1P*, which have roles in immune function regulation, cell growth and cell proliferation. A list of significant differentially methylated genes is presented at the promoter-level in Table 16.

Table 16: Gene list derived from promoter-level analysis of DNA methylation in Gambian infants*.

Number	Mean in High AFB_1 group	Mean in Low AFB_1 group	Mean Difference	Adjusted *P* value	Gene Symbol
1	0.2286	0.2935	-0.0649	0.0009	CREB3L3
2	0.4923	0.5295	-0.0373	0.0018	AREG
3	0.6996	0.6708	0.0288	0.0024	TRAJ33
4	0.2455	0.2636	-0.0181	0.0036	SLC17A9
5	0.3190	0.3370	-0.0181	0.0041	GALNT5
6	0.8607	0.8257	0.0350	0.0049	USP17L1P
7	0.2859	0.2569	0.0291	0.0053	TRGV3
8	0.3004	0.3245	-0.0241	0.0059	PCYOX1
9	0.6724	0.6402	0.0321	0.0061	TRAC
10	0.6047	0.5689	0.0358	0.0062	CD3G
11	0.4748	0.4906	-0.0157	0.0064	ALOX5AP
12	0.6754	0.6475	0.0279	0.0080	TRAJ24
13	0.6115	0.5772	0.0343	0.0081	CD3D
14	0.4773	0.5008	-0.0235	0.0085	CHST4
15	0.7798	0.7455	0.0343	0.0115	PSMA8
16	0.6574	0.6897	-0.0323	0.0132	CTAGE5
17	0.1650	0.1774	-0.0125	0.0135	BCL2L2-PABPN1
18	0.5990	0.6260	-0.0270	0.0140	MIR1284
19	0.6836	0.6564	0.0273	0.0142	MIR4761
20	0.3362	0.3535	-0.0173	0.0149	HORMAD2
21	0.6512	0.6055	0.0457	0.0151	CRYGEP
22	0.2379	0.2823	-0.0444	0.0159	HADHB
23	0.2197	0.2335	-0.0138	0.0160	HOXB-AS1
24	0.4844	0.4531	0.0313	0.0161	LTA
25	0.5312	0.5079	0.0233	0.0161	SIT1
26	0.2784	0.2941	-0.0157	0.0161	TYROBP
27	0.6567	0.5782	0.0785	0.0164	RRM2P2
28	0.2966	0.2801	0.0165	0.0169	PGM5P2

29	0.2752	0.2991	-0.0239	0.0186	GSN-AS1
30	0.2020	0.2440	-0.0420	0.0187	IL8
31	0.8985	0.8389	0.0596	0.0193	OR2T11
32	0.2539	0.2292	0.0247	0.0213	AGPAT2
33	0.4007	0.4204	-0.0197	0.0216	NTMT1
34	0.4649	0.4912	-0.0263	0.0217	FLI1
35	0.5584	0.5361	0.0224	0.0221	TRAJ25
36	0.5584	0.5361	0.0224	0.0221	TRAJ26
37	0.3507	0.3758	-0.0250	0.0226	RTN4
38	0.7409	0.7715	-0.0306	0.0234	ATP5A1P3
39	0.5649	0.5942	-0.0294	0.0237	RPL17P40
40	0.4046	0.4238	-0.0192	0.0263	FES
41	0.6172	0.6410	-0.0238	0.0265	CCL26
42	0.6022	0.6332	-0.0310	0.0265	LYZ
43	0.3822	0.3995	-0.0173	0.0279	ZNF124
44	0.4496	0.4617	-0.0121	0.0288	C21orf62
45	0.4682	0.4982	-0.0300	0.0291	EGOT
46	0.1764	0.1947	-0.0183	0.0295	LINGO3
47	0.1705	0.1552	0.0154	0.0304	UBXN11
48	0.3553	0.3692	-0.0139	0.0310	CASP10
49	0.2287	0.2454	-0.0168	0.0313	BIN2P1
50	0.5144	0.5380	-0.0236	0.0315	IPCEF1
51	0.4639	0.4885	-0.0246	0.0319	GPR21
52	0.2638	0.2847	-0.0209	0.0328	CD9
53	0.3456	0.3600	-0.0144	0.0332	WDFY4
54	0.2240	0.2432	-0.0193	0.0338	TRIM13
55	0.4405	0.4533	-0.0128	0.0346	SIRPB2
56	0.5719	0.5443	0.0276	0.0351	GP1BB
57	0.1823	0.2013	-0.0190	0.0369	INTS1
58	0.3986	0.4144	-0.0158	0.0374	SPI1
59	0.2265	0.2062	0.0203	0.0389	HSD17B7P2
60	0.4994	0.5182	-0.0188	0.0395	KDM6B

61	0.3590	0.3777	-0.0187	0.0400	ATP2C1
62	0.3142	0.3315	-0.0173	0.0414	CD302
63	0.5951	0.6204	-0.0253	0.0424	ANKRD22
64	0.4503	0.4776	-0.0273	0.0433	C1orf192
65	0.2851	0.2983	-0.0132	0.0435	MPG
66	0.2771	0.2556	0.0215	0.0437	GPR56
67	0.2675	0.2881	-0.0206	0.0444	HPGDS
68	0.2723	0.2895	-0.0172	0.0453	LMO2
69	0.3521	0.3650	-0.0129	0.0454	WSB2
70	0.3717	0.3559	0.0158	0.0459	ZAP70
71	0.5357	0.5574	-0.0217	0.0460	IL18
72	0.2580	0.2735	-0.0155	0.0465	MTMR9LP
73	0.2935	0.2764	0.0171	0.0475	PGM5P1
74	0.4808	0.4944	-0.0136	0.0479	MMP14
75	0.5368	0.5587	-0.0219	0.0485	FUT7
76	0.2897	0.3221	-0.0324	0.0487	ADAM28
77	0.2171	0.2312	-0.0140	0.0489	C15orf62
78	0.3706	0.3873	-0.0168	0.0489	FCGRT

*Only genes with a combined rank less than 1000 are included in the table.

A heatmap of all significant probes at the promoter region level was also produced (Figure 10). Differences between the two exposure groups were not very clear due to the significant changes between groups being relatively small. However, some patterns can be observed in healthy infants exposed to AFB_1.

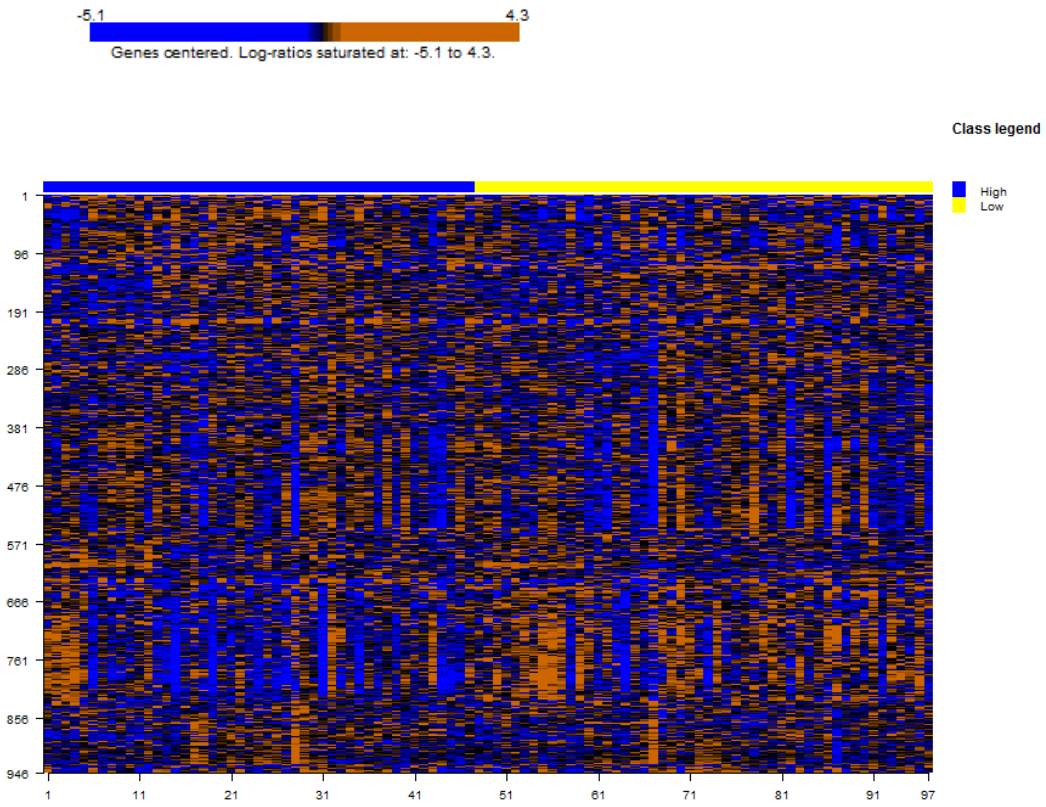

Figure 10: Heatmap of all significant probes at the promoter region level across all 97 samples when comparing high versus low *in utero* exposure in Gambian infants. Within the heatmap, blue represents hypomethylation while orange represents hypermethylation. The samples are divided by exposure groups: high (blue) and low (yellow).

5.2.2.1.3 Gene-level analysis

The comparison of high versus low AFB_1 exposure was also repeated at the gene level in all samples adjusted for season of conception. There were 261 differentially methylated genes (96 excluding non-annotated genes (NAs)) when comparing high

and low AFB_1 exposure groups. Twenty-five (11 excluding NAs) of these had a combined rank of less than 1000 and a mean difference of at least 3% between groups in either direction. The number of hypomethylated genes in the higher AFB_1 exposure group compared to the low exposure group with at least a 3% difference in mean methylation levels was 10 and the number of hypermethylated genes was 15.

Some of the genes in the significant gene list included *BRWD1-AS1*, *CD3D*, *CREB3L3*, *GDF7*, *GPR21* and *HOXB2*. Some of these genes were also differentially methylated at the promoter-level. These genes are mostly involved in immune function regulation, cell cycle and transcription. A list of the significant differentially methylated genes at the gene-level is presented in Table 17.

Table 17: Gene list derived from gene-level analysis of DNA methylation in Gambian infants*.

Number	Mean in High AFB_1 group	Mean in Low AFB_1 group	Mean Difference	Adjusted P value	Gene Symbol
1	0.2614	0.2395	0.0219	0.0010	PGM5P2
2	0.1764	0.2014	-0.0249	0.0037	MIR628
3	0.7581	0.7108	0.0473	0.0041	PSMA8
4	0.4517	0.5057	-0.0540	0.0082	SULT1E1
5	0.2516	0.2322	0.0194	0.0089	PGM5P1
6	0.1934	0.2086	-0.0153	0.0106	MIR24-2
7	0.5932	0.5635	0.0297	0.0117	IGSF6
8	0.3543	0.3700	-0.0157	0.0120	HOXB2
9	0.7604	0.7337	0.0267	0.0121	SNORD31
10	0.3399	0.3577	-0.0178	0.0130	MIR27A
11	0.6482	0.6250	0.0231	0.0139	SIT1
12	0.6255	0.5963	0.0291	0.0153	CD3D
13	0.2862	0.3032	-0.0170	0.0156	NEK4P2
14	0.5093	0.4807	0.0286	0.0158	LTA
15	0.6567	0.5782	0.0785	0.0164	RRM2P2
16	0.3224	0.2808	0.0416	0.0171	GDF7
17	0.7244	0.7543	-0.0299	0.0173	TNFAIP6
18	0.4506	0.4787	-0.0281	0.0190	BRWD1-AS1
19	0.7069	0.6824	0.0244	0.0194	OR10T2
20	0.8419	0.8102	0.0318	0.0221	ABCD1P3
21	0.7030	0.6776	0.0254	0.0236	SNORD12
22	0.5939	0.5628	0.0311	0.0252	KLHL35
23	0.7495	0.7091	0.0404	0.0272	ANKRD20A19P
24	0.6250	0.5965	0.0285	0.0298	SNORD97

25	0.6379	0.6636	-0.0257	0.0301	SLPI
26	0.4030	0.3629	0.0401	0.0306	OR4N5
27	0.3552	0.3754	-0.0202	0.0309	EMR4P
28	0.3977	0.4384	-0.0407	0.0311	CREB3L3
29	0.2644	0.2456	0.0187	0.0316	RXFP3
30	0.1922	0.2122	-0.0200	0.0338	MIR647
31	0.6485	0.6779	-0.0295	0.0354	CCIN
32	0.5402	0.5737	-0.0335	0.0377	MIR140
33	0.2091	0.1940	0.0151	0.0379	CD52
34	0.4921	0.5158	-0.0237	0.0388	GPR21
35	0.2529	0.2415	0.0114	0.0400	SNORA14B
36	0.7647	0.7414	0.0233	0.0418	FASLG
37	0.6272	0.6546	-0.0274	0.0421	SNORA38
38	0.7292	0.6970	0.0322	0.0464	PAEP
39	0.3284	0.3115	0.0169	0.0465	RNU7-27P
40	0.1509	0.1646	-0.0137	0.0478	CYTL1

*Only genes with a combined rank less than 1000 are included in the table.

A heatmap of all significant probes at the gene region level was produced (Figure 11). Differences between the two exposure groups were not very clear due to the significant changes between groups being relatively small. However, some patterns in gene methylation can be observed in healthy infants exposed to AFB_1.

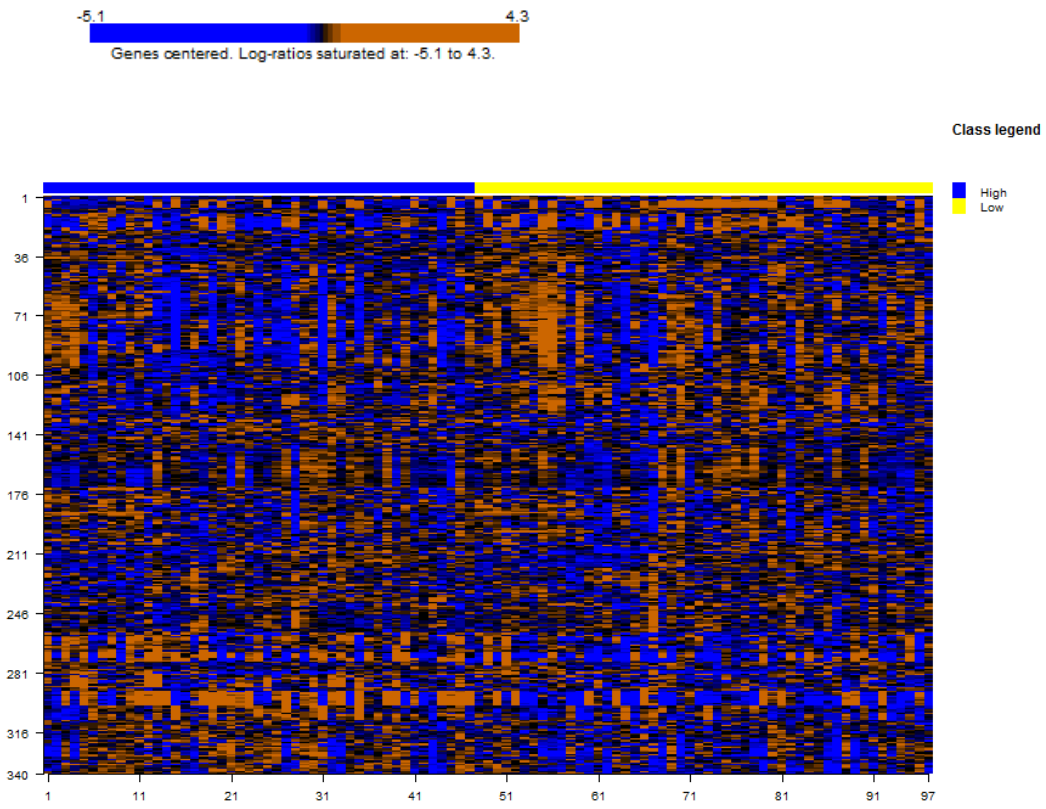

Figure 11: Heatmap of significant probes at the gene region level across all 97 samples when comparing high versus low *in utero* exposure in Gambian infants. Within the heatmap, blue represents hypomethylation while orange represents hypermethylation. The samples are divided by exposure groups: high (blue) and low (yellow).

5.2.2.2 Pathway analysis

Pathway analysis using DAVID v.6.7 revealed significant pathway categories using the Biocarta and KEGG databases both at the promoter and the gene level (Table 18).

Table 18: Summary of DAVID pathway analysis for gene lists derived from methylation analyses at the promoter and gene region-level.

	Pathway term	Pathway Category	Number of genes	P Value	Genes
		Promoter level			
Biocarta	h_il17Pathway	IL 17 Signalling Pathway	5	2.44E-06	*CD3G, CD3D, IL8, CD2, CD4*
	h_tcraPathway	Lck and Fyn tyrosine kinases in initiation of TCR Activation	4	5.33E-05	*CD3G, CD3D, ZAP70, CD4*
	h_thelperPathway	T Helper Cell Surface Molecules	4	7.08E-05	*CD3G, CD3D, CD2, CD4*
	h_CSKPathway	Activation of Csk by cAMP-dependent Protein Kinase Inhibits Signalling through the T Cell Receptor	4	2.14E-04	*CD3G, CD3D, ZAP70, CD4*
	h_tcapoptosisPathway	HIV Induced T Cell Apoptosis	3	0.001864	*CD3G, CD3D, CD4*
	h_tcytotoxicPathway	T Cytotoxic Cell Surface Molecules	3	0.003374	*CD3G, CD3D, CD2*
	h_IL12Pathway	IL12 and Stat4 Dependent Signalling Pathway in Th1 Development	3	0.009393	*CD3G, CD3D, IL18*
	h_cytokinePathway	Cytokine Network	3	0.009393	*IL8, IL18, LTA*
	h_inflamPathway	Cytokines and Inflammatory Response	3	0.015667	*IL8, CD4, LTA*
	h_tcrPathway	T Cell Receptor Signalling Pathway	3	0.026153	*CD3G, CD3D, ZAP70*
	h_tcrmolecule	T Cell Receptor and CD3 Complex	2	0.030301	*CD3G, CD3D*

KEGG	hsa04640	Hematopoietic cell lineage	6	4.51E-04	*CD9, CD3G, CD3D, GP1BB, CD2, CD4*
	hsa05340	Primary immunodeficiency	3	0.029086	*CD3D, ZAP70, CD4*
	hsa04660	T cell receptor signalling pathway	4	0.04901	*CD3G, CD3D, ZAP70, CD4*
		Gene level			
Biocarta	h_tcapoptosisPathway	HIV Induced T Cell Apoptosis	2	0.043115	*CD3D, FASLG*
KEGG	hsa04740	Olfactory transduction	6	0.008521	*OR2A7, OR10T2, OR4N5, OR52E4, OR13C8, OR4D6*

Most of the identified pathways were related to the regulation of the immune response including cytokine signalling with *CD3D*, *CD3G*, *CD4*, *IL8* and *IL18* featuring in most significant pathways.

5.2.2.3 Validation of differential DNA methylation by pyrosequencing

Validation was done in the 97 DNA samples, already analysed for whole genome DNA methylation changes. This was, therefore, a technical validation of the changes in specific genes using an alternate method i.e. pyrosequencing.

Twelve genes with a total of 29 CpG sites were selected for validation by pyrosequencing for all 97 DNA samples. This was done to determine if the observed changes were present when analysing using an alternate method. Of these, 12 CPG sites in *CD3D*, *CREB3L3*, *HORMAD2*, *HOXB2*, *HOXB2* (body), *PSMA8* and *SLC17A9* were significantly different between high and low AFB_1 exposure groups (Table 19 and Figure 12).

Table 19: Number of significant and non-significant CpG sites in 12 genes selected for validation by pyrosequencing.

Gene	Number of CpG sites		
	Total	**Significant**	**Not Significant**
BRWD1	1	0	1
CD3D	3	1	2
CREB3L3	2	2	0
USP17L1P	3	0	3
HORMAD2	4	2	2
HOXB2	7	5	2
MIR24	2	0	2
PSMA8	3	1	2
SLC17A9	4	1	3

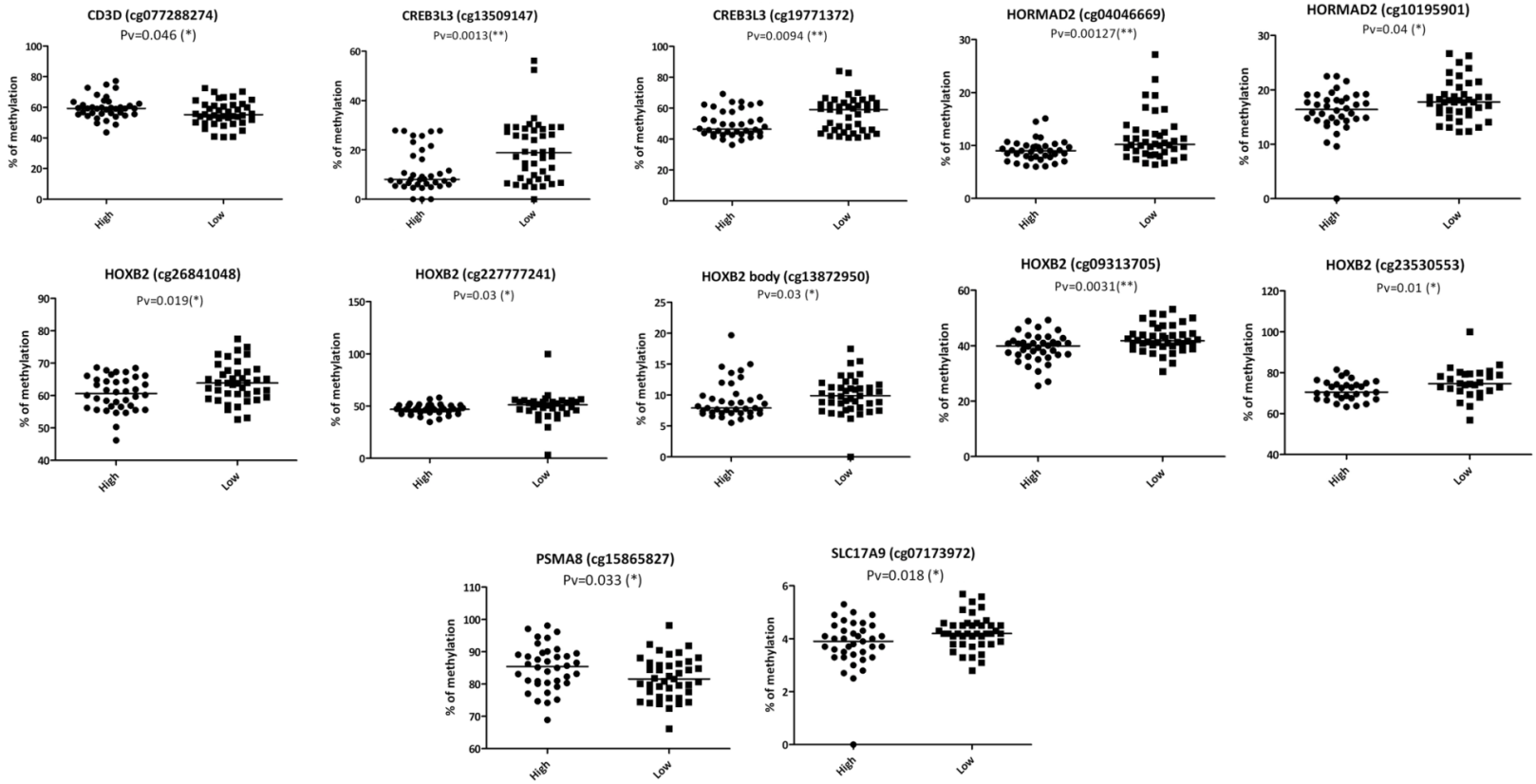

Figure 12: Graphs of CpG sites successfully validated by pyrosequencing. Methylation percentages are presented per AFB_1 exposure group per graph. Each dot per graph represents a sample with the median AFB_1 level per exposure group represented by a line in each cluster. The *P* value per comparison is displayed beneath the gene symbol and CpG unique Illumina ID. *CD3D* and *PSMA8* are significantly hypermethylated in the high AFB_1 exposure group relative to methylation in the low exposure group while the remaining genes (*CREB3L3*, *HORMAD2*, *HOXB2* and *SLC17A9*) are hypomethylated.

5.3 Gambian infant gene expression

5.3.1 Methods

5.3.1.1 Sample selection and preparation

A total of 52 infant whole blood-cell samples collected at 3-6 months of age were available for analysis. Total RNA was extracted from the samples and stored in RNAlater immediately, as described in Chapter 2, Section 2.4.2.1. RNA quality was determined using an Agilent 2100 Bioanalyser (Agilent Technologies, Berkshire, UK), as described in Chapter 2, Section 2.4.2.2. All RNA samples had an RNA integrity number (RIN) > 6.

Twenty-two of these samples belonging to the dry season were selected for whole genome gene expression analysis. Information on the corresponding maternal aflatoxin exposure and season of blood sample collection is displayed in Table 20.

Table 20: Geometric mean levels of AF-alb in AFB_1 exposure groups included in the whole genome gene expression analysis.

	N	AF-alb GM (95% confidence intervals)
AFB_1 exposure group		
Low	11	8.9 (7.0, 11.2)
High	11	107.5 (76.5, 151.1)**

N number of samples per group

** $P < 0.001$ between high and low AFB_1 exposure groups

Two samples were duplicated to measure accuracy of the whole genome gene expression analysis. Samples were assigned a randomised code ID and added to the bead chips, ordered by ID. All samples were analysed in one batch precluding the effects of batch variability. Samples were then analysed using the Illumina Human HT12-v4 Expression BeadChip Kit (Illumina, Freddy van Riemsdijkweg, The Netherlands), described in Chapter 2, Section 2.4.2.2.

5.3.1.2 Data analysis

Raw data in the form of expression values were imported and analysed on BRB ArrayTools software (version 4.3.0, developed by Dr. Richard Simon and BRB-ArrayTools Development Team) using the *lumi* package, which is specifically designed for Illumina data (Du *et al.* 2008). The package is installed through R statistical software (version i386 2.15.2, RStudio, Massachusetts, USA). This data analysis was done with input and advice from Dr. Hector Hernandez-Vargas, IARC, Lyon, France.

Each probe on the BeadChips was identified via a Nucleotide Universal Identifier (nuID), which is unique to the oligonucleotide sequence, globally accepted as unique indentifiers and includes an algorithm to check for errors (Du *et al.* 2007b). The *lumi* package makes use of Variance Stabilization Transformation (VST), which takes advantage of the large number of technical replicates per array (Du *et al.* 2008). VST stabilises the spatially random technical replicates, reduces both the signal-to-noise ratio and the number of false positive identifications (Lin *et al.* 2008).

Following the pre-processing steps using *lumi,* the data was first $\log_2$ transformed followed by normalisation using Robust Spline Normalisation (RSN), which utilises

continuous mapping, preserves the rank order and is not affected by a large number of differentially expressed genes as compared to other methods of normalisation including Quantile and Loess normalisation. With the RSN form of normalisation, the first array is calibrated against a reference array and the intensities of probes of differentially expressed genes are down-weighted (Du *et al.* 2007a). Data was also corrected for multiple testing using t-statistics and multivariate permutation analysis, which generates a q value per loci per comparison. Only q values < 0.05 were accepted.

5.3.1.2.1 Scatter plots and unsupervised clustering

To check quality control, scatter plots of the technical replicates were produced and unsupervised clustering was done to determine if the samples grouped according to AFB_1 exposure.

5.3.1.2.2 Data filtering

Following normalisation, the data were then filtered to exclude less than 25% of genes that have at least a 1.3-fold change in expression data in either direction of the gene's median expression value. Also, probes with more than 50% of samples missing expression values for that probe were excluded from the analysis. These settings improved the number of hits obtained following class comparison and quantitative trait analyses (Table 21).

Table 21: Comparison of different filtering conditions in terms of hits obtained following class comparison analysis.

Data filtering conditions	*N*
No filter	0
Excluding genes with more than 50 % missing data	0
and 20 % genes with less than 2-fold change	0
and 20 % genes with less than 1.5-fold change	8
and 20 % genes with less than 1.3-fold change	228
and 25 % genes with less than 1.5-fold change	7
and 25 % genes with less than 1.3-fold change	138
and 30 % genes with less than 1.5-fold change	5
and 30 % genes with less than 1.3-fold change	15

N number of probes with a false discovery rate (FDR) or q value less than 10 %

5.3.1.2.3 Class comparison analysis

Using the filtering conditions of excluding genes with more than 50% missing data and 20% genes with at least a 1.3-fold difference in either direction between groups and with a q value cut-off at 0.1, a class comparison was conducted to compare the high and low AFB_1 exposure groups. Unsupervised and supervised clustering and heatmaps were then produced for a gene list containing only probes repeated more than once and probes with a high fold-change.

5.3.1.2.4 Quantitative trait analysis

To further analyse the relationship between gene expression and AFB_1 exposure, Quantitative Trait Analysis (QTA) was conducted using both early and later AF-alb levels. This analysis determines the extent of correlation between expression values of probes and actual AF-alb levels.

5.3.1.3 Pathway analysis

Genes identified as being differentially expressed between the two AFB_1 classes, were examined to determine if they belonged to any pathways involved in varying biological processes. The final gene list (40 genes), consisting of genes with at least two significant probes or at least a 1.5-fold difference between classes, was used to in the pathway analysis with DAVID v.6.7.

5.3.1.4 Validation of gene expression changes by qPCR

Using specific criteria, twelve genes were selected for validation from the gene list consisting of only significant probes that were repeated more than once and with a high fold-change between exposure groups. These genes along with the selection criteria are described in Table 22.

Table 22: Gene selection for gene expression validation.

	Gene Symbol	Fold-change*	Selection criteria
1	*BOLA3*	1.29	More than one probe; interest
2	*C19orf60*	1.36	High fold-change, more than one probe and low FDR
3	*CD3D*	1.51	High fold-change and more than one probe
4	*CENPT*	1.33	More than one probe and low FDR
5	*ID2*	1.26	More than one probe; interest
6	*Il27RA*	1.42	High fold change
7	*MBP*	0.62	Low fold-change and more than one probe
8	*NCOR2*	1.42	High fold change
9	*PTPRC*	0.61	Low fold-change and more than one probe
10	*RIOK3*	0.37	High fold change and more than one probe
11	*STAT3*	0.75	More than one probe; interest
12	*USP4*	0.82	Top gene in Quantitative Trait Analysis (correlation coefficient = 0.839)

* Fold change derived by comparing expression in the low AFB_1 exposure group to that in the high exposure group. For example, with a fold-change of 1.29, *BOLA3* expression is higher in the low exposure group than in the high exposure group.

Selected genes were validated by qPCR, described in Chapter 2, Section 2.3.6. The primer sequences and PCR conditions for each gene are described in Table 23.

Table 23: Primer sequences and annealing temperatures for genes selected for validation by qPCR.

Gene Symbol	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
BOLA3	TTCCACGAGCTACAGCTATAAAAG	CATCCAAGGTCTTAAGCAGCA
C19orf60	TTGTGTGGCTGCAGAGTCAGAT	AGAGAGAAGCCACGAAGAGTGAGT
CD3D	CACCTATATATTCCTCGTGGGTCC	ATACCAGCATCACATGGGTAGAGG
CENPT	CCAACCCTCCAGACAAGAGA	GGCTCGAGCTCAGGAAGTT
MBP	TTTGGCATCACGCTGACTACTCCT	TATCCTTCTTAACACCCGCTGGCA
NCOR2	GCACGAGGTGTCAGAGATCA	CTGGCGCATCTGCTTCTC
PTPRC	TGAACTGAGACATTCCAAGAGG	TGTTTGACGACCTGAATCATAGA
STAT3	AACTTCAGACCCGTCAACAAA	GGGTCCCCTTTGTAGGAAAC
ID2	TGTCAAATGACAGCAAAGCAC	GTTGTTGTTGTGCAAAGAATAAAAG
IL27	ATCCTGGAAGTGGAGGAGAT	GCTTCTCATACCCAGAGTCAAG
RIOK3	CCATGATTGTGCCACTGTACT	CCACCTCACCTGGCTTATTT
USP4	GATCGAGTTATGGAGGTTTTCCT	CGGCACAGTCACACGGTA
HPRT1	CATTGTAGCCCTCTGTGTGC	CACTATTTCTATTCAGTGCTTTGATGT

5.3.1.5 Influence of season of measurement on *USP4* and *STAT3* expression

Univariate regression and correlation between *USP4*, *STAT3* (3 probes); season of measurement; and AF-alb levels were tested using Stata v.11 (StataCorp, College Station, TX, USA). Season of measurement was defined as the season of blood collection for AF-alb measurement. November-May was considered to be the dry season while June-October was classed as the rainy season.

5.3.2 Results

The initial stages of the analysis involved determining the quality control of the data. A boxplot of the samples with and without RSN normalisation is shown in Figure 13. One sample appeared to be an outlier compared to the other samples prior to normalisation and so was removed from further analysis. This analysis was compared to analysis on all samples and was found to be similar. Therefore, for all reported analysis, all samples were included.

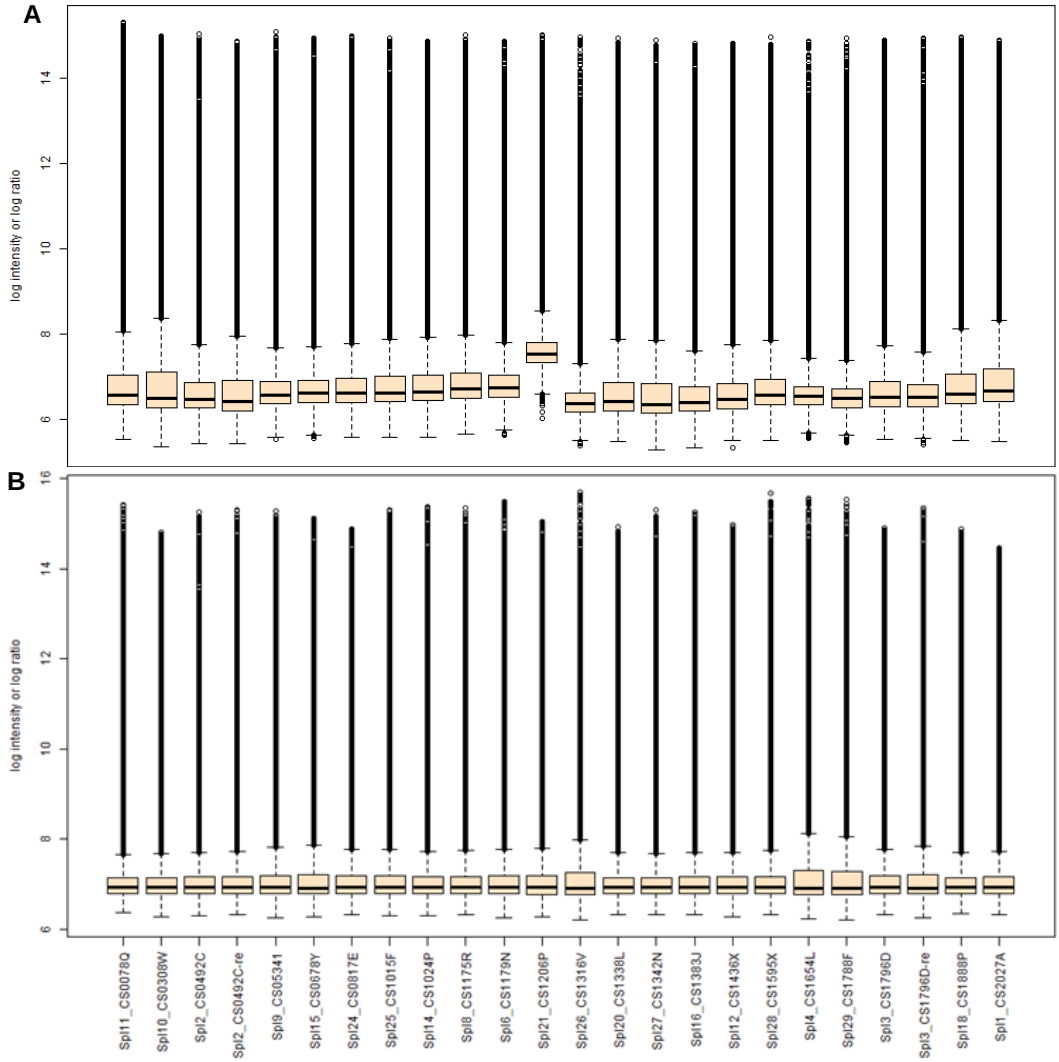

Figure 13: Boxplot of the log intesities of ratios of all genes on the bead chips for 22 (+2 technical replicates) samples showing raw data (A) and robust spline normalised data (B). Several outliers are present potentially due to the variability in gene expression by treatment group. Normalisation removes any technical variability by calibrating against expression intensities on a reference array.

5.3.2.1 Scatter plots and unsupervised clustering

Scatter plots comparing overall gene expression in the technical replicates included in the analysis (Figure 14) reveal strong correlation of gene expression between them.

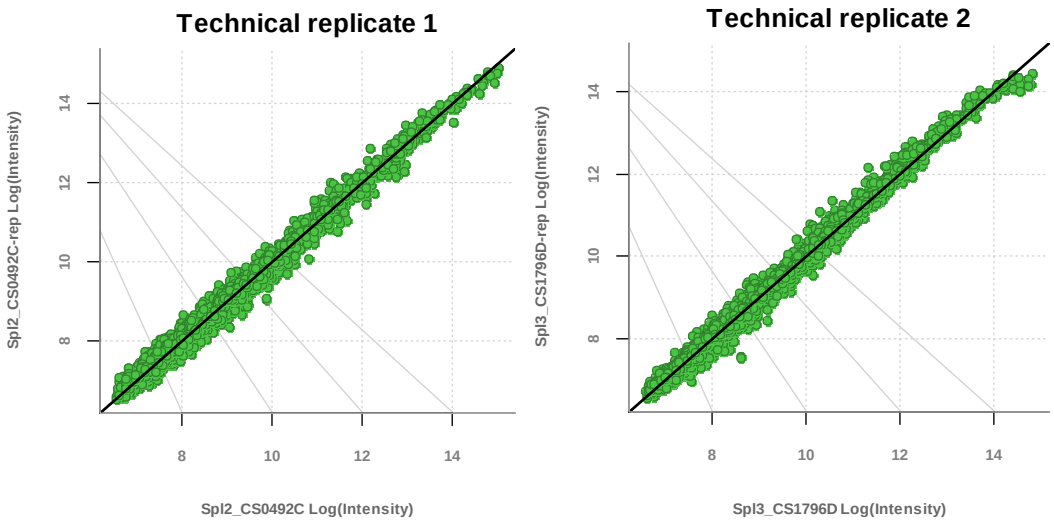

Figure 14: Scatter plot of technical replicates showing strong correlation of gene expression between replicates.

The dendrogram, displayed in Figure 15, shows expected mixed clustering with low correlation coefficients according to AFB_1 exposure grouping. As the analysed RNA samples were from relatively healthy infants, major changes in gene expression patterns would not be expected. However, *in utero* exposure to AFB_1 could alter gene expression profiles resulting in a AFB_1-specific signature.

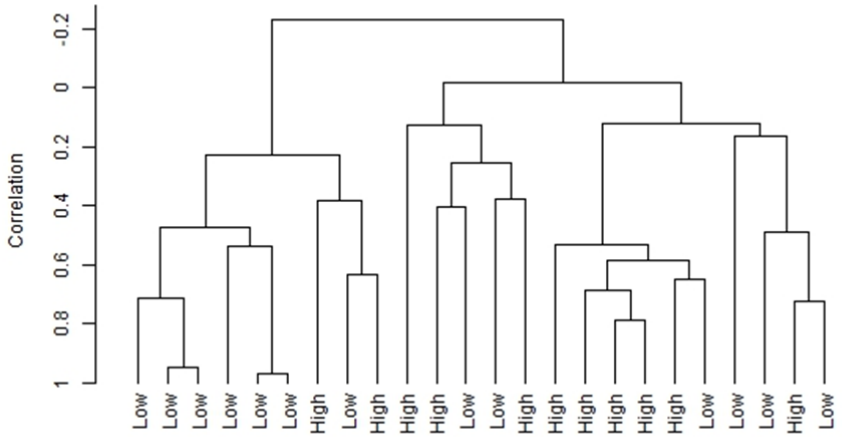

Figure 15: Dendrogram of unsupervised sample clustering showing correlation between sample linkages. Most samples are only weakly correlated to each other. A few samples (including the technical replicates) cluster closely together with relatively higher correlation coefficients.

5.3.2.2 Class comparison analysis

The complete list of genes that are differentially expressed between the two groups using filtering conditions of excluding genes with more than 50% missing data and 20% genes with at least a 1.3-fold difference in either direction between groups conditions and with an FDR below 10% is presented in Table 24.

A total of 30 probe IDs did not correspond to coding genes and 197 probes corresponding to coding genes with differential expression have been associated with AFB_1 exposure groups.

Table 24: Class comparison analysis with at least a 1.3-fold change in differential gene expression between AFB_1 exposure groups and with FDR/q value below 10 %.

No.	Parametric p-value	FDR	Geometric mean of intensities in Low AFB_1 exposure group	Geometric mean of intensities in High AFB_1 exposure group	Fold-change	Gene Symbol	Gene Name	Entrez ID
1	3.30E-05	0.0387	198.92	274.08	0.73	*CENPT*	centromere protein T	80152
2	3.98E-05	0.0387	32121.21	24300.87	1.32	*CLUAP1*	clusterin associated protein 1	23059
3	4.22E-05	0.0387	35911.06	27147.09	1.32	*LAIR1*	leukocyte-associated immunoglobulin-like receptor 1	3903
4	4.92E-05	0.0387	27566.32	20476.92	1.35	*SLC7A5P2*	solute carrier family 7 (amino acid transporter light chain, L system), member 5 pseudogene 2	387254
5	7.24E-05	0.0413	303.9	203.71	1.49	*CDK11A*	cyclin-dependent kinase 11A	728642
6	9.51E-05	0.0468	405.98	579.94	0.7	*C19orf60*	chromosome 19 open reading frame 60	55049
7	0.000156	0.0594	225.38	177.47	1.27	*MOSPD2*	motile sperm domain containing 2	158747
8	0.000166	0.0594	1024.87	1823.48	0.56	*RPL10A*	ribosomal protein L10a	4736
9	0.000209	0.062	374.08	498.89	0.75	*MALSU1*	mitochondrial assembly of ribosomal large subunit 1	115416
10	0.000228	0.062	595.75	782.73	0.76	*POLR3GL*	polymerase (RNA) III (DNA directed) polypeptide G (32kD)-like	84265
11	0.000244	0.062	25206.81	19177.2	1.31	*NAG18*	NAG18 mRNA	57051
12	0.000299	0.062	1059.28	599.66	1.77	*YY1*	YY1 transcription factor	7528

13	0.000327	0.062	433.72	246.5	1.76	*PTPRC*	protein tyrosine phosphatase, receptor type, C	5788
14	0.000352	0.062	245.08	343.19	0.71	*ZNF266*	zinc finger protein 266	10781
15	0.00037	0.062	632.48	812.37	0.78	*CCDC130*	coiled-coil domain containing 130	81576
16	0.000415	0.062	220.82	312.82	0.71	*ZP3*	zona pellucida glycoprotein 3 (sperm receptor)	7784
17	0.000443	0.062	958.48	1230.7	0.78	*UBXN1*	UBX domain protein 1	51035
18	0.000465	0.062	481.19	649.74	0.74	*ARHGEF1*	Rho guanine nucleotide exchange factor (GEF) 1	9138
19	0.00047	0.062	25268.34	18534.95	1.36	*F2R*	coagulation factor II (thrombin) receptor	2149
20	0.000471	0.062	336.25	209.3	1.61	*ZNF148*	zinc finger protein 148	7707
21	0.000475	0.062	707.79	523.49	1.35	*GATAD2A*	GATA zinc finger domain containing 2A	54815
22	0.000478	0.062	571.54	777.45	0.74	*LSM3*	LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae)	27258
23	0.000489	0.062	384.9	518.31	0.74	*MRPL21*	mitochondrial ribosomal protein L21	219927
24	0.000513	0.063	518.4	335.49	1.55	*CCNY*	cyclin Y	219771
25	0.000592	0.0692	510.05	726.2	0.7	*IL27RA*	interleukin 27 receptor, alpha	9466
26	0.000598	0.0692	192.86	246.14	0.78	*CENPT*	centromere protein T	80152
27	0.000641	0.0704	41012.61	31243.12	1.31	*HBA1*	hemoglobin, alpha 1	3039
28	0.000844	0.0861	1037.41	807.77	1.28	*SPOP*	speckle-type POZ protein	8405

29	0.000854	0.0861	545.17	319.18	1.71	*MBP*	myelin basic protein	4155
30	0.000963	0.0874	444.06	285.27	1.56	*MBP*	myelin basic protein	4155
31	0.000971	0.0874	299.26	446.11	0.67	*FLT3LG*	fms-related tyrosine kinase 3 ligand	2323
32	0.000997	0.0874	795.13	1116.6	0.71	*ID2*	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	3398
33	0.001024	0.0874	834.92	1182.37	0.71	*AES*	amino-terminal enhancer of split	166
34	0.001032	0.0874	213.59	171.49	1.25	*GTF2I*	general transcription factor IIi	2969
35	0.001079	0.0874	852.26	1118.78	0.76	*COMMD3*	COMM domain containing 3	23412
36	0.001116	0.0874	321.34	240.64	1.34	*AMD1*	adenosylmethionine decarboxylase 1	262
37	0.001128	0.0874	301.97	223.13	1.35	*EIF4E3*	eukaryotic translation initiation factor 4E family member 3	317649
38	0.001149	0.0874	533.92	292.85	1.82	*PTPRC*	protein tyrosine phosphatase, receptor type, C	5788
39	0.00118	0.0874	454.88	335.45	1.36	*ARID4B*	AT rich interactive domain 4B (RBP1-like)	51742
40	0.001209	0.0874	287.5	220.12	1.31	*CAMK1D*	calcium/calmodulin-dependent protein kinase ID	57118
41	0.001227	0.0874	181.95	232.03	0.78	*TMEM141*	transmembrane protein 141	85014
42	0.001244	0.0874	234.41	297.25	0.79	*LMF2*	lipase maturation factor 2	91289
43	0.001244	0.0874	459.18	563.86	0.81	*DPM1*	dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit	8813
44	0.001249	0.0874	454.54	563.23	0.81	*RABAC1*	Rab acceptor 1 (prenylated)	10567

45	0.001278	0.0874	42967.7	33338.66	1.29	*HBA2*	hemoglobin, alpha 2	3040
46	0.001288	0.0874	248.09	320.5	0.77	*SSBP4*	single stranded DNA binding protein 4	170463
47	0.001355	0.0889	185.21	252.4	0.73	*ZNF33B*	zinc finger protein 33B	7582
48	0.001368	0.0889	243.11	319.35	0.76	*FYN*	FYN oncogene related to SRC, FGR, YES	2534
49	0.001425	0.0893	174.23	230.7	0.76	*CCDC84*	coiled-coil domain containing 84	338657
50	0.001515	0.0893	1593.08	2116.22	0.75	*EDF1*	endothelial differentiation-related factor 1	8721
51	0.001537	0.0893	147.18	189.62	0.78	*NPHP3*	nephronophthisis 3 (adolescent)	27031
52	0.00156	0.0893	795.04	1073.35	0.74	*SRP14P1*	signal recognition particle 14kDa (homologous Alu RNA binding protein) pseudogene 1	390284
53	0.001569	0.0893	4339.92	5566.81	0.78	*SNRPD2*	small nuclear ribonucleoprotein D2 polypeptide 16.5kDa	6633
54	0.001572	0.0893	2111.97	1632.35	1.29	*ALKBH5*	alkB, alkylation repair homolog 5 (E. coli)	54890
55	0.001578	0.0893	151.37	206.62	0.73	*MIAT*	myocardial infarction associated transcript (non-protein coding)	440823
56	0.001624	0.0893	447.07	611.5	0.73	*ACAP1*	ArfGAP with coiled-coil, ankyrin repeat and PH domains 1	9744
57	0.001629	0.0893	39455.17	30685.92	1.29	*HBB*	hemoglobin, beta	3043
58	0.001638	0.0893	138	184.58	0.75	*ZNF589*	zinc finger protein 589	51385
59	0.001726	0.0893	323.31	415.53	0.78	*C19orf60*	chromosome 19 open reading frame 60	55049
60	0.001729	0.0893	360.61	471.91	0.76	*ATP1A1*	ATPase, Na+/K+ transporting, alpha 1 polypeptide	476

61	0.001763	0.0893	307.76	388.43	0.79	*EMG1*	EMG1 nucleolar protein homolog (S. cerevisiae)	10436
62	0.001774	0.0893	382.53	234.2	1.63	*EIF2C2*	eukaryotic translation initiation factor 2C, 2	27161
63	0.001803	0.0893	321.91	423.21	0.76	*NGDN*	neuroguidin, EIF4E binding protein	25983
64	0.001822	0.0893	184.15	221.39	0.83	*MDP1*	magnesium-dependent phosphatase 1	145553
65	0.00184	0.0893	2876.88	4314.84	0.67	*HCST*	hematopoietic cell signal transducer	10870
66	0.001849	0.0893	313.49	421.55	0.74	*PPP1R35*	protein phosphatase 1, regulatory subunit 35	221908
67	0.001861	0.0893	181.28	223.75	0.81	*C18orf21*	chromosome 18 open reading frame 21	83608
68	0.001872	0.0893	329.35	252.34	1.31	*STAT3*	signal transducer and activator of transcription 3 (acute-phase response factor)	6774
69	0.001926	0.0893	238.27	303.24	0.79	*RPL34*	ribosomal protein L34	6164
70	0.001931	0.0893	196.97	259.16	0.76	*RRP7A*	ribosomal RNA processing 7 homolog A (S. cerevisiae)	27341
71	0.001955	0.0894	144.59	183.23	0.79	*CA5B*	carbonic anhydrase VB, mitochondrial	11238
72	0.001988	0.0899	210.3	157.22	1.34	*TRIM23*	tripartite motif containing 23	373
73	0.002034	0.0901	1131.18	1517.07	0.75	*LTB*	lymphotoxin beta (TNF superfamily, member 3)	4050
74	0.002097	0.0901	40246.96	31091.59	1.29	*HBG2*	hemoglobin, gamma G	3048
75	0.002108	0.0901	170.38	217.72	0.78	*ACYP2*	acylphosphatase 2, muscle type	98
76	0.002148	0.0901	757.34	1065.26	0.71	*NDUFS8*	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)	4728

77	0.002158	0.0901	816.17	1165.21	0.7	*RPS27L*	ribosomal protein S27-like	51065
78	0.002243	0.0901	394.5	493.86	0.8	*MRPL21*	mitochondrial ribosomal protein L21	219927
79	0.002255	0.0901	36973.49	29498.22	1.25	*TMSB4X*	thymosin beta 4, X-linked	7114
80	0.002261	0.0901	1052.3	1313.62	0.8	*AKNA*	AT-hook transcription factor	80709
81	0.002269	0.0901	244.88	176.88	1.38	*PTPRC*	protein tyrosine phosphatase, receptor type, C	5788
82	0.002295	0.0901	671.32	466.92	1.44	*WSB1*	WD repeat and SOCS box containing 1	26118
83	0.002296	0.0901	414.94	550.56	0.75	*CCNK*	cyclin K	8812
84	0.002307	0.0901	339.98	483.89	0.7	*ECHDC2*	enoyl CoA hydratase domain containing 2	55268
85	0.002313	0.0901	353.78	236.77	1.49	*AKIRIN1*	akirin 1	79647
86	0.002328	0.0901	318.22	422.73	0.75	*TSPAN32*	tetraspanin 32	10077
87	0.002356	0.0901	229.81	310.7	0.74	*DGKQ*	diacylglycerol kinase, theta 110kDa	1609
88	0.002401	0.0901	138.51	170.88	0.81	*RPL23AP5*	ribosomal protein L23a pseudogene 5	729480
89	0.002406	0.0901	1529.2	2052.18	0.75	*NDUFB2*	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2, 8kDa	4708
90	0.002441	0.0906	1435.51	1870.49	0.77	*SS18L2*	synovial sarcoma translocation gene on chromosome 18-like 2	51188
91	0.002476	0.091	351.48	251.68	1.4	*BIRC2*	baculoviral IAP repeat containing 2	329
92	0.002598	0.0915	349.22	218.1	1.6	*LBR*	lamin B receptor	3930

93	0.002652	0.0915	212.75	263.14	0.81	*FAM111A*	family with sequence similarity 111, member A	63901
94	0.002694	0.0915	352.89	467.06	0.76	*PPCS*	phosphopantothenoylcysteine synthetase	79717
95	0.002702	0.0915	485.35	605.97	0.8	*DAP3*	death associated protein 3	7818
96	0.00271	0.0915	277.98	348.19	0.8	*CCDC167*	coiled-coil domain containing 167	154467
97	0.002717	0.0915	802.17	1105.36	0.73	*C9orf142*	chromosome 9 open reading frame 142	286257
98	0.002726	0.0915	163.47	212.64	0.77	*TPM2*	tropomyosin 2 (beta)	7169
99	0.002768	0.0915	159.17	197.69	0.81	*IDUA*	iduronidase, alpha-L-	3425
100	0.002772	0.0915	674.6	845.39	0.8	*DCTN3*	dynactin 3 (p22)	11258
101	0.002776	0.0915	348.17	450.25	0.77	*PEBP1*	phosphatidylethanolamine binding protein 1	5037
102	0.002886	0.0915	1083.9	1540.81	0.7	*SNRNP70*	small nuclear ribonucleoprotein 70kDa (U1)	6625
103	0.002899	0.0915	1907.71	2863.32	0.67	*CD3D*	CD3d molecule, delta (CD3-TCR complex)	915
104	0.002904	0.0915	868.06	1196.05	0.73	*SNRPB2*	small nuclear ribonucleoprotein polypeptide B	6629
105	0.002932	0.0915	246.53	305.57	0.81	*CES2*	carboxylesterase 2	8824
106	0.002937	0.0915	377.57	527.02	0.72	*RAB37*	RAB37, member RAS oncogene family	326624
107	0.003064	0.0915	458.96	640.79	0.72	*PILRB*	paired immunoglobin-like type 2 receptor beta	29990
108	0.003075	0.0915	395.09	502.08	0.79	*SIVA1*	SIVA1, apoptosis-inducing factor	10572

109	0.003076	0.0915	611.59	392.21	1.56	*USP10*	ubiquitin specific peptidase 10	9100
110	0.0031	0.0915	2017.17	644.26	3.13	*ALPL*	alkaline phosphatase, liver/bone/kidney	249
111	0.003107	0.0915	375.29	491.1	0.76	*BOLA3*	bolA homolog 3 (E. coli)	388962
112	0.00315	0.0915	302.88	402.22	0.75	*MAPK8IP3*	mitogen-activated protein kinase 8 interacting protein 3	23162
113	0.003151	0.0915	389.45	489.27	0.8	*PYCR2*	pyrroline-5-carboxylate reductase family, member 2	29920
114	0.003187	0.0915	370.3	493.79	0.75	*CELF2*	CUGBP, Elav-like family member 2	10659
115	0.003224	0.0915	384.9	508.94	0.76	*ATRAID*	all-trans retinoic acid-induced differentiation factor	51374
116	0.003227	0.0915	555.89	744.08	0.75	*NHP2*	NHP2 ribonucleoprotein homolog (yeast)	55651
117	0.003261	0.0915	321.34	418.82	0.77	*C7orf55*	chromosome 7 open reading frame 55	154791
118	0.003291	0.0915	20973.88	15402.58	1.36	*ANXA2P2*	annexin A2 pseudogene 2	304
119	0.003332	0.0915	435.08	534.84	0.81	*DIABLO*	diablo, IAP-binding mitochondrial protein	56616
120	0.003335	0.0915	2232.09	3384.84	0.66	*CD3D*	CD3d molecule, delta (CD3-TCR complex)	915
121	0.003345	0.0915	331.03	435.94	0.76	*PSMB5*	proteasome (prosome, macropain) subunit, beta type, 5	5693
122	0.003349	0.0915	164	210.9	0.78	*NUDT14*	nudix (nucleoside diphosphate linked moiety X)-type motif 14	256281
123	0.003385	0.0918	206.44	250.42	0.82	*FAM195B*	family with sequence similarity 195, member B	348262
124	0.003443	0.0918	230.95	303.36	0.76	*LOC338799*	uncharacterized LOC338799	338799

125	0.003463	0.0918	278.29	361.69	0.77	*DPM3*	dolichyl-phosphate mannosyltransferase polypeptide 3	54344
126	0.003472	0.0918	2629.45	3473.26	0.76	*HINT1*	histidine triad nucleotide binding protein 1	3094
127	0.003476	0.0918	514.54	371.74	1.38	*STAT3*	signal transducer and activator of transcription 3 (acute-phase response factor)	6774
128	0.003518	0.0921	430.85	552.78	0.78	*TMEM147*	transmembrane protein 147	10430
129	0.003569	0.0921	142.65	184.87	0.77	*TNFRSF13C*	tumor necrosis factor receptor superfamily, member 13C	115650
130	0.003587	0.0921	377.9	557.6	0.68	*PVRIG*	poliovirus receptor related immunoglobulin domain containing	79037
131	0.003591	0.0921	122.29	150.14	0.81	*MYL5*	myosin, light chain 5, regulatory	4636
132	0.003606	0.0921	342.44	474.92	0.72	*ZMYM6*	zinc finger, MYM-type 6	9204
133	0.003649	0.0922	409.4	554.63	0.74	*VAMP2*	vesicle-associated membrane protein 2 (synaptobrevin 2)	6844
134	0.00377	0.0922	908	1357.91	0.67	*SRRM2*	serine/arginine repetitive matrix 2	23524
135	0.003783	0.0922	371.98	469.99	0.79	*BOLA3*	bolA homolog 3 (E. coli)	388962
136	0.003805	0.0922	195.2	241.21	0.81	*PAAF1*	proteasomal ATPase-associated factor 1	80227
137	0.003849	0.0922	3723.92	5291.54	0.7	*RPL36*	ribosomal protein L36	25873
138	0.003864	0.0922	1981.53	2700.8	0.73	*SEC61G*	Sec61 gamma subunit	23480
139	0.003913	0.0922	245.07	177.82	1.38	*GNL3L*	guanine nucleotide binding protein-like 3 (nucleolar)-like	54552
140	0.00392	0.0922	175.44	222.07	0.79	*C3orf78*	chromosome 3 open reading frame 78	440957

141	0.003921	0.0922	1064.55	1384.14	0.77	*IMP3*	IMP3, U3 small nucleolar ribonucleoprotein, homolog (yeast)	55272
142	0.003921	0.0922	1399.33	1782.58	0.79	*ZNFX1-AS1*	ZNFX1 antisense RNA 1	441951
143	0.003938	0.0922	309.49	416.42	0.74	*CCDC53*	coiled-coil domain containing 53	51019
144	0.004044	0.0923	1006.75	552.51	1.82	*NBPF9*	neuroblastoma breakpoint family, member 9	400818
145	0.004068	0.0923	457.62	572.65	0.8	*TMEM160*	transmembrane protein 160	54958
146	0.004074	0.0923	377.26	494.11	0.76	*LOC148413*	uncharacterized LOC148413	148413
147	0.004132	0.0923	362.24	247.02	1.47	*RAD21*	RAD21 homolog (S. pombe)	5885
148	0.004142	0.0923	327.44	415.7	0.79	*CCDC115*	coiled-coil domain containing 115	84317
149	0.004151	0.0923	465.89	588.16	0.79	*CYB561D1*	cytochrome b-561 domain containing 1	284613
150	0.004199	0.0928	173.87	215.83	0.81	*SOCS4*	suppressor of cytokine signaling 4	122809
151	0.004296	0.0944	464.77	628.42	0.74	*SNRPN*	small nuclear ribonucleoprotein polypeptide N	6638
152	0.00441	0.0961	4175.18	5729.43	0.73	*COX7C*	cytochrome c oxidase subunit VIIc	1350
153	0.004464	0.0961	1688.62	2149.52	0.79	*SSR4*	signal sequence receptor, delta	6748
154	0.004484	0.0961	361.15	484.76	0.75	*CD3E*	CD3e molecule, epsilon (CD3-TCR complex)	916
155	0.004493	0.0961	602.81	754.05	0.8	*U2AF2*	U2 small nuclear RNA auxiliary factor 2	11338
156	0.004498	0.0961	213.58	278.62	0.77	*SUGP2*	SURP and G patch domain containing 2	10147

157	0.004589	0.0975	216.86	262.01	0.83	*PPP1R8*	protein phosphatase 1, regulatory subunit 8	5511
158	0.004611	0.0975	189.54	141.12	1.34	*USF1*	upstream transcription factor 1	7391
159	0.004646	0.0977	717.47	941.15	0.76	*MYO1G*	myosin IG	64005
160	0.004695	0.0982	39016.04	29688.06	1.31	*HBG1*	hemoglobin, gamma A	3047
161	0.004751	0.0983	358.19	241.47	1.48	*AKIRIN1*	akirin 1	79647
162	0.004839	0.0983	591.88	782.88	0.76	*RPAIN*	RPA interacting protein	84268
163	0.004862	0.0983	674.92	855.25	0.79	*RASGRP2*	RAS guanyl releasing protein 2 (calcium and DAG-regulated)	10235
164	0.00491	0.0983	1030.44	1345.85	0.77	*CUTA*	cutA divalent cation tolerance homolog (E. coli)	51596
165	0.004929	0.0983	595.65	765.75	0.78	*DYNLRB1*	dynein, light chain, roadblock-type 1	83658
166	0.004939	0.0983	149.2	186.14	0.8	*MRPL2*	mitochondrial ribosomal protein L2	51069
167	0.004969	0.0983	301.43	381.43	0.79	*RRP36*	ribosomal RNA processing 36 homolog (S. cerevisiae)	88745
168	0.005025	0.0983	451.44	575.02	0.79	*DPM1*	dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit	8813
169	0.005041	0.0983	399.06	506.22	0.79	*NHP2*	NHP2 ribonucleoprotein homolog (yeast)	55651
170	0.005086	0.0983	200.22	249.58	0.8	*ICT1*	immature colon carcinoma transcript 1	3396
171	0.005099	0.0983	312.05	387.84	0.8	*STRA13*	stimulated by retinoic acid 13 homolog (mouse)	201254
172	0.005129	0.0983	199.04	262.27	0.76	*MIB2*	mindbomb E3 ubiquitin protein ligase 2	142678

173	0.00513	0.0983	442.77	549.38	0.81	*C19orf43*	chromosome 19 open reading frame 43	79002
174	0.00515	0.0983	240.06	181.97	1.32	*LMNB1*	lamin B1	4001
175	0.005196	0.0983	956.52	1194.24	0.8	*PSMB4*	proteasome (prosome, macropain) subunit, beta type, 4	5692
176	0.005221	0.0983	329.11	468.77	0.7	*NCOR2*	nuclear receptor corepressor 2	9612
177	0.005252	0.0983	283.42	343.01	0.83	*DSTN*	destrin (actin depolymerizing factor)	11034
178	0.005314	0.0983	636.14	863.73	0.74	*TECR*	trans-2,3-enoyl-CoA reductase	9524
179	0.005339	0.0983	299.88	383.16	0.78	*C1orf131*	chromosome 1 open reading frame 131	128061
180	0.005359	0.0983	325.98	413.56	0.79	*ZBTB4*	zinc finger and BTB domain containing 4	57659
181	0.005379	0.0983	824.87	1057.83	0.78	*RPP21*	ribonuclease P/MRP 21kDa subunit	79897
182	0.005381	0.0983	342.66	421.94	0.81	*KLHL22*	kelch-like 22 (Drosophila)	84861
183	0.005415	0.0983	4122.36	5473.07	0.75	*RPL26*	ribosomal protein L26	6154
184	0.005423	0.0983	916.97	1187.79	0.77	*DYNLRB1*	dynein, light chain, roadblock-type 1	83658
185	0.005449	0.0983	210.64	264.99	0.79	*ID2*	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	3398
186	0.005451	0.0983	777.85	1057.66	0.74	*LIME1*	Lck interacting transmembrane adaptor 1	54923
187	0.005469	0.0983	287.98	354.89	0.81	*GGNBP2*	gametogenetin binding protein 2	79893
188	0.005513	0.0983	223.28	179.13	1.25	*AGFG1*	ArfGAP with FG repeats 1	3267

189	0.005517	0.0983	1575.42	931.63	1.69	*NBPF10*	neuroblastoma breakpoint family, member 10	100132406
190	0.005522	0.0983	308.77	377.62	0.82	*MED30*	mediator complex subunit 30	90390
191	0.005525	0.0983	604.38	752.11	0.8	*C11orf48*	chromosome 11 open reading frame 48	79081
192	0.00562	0.0996	730.44	868.54	0.84	*UFC1*	ubiquitin-fold modifier conjugating enzyme 1	51506
193	0.005707	0.0998	285.98	348.91	0.82	*MRPL11*	mitochondrial ribosomal protein L11	65003
194	0.005713	0.0998	241.26	306.84	0.79	*SEPT6*	septin 6	23157
195	0.005734	0.0998	2511.24	1829.86	1.37	*SQSTM1*	sequestosome 1	8878
196	0.005746	0.0998	175.4	220.72	0.79	*EPM2AIP1*	EPM2A (laforin) interacting protein 1	9852
197	0.005758	0.0998	211.15	262.79	0.8	*FOXK1*	forkhead box K1	221937

When using a cut-off fold change of at least 1.5 in either direction and including genes with at least 2 probes in the significant gene list, there were 40 significant probes that corresponded to coding genes of which 22 were upregulated and 18 probes were downregulated in the high AFB_1 exposure group compared to the low AFB_1 exposure group.

Unsupervised clustering of the samples within the complete significant gene list showed almost complete separation of the low and high AFB_1 exposure groups although with correlation coefficients ranging from 0.8 to -0.2. This dendrogram was partnered with a supervised heatmap to visualise gene expression differences between AFB_1 exposure groups (Figure 16).

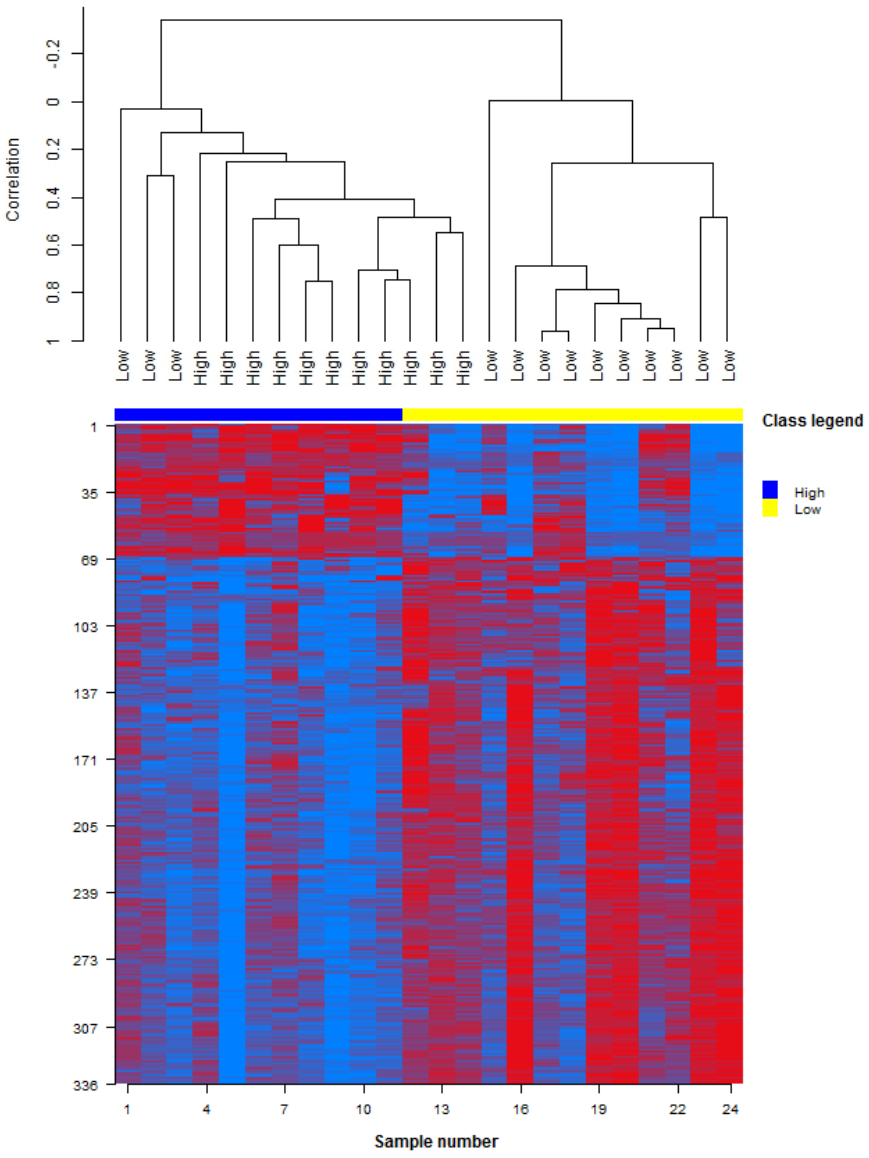

Figure 16: Unsupervised dendrogram and supervised heatmap of gene expression patterns by AFB_1 exposure groups within the significant gene list (including non-coding probes). Blue represents lower expression relative to red.

Several of the significantly different genes are downregulated in the high AFB_1 exposure group compared to the low exposure group. The heatmap for gene expression by AFB_1 groups in the smaller subset of significant genes is shown in Figure 17.

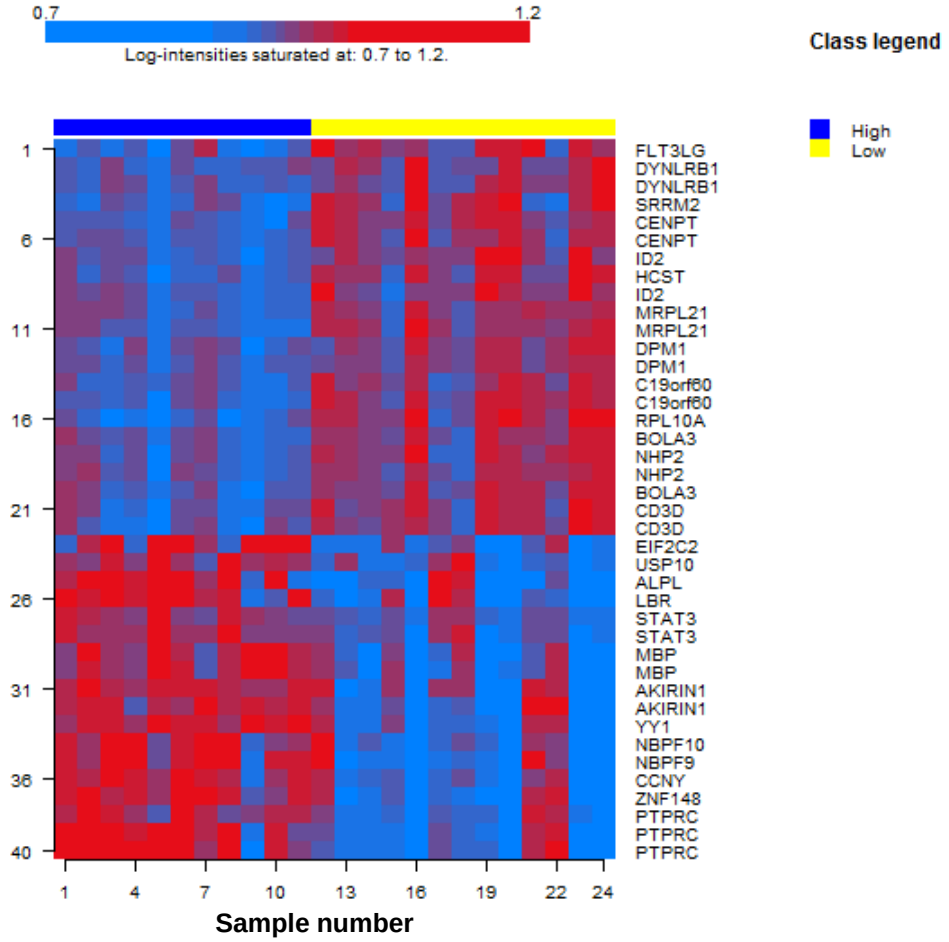

Figure 17: Supervised clustering of a small subset of differentially expressed genes between high and low AFB_1 exposure groups.

Clear patterns can be observed from the heatmaps in both figures with more than 50% of the genes, including *ID2* and *CD3D*, being upregulated in the low AFB_1 exposure group relative to expression in the high AFB_1 exposure group. However, genes including *STAT3* and *PTPRC* are upregulated in the high exposure group compared to the low exposure group. Additionally, there was no correlation between the DNA methylation and gene expression changes observed in Gambian infants.

5.3.2.3 Quantitative trait analysis

QTA was done to determine if actual AF-alb values correlated with gene expression. Early maternal AFB_1 exposure was selected for this analysis and resulted in one gene, ubiquitin-specific protease-4 (*USP4*), being significantly correlated with AFB_1 exposure values (correlation coefficient: 0.839; $P = 9 \times 10^{-7}$; FDR-adjusted *P* value = 0.043) (Figure 18).

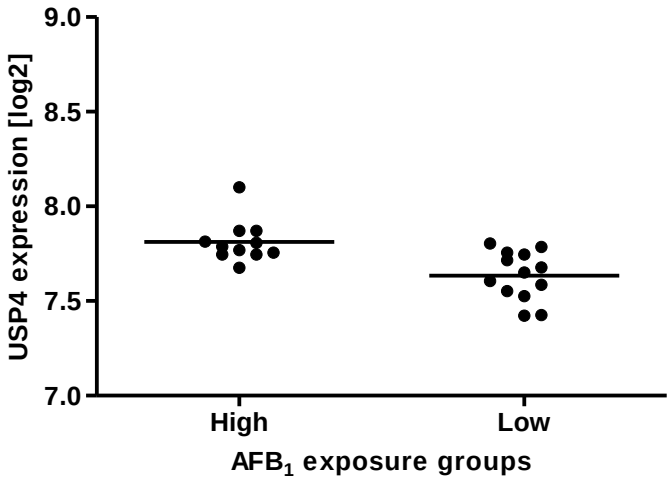

Figure 18: Dot plot of log-transformed *USP4* gene expression by AFB_1 exposure groups.

A two-tailed t-test with Welch's correction for unequal variances done on the log transformed expression values for *USP4* between AFB_1 exposure groups revealed a significant difference between groups (R^2 = 0.388; *P* = 0.002). The QTA was repeated with later-gestational-stage AF-alb exposure levels but no significant genes with less than 5% FDR were identified.

5.3.2.4 Pathway analysis

Pathway analysis using DAVID v.6.7 revealed significant pathway categories using the Biocarta database but none with the KEGG database. Of the 40 significantly different genes, only two genes belonged to three pathway categories (Table 25).

Table 25: Differentially expressed genes in the significant gene list with at least a 2-fold change or repeated probes represented by their associated pathways.

Biocarta Pathway	Pathway Descriptor	Genes	Number of Genes	P value
h_tcraPathway	Lck and Fyn tyrosine kinases in initiation of TCR Activation	*PTPRC, CD3D*	2	0.038
h_thelperPathway	T Helper Cell Surface Molecules	*PTPRC, CD3D*	2	0.041
h_tcytotoxicPathway	T Cytotoxic Cell Surface Molecules	*PTPRC, CD3D*	2	0.041

5.3.2.5 Validation of gene expression changes by qPCR

Genes selected for validation were analysed by qPCR on 52 total RNA samples. As the validation was done on several samples that were not analysed for whole genome gene expression changes, this was a validation of the specific gene expression change in mostly independent samples. The samples were divided into high and low AFB_1 exposure groups using 33.1 pg/mg as the cut-off value. This value was selected on the basis that it is the median of the complete AF-alb data set for early exposure. Figure 19 presents the dot plots of the mRNA expression of genes selected for validation in the two AFB_1 exposure groups. Ten of the 12 genes are not significantly different between the two groups. However, *USP4* and *STAT3* gene expression were both significantly upregulated in the high AFB_1 exposure group compared to the low exposure group ($P = 0.01$ and $P = 0.03$, respectively), with a similar trend to results obtained from the whole genome study.

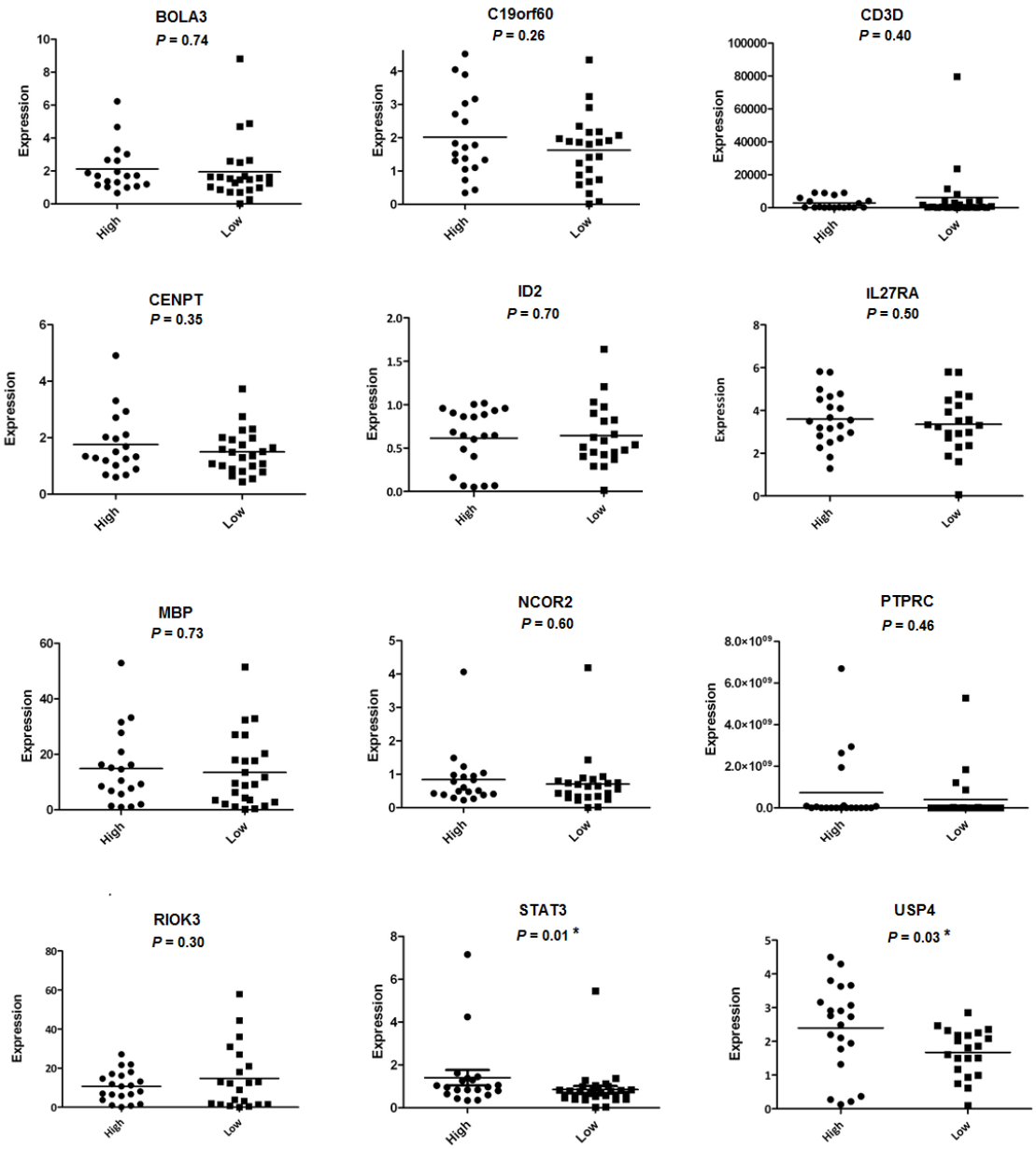

Figure 19: Validation of qPCR results for 12 genes comparing gene expression per AFB_1 exposure group. The mean per group is marked in each cluster per graph and the *P* value per graph is displayed below the gene name.

5.3.2.6 Influence of season of measurement on *USP4* and *STAT3* expression

Univariate regression with *USP4* log expression values and season (classed as dry and rainy) found no significant difference in expression by season (P = 0.749). However, when dichotomising by season, the correlation between AF-alb values and *USP4* expression was only significant during the dry season (R^2 = 0.918; P < 0.001 for dry season and R^2 = 0.599; P = 0.09 for rainy season) (Table 26).

Three probes for *STAT3* were significantly different in the gene list and were therefore all individually considered in the analysis. Univariate regression did not determine any significant differences in *STAT3* expression by season (P > 0.05). When dichotomising by season, correlation between AF-alb values and *STAT3* probe expression were only significant during the dry season (Table 26).

Table 26: Correlation coefficients for *USP4* and *STAT3* per season of measurement.

Gene	Dry Season		Rainy Season	
	Correlation coefficient (R^2)	*P* value	Correlation coefficient (R^2)	*P* value
USP4	0.918	< 0.001	0.599	0.089
STAT3 probe 1	0.791	0.001	0.412	0.270
STAT3 probe 2	0.856	< 0.001	0.171	0.661
STAT3 probe 3	0.722	0.002	0.172	0.659

5.4 Discussion

Exposures to environmental toxins and factors during conception and early life can drastically affect the developing epigenome, which can increase the risk of adverse health outcomes in later life (Lucas 1991). As pregnant women are exposed to AFB_1 through contaminated diet, the ingested toxin can cross the placenta and can be activated to the reactive aflatoxicol at the placental interface (Partanen *et al.* 2010). In this project, a whole genome approach was used to identify altered DNA methylation and gene expression patterns in infants exposed to AFB_1 *in utero*. These patterns can contribute to our understanding of the mechanistic action of AFB_1 exposure on human health.

5.4.1 Timing of environmental exposures

While a lot of research has focused on conception being a crucial time of fetal vulnerability to exposures, a number of studies have also discovered an association between environmental exposures, factors or events and changes to the epigenome or genome at different time points of development during pregnancy. For instance, prenatal famine has been associated with significant health outcomes in adulthood and the effects can be dependent on the time of exposure (Tobi *et al.* 2009). In Dutch people exposed to a famine occurring towards the end of the Second World War, differences in methylation of growth and development-related genes *INSIGF*, *IL10*, *LEP*, *ABCA1* and *GNASAS* was observed in individuals exposed to famine periconceptionally compared to their unexposed siblings (Tobi *et al.* 2009). However,

in people exposed to famine during late gestation, differential methylation of *GNASAS* and *LEP* was observed (Tobi *et al.* 2009).

AFB_1 exposure during late gestation has been shown to inhibit growth in rats while having no obvious effects when administered earlier during pregnancy (Butler and Wigglesworth 1966). This has important implications in humans particularly if exposure during mid-late gestation can also affect the epigenome and genome, potentially predisposing the child to adverse health outcomes in later life. Of course, it cannot be assumed that similar effects will be seen in different species but the rat data shows the possibility that exposure during different times of exposure during pregnancy could have different outcomes for child health.

5.4.2 DNA methylation differences associated with aflatoxin exposure

One important confounder that has been included in the DNA methylation analysis is the season at the time of conception. Season has been identified as a confounder in previous studies associating environmental exposures with DNA methylation changes (Breton *et al.* 2009; Madrigano *et al.* 2011). Indeed, the season of conception in The Gambia has been associated with nutritional differences in pregnant women, which can influence fetal development, particularly DNA methylation at this crucial early time point (Waterland *et al.* 2010). Due to the propensity to alter DNA methylation in the foetus, it is necessary to adjust for season of conception in DNA methylation analyses in order to avoid identifying season-associated changes attributed aflatoxin exposure. However, there is a potential of over-adjustment, which may mask the true effects of aflatoxin exposure. For this

reason, the analysed data was compared with and without adjustment for season of conception. As no major differences were observed between the two groups, and due to the potential biological relevance to DNA methylation, the data was adjusted for season of conception in the final analysis.

In this study, no significant differences in DNA methylation were observed at the probe level. However, a number of significant differentially methylated genes were identified at the gene and promoter level. While changes at individual sites are interesting especially if changes occur in the shore (regions near the CpG islands) or open seas (isolated CpGs in the genome), consistent alterations to the methylation status within the gene body or promoter region would potentially have a greater influence on gene expression.

Several of the identified genes are involved in immune regulation and cytokine signalling suggesting that *in utero* AFB_1 exposure can alter methylation patterns that may affect the immune response.

5.4.2.1 Association of validated DNA methylation changes in genes with health effects

CpG sites within 6 genes (*CD3D*, *CREB3L3*, *HORMAD2*, *HOXB2*, *PSMA8* and *SLC17A9*) were successfully validated by pyrosequencing. Of these, *CD3D* is involved in the immune response, particularly in T cell development and signal transduction. It has recently been proposed as one of many markers of breast cancer prognosis where high gene expression is associated with a better clinical outcome (Dedeurwaerder *et al.* 2011b). *HORMAD2* is important for meiotic progression and is essential during meiosis. It was also identified in a genome-wide association study

as a contributing factor to the risk of lung cancer in Han Chinese individuals (Liu *et al.* 2012). *PSMA8* encodes for a proteasome subunit, which cleaves peptides in the cell. Hypermethylation may result in lower expression of this subunit, which may then slow-down the destruction of faulty peptides or invading viruses. *SLC17A9* encodes for a solute carrier gene, which is involved in the vesicular storage and the exocytosis of ATP. It is significantly hypomethylated in the higher AFB_1 exposure group in the current study suggesting that *in utero* AFB_1 exposure can alter and potentially disrupt normal cellular processes.

HOXB2 and *CREB3L3*, which encode transcription factors, were hypomethylated in Gambian infants exposed to higher AFB_1. Interestingly, *HOXB2* overexpression has been observed in lung tumour tissues and has been proposed as prognostic marker for stage I lung adenocarcinomas (Inamura *et al.* 2007). *CREB3L3* is a liver-specific transcription factor and in mice has been found to encode a growth suppressor protein and is under-expressed in hepatoma cells and overall in HCC (Chin *et al.* 2005).

5.4.3 Gene expression differences associated with aflatoxin exposure

5.4.3.1 Quality of mRNA for whole genome gene expression analysis

A number of factors can influence or interfere with transcriptomic analysis including varied bench time, storage temperatures and time in cold storage (Hebels *et al.* 2013). In the current study, blood was collected from infants at varying time points between 3-6 months following birth. Blood cell separation and preparation prior to storage

was conducted by trained individuals in the MRC unit, Keneba, The Gambia. Inter-individual variation in sample handling could be an additional factor in introducing some differences in RNA transcripts within the samples. Also, as infants were born at different times, variation in time in cold storage existed for these samples, although the range of storage time did not exceed more than a few months. In order to avoid much of this variation in RNA transcript levels, blood cells were separated and immediately stored in RNAlater reagent, which stabilises RNA transcripts. RNA extraction from blood cells stored in RNAlater was undertaken by trained technicians at the Epigenetics Group, IARC, Lyon, France, where conditions of storage and bench time could be controlled more accurately.

5.4.3.2 Changes in gene expression associated with AFB_1 exposure

We have found significant differences in whole genome gene expression when comparing AFB_1 exposure groups based on early exposure but none when looking at exposure during later pregnancy. All highlighted pathways were involved in the T cell mediated immune response, which provides some evidence that *in utero* AFB_1 exposure affects the immune system components.

Interestingly, *BOLA3*, which was found to be upregulated in the high AFB_1 exposure group, has been suggested to be a metastable epiallele in humans following a study also based in The Gambia. The study observed that *BOLA3* was hypomethylated during the dry season at the time of conception (Waterland *et al.* 2010), which is also a period of higher AFB_1 exposure (Chapter 4).

There was no correlation between the observed DNA methylation and gene expression changes in these Gambian infants, although a positive correlation would

be expected (Hellman and Chess 2007). This could be due to technical or biological reasons. While the blood from which both DNA and RNA was extracted was collected at a similar time, the actual extraction was carried out at two different time points and at two different locations: DNA was extracted at the MRC Unit, Keneba, The Gambia shortly after blood collection and blood stored in RNAlater was first shipped to IARC, Lyon, France, where RNA extraction was carried out. Any other sources of variation including bench time and storage conditions for the two sets of samples could have introduced further differences in DNA methylation and gene expression.

As gene expression is not only modulated by DNA methylation but also by other epigenetic mechanisms or by mutations (Portela and Esteller 2010), it is possible that the significant gene expression changes observed in the current work are controlled by mechanisms other than DNA methylation. Furthermore, the actual observed changes in the current work do not exceed 2-fold and are indeed changes of a relatively low magnitude. In previous studies that have correlated DNA methylation with gene expression have done so at loci with at least a 2-fold change between groups (Schmelz *et al.* 2004; Fan *et al.* 2006). Therefore, this may additionally explain the lack of correlation between the observed significant DNA methylation and gene expression changes.

We have successfully validated expression changes in *USP4* and *STAT3*, although a total of 12 genes were selected. This may be because of the relatively small changes in expression observed. While these changes are statistically significant, it is not known if they are biologically relevant and therefore may not reflect real AFB_1-associated changes.

Additionally, the effect of season of blood measurement was examined. Seasonality is known to be one factor in the variation of AF-alb levels (See Chapter 4). Interestingly, both *USP4* and *STAT3* expression was more significantly associated with AF-alb during the dry season than during the rainy season. However, this may be attributed to the fact that AF-alb levels are higher during the dry season. Univariate analyses did not find any associations between gene expression and season suggesting it is not relevant to variations in expression levels.

5.4.3.3 Role of USP4 in carcinogenesis

USP4 is an enzyme responsible for deubiquitinating the A_{2A}-adenosine receptor, among other targets. USP4 binds to the carboxyl terminus of the A_{2A} receptor, which then relaxes the stringent quality control of the endoplasmic reticulum and enhances cell surface expression. This then allows misfolded proteins to move out of the ER to the plasma membrane (Milojević *et al.* 2006). *USP4* gene expression has been consistently elevated in prostate and urinary bladder cancers, suggesting a role in cancer development. Indeed, it has also been shown to lead to the inhibition of p53 by binding ARF-BP1, which is involved in the degradation of p53 (Zhang *et al.* 2011).

5.4.3.4 The role of STAT3 in development

STAT3 or signal transducer and activator of transcription-3 is an important member of the JAK/STAT signalling family. In response to cytokines and growth factors (for example. IL6 and human growth factor), STATs are phosphorylated, form dimers

and translocate to the nucleus to activate transcription of genes responsible for cell growth and apoptosis (Rawlings *et al.* 2004).

STAT3 plays an important role in tumour cell proliferation, survival and invasion as well as restraining the anti-tumour immune responses. However, it also induces the expression of cytokines and growth factors, with the respective receptors activating STAT3 resulting in a feedforward loop between the tumour and the immune system (Yu *et al.* 2009).

De Benedetti et al. (De Benedetti *et al.* 1997) studied transgenic mice, which carry the neurospecific enolase promoter overexpressing human IL6 cDNA. The study found that the overexpression of IL6, which activates the STAT3 pathway, resulted in a reduced growth rate with transgenic mice 50-70% the size of non-transgenic littermates. When the IL6 receptor was blocked with a monoclonal antibody, the growth impairment was partly reverted. Interestingly, circulating IGF1 levels were significantly lower in the transgenic mice. Furthermore, administration of the same strain of IL6 to non-transgenic mice led to a reduction of circulating IGF1 levels. Also, in 21 children with systemic juvenile rheumatoid arthritis, circulating IL6 levels were negatively correlated with IGF1 levels (De Benedetti *et al.* 1997).

In transgenic mice overexpressing IL6 and with growth impairment, gene expression of the suppressor of cytokine signalling-3 (*SOCS*-3) was elevated. SOCS are also inhibitors of the STAT and GH signalling. IL6 induces expression of STAT3, which in turn activates SOCS, which is thought to be part of the mechanism of growth impairment by IL6 (Lieskovska *et al.* 2003).

DON, a *Fusarium* mycotoxin, which has been associated with growth impairment in mice (Amuzie and Pestka 2010), has been associated with increased mRNA and/or

protein expression of IL6 in macrophage cell lines and in mice (Sugita-Konishi and Pestka 2001; Amuzie *et al.* 2009). Moreover, following IL6 upregulation, SOCS-3 mRNA expression was also increased in the liver, muscle and spleen of DON-exposed mice (Amuzie *et al.* 2009). Also, hepatic SOCS expression was significantly associated with a 75% suppression of IGF-ALS subunit, which together with IGFBP3 is important in prolonging the half-life of IGF1 in circulation (Amuzie *et al.* 2009). Indeed, SOCS proteins are known to modulate the GH-IGF1 growth axis and consequently play a role in linear growth (Ahmed and Farquharson 2010).

In the present work, *STAT3* expression was upregulated in the high AFB_1 exposure group. Therefore, it is possible that mechanism of AFB_1-associated growth impairment is through elevated IL6 levels, which in turn induce expression of STAT3. It may accomplish this either by altering the IGF growth axis and/or through the activation of SOCS. Identification of *STAT3* upregulation may be an important clue to aid our understanding of the mechanism of growth impairment in children exposed to high levels of AFB_1.

5.4.4 Conclusions

Patterns of DNA methylation and gene expression changes associated with *in utero* AFB_1 exposure have been identified. Most of the altered genes are involved in the immune response and cell cycle progression while a few including *CD3D*, *HOXB2*, *USP4* and *STAT3* have been previously associated with carcinogenesis and growth impairment. While the observed changes in DNA methylation and gene expression were statistically significant, they must be considered with caution. The differences in methylation and gene expression levels are not very high, which is expected as the

infants in the study were relatively healthy. Additionally, these changes were observed in white blood cells, which can have different patterns of DNA methylation depending on cell type (Reinius *et al.* 2012). However, the identification of some key altered genes does contribute to our understanding of the effects of early life AFB_1 exposure on human health and potentially represent markers of adverse health outcomes in later life.

6 Genome-wide effects of AFB_1 exposed cultured liver cells

6.1 Introduction

The use of blood as a surrogate for liver-specific changes following AFB_1 exposure has already been discussed. However, a system whereby changes in the liver can be explored is also important in building a picture of the underlying mechanisms involved in AFB_1-associated health effects. Studies using a cell-based model have been widely used to explain the mechanistic action of toxins. For instance, using an insulin-producing β cell line, INS-1 (832/13), Fu et al were able to show that low arsenic exposure induced an adaptive oxidative stress response. This increased antioxidant levels and disrupted reactive oxygen species (ROS) signalling involved in glucose-stimulated glucose secretion. These events resulted in altered β-cell function, thereby linking them to arsenic exposure-associated type 2 diabetes (Fu *et al.* 2010).

The use of cells enables the study of the effects of acute and chronic exposures in a tissue-specific manner. Additionally, time-course studies are easily designed and can be rapidly accomplished in an *in vitro* study compared to an epidemiological study. However, some limitations do exist including the difficulty in mimicking *in vivo* exposures and the removal of the cells from their 'normal' environment, which ignores any contribution of surrounding cells in exposure-related effects (Devlin *et al.* 2005).

Despite these failings, *in vitro* work with cells can contribute greatly to our understanding of various pathways and systems involved in AFB_1-associated carcinogenesis and growth impairment. Additionally, using a cell-based model may also reveal important markers of exposure and effects.

Indeed, global hypomethylation and hypermethylation of the *p53* gene were observed in caco-2 cells (a human colon cancer cell line exposed to selenium and arsenic for 7 days (Davis *et al.* 2000). Environmental exposures have also been associated with significant changes to gene expression patterns. Benzo(a)pyrene altered expression of several genes involved in xenobiotic metabolism, cell cycle regulation and DNA repair in MCF-7 (a human breast cancer cell line) and HepG2 cells. Furthermore, it was identified that a large number of changes were associated with p53 signalling (Hockley *et al.* 2007).

AFB_1 exposure for 24 hours has been associated with early gene expression changes to non-tumourigenic liver cells, HepaRG, including alterations to p53 signalling and DNA repair. Additionally, a direct effect on p53 protein levels upon AFB_1 exposure was identified using a small interfering RNA against p53 (Josse *et al.* 2012).

A whole genome approach was adopted to identify early events in DNA methylation and gene expression associated with AFB_1 exposure in non-tumourigenic liver cells (HHL-16). Cells exposed to three varied AFB_1 doses at two time points were analysed using Illumina arrays to determine early patterns of exposure that could provide a better understanding of aflatoxin-induced health effects.

6.2 Cultured cell DNA methylation analysis

6.2.1 Methods

6.2.1.1 Sample preparation

HHL-16 non tumourigenic cells at passage 11 were treated with AFB_1 and DNA was extracted from them as described in Chapter 2, Section 2.3.4. Samples were prepared in triplicate and incubated with AFB_1 for 24 and 48 hours (Table 27).

Table 27: Description of cell sample control and treatment groups.

Group	Description
NT	Untreated cells (no treatment)
D5	Cells treated with 0.0025% DMSO (control for A0.5 and A5)
D20	Cells treated with 0.1% DMSO (control for A20)
A0.5	Cells treated with 0.5 μg/ml AFB_1 (≈ 1.09 μM)
A5	Cells treated with 5 μg/ml AFB_1 (≈ 10.94 μM)
A20	Cells treated with 20 μg/ml AFB_1 (≈ 43.76 μM)

Prior to further analysis, samples were assigned a randomised code ID and added to the bead chips, ordered by ID. All samples were analysed in one batch precluding the effects of batch variability. Samples were bisulfite treated and analysed by pyrosequencing to ascertain the success of bisulfite treatment. This was done to assess methylation in *LINE1* at this stage prior to analysis using the Illumina Infinium HD 450K DNA Methylation array (Illumina, Freddy van Riemsdijkweg,

The Netherlands), as described in Chapter 2, Sections 2.4.1.2 and 2.4.1.3. The whole genome work was done by Geoffroy Durand, IARC, Lyon, France.

6.2.1.2 Data analysis and normalisation

Illumina's GenomeStudio Methylation module version 1.9 (Illumina, Freddy van Riemsdijkweg, The Netherlands) was used to calculate the methylation level at each CpG site as the beta (β) value. The data analysis was done with input and advice from Dr. Hector Hernandez-Vargas, IARC, Lyon, France.

The detection *P* value for each β value was also calculated. β values were then log transformed to obtain the M value. The M values were analysed using RnBeads. The methylumi package was also used in conjunction to normalise the data. The analysis produced six modules, which are described in Table 28.

Table 28: Description of RnBeads analysis modules.

Module	Description
Loading and normalisation	Summary of sample loading. Data was normalised using the methylumi package
Quality control	Box plots of all quality controls included in the 450K bead chips
Filtering	Utilises a GreedyCut algorithm which filters out probes and samples of low purity. Also removes unreliable measurements with *P* value above 0.01
Batch effects	Determines if any batch effects exist. In the current work, all samples were analysed in the same batch
Methylation profiles	Displays the distribution of methylation values over Infinium I and II probes
Differential methylation	Determines differentially methylated probes either at the individual level or at the region level between sample groups

6.2.1.2.1 Analysis of differential methylation

The data were corrected for multiple comparisons using a multivariate permutation test, which provided 95% confidence that the false discovery rate (FDR) or q value was less than 5%. Random variance t-statistics were used for each CpG site (Wright and Simon 2003).

Lists of probes, genes and promoter-level differential methylation were produced through RnBeads following correction for multiple testing to determine differential methylation patterns in cells treated with AFB_1. Lists were filtered to remove all probes/genes with a q value above 0.05. Additionally, a shorter list was produced only including probes/genes with at least a 5% difference in methylation levels.

6.2.1.3 Pathway analysis

Pathway analysis was performed using DAVID v.6.7, which uses sources including GenBank, UniGene, KEGG, and GO to compare input gene lists with large databases.

6.2.1.4 Comparison with Gambian DNA methylation changes

Each gene list from different treatment versus control comparisons at the promoter or gene level was compared with the gene list derived from Gambian infants exposed to AFB_1 during fetal development to determine if any of the differentially methylated genes match between gene lists.

6.2.2 Results

6.2.2.1 Differential methylation

6.2.2.1.1 Probe-level analysis

Each comparison of treatment versus control group was done at the probe level to determine if AFB_1 exposure *in vitro* affected individual CPG site methylation status. However, none of the differentially methylated probes were significantly different, with q values (equivalent to FDR) above 0.05. This suggests that changes at the individual probe level may not be apparent at the doses and time points assessed in this study.

6.2.2.1.2 Gene-level analysis

The comparisons of treatment versus control groups were repeated at the gene region level to identify changes to the methylome that potentially affect gene expression. The results from this analysis are displayed in Table 29. The differential methylation observed following a 24-hour treatment was dose-dependent with the number of differentially methylated genes increasing with increasing dose. Following a 48-hour treatment, differential methylation only appeared to be dose-dependent up to A5 with the number of differentially methylated genes being much lower at the highest dose. Additionally, at both time points, the number of hypomethylated genes in the AFB_1-treated cells compared to the DMSO controls was much higher than the number of hypermethylated genes at all three doses.

Table 29: Number of significant differentially methylated genes at the gene region level for different control versus treatment comparisons in cultured liver cell DNA.

Time Point	Comparison	Number of significant gene regions	Number of significant gene regions with at least 5% difference in methylation (including NAs)	Number of Hypomethylated gene regions	Number of Hypermethylated gene regions	Significant gene regions with at least 5% difference in methylation (excluding NAs)
24 hours	D5 vs A0.5	65	15	13	2	*MAS1, MIR943, PABPC1P6, POTEJ, VCX2*
	D5 vs A5	105	32	29	3	*DNM1P32, GSN-AS1, HIST3H3, MIR4297, PABPC1P6, PPP1R2P2, PPY2, RNASE3, RPL15P3, TBC1D3P4, USP17L7*
	D20 vs A20	188	52	40	12	*ANKRD20A14P, C19orf76, CCNJP1, CFHR1, E2F3-IT1, GOLGA6B, GPR42, KRTAP20-3, LINC00341,*

						MIR24-2, MIR4508, MIR551B, OLR1, OR2D2, OR4L1, OR5AC2, OR5H1, OXCT2P1, PFN1P1, PROL1, RN5-8S3, SCARNA3, STC1, TGM7, TUG1
48 hours	D5 vs A0.5	158	14	14	0	*CYLC2, NTM-IT1, OR5W2, OR6B1, RHOXF2, RPS26P34, SPTLC3*
	D5 vs A5	221	33	33	0	*ABCA6, AK4P5, C5, CYLC2, KERA, KIF4B, MIR2276, PABPC1P1, RNU5F-2P, SLC15A5, SPTLC3, SUMO4, TAS2R43, UQCRFS1P1*
	D20 vs A20	58	34	24	10	*FOXQ1, IL2, KIAA0125, LINC00413, LINC00596, LINC00615, MBL2, MIR410, MIR4458, NT5CP2, SNORD1140-31*

NA – probes in non-annotated regions

Gene lists, consisting of significant differentially methylated genes, were analysed by comparing treatment versus control groups. A comparison of these significant gene lists from each set shows that few genes are in common between the lists (Figure 20).

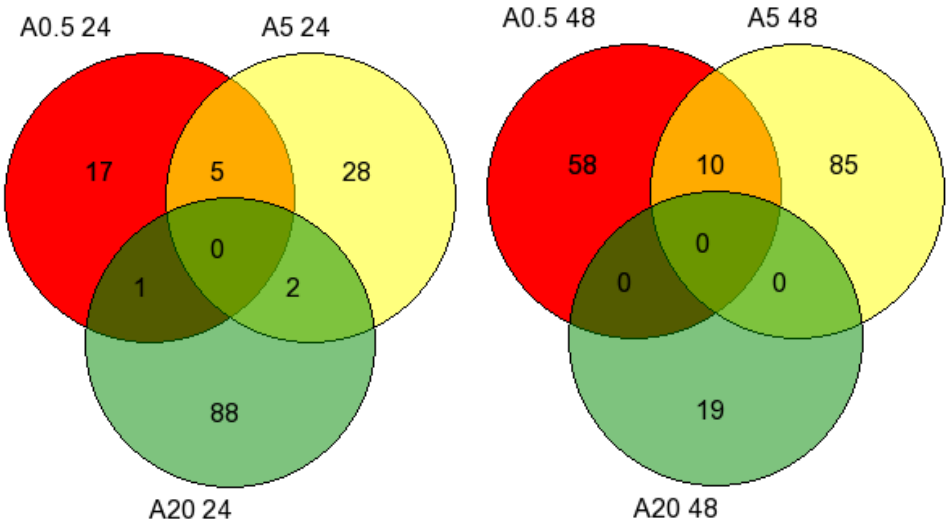

Figure 20: Venn diagrams comparing gene lists derived from treatments versus control groups at 24 and 48 hour time points. Very few genes were in common between the three gene lists.

The five genes in common between A0.5 and A5 (24hr) were: *PABPC1P6*, *RNASE13*, *RRH*, *SNORA52*, *USP17L7*. The one gene in common between A0.5 and A20 (24hr) was: *MIR24*-2. The two genes in common between A5 and A20 (24hr) were: *ANKRD20A14P* and *PPY2*. The ten genes in common between A0.5 and A5 (48hr) were: *AGR3*, *CFC1B*, *CYLC2*, *KRTAP10*-9, *KRTAP4*-2, *SPTLC3*, *TAS2R43*, *TLR12P*, *TNFS8* and *UQCRFS1P1*.

6.2.2.1.2.1 Gene-level pathway analysis

Pathway analysis by DAVID identified only one significant pathway (Olfactory transduction) for the D20 versus A20 comparison at 24 hours and the D5 versus A5 comparison at 48 hours (Table 30).

Table 30: Pathway analysis by DAVID at the gene region-level in gene lists derived from AFB_1 treatment versus control comparisons.

Comparison	Pathway Descriptor		Number of genes	Genes	P value
D20 vs A20 (24hr)	hsa04740	Olfactory transduction	8	*OR56A1, OR10G8, OR52I2, OR2D2, OR4L1, OR7D4, OR51I1, OR5AC2*	2.62E-05
D5 vs A5 (48hr)	hsa04740	Olfactory transduction	6	*OR5P2, OR4C15, OR2T12, OR3A2, OR2T6, OR10H4*	0.001746

6.2.2.1.3 Promoter-level analysis

The comparisons of treatment versus control groups were performed at the promoter region level. The results from this analysis are displayed in Table 31.

Table 31: Number of significant differentially methylated genes at the promoter-region level for different control versus treatment comparisons in cultured liver cell DNA.

Time Point	Comparison	Number of significant promoter regions	Number of significant promoter regions with at least 5% difference in methylation (including NAs)	Number of hypomethylated significant promoters	Number of hypermethylated significant promoters	Significant promoter regions with at least 5% difference in methylation (excluding NAs)
24 hours	D5 vs A0.5	59	16	11	5	*ANKRD20A4, LRRC3C, MIR4758, PABPC1P6, PPP1R3A, PRINS, SFXN5, SNORD113-3, TRIM48, ZNF211*
	D5 vs A5	114	25	20	5	*ACTG1, AMY1B, CTAGE9, FAM90A24P, MIR455, PABPC1P6, PRINS, RPL15P3, RPL18AP2, SFXN5, SNORD113-3*

	D20 vs A20	353	48	36	12	*ARPC3P1, C12orf59, C5, GPR42, HSD3BP5, KRT8P33, KRTAP5-11, LCE1A, PR5AC2, RN5-8S3, RPS3AP3, SNORDA65, SNORD71, SYS1, TRAV22, ZNF806*
48 hours	D5 vs A0.5	159	14	11	3	*CFC1, CFC1B, RPS26P34*
	D5 vs A5	265	44	41	3	*ABCA6, C4BPB, COL3A1, EAF1-AS1, EMB, EVI2B, GATA4, LRIT3, MRS2P2, OR13J1, PABPC1L2A, PABPC1P1, RASGRP3, SKOR1, SMEK2, TRGV10, TTC18, UQCRFS1P1*
	D20 vs A20	96	46	28	18	*AMELX, CYP2C19, EAF1-AS1, GSTA3, HBE1, IGHD7-27, IGHJ1, IGHJ1P, IGHJ2, IGHJ2P, IGHJ3, IGHJ4, IQCA1P1, LINC00413, MIR370, MIR4458, MIR5683, NPBWR1, NT5CP2, OPRK1, RPA3, RSPH10B, SGCZ, SNORD113-3, TRGV2*

At the promoter region-level, a similar trend to the gene region-level analysis in the numbers of differentially methylated genes was observed. Following a 24-hour treatment, differential methylation was dose-dependent with increasing number of differentially methylated genes with increasing dose. However, following a 48-hour treatment, the number of differentially methylated genes was higher in A5 cells than in A0.5 cells but much lower in A20 cells. Once again, AFB_1 exposure was associated with higher levels of hypomethylation than of hypermethylation. A comparison of the significant gene lists from each set showed that very few genes were in common between the lists (Figure 21).

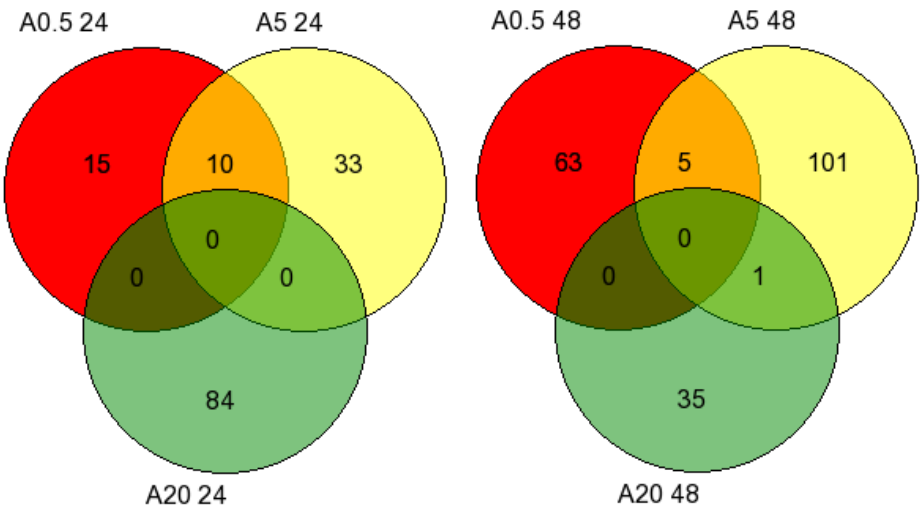

Figure 21: Venn diagrams of DNA methylation changes comparing gene lists derived from treatments versus control groups at 24 and 48 hour time points. Very few genes were in common between the three gene lists.

The ten genes in common between D5 vs A0.5 and D5 vs A5 at 24 hours were: *ANKRD20A4*, *C9orf131*, *DEFB124*, *LINC00319*, *PABPC1P6*, *PPP1R3A*, *PRINS*, *SFXN5*, *SNORD113*-3 and *ZNF883*.

The one gene in common between D5 vs A5 and D20 vs A20 at 48 hours was *EAF1*-AS1. The five genes in common between D5 vs A0.5 and D5 vs A5 at 48 hours were: *DCX*, *EPT1*, *GPR56*, *SETP10* and *UQCRFS1P1*.

6.2.2.1.3.1 Promoter-level pathway analysis

Pathway analysis by DAVID did not identify any significant pathways for gene lists derived from each comparison between controls and treatment groups at both time points.

6.2.2.2 Comparison with Gambian DNA methylation changes

A few genes were in common with a significant gene list derived from Gambian infant samples (Table 32). At the promoter level, G-protein coupled receptor 56 (*GPR56*) was differentially methylated in cells treated with 5 µg/ml AFB_1 at both 24- and 48-hour time points as well as in Gambian infants. At the gene level, microRNA 24-2 (*MIR24-2*) was differentially methylated in cells treated for 24-hours with 0.5 and 20 µg/ml AFB_1 and in Gambian infants. Also at the gene level, mannose-binding lectin-2 (*MBL2*) was differentially methylated in cells treated with 20 µg/ml AFB_1 at both time points. However, of these three genes, only one (*MIR24-2*) showed the same type of change in methylation (hypomethylation in this case) in both gene lists.

Table 32: Differentially methylated genes identified in Gambian infants and in cultured liver cells following AFB_1 exposure.

Gene Symbol	Gene Name	Change in Gambian infants (high exposure versus low exposure)	Change in liver cells (high exposure versus low exposure)	Region level
GPR56	G-protein coupled receptor 56	Hypomethylated	Hypermethylated	Promoter
MBL2	mannose-binding lectin-2	Hypomethylated	Hypermethylated	Gene
MIR24-2	microRNA 24-2	Hypomethylated	Hypomethylated	Gene

6.3 Cultured cell gene expression analysis

6.3.1 Methods

6.3.1.1 Sample preparation

Total RNA was extracted from AFB_1-treated HHL-16 cells following the method described in Chapter 2, Section 2.3.5. Samples were prepared in triplicate and incubated with AFB_1 for 24 or 48 hours.

Prior to further analysis, samples were assigned a randomised code ID and added to the bead chips, ordered by ID. All samples were analysed in one batch precluding the effects of batch variability. Samples were analysed using the Illumina Human HT12-v4 Expression BeadChip Kit (Illumina, Freddy van Riemsdijkweg, The Netherlands), described in Chapter 2, Section 2.4.2.2. This whole genome analysis was done by Geoffroy Durand, IARC, Lyon, France.

6.3.1.2 Data analysis and normalisation

Raw data in the form of expression values was imported and analysed on BRB ArrayTools software (version 4.3.0, developed by Dr. Richard Simon and BRB-ArrayTools Development Team) using the *lumi* package, which is specifically designed for Illumina data (Du *et al.* 2008). This data analysis was done with input and advice from Dr. Hector Hernandez-Vargas, IARC, Lyon, France.

Each probe on the BeadChips was identified via a nuID. Following the pre-processing steps using *lumi*, the data was first $\log_2$ transformed followed by normalisation using RSN. Data was also corrected for multiple testing using t-statistics and multivariate permutation analysis, which generates a q value per loci per comparison. Only q values < 0.05 were accepted.

6.3.1.3 Clustering

Unsupervised clustering was done to determine the distribution and correlation between the treated samples and controls at the two time points.

6.3.1.4 Data filtering

Following normalisation, the data were then filtered to exclude less than 20 % of genes that have at least a 1.3-fold change in expression data in either direction of the gene's median expression value. Also, genes with more than 50 % missing data were excluded from the analysis. These settings improved the number of hits obtained following class comparison analysis while excluding genes with missing values or with a very small change in expression between classes (Table 33).

Table 33: Comparison of different filtering conditions in terms of hits obtained following class comparison analysis of the 48-hour D5 vs A5 comparison.

Data filtering conditions	N
No filter	2007
Excluding genes with more than 50 % missing data	2007
and 20 % genes with less than 2-fold change	125
and 20 % genes with less than 1.5-fold change	488
and 20 % genes with less than 1.3-fold change	1116
and 25 % genes with less than 1.5-fold change	451
and 25 % genes with less than 1.3-fold change	1009
and 30 % genes with less than 1.5-fold change	368
and 30 % genes with less than 1.3-fold change	850

N number of probes with a q value less than 5%

6.3.1.5 Class comparison analysis

Using the filtering conditions of excluding genes with more than 50% missing data and 20% genes with at least a 1.3-fold difference in either direction between groups, and with a significance threshold of 0.01, a class comparison was conducted to compare the different control versus treatment groups.

6.3.1.6 Pathway analysis

Pathway analysis was done DAVID v.6.7, which uses sources including GenBank, UniGene, KEGG, and GO to compare input gene lists with large databases.

6.3.1.7 Transcription factor gene set comparison

Gene set expression comparison was done to determine if any transcription factor targets and pathways were differentially expressed due to AFB_1 exposure. This analysis was only produced for the 48-hour D5 vs A5 and D20 vs A20 comparisons as a result of the higher number of significant changes in gene expression.

6.3.1.8 Comparison of gene expression changes between liver cells and Gambian infants

The list of significantly different expression of genes in AFB_1-exposed Gambian infants (Chapter 5) was compared with gene lists derived from AFB_1-exposed liver cells at 5 µg/ml and 20 µg/ml doses following 48-hour treatments.

6.3.2 Results

6.3.2.1 Normalisation

Raw data was first compared with normalised data to check if any extraneous variation was removed. A boxplot of the samples with and without RSN normalisation is shown in Figure 22.

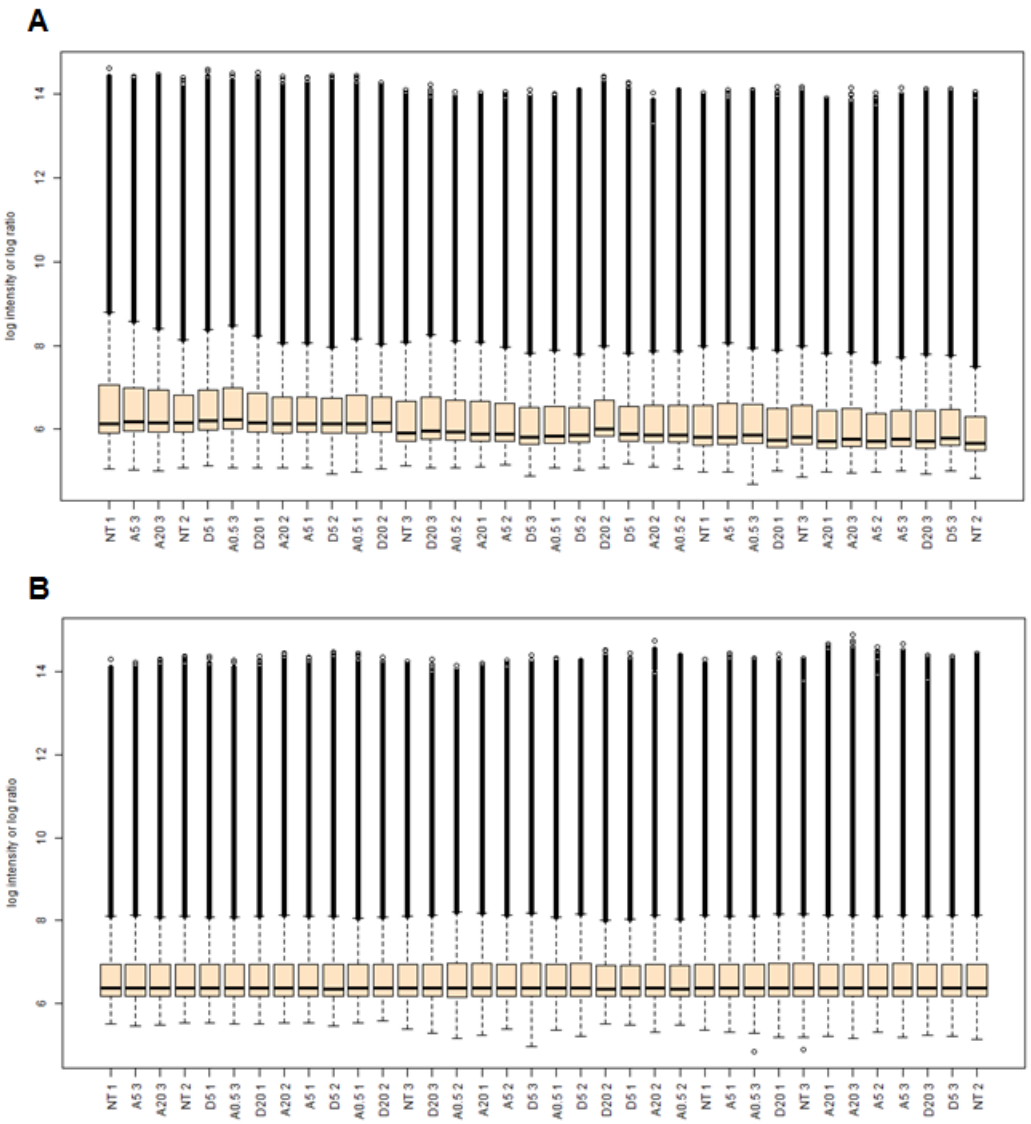

Figure 22: Boxplot of the log intensity of log ratios of all genes on the Bead Chips for 36 samples showing raw data (A) and robust spline normalised data (B). Several outliers are present potentially due to the variability in gene expression by treatment group. Normalisation removes any technical variability by calibrating against expression intensities on a reference array.

6.3.2.2 Clustering

Unsupervised clustering (see Figure 23) showed separation of the two treatment time points with a correlation coefficient (R^2) of -0.6. Most samples clustering together display strong correlation with correlation coefficients between 0.7 and 1. Within the

24 hours treatment time, the controls and treatments clustered together without any obvious separation between the type of treatments. However, duplicates of all controls and treatment groups did cluster together with the third sample per group clustering separately. Within the 48 hours treatment time, there was a clearer separation between controls and A5 and A20 treatments. A weak correlation existed between the three A20 treatments and the rest of the sample groups (R^2 = 0.2). Additionally, the A5 triplicates clustered together but away from the other groups with a correlation coefficient of 0.7.

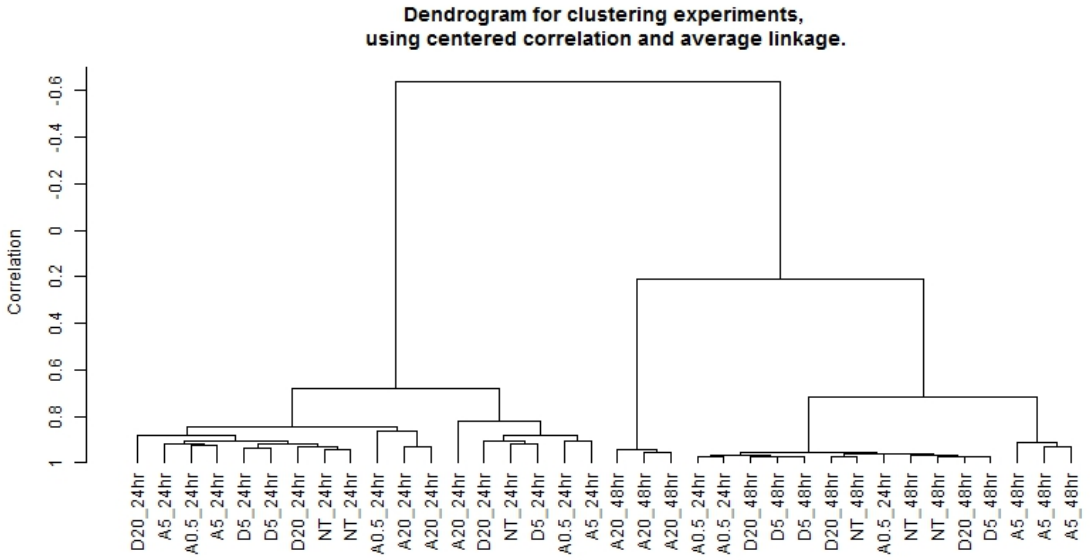

Figure 23: Unsupervised clustering of controls and treatment groups at two time points showing correlation between sample linkages. Samples clustering together are strongly correlated to one another, while samples at 24 hours are negatively correlated to those at 48 hours (R^2 = -0.6). Within the 48 hour treatment time, A20 and A5 cluster separately to the rest of the sample groups (R^2 = 0.2; 0.7 respectively).

6.3.2.3 Class comparison analysis

Table 34 displays the number of significant differentially expressed probes between classes with at least a 2-fold change is displayed.

Comparisons between NT and both DMSO controls at 24 hours showed that no probes were significantly different between the groups suggesting that the vehicle of AFB_1 treatment had no effect on gene expression patterns. Furthermore, only one probe corresponding to *EGR1* was identified as being differentially expressed between NT and DMSO controls following 48 hours treatment.

Very few to no significant differently expressed genes were observed following a 24-hour treatment. After a 48 hour treatment, several gene expression changes were observed between controls and treatment groups. These changes were more apparent with at least a 2-fold change in either direction in cells treated with 5 µg/ml and 20 µg/ml AFB_1. Several changes were unique to the 48 hour treatment period. There was a dose dependent difference in gene expression with increasing numbers of genes being differentially expressed in cells treated with 5 µg/ml compared to those treated with 0.5 µg/ml, and again in cells treated with 20 µg/ml compared to those treated with 5 µg/ml AFB_1.

A few key genes *IL6*, *IL8*, *GADD45A*, *HBEGF* and *CCL20* were differentially expressed at both 24- and 48-hour treatment times and are described in Table 35.

Table 34: Number of significant probes with at least a 2-fold change in either direction following exposure to AFB_1 at two time points.

Time Point	Data Set	No. of significant upregulated probes (at least 2-fold change)	No. of significant downregulated probes (at least 2-fold change)	Total no. of significant probes (at least 2-fold change)	Top Genes
24 hours	D5 vs A0.5	0	0	0	-
	D5 vs A5	0	0	0	-
	D20 vs A20	1	0	1	*IL6*
48 hours	D5 vs A0.5	0	0	0	-
	D5 vs A5	33	10	43	*IL8*, CXCL1, CSF2, BIRC3, HMOX1, HSPA1A, HSPA1B, HSPA6, LAMP3*
	D20 vs A20	280	183	463	*IL8*, CSF2, PTGS2, BIRC3, HMOX1, INHBE, ALOX5AP, HAPLN1, HSPA1A*

* probe repeated twice in top gene list

Table 35: Description of differentially expressed genes common to all AFB_1 treatments versus controls.

Gene Symbol	Gene Name	Function	Expression change in exposed cells versus controls	Reference(s)
CCL20	Chemokine (C-C motif) ligand 20	Also known as liver and activation-regulated chemokine. Elevated protein expression observed in HCC	Upregulated	(Soliman *et al.* 2012)
GADD45A	Growth arrest and DNA-damage-inducible, alpha	Involved in DNA repair and cell growth suppression. Increased protein expression observed in HCC	Upregulated	(Gramantieri *et al.* 2005)
HBEGF	Heparin-binding EGF-like growth factor	Involved in wound healing. Increased mRNA expression observed in HCC	Upregulated	(Inui *et al.* 1994)
IL6	Interleukin-6	Pro- and anti-inflammatory cytokine involved in the immune response. Elevated serum levels observed in HCC and in growth suppressed mice	Upregulated	(Giannitrapani *et al.* 2002; Lieskovska *et al.* 2002)
IL8	Interleukin-8	Chemokine involved in angiogenesis. Potentially involved in the progression of HCC	Upregulated	(Ren *et al.* 2003)

6.3.2.3.1 Comparison of D5vsA5 and D20vsA20 analyses

A Venn diagram was prepared comparing the gene lists derived from the D5 versus A5 and D20 versus A20 comparisons (Figure 24).

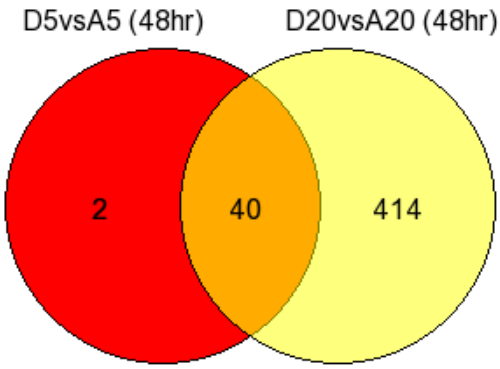

Figure 24: Venn diagram depicting the number of genes in common or unique to each gene list derived from D5 versus A5 and D20 versus A20 comparisons.

The 40 probes in common between the two gene lists included *IL8*, *IL6*, *IL7R*, *BIRC3*, *GADD45A*, *E2F2*, *NFKB1* and *MCM7*. The two probes unique to D5 versus A5 were *CXCL6* and *THBS1* while some of the 414 genes unique to D20 versus A20 were *AEN*, *ASS1*, *CD44*, *CD83*, *CYP1A1*, *CYP1B1*, *IL24*, *MGMT*, *RIOK3*, *TGFBR2* as well as additional probes for *BIRC3* and *GADD45A*.

6.3.2.4 Pathway analysis

The 43 genes, identified as having at least a 2-fold change in expression in the D5 versus A5 comparison, were imported into DAVID v. 6.7 to determine the associated KEGG and Biocarta pathway descriptors. These pathway categories are displayed in Table 36**.**

Table 36: Differentially expressed genes in the D5 versus A5 comparison with at least a 2-fold change represented by their associated pathways.

Pathway Descriptor			Genes	Number of Genes	P value
KEGG	hsa04621	NOD-like receptor signalling pathway	*CXCL1, IL6, CCL2, IL8, CXCL2, NFKB1, TNFAIP3, BIRC3*	8	2.08E-08
	hsa04060	Cytokine-cytokine receptor interaction	*CXCL1, CSF2, IL6, CCL2, IL8, CCL20, CXCL2, CXCL6, IL7R*	9	4.21E-05
	hsa04062	Chemokine signalling pathway	*CXCL1, CCL2, IL8, CCL20, CXCL2, NFKB1, CXCL6*	7	3.54E-04
	hsa05120	Epithelial cell signalling in Helicobacter pylori infection	*CXCL1, IL8, HBEGF, NFKB1*	4	0.005318
	hsa05222	Small cell lung cancer	*E2F2, PTGS2, NFKB1, BIRC3*	4	0.009557
	hsa05219	Bladder cancer	*E2F2, IL8, THBS1*	3	0.020516
	hsa05200	Pathways in cancer	*E2F2, IL6, PTGS2, IL8, NFKB1, BIRC3*	6	0.026985
	hsa04115	p53 signalling pathway	*RRM2, THBS1, GADD45A*	3	0.0499
Biocarta	h_stemPathway	Regulation of haematopoiesis by cytokines	*CSF2, IL6, IL8*	3	0.014135

	h_p53hypoxiaPathway	Hypoxia and p53 in the Cardiovascular system	*HSPA1A, HSPA1B, NQO1, GADD45A*	3	0.034759
	h_inflamPathway	Cytokines and Inflammatory Response	*CSF2, IL6, IL8*	3	0.040329

A similar analysis was done for the 463 differentially expressed genes with at least a 2-fold change derived from the D20 versus A20 comparison (Table 37).

Table 37: Differentially expressed genes in the D20 versus A20 comparison with at least a 2-fold change represented by their associated pathways.

	Pathway Descriptor		Genes	Number of Genes	P value
KEGG	hsa04621	NOD-like receptor signaling pathway	*CXCL1, IL6, CCL2, IL8, CXCL2, NFKBIA, IL1B, NFKB1, TNFAIP3, BIRC3, BIRC2*	11	1.42E-05
	hsa05222	Small cell lung cancer	*TRAF1, CCNE2, E2F2, LAMB3, PTGS2, LAMC3, LAMA5, NFKBIA, NFKB1, BIRC3, BIRC2*	11	2.06E-04
	hsa04512	ECM-receptor interaction	*LAMB3, CD44, LAMC3, LAMA5, COL1A2, COL1A1, COL11A1, THBS3, COL5A1, HMMR*	10	9.38E-04
	hsa04110	Cell cycle	*CCNE2, E2F2, CDC45, MCM7, CDKN2D, PKMYT1, CHEK1, PTTG1, GADD45B, MCM4, GADD45A, CDC25A*	12	0.001372
	hsa03030	DNA replication	*RFC3, MCM7, POLE, POLA2, MCM4, FEN1*	6	0.004332
	hsa04060	Cytokine-cytokine receptor interaction	*CXCL1, CSF2, IL6, CCL2, IL8, CXCL5, CXCL2, TGFBR2, IL24, CX3CL1, IL7R, IL11, CCL20, IL20RB, INHBE, IL1RAP, IL1B*	17	0.005763

	hsa05200	Pathways in cancer	*TRAF1, E2F2, IL6, FGFR3, IL8, PTGS2, MITF, TGFBR2, NFKBIA, CDH1, NFKB1, NFKB2, BIRC3, BIRC2, CCNE2, LAMB3, LAMC3, LAMA5, SLC2A1*	19	0.010304
	hsa05020	Prion diseases	*EGR1, IL6, IL1B, HSPA1A, HSPA5, HSPA1B*	5	0.020858
	hsa00330	Arginine and proline metabolism	*ODC1, PYCR1, ASS1, P4HA2, P4HA1, AMD1*	6	0.021823
	hsa00240	Pyrimidine metabolism	*RRM2, RRM1, POLE, UPP1, TXNRD1, POLA2, TK1, POLR2A*	8	0.025121
	hsa04210	Apoptosis	*IRAK2, IL1RAP, NFKBIA, IL1B, NFKB1, BIRC3, BIRC2*	7	0.048622
Biocarta	h_il1rPathway	Signal transduction through IL1R	*IRAK2, IL6, MAP2K3, IL1RAP, NFKBIA, IL1B, NFKB1*	7	0.001201
	h_nthiPathway	NFkB activation by Nontypeable Hemophilus influenzae	*IL8, MAP2K3, TGFBR2, NFKBIA, IL1B, NFKB1*	6	0.003077
	h_stemPathway	Regulation of hematopoiesis by cytokines	*CSF2, IL6, IL8, IL11*	4	0.025061
	h_LDLpathway	Low-density lipoprotein (LDL) pathway during atherogenesis	*IL6, CCL2, LDLR*	3	0.025323
	h_tnfr2Pathway	TNFR2 Signaling Pathway	*TRAF1, NFKBIA, NFKB1, TNFAIP3*	4	0.03519

Several of the pathways associated with the gene lists derived from the A5 versus D5 and A20 versus D20 groups involve immune regulation, cytokine signalling and cancer development. Additionally, transcription factor pathways, including NF-κB, are significantly associated with AFB_1-associated gene lists.

6.3.2.5 Transcription factor gene set comparison

In order to further explore the involvement of transcription factors (TF) in AFB_1-associated gene expression changes, gene set enrichment was carried out. When comparing gene sets associated with known TFs, it was determined that several significant differentially expressed genes belonged to 15 TF pathways for the D5 vs A5 comparison and to 17 TF pathways for the D20 vs A20 comparison (Table 38).

Table 38: Genes related to A5 and A20 exposures with associated transcription factor pathways (*P* < 0.001).

Transcription Factor Pathway	Number of significant genes	Genes in pathway
D5 vs A5		
E2F-1_T01542	3	*EDN1, ALOX5AP, POLA2*
E2F-4_T01546	3	*E2F2, GADD45A, POLA2*
ETS1_T00112	2	*CSF2, HMOX1*
ETS2_T00113	1	*CSF2*
FOS_T00123	2	*HSPA1A, NQO1*
NFIC_T00176	4	*EDN1, HMOX1, HSPA1A, IL6*
NFKB1_T00591	11	*BIRC3, CXCL1, EDN1, HMOX1, IL6, IL8*, NQO1, GADD45A, CCL2, CXCL2*
POU2F1_T00641	4	*CSF2, IL8*, GADD45A*

RARA_T00719	4	*IL6, IL8*, GADD45A*
REL_T00168	6	*CSF2, CXCL1, IL6, IL8*, CCL2, CXCL2*
RELA_T00594	9	*BIRC3, CSF2, CXCL1, IL6, IL8*, CCL2, CXCL2, CCL20*
SP3_T02338	3	*CXCL1, EXO1**
TFAP2A_T00035	8	*HMOX1, HSPA1A, IL6, IL8*, ALOX5AP, NQO1, IL7R*
TP53_T00671	11	*BIRC3, E2F2, HSPA1A, IL6, IL8*, THBS1, NDRG1, GADD45A, MCM7, DKK1*
WT1_T00899	2	*HSPA1A, THBS1*
D20 vs A20		
CEBPB_T00581	4	*IL1B, IL6, IL8**
CEBPD_T00583	3	*ALOX5AP, IL1B, IL6*
E2F-1_T01542	22	*ALOX5AP, CDC25A, FEN1*, GCLM*, IL1B, INHBE, NPTX2, PLAU, SLC13A5, TK1, IER3, POLA2, RAD54L, EDN1,*
E2F-2_T01544	1	*CX3CL1, RRM1, HERPUD1*, RFC3*, PLAU*
E2F-4_T01546	19	*CDC25A, E2F2, FEN1*, GADD45A*, TK1, AURKB, BARD1, CYP1B1, POLA2, RAD54L, RRM1, CHEK1, KIF4A, RFC3*, HMMR, PTTG1*
ERG_T00265	3	*HMOX1, TGFBR2**
ESR1_T00261	7	*CDH1, GADD45A*, IL8*, CYP1B1, GADD45B*
FLI1_T02066	6	*COL1A1, HMOX1, BARD1, TGFBR2*, UPP1*
LEF1_T02905	2	*MMP7**
NFIC_T00176	9	*COL18A1, COL1A1, HMOX1, HSPA1A, IL6, EDN1, PCK2*, SCD*
NFKB1_T00591	26	*BIRC3*, COL1A1, CXCL1, CXCL2, GADD45A*, HMOX1, IL1B, IL6, IL8*, NQO1, PLAU, IER3, NFKBIA, PTHLH*,*

POU2F1_T00641	9	*EDN1, CCL2, CD83*, BIRC2*, CXCL5*, CSF2, GADD45A*, IL8*, PLAU, TXNRD1*, AKR1C3*
RARA_T00719	12	*GADD45A*, IL6, IL8*, PLAU, PTHLH*, SCD, RARRES3, GADD45B, SERPINF1*
REL_T00168	9	*CSF2, CXCL1, CXCL2, IL1B, IL6, IL8*, NFKBIA, CCL2*
RELA_T00594	17	*BIRC3*, CCL20, COL1A1, CSF2, CXCL1, CXCL2, IL1B, IL6, IL8*, NFKBIA, PCK2*, CCL2, BIRC2**
TFAP2A_T00035	23	*ALOX5AP, CA9, COL11A1*, COL1A1, EGR1, HMOX1, HSPA1A, IL6, IL8*, MMP7*, NQO1, TK1, IL7R, LOX, ODC1, HK2, PDE5A, CXCL5*, FGFR3*
TP53_T00671	33	*BIRC3*, E2F2, EGR1, GADD45A*, GCLM*, HSPA1A, IL1B, IL6, IL8*, NDRG1, TK1, DKK1, FBLN2*, IER3, PTHLH*, CX3CL1, TIMP3, CHEK1, MCM7*, BIRC2*, EEF1A1, ANTXR1, PTTG1*

Genes marked with * have more than one probe that is significantly different

The majority of the genes (92% for D5 vs A5 and 72% for D20 vs A20) within these pathways were upregulated and therefore activated in AFB_1-treated cells compared to the DMSO controls. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway including NF-κB1, Rel and RelA was particularly highlighted in both A5 and A20 analyses with a large number of genes associated with these TFs.

6.3.2.6 Comparison of gene expression changes between liver cells and Gambian infants

The gene symbols as well as the direction of changes were compared between gene lists derived from the Gambian infant comparison and liver cell D5 versus A5 and D20 versus A20 comparisons. The gene lists used for this purpose were the complete lists with FDR below a threshold (5% for cells and 10% for Gambian infants) including probes repeated only once and with lower fold change.

The Venn diagram in Figure 25 depicts this comparison with a table (Table 39) describing the genes in common between both lists as well as the direction of expression changes. Fifty % of the genes in common between the lists show the same trend in gene expression changes. These genes were *COMMD3*, *FAM111A*, *ID2*, *LSM3* and *WSB1*. Comparison of gene lists consisting only of genes with repeated probes and/or a high fold change did not identify any genes in common.

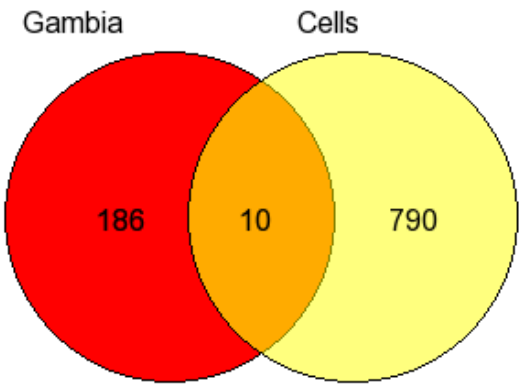

Figure 25: Venn diagram showing the relation between gene lists derived from Gambian infants and liver cells exposed to AFB_1.

Table 39: Description of the trend of gene expression direction in genes common to Gambian infants and liver cells. Genes displaying similar trend in gene change direction are highlighted.

	Direction of change (high exposure versus low exposure)	
Gene Symbol	**Gambian infants**	**Liver cells**
BIRC2	Downregulated	Upregulated
COMMD3	Upregulated	Upregulated
DSTN	Upregulated	Downregulated
FAM111A	Upregulated	Upregulated
FYN	Upregulated	Downregulated
ID2	Upregulated	Upregulated
LSM3	Upregulated	Upregulated
NBPF10	Downregulated	Upregulated
TMEM141	Upregulated	Downregulated
WSB1	Downregulated	Downregulated

6.4 Discussion

A genome-wide approach with the aim to identify the effects of *in vitro* AFB_1 exposure in liver cell DNA methylation and gene expression patterns was explored.

6.4.1 Whole genome DNA methylation in AFB_1-exposed liver cells

Illumina's 450K bead chip arrays explore DNA methylation changes at the individual CpG site level. While a site-level analysis would enable identification of minute changes to a single site in the methylome following an environmental exposure, it vastly increases the number of multiple comparisons necessary (Wang *et al.* 2012). Additionally, a change at the individual site level may not reflect any biological changes. For instance, a change occurring in one CpG site in the gene body may not be sufficient to produce an alteration in gene expression. In this case, a region-level analysis would be informative as it would take into account the changes occurring within the gene body or promoter region, which could reflect consistent changes related to the phenotype of interest. This would reduce the number of multiple comparisons necessary and would potentially reflect real changes within the mechanics of gene expression regulation by DNA methylation (Wang *et al.* 2012).

6.4.2 DNA methylation differences associated with aflatoxin exposure

In the current study, no significant changes were observed at the probe or site-level suggesting that changes at individual CpG sites were not associated with AFB_1 exposure. However, when grouping these sites by gene or promoter level, a number of significant changes were observed.

Some of the genes identified in the gene lists are involved in immune function regulation (*IL2*, *IGHJ2*, *IGHJ3*), while pathway analysis revealed that some genes belonged to olfactory transduction. However, there were no genes in common between all three treatments versus controls. Of the genes in common between any two of the comparisons at either time points, a few have a potential role in the mechanism of AFB_1-related health effects.

USP17 is a subfamily of deubiquitinating enzymes, which are induced by cytokine stimulation and can influence cell growth and survival (Burrows *et al.* 2010). Indeed, *USP17* overexpression has been observed in biopsies from colon, lung and cervix tumours as well as in a number of tumour-derived cell lines (Mcfarlane *et al.* 2010). In the present study, *USP17L7* was hypomethylated in AFB_1 exposed cells compared to the DMSO controls. It is possible that deubiquitinating enzymes may play a role in AFB_1-associated carcinogenesis.

Another gene in common to the gene lists was *GPR56*, which was hypomethylated in AFB_1-treated cells compared to controls. Overexpression of *GPR56* was observed in glioblastoma tumours and was found to inhibit glioma cell adhesion causing cell rounding. Furthermore, upregulation of *GPR56* was observed to activate the NF-κB signalling pathway (Shashidhar *et al.* 2005).

Interestingly, a comparison of the differentially methylated genes in Gambian infants and in cultured treated liver cells revealed that a few genes (*GPR56, MIR24-2* and *MBL2*) were altered in both cases with only *MIR24-2* showing the same trend in methylation change. MicroRNA-24 (miR24) has been identified as a foetal liver microRNA (Fu *et al.* 2005). Also, overexpression of miR24 has been shown to suppress p16 activity in cervical carcinoma and human diploid fibroblast cells (Lal *et al.* 2008) and to inhibit cell cycle regulatory genes such as *MYC* and *E2F2* and prevented differentiation of HepG2 cells (Lal *et al.* 2009). The latter effect is most likely due to the inhibition of hepatocyte nuclear factor 4α, an important factor in hepatocyte differentiation, in HepG2 cells with overexpressed miR24 (Takagi *et al.* 2010).

In this study, *MIR24-2* was hypomethylated in high exposure groups suggesting that mir24-2 expression is higher in these infants/cells. It is possible that at an early stage of AFB_1 exposure, changes occur in response to treatment including survival mechanisms that prevent the differentiation of damaged cells. Interestingly, miR24 clearly has an important role in hepatocyte differentiation but also in disruption of tumour suppression at the cellular level.

Overall, it appears that AFB_1 exposure *in vitro* does affect the liver cell methylome resulting mainly in hypomethylation of several genes encoding immunoglobulins, interleukins and some cancer-associated genes. Additionally, the observed changes to DNA methylation in Gambian infants (Chapter 5) exposed to AFB_1 *in utero* were also involved in the immune response. Therefore, the current results provide some support to the significant differentially methylated genes identified in Gambian infants.

However, no dose-dependent genes have been identified. This may suggest that the changes observed are potentially temporary. Additionally, pathway analysis did not reveal any realistic patterns of AFB_1-induced differences. It is possible that at alternate time points, cells display a clear signature pattern of AFB_1 exposure on DNA methylation. Furthermore, long-term exposure assessment may replicate changes occurring in humans and may reveal a DNA methylome signature of exposure.

6.4.3 Whole genome gene expression in AFB_1-exposed liver cells

While very few changes were observed following a 24-hour treatment, several genes were differentially expressed after 48 hours of exposure, with these changes being dose dependent. However, five genes common to all AFB_1 treatments were identified suggesting a general mechanism of exposure-associated effects. All five genes (*COMMD3*, *FAM111A*, *ID2*, *LSM3* and *WSB1*) have been previously linked to HCC development, prognosis or progression (Ren *et al.* 2003; Giannitrapani *et al.* 2002) suggesting that these changes occur at an early stage following exposure and can be potential markers of carcinogenesis. Additionally, increased *IL6* expression has been observed in these cells, which has previously been associated with growth impairment (Lieskovska *et al.* 2002; Lieskovska *et al.* 2003).

6.4.4 Pathways involved in AFB_1 exposure-associated effects

Several of the differentially expressed genes were associated with p53 and cytokine signalling and cancer pathways. These alterations represent early changes to the genome in normal liver cells in response to AFB_1 exposure. Chronic exposure can therefore induce repeated insults, which can ultimately lead to carcinogenesis as well as disruption to other important biological pathways. This type of alteration by chronic exposure to an environmental carcinogen has been previously shown for arsenic exposure in normal human keratinocytes. At levels lower than the limit permitted in drinking water, chronic arsenic exposure induced alterations to gene expression, some of which were then maintained throughout the treatment period of up to 14 days (Rea *et al.* 2003).

6.4.4.1 The NF-κB pathway

Notably, the NF-κB pathway was activated in AFB_1-exposed liver cells, after 48 hours. The NF-κB family of transcription factors while traditionally thought to only regulate the inflammatory and immune response, have also been found to influence transcriptional regulation of cell survival, differentiation and proliferation (Hayden and Ghosh 2008). The NF-κB family comprises 5 members: p50 (precursor p105), p52 (precursor p100), p65, c-Rel and RelB, which are encoded by *NF-κB1*, *NF-κB2*, *RELA*, *REL* and *RELB*. These transcription factors all have an N-terminal Rel homology (RHD) in common, which is responsible for DNA binding and dimerization. NF-κB hetero- or homo-dimers regulate transcription by binding to the κB sites in the promoters or enhancers of target genes. p65, c-Rel and RelB are thought to activate transcription due to the presence of a transcriptional activation

domain (TAD). However, p50 and p52 lack a TAD and are thought to repress transcription unless associated with a transcriptional activating member of the NF-κB family (Dolcet *et al.* 2005).

NF-κB members are inactivated by binding to IκB proteins, IκBα, IκBβ or IκBƐ encoded by *IKBA*, *IKBB* or *IKBE* genes respectively. Initially, it was thought that this inactivation prevented the NF-κB dimers from moving from the cytoplasm to the nucleus. However, it has been noted that the IκBα, while bound to the p65/p50 dimer, masks the nuclear localisation sequence (NLS) of p65 leaving the NLS of p50 exposed. It has also been discovered that this complex shuttles between the cytoplasm and nucleus, although the presence of a nuclear export signal (NES) on IκBα immediately expels the complex forcing it to return to the cytoplasm (Ghosh and Karin 2003).

In response to inducing stimuli, the two serines present in IκBs are phosphorylated by IκB kinases (IKKs), which then marks them for ubiquitination and consequently proteasomal degradation (Hayden and Ghosh 2008). There is a broad range of stimuli including cytokines and growth factors that have been identified as factors initiating the degradation of the IκBs and therefore releasing NF-κB dimers, which are then free to regulate transcription of target genes (Dolcet *et al.* 2005).

NF-κB plays a key role in the progression of liver disease that can be brought about by chronic injury or exposure. In response to injury, the NF-κB pathway is activated to initiate an inflammatory response as a protective measure. However, if the balance of this pathway is tipped in either direction, it can contribute to the onset of hepatocarcinogenesis (Luedde and Schwabe 2011).

Regardless of aetiology, the activation of the NF-κB pathway seems to be a consistently early event in human liver cancers (Liu *et al.* 2002). Also, it has been shown that in HepG2 cells treated with 10 μM AFB_1 (similar in concentration to A5 in the current work) there was an 8-fold increase in NF-κB transcription factor and a 6-fold increase in AP1 transcription factor following a 24 hour treatment. Additionally, it was observed that incubation with AFB_1 resulted in increased induction of p65 (Rel-A), p50, c-JUN and c-FOS complexes compared to the control (Banerjee *et al.* 2000).

6.4.4.2 Role of NF-κB and STAT3 pathways in HCC

The STAT3 pathway, activated by growth factors and cytokines including IL6, has been linked to HCC development. In fact, phosphorylated nuclear STAT3, indicative of its activation, has been observed in 60 % of human HCCs (He *et al.* 2010). A number of the transcription targets for both NF-κB and STAT3 pathways overlap and are involved in cell proliferation, survival and the stress response (Karin 2009). In carcinogen-induced hepatocyte death, IL1α is produced, which activates the NF-κB pathway in Kupffer cells. In response, these cells then produce IL6, which activates STAT3 in hepatocytes, resulting in cell proliferation compensatory to the initial hepatocyte death- events eventually leading to HCC development (He and Karin 2010). However, NF-κB can also act as a negative regulator of STAT3 in HCC cells. Suppression of IKKβ, which is responsible for the activation of NF-κB, results in the accumulation of ROS. Oxidation of Src homology region 2 domain-containing phosphatase-1 (SHP1) or SHP2, which dephosphorylate JAK2 and STAT3, inhibits their catalytic activity leading to an accumulation of activated JAK2 and STAT3. These then lead to hepatocyte proliferation and tumourigenesis (He *et al.* 2010).

In the present work, *STAT3* expression was higher in infants exposed to higher AFB_1 *in utero*, while no significant change in *IL6* was observed. In normal liver cells exposed to AFB_1, *IL6* and *NFKB1A* expression were elevated compared to controls. These results suggest a positive interaction between the NF-κB and STAT3 pathways in AFB_1-induced changes to the genome. Furthermore, these observed differences in gene expression in cultured liver cells support the identified genes and pathways in Gambian infants.

In a more direct exposure system i.e in the liver cells, AFB_1 potentially activates STAT3 via increased expression of IL6 and NF-κB family members. These initial changes following a 2-day exposure could contribute to hepatocarcinogenesis leading to HCC development. In Gambian infants, potentially chronic exposure during gestation can also activate the STAT3 signalling either via a similar mechanism involving IL6 or through alternate pathways. However, the limitations of the Gambian infant study must be taken into account when considering the implications of these findings. One of the main limitations was the use of RNA derived from whole blood with potential disruption from globin RNA to mRNA expression. Also, as infant blood was collected 3-6 months after birth, other factors such as viruses, may have influenced the methylation and expression of genes.

Nevertheless, they do provide a link between AFB_1 exposure and activation of the STAT3 and NF-κB pathways, which could potentially be implicated in hepatocarcinogenesis. Additionally, the association between AFB_1 exposure and IL6 induced STAT3 signalling, with SOCS-3 activation and consequently IGF1 down-regulation, could be part of the mechanism of AFB_1-related growth impairment.

6.4.5 Conclusions

AFB_1 exposure in cultured non-tumourigenic liver cells indeed alters the methylome and gene expression patterns. These findings suggest that exposure to AFB_1 can induce early events and cellular disruptions that can lead to aberrant mechanisms of cell survival, growth and development. Although a DNA methylation signature was not identified, specific changes in the regulation of expression of key genes, including those encoding immunoglobulins, indicate a disruption of the immune response.

A clearer signature of gene expression changes in liver cells was determined. Activation of the NF-κB and STAT3 signalling pathways imply that these early events mark the start of important changes to the genome. These key alterations potentially reflect a predisposition to cancer development and also provide some evidence for mechanisms of AFB_1-associated growth impairment.

7 Overall discussion and conclusions

7.1 Introduction

Following the discovery and characterisation of aflatoxins in the 1960s, several studies have focussed on the impact they have on animal and human health. The identification of AFB_1 adducts in the blood and urine as biomarkers of exposure have furthered our understanding of the various health effects associated with AFB_1 exposure. The mechanisms of AFB_1-associated liver carcinogenesis, growth and immune function impairment have been widely explored. A major finding about the mechanism of AFB_1 liver carcinogenesis was the G->T transversion in codon 249 of the *p53* gene observed in liver cancers in regions of high AFB_1 exposure. However, the mechanism of exposure-associated growth and immune function impairment is not understood. Additionally, AFB_1 exposure occurring during foetal development can cause growth retardation in new-borns through an unknown mechanism. In the present work, we have 1) tested a hypothesis exploring the role of IGF in AFB_1-associated child growth impairment; 2) determined AFB_1 exposure at two time points during pregnancy and examined the effects of seasonality on exposure; and 3) identified potential signatures of DNA methylation and gene expression changes following AFB_1 exposure in both Gambian infants and cultured liver cells.

7.2 AFB_1-associated growth impairment

At the start of this study, little was known about the mechanism of AFB_1-associated growth impairment, but based on the results presented here, it can be seen that this mechanism is multi-faceted with the involvement of a few key factors that can

disrupt normal developmental processes. We have found that approximately 16% of the overall effect of AFB_1 exposure on height reduction in a population of Kenyan school children was through an indirect mechanism involving reduced IGF1 protein levels. In non-tumourigenic liver cells, a direct effect of AFB_1 exposure on IGF axis component gene and protein expression was also observed, providing supporting evidence for the role of an altered IGF axis in AFB_1-associated growth impairment.

While these findings explain a part of the effect of AFB_1 on growth impairment, other routes of growth impairment are probably involved in the overall mechanism. In Gambian infants with *in utero* exposure and in AFB_1-treated liver cells, *IL6*, *STAT3* and *NFKB1A* were upregulated in the higher exposure groups. These genes belong to the interlinked JAK/STAT and NF-κB pathways, which are involved in several developmental processes including growth, cell proliferation and apoptosis. The STAT pathway, via STAT3, is activated either directly through elevated IL6 levels or indirectly through the NF-κB pathway. This, in turn, increases the expression of SOCS-3, which is a negative regulator of the GH-IGF1 signalling pathway (Lieskovska *et al.* 2003). Indeed, exposure to another mycotoxin, DON, has been observed to overexpress IL6 followed by upregulation of SOCS-3 in the liver, which then lowers IGF-ALS levels in mice (Amuzie *et al.* 2009). This has been suggested to be the mechanism through which DON induces growth impairment in mice (Amuzie and Pestka 2010).

Taken together, the changes in the expression of key genes involved in JAK/STAT and NF-κB signalling appear to contribute to the mechanism of growth impairment in infants highly exposed to aflatoxins during development. The fact that these changes are observed following *in utero* exposure in infants and also in non-tumourigenic cells treated with AFB_1, we may conclude that the upregulation of

STAT3, *IL6* and *NFKB1A* occurs in response to early life exposure or as an early event of AFB_1 exposure. If dietary AFB_1 exposure persists, as a result of important changes to the genome, a significant alteration to the phenotype is probable. Additionally, the timing of AFB_1 exposure may have an important bearing on the consequences of exposure. The earliest stages of foetal development are a crucial time during which any exposure or external insults can alter the development of the methylome (Chmurzynska 2010). While we have observed changes in some key genes potentially involved in a mechanism of AFB_1-associated health effects, further enquiry into the changes immediately following birth with assessment of cord blood AF-alb levels may provide more clues into the mechanisms involved. Furthermore, exposure at alternate times may be reflected by different mechanisms ultimately affecting health outcomes.

7.3 Effect of AFB_1 on immune function and inflammation-related liver carcinogenesis

The mechanism of AFB_1-associated liver carcinogenesis has been explored previously resulting in the identification of the G->T transversion in *p53* (Lasky and Magder 1997) as well as in hypermethylation of key tumour suppressor genes including *RASSF1A* and *p16* (Zhang *et al.* 2002; Zhang *et al.* 2003; Zhang *et al.* 2006). However, very little is known about the impact of early life AFB_1 exposure on health.

An important finding of this study was the global increase in expression of factors involved in the immune response. These factors included immunoglobulins (*IGHJ1*, *IGHJ3*) and cytokines (*IL2*, *IL6*, *IL8*, *IL18*). These factors were either significantly hypomethylated or highly expressed in Gambian infants or cells exposed to high levels of AFB_1. Also, cell-surface markers (*CD3D*, *CD2*, *CD4*, *CD3G*) were hypermethylated in Gambian infants in the higher AFB_1 exposure group.

These changes are similar to previous findings where *IL6* expression was increased and *CD14* expression was decreased in a murine macrophage cell line, J774A.1 exposed to low doses of AFB_1 (Bruneau *et al.* 2012). Indeed, IL6 overexpression was also observed in pigs fed diets containing AFB_1 (Meissonnier *et al.* 2008).

The alteration of the factors responsible for the immune response can affect susceptibility to infections and can also promote inflammation eventually leading to cancer development. Indeed, inflammation manifesting through NF-κB signalling can exert pro-tumourigenic effects (Karin and Greten 2005). In hepatocarcinogenesis, the NF-κB pathway is activated as a result of chronic injury or insult in hepatocytes, which in turn activate the Kupffer cells. IL6 is produced by activated Kupffer cells and plays a very important role in inflammation. Under normal conditions, IL6 is present in low quantities in the serum but is overexpressed under stress. Increased amounts of IL6 then activate the JAK/STAT pathway resulting in compensatory proliferation, which eventually leads to malignant transformation (Naugler and Karin 2008). These pathways potentially form the basis of the mechanism of AFB_1-associated liver carcinogenesis. A model of inflammation-induced hepatocarcinogenesis due to chronic insult is shown in Figure 25.

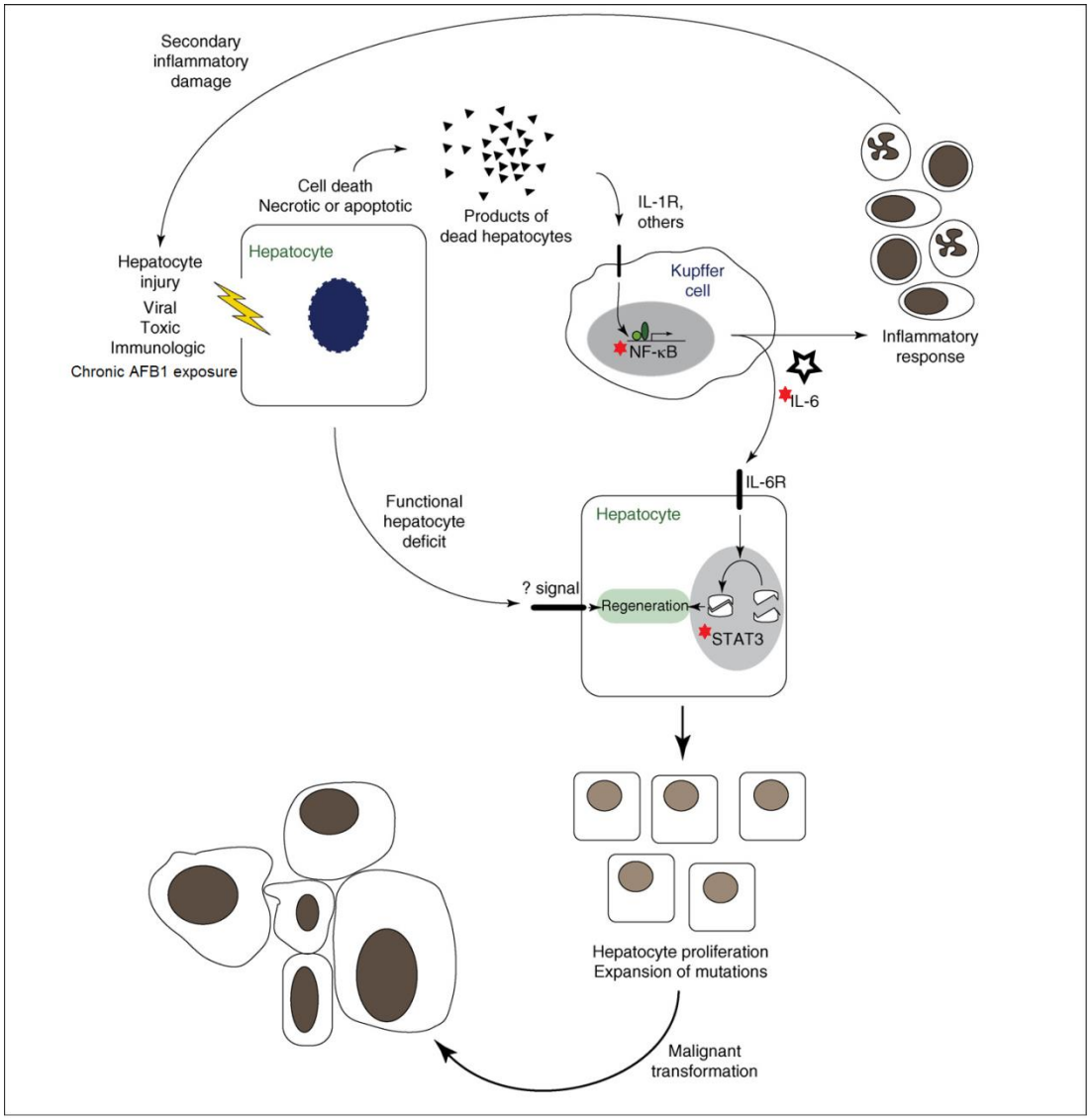

Figure 26: Model of hepatocarcinogenesis induced by chronic injury or insult including AFB_1 exposure resulting in an inflammatory response. Chronic injury or exposures result in death of vulnerable hepatocytes, which trigger signals in the form of IL1R or other factors. These signals activate the NF-κB signalling pathway in Kupffer cells prompting the production of IL6, which contributes to the inflammatory response and also activates JAK/STAT signalling. This pathway together with a trigger from dead hepatocytes promotes the compensatory production of new cells, which can eventually lead to malignant transformation through mutations. Components potentially altered via aberrant gene expression or methylation as a result of early life or *in vitro* exposure to AFB_1 are marked with a red star. Image modified from (Naugler and Karin 2008).

A common element in these reported changes is inflammation, which may be the driver in AFB_1-induced health effects. Exposure to AFB_1 can trigger inflammatory events with overexpression of IL6 and NFKB1A, which in turn activate the

JAK/STAT and NF-κB pathways. Repeated insult would eventually lead to carcinogenesis, growth and immune function impairment.

Further work on the mechanism of AFB_1 exposure-associated health effects through the NF-κB, IL6 and STAT signalling pathways is necessary to confirm their involvement and to fully understand the association with growth impairment and the conversion of a normal, healthy cell into a cancerous cell. This work could include *in vitro* analysis to study the pathways at the protein level using techniques such as immunofluorescence. Further work would also potentially identify additional markers of AFB_1 exposure.

7.4 Limitations of this study

A number of factors may have influenced the results obtained throughout this work and therefore need to be addressed.

In order to ascertain the role of a disrupted IGF growth axis on child growth impairment, a population of children with a more homogeneous age range would be beneficial. In the work presented in Chapter 3, children were aged 6-17, which included children undergoing puberty thereby becoming a major confounder to the impact of dietary AFB_1 on growth. Additionally, infection status and other health effects would also have to be taken into account as potential confounders to growth impairment and alteration to IGF levels and expression. To overcome these issues, a cell model was added to the study providing additional evidence for AFB_1-induced disruption of IGF. However, cell models may not always reflect the exact nature of the effects of a toxin due to altered expression of cell surface molecules or the fact

that the cells are not in their natural environment and may behave differently (Kaur and Dufour 2012).

Another major limitation of this study was that all whole genome analyses were done using whole blood cells rather than the target tissue. As it is unethical to obtain and study liver tissue from new born babies, an easily accessible source i.e. blood, was utilised instead. However, it is important to note that DNA methylation patterns vary by blood cell type. For instance, in healthy males it was found for that for methylation levels were different for 22% of the probes analysed for mononuclear and granulocyte cells (Reinius *et al.* 2012). Therefore, any differences in WBC counts between infants due to an infection or due to natural variation would skew the DNA methylation results considerably. The total white blood cell count was unavailable for the infant blood samples analysed in the present study and therefore all significant differences in DNA methylation should be considered with caution.

Blood samples analysed for whole genome DNA methylation and gene expression were obtained from infants aged 3-6 months. Preferably, cord blood samples would have provided a better idea of the changes occurring as a result of *in utero* exposure. Due to the amount of time between birth and sample collection, other factors including infection, may have confounded the results.

Additionally, the moment of conception would have been the ideal point at which changes could occur in the methylome and genome of developing embryos following an exposure (Heijmans *et al.* 2008) including AFB_1. In the current study, AFB_1 exposure at an early point during pregnancy i.e. 0-15 weeks was analysed. Therefore, for some infant blood samples, exposure would have been measured towards the end of the 'early' period. Another important variable that could skew the results at this

stage would be season of exposure. The study was conducted with the assumption that a woman belonging to a high AFB_1 exposure group during conception would likely remain in the same group a few months into pregnancy. The season at the time of conception would also influence DNA methylation (Khulan *et al.* 2012). However, the season during a later time point following conception may be different to the season at the time of conception. For this reason, only women who conceived during a season that matched the season at the time of blood collection for AF-alb measurement were analysed separately. Nevertheless, the observed DNA methylation changes were similar to changes observed when including all women in the analysis.

Another potential limitation of the study was the relatively high *P* value threshold used in the whole genome analyses. In DNA methylation or gene expression analyses comparing only a few loci between groups, a *P* value less than 0.05 or 0.01 is usually acceptable. However, when comparing large numbers of loci as is the case in genome-wide analyses, the proportion of false positives among the list of total positives is greatly increased. Therefore, a more stringent cut-off (such as $P < 0.0001$ or lower) is necessary to reduce the chance of identifying false positives (Storey and Tibshirani 2003). In the current study, a cut-off of 0.01 was used to filter out genes with a *P* value above this value. This is a particularly high threshold, which may have increased the number of false positives in the final gene lists. The validations for DNA methylation and gene expression in Gambian infants may have been unsuccessful for some loci due to the observed changes being false positives. However, an acceptable FDR (q value) of < 0.10 or < 0.05 was adopted to minimise the proportion of false positives. Additionally, while a higher threshold would

increase the number of false positives, it would also increase the chances of identifying small changes in biologically sensible loci that can be explored further.

7.5 Conclusion

AFB_1 exposure at an early stage in life can affect normal protein levels (IGF1, IGFBP3) and is associated with significant DNA methylation and gene expression changes in infants exposed to AFB_1 *in utero* as well as in non-tumourigenic cultured liver cells.

Despite the limitations in this study, key genes involved in inflammation, growth and cell proliferation have been identified as being differentially methylated or expressed following AFB_1 exposure. This is the first time that such altered epigenetic and transcriptomic patterns in association with aflatoxin have been observed in exposed children. Interestingly, these important changes have been observed in both Gambian infants and in cultured liver cells. A mechanism involving NF-κB, IL6 and STAT3 potentially play a central role in AFB_1-associated immune function and growth impairment and liver carcinogenesis. The involvement of the IGF growth axis also provides a better understanding of the mechanism of growth impairment.

Overall, the current study has identified potentially key players in AFB_1 exposure-related health effects. The involvement of these important proteins in the manifestation of the adverse health effects associated with aflatoxin contributes to our understanding of the impact of early life exposure to aflatoxins.

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