Impacts of Aflatoxin Exposure on Human Health and Mechanism Investigation of Aflatoxin and Fumonisin Health Effect *in vitro*

Hang Wu

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This candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Abstract

Aflatoxin is one of the major types of mycotoxins, and frequently contaminates foods in tropical or sub-tropical areas. Populations with regular consumption of the contaminated foods have frequent exposure to aflatoxin. Dietary exposure to aflatoxin has been reported to cause acute aflatoxicosis and liver cancer and has been implicated with immune suppression and child growth impairment. However, the effects of aflatoxin exposure on child growth impairment are inconclusive and mechanisms of aflatoxin health effects are not fully understood. Evidence shows a high prevalence of co-exposure of aflatoxin and fumonisin, another important mycotoxin, in many populations. The combined toxicity of aflatoxin and fumonisin has been investigated less frequently than aflatoxin exposure alone.

In this work, aflatoxin exposure levels in acutely ill children in Africa and South Asia were assessed by measuring the biomarker, aflatoxin-albumin adduct, in serum samples. The relationship of aflatoxin exposure to child growth and child mortality was determined. Aflatoxin exposure levels in the children varied by geographic factor and age. African children had higher aflatoxin exposure levels than South Asian children, and aflatoxin exposure levels increased with increasing age. Aflatoxin exposure was negatively associated with child growth, and it was a risk factor contributing to child mortality.

An *in vitro* model was used to investigate the mechanisms that potentially explain aflatoxin health effects, particularly the effects on gene expression and DNA methylation caused by aflatoxin alone or in combination with fumonisin. Aflatoxin caused significant modulation of gene expression of growth factors and immune-related genes, such as: *IL6*, *CCL20*, *BMP2*, and *NDP*. And one CpG site near the transcription start site of *CCL20* was found to have hypomethylation associated with aflatoxin exposure. RNA-seq results indicated that aflatoxin caused differentially expressed genes were significantly enriched in the pathways such as: cytokine-related/TNF/NF-kB pathways. In the presence of fumonisin, there were synergistic effects of aflatoxin and fumonisin on cytotoxicity and gene expression modulation of *IL6* and *BMP2*.

In the acutely ill children in Africa and South Aisa, metabolomic levels in those children, in relation to aflatoxin exposure, were conducted. Aflatoxin exposure was significantly associated with several metabolites, and the affected metabolites were enriched in the pathways that have important roles in oxidative stress-related disease, protein synthesis, and growth.

Overall, this study strengthens the evidence that aflatoxin exposure contributes to child growth impairment and illustrates that aflatoxin exposure is a potential risk factor to child mortality. Aflatoxin modulates expression of genes having roles in inflammatory, growth, and cancers, also has effects on DNA methylation, which are potential mechanisms responsible for aflatoxin health effects. Evidence of synergistic effects of combined aflatoxin and fumonisin on cytotoxicity and gene expression modulation found in this study also emphasises the importance to avoid potential hazards of the co-exposure. Aflatoxin affects metabolites levels *in vivo*, which are significantly enriched in the pathways that have crucial roles in oxidative stress-related diseases, protein synthesis, and growth. Therefore, this study has explored the health effects of aflatoxin in children and contributed to a better understanding of the mechanisms of aflatoxin exposure both *in vitro* and *in vivo*, and supplemented evidence of combined toxicity of aflatoxin and fumonisin.

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Abbreviations

ACTB	Beta-actin
AF-alb	Aflatoxin-albumin adducts
AFB_1	Aflatoxin B_1
AFB_1-lys	Aflatoxin B_1 lysine
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFM_2	Aflatoxin M_2
BMP2	Bone Morphogenetic Protein 2
CCL20	Chemokine (C-C motif) ligand 20
CHAIN	Childhood Acute Illness and Nutrition
CI	Confidence interval
CNCC	CHAIN Nested Case-Cohort
CV	Coefficient of variation
DEGs	Differentially expressed genes
DMSO	Dimethyl sulfoxide
DNMT1	DNA methyltransferases 1
DNMT3a	DNA methyltransferase 3a
DNMT3b	DNA methyltransferase 3b
DNMTs	DNA methyltransferases
EC	Esophageal cancer
ELISA	Enzyme-linked immunosorbent assay
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FBS	Foetal bovine serum

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSH	Glutathione
HAZ	Height-for-age Z-score
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HHL-16	Human hepatocyte line 16
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
IDMS	Isotope dilution mass spectrometry
IGF	Insulin-like growth factor
IGF1	Insulin-like growth factor 1
IGFBP3	Insulin Like Growth Factor Binding Protein 3
IL6	Interlukin-6
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
MEM	Minimum Essentia Media
MTT	3-(4.5-dimethylathiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUAC	Mid-upper arm circumference
NB GLMM	Negative Binominal Generalised Linear Mixed Models
NDP	Norrin Cystine Knot Growth Factor
NF-kB	Nuclear Factor Kappa B
NTD	Neural tube defects
ORs	Odds ratios
PBS	Phosphate buffered saline
RNA-Seq	RNA-sequencing
ROS	Reactive oxygen species

Sa	Sphinganine
So	Sphingosine
TDI	Tolerable daily intake
TNF-α	Tumour necrosis factor alpha
UFB_1	Urinary FB_1
WAZ	Weight-for-age Z-score
WHO	World Health Organization
WHZ	Weight-for-height Z-score

Chapter 1 Introduction

1.1 Background of mycotoxin exposure

Mycotoxins are secondary metabolites produced by filamentous fungi, and are widely distributed in cereal, cereal-based foods, and feedstuffs, posing great threats to human health (Alshannaq and Yu, 2017). Mycotoxins can contaminate different foods or animal feeds including maize, cereals, sorghum and peanuts, especially in tropical and sub-tropical countries, where the warm humid climate provides favourable environmental growing conditions for fungi to produce mycotoxins (Darwish et al., 2014). Consumption of contaminated foods directly, or indirectly from animal-derived products such as milk, meat, and eggs from animals that fed on contaminated feedstuffs, can result in mycotoxin dietary exposure (Alshannaq and Yu, 2017). Exposure to mycotoxins is mostly from ingestion, but also occurs through the dermal and inhalation routes (Zain, 2011). Dietary exposure to mycotoxins exerts both acute and chronic effect on humans, causing adverse health effects (Hussein and Brasel, 2001). Numerous studies reported that the effects of dietary exposure to mycotoxins on health are associated but not limited to carcinogenic, hepatotoxic, nephrotoxic, teratogenic toxicity, immune suppression and growth impairment (Reddy et al., 2010; Marroquín-Cardona et al., 2014; Lee and Ryu, 2015; Cimbalo et al., 2020). Among all the mycotoxins, aflatoxin and fumonisin are considered as the most important because of their frequent occurrence in nature and the effects on public health.

1.2 Aflatoxins

Aflatoxins are the most poisonous mycotoxins produced by *Aspergillus flavus* and *A. parasiticus*, commonly contaminating various foods in tropical regions, which shows greater health concerns in low-income countries, particularly in sub-Saharan Africa and South Asia (Wild and Gong, 2010). Four major types of aflatoxins are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2), named because of their blue (B) and yellow green (G) fluorescence emitted under UV light (Figure 1-1) (Dhanasekaran et al., 2011). Two hydroxylated metabolites of AFB_1 and AFB_2, aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2) respectively, are commonly found in milk and dairy products (Figure 1-1) (Gong et al., 2016). AFB_1 is the most prevalent, also the most toxic form, which has been classified as Group 1 carcinogen to humans by International Agency for Research on Cancer (IARC) (Ostry et al., 2017). Dietary exposure to AFB_1 has been reported to associated with acute aflatoxicosis, liver cancer, immune suppression and growth retardation (Liu et al., 2012).

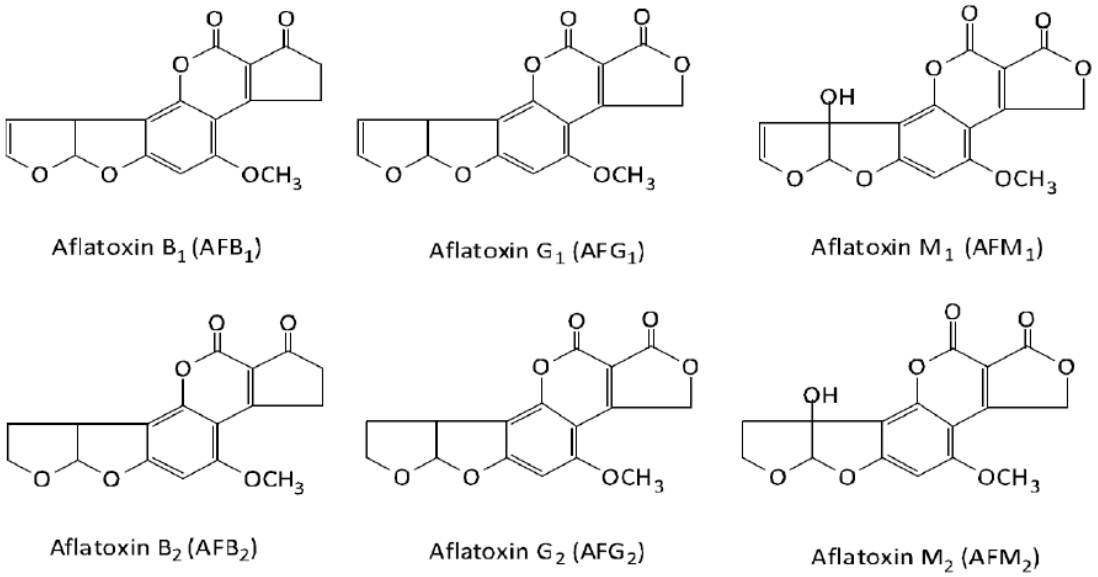


Figure 1-1 Chemical structure of aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2) (Sheng et al., 2014).

1.2.1 Assessment of aflatoxin exposure in humans

Analysis of aflatoxin contamination in foods combined with dietary questionnaire data is one method to evaluate human exposure levels, but the heterogeneous distribution of aflatoxins in foods makes it difficult to get an accurate estimation of individual exposure by this method. However, molecular biomarkers of aflatoxin exposure, particularly aflatoxin metabolites or reaction products formed in body fluids or tissues, which indicate the presence and magnitude of short-term or long-term exposure have been developed (Marín et al., 2021). Biomarkers are considered as more accurate indicators to reflect the actual internal and biologically effective levels of individual aflatoxin exposure compared to the food based assessment (Schmidt, 2006).

1.2.1.1 Aflatoxin metabolism

Since aflatoxin was first discovered in the early 1960s, the toxic mechanism of aflatoxin exposure in the body has been established by extensive studies. Aflatoxins are predominantly metabolised by cytochrome P450 (CYP450) enzymes, such as CYP 3A4, 3A5 and 1A2, in the liver, generating several hydroxylation metabolites including AFP_1, AFQ_1 and AFM_1, as well as two epoxides, AFB_1-*exo*-8,9-epoxide and AFB_1-*endo*-8,9-epoxide (Figure 1-2) (Wild and Turner, 2002). AFB_1-*exo*-8,9-epoxide is highly reactive and has higher affinity compared to the *endo* isomer to bind with DNA and proteins to

form covalent adducts. AFB_1-*exo*-8,9-epoxide preferentially binds with DNA at the N-7 position of guanine residues to form a primary DNA adduct (AFB_1-N^7-Gua). The adduct is not stable and will subsequently undergo a depurination to be metabolised into an apurinic site and a stable ring-opened adduct, AFB_1-formamidopyrimidine (AFB_1-FAPY) and excreted in urine (Bennett et al., 1981; Raney et al., 1993). AFB_1-N^7-Gua has mutagenic properties and plays an important role in the formation of human liver tumors (Bailey et al., 1996). Evidence shows that AFB_1-FAPY is capable of inducing 6-times higher mutation frequency compared to AFB_1-N^7-Gua, and a block of DNA replication caused by AFB_1-FAPY was found, which might be one of reasons explaining the toxic effects of AFB_1 (Smela et al., 2002). Compared to AFB_1-*exo*-8,9-epoxide, AFB_1-*endo*-8,9-epoxide is more stable and does not tend to form DNA adducts, therefore the *endo*-8,9-epoxide is not mutagenic (Iyer et al., 1994). Both AFB_1-*endo*-8,9-epoxide and AFB_1-*exo*-8,9-epoxide can go through hydrolysis to form a dihydrodiol, which reacts with serum albumin to form aflatoxin-albumin adducts (AF-alb) (Sabbioni et al., 1987).

In addition, conjugation of AFB_1-8,9-epoxide with glutathione (GSH) catalysed by glutathione-S-transferase is considered as a critical determinant of the detoxification of AFB_1, which reduces the amount of bioactive AFB_1-8,9-epoxide to bind with DNA (Johnson et al., 1997; Guengerich et al., 1998). Indeed, the extent of conjugation of AFB_1-8,9-epoxide with GSH is a key factor determining variation in risk to AFB_1 between species, for example, mouse and rat having greater GSH conjugation levels than humans (Raney et al., 1992).

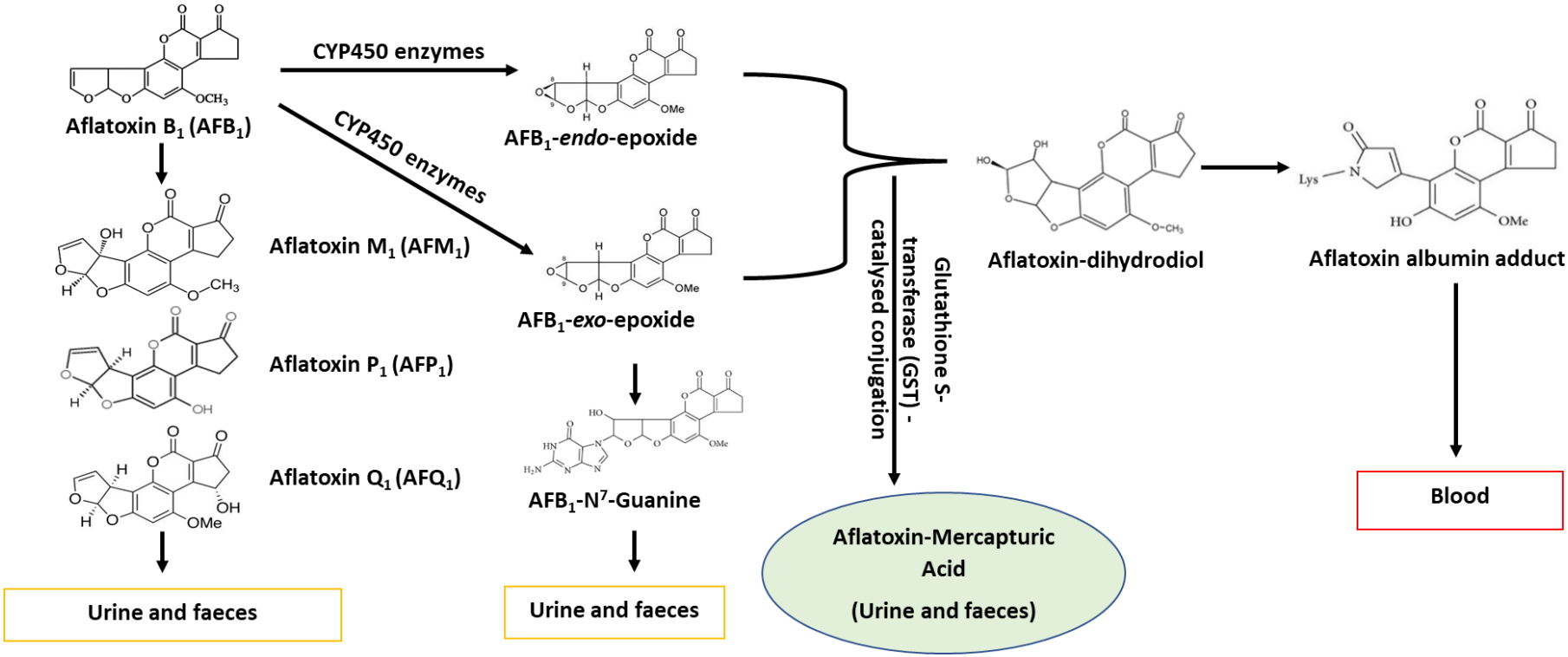


Figure 1-2 Mechanism of aflatoxin metabolism. AFB_1: aflatoxin B_1; AFM_1: aflatoxin M_1; AFP_1: aflatoxin P_1; AFQ_1: aflatoxin Q_1; CYP: cytochrome P450; GST: glutathione S-transferase.

1.2.1.2 Development of molecular biomarkers of aflatoxin exposure

A series of studies have examined the association between aflatoxin ingestion and levels of aflatoxin metabolites, which indicates that urinary AFB_1-N^7-Gua, urinary AFM_1 and AF-alb in serum are validated as good biomarkers (Zhu et al., 1987; Groopman, Hall, et al., 1992; Groopman et al., 1993; Routledge et al., 2014; Xu et al., 2018). AFB_1-N^7-Gua and AFM_1 in urine are evaluated as indicators for short-term exposure. A study used high-performance liquid chromatography (HPLC) to detect AFB_1-N^7-Gua excretion in the urine of a population from The Gambia, and it was found that the correlation coefficient was 0.82 (p<0.0001) between aflatoxin intake and AFB_1-N^7-Gua in urine (Groopman et al., 1992). A relatively less but significant association (correlation coefficient=0.65, p<0.000001) between aflatoxin intake and AFB_1-N^7-Gua in urine was also found in the participants from China (Groopman et al., 1992). In the latter study, a correlation coefficient of 0.55 between AFM_1 excretion in urine and AFB_1 intake was also found (Groopman et al., 1992). The studies combined indicate that both urinary AFB_1-N^7-Gua and urinary AFM_1 serve as good biomarkers to assess recent aflatoxin exposure. Additionally, AF-alb has a longer half-life of around 21 days, and is a reliable biomarker for the indication of long-term exposure over previous 2-3 months (Scholl and Groopman, 2008). AF-alb was assessed by enzyme-linked immunosorbent assay (ELISA) and HPLC techniques, and a significant correlation between dietary intake of aflatoxin assessed over a 7-day food diary and AF-alb was observed (correlation coefficient=0.55, p<0.05). These studies indicate the validation of AFB_1-N^7-Gua and AFM_1 in urine, as well as AF-alb in peripheral blood as biomarkers of aflatoxin exposure (Wild et al., 1992).

With the application of biomarkers, different methods including HPLC, isotope dilution mass spectrometry (IDMS), LC-MS/MS and ELISA have been mainly used to assess aflatoxin exposure in biological fluids in humans. HPLC is applied based on components separation in a liquid mixture and following to identify and quantify the level of biomarkers, such as urinary AFM_1, by fluorescence detection (Schwartzbord et al., 2017). IDMS is established as a radiochemical method to analyse the mass and quantity of aflatoxin metabolites, such as AF-lys (Scholl and Groopman, 2008). LC-MS/MS performed chromatographic separation and equipped with electrospray ionization to detect the level of urinary AFM_1 has been reported (Xia et al., 2020). ELISA using antibodies raised against aflatoxin adducts or metabolites is widely applied to the assessment of aflatoxin exposure in epidemiology studies, detecting the

level of AF-alb or AFM_1 in blood samples of humans (Sadeghi et al., 2009; Xu et al., 2018).

A study has reported the variation of different methods by assessing the same serum samples collected from an acute aflatoxicosis outbreak in Kenya in 2004, and the results showed that IDMS has highest sensitivity, followed by ELISA and HPLC-f. IDMS has the lowest limit of detection (LOD) of 0.25 pg/mg and the LOD of ELISA and HPLC-f are 3 pg/mg albumin and 9 pg/mg, respectively (McCoy et al., 2008). It is apparent that IDMS and HPLC-f have higher sensitivity to aflatoxin assessment than ELISA, but ELISA also offers a high degree of sensitivity and least cost. The methods above are all validated for the evaluation of aflatoxin exposure, but selection of appropriate methods depends on the number of samples as well as the consideration of cost.

Notably, an ELISA method using a polyclonal antibody was developed by Chapot and Wild, has frequently been applied to assess the level of AF-alb in serum samples in humans (Wild et al., 1990). The ELISA method has also been compared to IDMS assay measuring AF-lys in the same batch of samples, it was found that the level of AF-alb measured in ELISA is approximately three times higher than the level of AF-lys assessed in IDMS method (Scholl et al., 2006; McCoy et al., 2008). The reason for higher concentration measured in ELISA probably is that other aflatoxin moieties in addition to AF-lys could also react with the antibody in this technique (Wild et al., 1990). However, the advantages of high sample throughput and sensitivity (3 pg AF-lys equivalents/mg albumin) led to the ELISA method being also used in epidemiological studies (Gong et al., 2003).

1.2.1.3 Aflatoxin exposure levels in Africa and South Asia populations

With the application of various biomarkers and methods, aflatoxin exposure has been detected worldwide. As previously discussed, aflatoxin exposure is more frequent in tropical and sub-tropical countries, so high levels of aflatoxin exposure were observed in populations from Africa and South Asia, in particular. Table 1 summarizes the studies that determined aflatoxin exposure levels in populations in Africa and South Asia during the last decade.

1.2.1.4 Geographical factor in the variations of aflatoxin exposure

Studies in Table 1-1 have reported the high levels of aflatoxin exposure in populations, which is in consistent with the observed high levels of aflatoxin contamination in those countries (Gnonlonfin et al., 2013). The levels of aflatoxin exposure vary by countries and may be also different between regions

within one country. Evidence of aflatoxin exposure in populations from Europe showed much lower frequency and levels compared to in African and Asian populations. Wild and colleagues assessed AF-alb in different populations of the world and found that the levels of AF-alb in either children or adults from France and Poland, were all below the LOD (5 pg/mg), while populations from Africa countries, including The Gambia, Senegal and Kenya, as well as South Asia country Thailand had higher AF-alb levels (Wild et al., 1990). Especially, some children from The Gambia and Kenya had AF-alb levels of over 200 pg/mg albumin (Wild et al., 1990).

Even in the same country, the levels of aflatoxin exposure are varied by regions. A study conducted in Senegal has assessed the AF-alb levels of residences in three villages, Nioro du Rip, Saint-Louis, and Mboro, it was found that people in Nioro du Rip had significantly higher AF-alb levels compared to the levels of participants in Saint-Louis and Mboro (80.0 pg/mg vs 15.6 and 33.3 pg/mg, respectively, $p<0.001$) (Watson et al., 2015). Therefore, geographical location is a strong determining factor of aflatoxin exposure.

1.2.1.5 Variations of aflatoxin exposure in different ages

Aflatoxin exposure can happen in every life stage of humans, starting from the fetal period, through childhood and during the whole of adulthood finally to old age. Evidence shows that human placenta has the capacity to transfer aflatoxin, which could cause the fetus to be exposed to aflatoxin in uterus (Partanen et al., 2010). AF-alb levels of 40.4 pg/mg, 10.1 pg/mg and 8.7 pg/mg were found in maternal, cord blood and infant blood, respectively, which indicated that maternal aflatoxin exposure during pregnancy results in early life exposure to aflatoxin in the infants (Turner et al., 2007). After birth, the infant could be exposed to aflatoxin through breastfeeding (in the form of AFM_1) with aflatoxin exposure levels rising when fed with complementary/weaning foods. Urinary AFM_1 was detected in Nigerian children aged 1-18 months, and it was found that the mean level of AFM_1 was 23 ng/l in exclusively breastfed children while non-exclusively breastfed infants had higher mean level (166 ng/l) of AFM_1 (Ezekiel et al., 2022).

Since children are gradually developed and forming the same diet as adults, aflatoxin exposure levels of them are increased with the increasing of age and finally reach similar levels as adults. Children in Tanzania had AF-alb levels of 4.7 pg/mg at recruitment, and the levels of AF-alb were increased to 12.9 and 23.5 pg/mg after 6 and 12-months, respectively (Shirima et al., 2015). Gong et al. assessed the AF-alb levels in children aged from 9 months to 5 years old,

comparing the aflatoxin exposure levels based on both age and weaning status. It was found that children younger than 1 year old had a geometric mean level of AF-alb of 9.5 pg/mg, and the level increased to 21.1 pg/mg when the children were 1 year old, finally stabilized at approximately 40 pg/mg after 2 years old. With regard of weaning status, frequency of maize consumption was positively correlated with the level of AF-alb in those children, which to a certain extent supports the idea that aflatoxin exposure levels increased in children with the increasing of age due to diet transformation (Gong et al., 2003).

Table 1-1 Aflatoxin exposure levels in populations in Africa and South Asia in recent 10 years (2012-2022).

Reference	Country	Age	Number, positive percentage (%)	Biomarkers	[a] Mean; [b] GM (95% CI); [c] Median	Range
			ELISA method assessed studies			
Asiki et al., 2014	Uganda	18-89 years old	100 (100%)	AF-alb	[b]11.5 (10.2-13.0) pg/mg	0-237.7 pg/mg
		0-3 years old	96 (100%)		[b]9.7 (8.2-11.5) pg/mg	
Ghantous et al., 2021	The Gambia	6, 12 and 18 months	6 months: 230	AF-alb	6 months: [b]3.78 (3.29-4.34) pg/mg	NR
			12 months: 220		12 months: [b]25.1 (21.67-29.13) pg/mg	
			18 months: 210		18 months: [b]49.48 (43.34-56.49) pg/mg	
Boshe et al., 2020	Ethiopia	Over 18 years old	360 (53.3%)	Urinary AFM_1	[C] 214 ppt	0-2582 ppt
Shirima et al., 2015	Tanzania	Recruited at 6-14 months	At recruitment: 146 (67%)	AF-alb	At recruitment: [b]4.7 (3.9-5.6) pg/mg	NR
		6-12 months follow up	6 months follow up: 146 (84%)		6 months follow up: [b]12.9 (9.9-16.7) pg/mg	
			12 months follow up: 146 (99%)		12 months follow up: [b]23.5 (19.9-27.7) pg/mg	
Shirima et al., 2013	Tanzania	12-22 months	148 (84%)	AF-alb	[b]12.9 (9.9-16.7) pg/mg	NR

Smith et al., 2017	Zimbabwe	26.8±8.1 years old	1580 (30%)	Urinary AFM_1	[c]162.5 pg/mg	31-6046 pg/mg
Gong et al., 2012	Kenya	6-17 years old	218	AF-alb	[b]114.5 (99.7-131.4) pg/mg	NR
Chen et al., 2017	Tanzania	6-14 months	84 (86%)	Urinary AFM_1	[b]36.5 (30.8-46.3) pg/ml	15-2840 pg/ml
Castelino et al., 2013	Kenya	6-17 years	180	AF-alb	[b]110.5 (95.4-127.9) pg/mg	NR
Watson et al., 2015	Guinea	28.8 ± 8.4 months	Harvest: 305 (88.2%) Postharvest: 288 (93.4%)	AF-alb	Harvest: [b]12.7 (10.9-14.7) pg/mg Postharvest: [b]16.3 (14.4-18.5) pg/mg	NR
Ezekiel et al., 2018	Nigeria	2-65 years	84 (99%)	Urinary AFM_1	[c]0.27 ng/ml	0.06-0.51 ng/ml
Xu et al., 2021	The Gambia	24 and 52 weeks	24 weeks: 352 (50%) 52 weeks: 331 (98%)	AF-alb	24 weeks: [b]3.52 (3.15-3.94) pg/mg 52 weeks: [b]25.39 (22.37-28.82) pg/mg	NR
Hernandez-Vargas et al., 2015	The Gambia	18-45 years	115 Dry season: 57 (49.6 %) Rainy season: 58 (50.4%)	AF-alb	Dry season: [b]34.36 (26.05-45.32) Rainy season: [b]35.23 (27.76-44.70)	3.9-458.4 pg/mg

Castelino et al., 2014	The Gambia	18-45 years	99 Dry season: 47 (100%) Rainy season: 52 (100%)	AF-alb	Dry season: [b]52.8 (42.8-65.2) Rainy season: [b]29.6 (25.8-34.0)	NR
Gebreegziabher et al., 2022	Ethiopia	6-12 years	408 (93%)	Urinary AFM_1	[C]480 pg/mg	NR
Routledge et al., 2014	Tanzania	12-22 months	148 (83%)	AF-alb	Storage visit: [b]12.9 (9.9-16.7) Harvest visit: [b]23.4 (19.9-27.7)	NR
HPLC-fluorescence assessed studies						
Andrews-Trevino et al., 2021	Nepal	Over 18 years old 3, 6, 12, and 18-22 months	Over 18 years old: 1652 (94.3%) 3 months: 1363 (80.5%) 6 months: 1297 (75.3%) 12 months: 1329 (81.1%) 18-22 months: 699 (85.1%)	AFB_1-lys	Over 18 years old: [b]1.54 (1.46-1.62) pg/mg 3 months: [b]0.83 (0.80-0.86) pg/mg 6 months: [b]0.86 (0.83-0.90) pg/mg 12 months: [b]1.08 (1.02-1.14) pg/mg 18-22 months: [b]1.27 (1.18-1.36) pg/mg	Over 18 years old: 0.4-147.32 pg/mg 3 months: 0.4-24.72 pg/mg 6 months: 0.4-41.6 pg/mg 12 months: 0.4-84.65 pg/mg 18-22 months: 0.4-128.07 pg/mg

Passarelli et al., 2019	Tanzania	Over 18 years old	400 (99%)	AFB_1-lys	[b]1.52 (1.42-1.64) pg/mg	0.42-84.78 pg/mg
Lauer et al., 2018	Uganda	18-45 years	220 (100%)	AFB_1-lys	[b]5.89 (5.25-6.60) pg/mg	0.71-95.60 pg/mg
Kang et al., 2015	Uganda	General Population Cohort (GPC): Infant to very old Rakai Community Cohort Study (RCCS): 15-49 years	GPC: 713 (90%) RCCS: 374 (92.5%)	AFB_1-lys	GPC: [c]1.58 pg/mg RCCS: [c]1.18 pg/mg	GPC: 0.40-168 pg/mg RCCS: 0.40-122.5 pg/mg
LC-MS/MS assessed studies						
Xia et al., 2020	Pakistan	4-80 years old	264 (69%)	Urinary AFM_1	[a]0.023±0.048 ng/ml	Nd- 0.393 ng/ml
Ayelign et al., 2017	Ethiopia	1-4 years	200 (17%)	Urinary AFM_1	[a]0.064 ng/ml	0.06-0.07 ng/ml
Chen et al., 2018	Tanzania	24 months	60	AFB_1-lys	[c]3.6 pg/mg	0.28-25.1 pg/mg

IDMS assessed studies						
Groopman et al., 2014	Nepal; Bangladesh	Over 14 years old 2 years old	Nepal: 141 (94%) Bangladesh: First trimester: 63 (100%) Third trimester: 63 (100%) 2 years old follow-up:63 (100%)	AFB_1-lys	Nepal: [b]25.28 (19.47-31.09) pg/mg Bangladesh: First trimester: [c]18.08 pg/mg Third trimester: [c]25.35 pg/mg 2 years old follow-up: [c]13.79 pg/mg	Nepal: 0.45-2939.30 pg/mg Bangladesh: NR
Mahfuz et al., 2018	Bangladesh	7, 15, 24, and 36 months	7 months: 208 (10%) 15 months: 196 (20%) 24 months: 173 (17%) 36 months: 167 (62%)	AFB_1-lys	7 months: [a]1.30±1.50 pg/mg 15 months: [a]1.52±1.47 pg/mg 24 months: [a]3.43±11.83 pg/mg 36 months: [a]3.70±12.99 pg/mg	7 months: 0.09-5.79 pg/mg 15 months: 0.06-6.35 pg/mg 24 months: 0.15-65.60 pg/mg 36 months: 0.09-126.54 pg/mg
McMillan et al., 2017	Nigeria	6-48 months	58 (100)		[C]2.6 pg/mg	0.2-59.2 pg/mg

AF-alb: aflatoxin-albumin adduct; AFM_1: aflatoxin M_1; AFB_1-lys: aflatoxin B_1-lysine adduct; ELISA: enzyme-linked immunosorbent assay; LC-MS/MS: liquid chromatography-mass spectrometry; HPLC: high performance liquid chromatography; IDMS: isotope dilution mass spectrometry; CI: confidence interval; NR: not reported.

1.2.2 Health effects of aflatoxin on humans

Aflatoxin exposure has both acute and chronic effects on human health, which is a heavy burden on public health in low-income or middle low-income populations in tropical areas, due to the high susceptibility of foods to aflatoxin contamination in those regions and often reliance on a contaminated staple food. High levels of aflatoxin exposure in a short time cause acute aflatoxicosis, which has been reported several times in Africa. For instance, 20 patients admitted to hospital with a case fatality rate of 60% because of acute aflatoxicosis was reported in the Machakos district of Kenya from March to June 1981 (Ngindu et al., 1982). Another larger outbreak of aflatoxin poisoning in Kenya during January-June 2004 showed 317 patients with hepatic injury admitted to hospital, of which 125 cases died due to acute aflatoxicosis (Gieseker, 2004). Subsequently, a case-control study conducted to compare the exposure levels of aflatoxin between patients involved in the Kenya outbreak 2004 and participants in control group, it was found that the geometric mean of AF-alb levels is significantly higher in the patients than it in the control group (1.2 ng/mg vs 0.15 ng/mg, p<0.001) (Azziz-Baumgartner et al., 2005). Note the high levels of exposure, which are more usually reported as pg/mg albumin.

However, chronic effects of aflatoxin exposure due to low concentration over a long period is more commonly seen. The best documented chronic health effect of aflatoxin exposure is hepatocellular carcinoma (HCC). The annual deaths from liver cancer in Africa associated with aflatoxin exposure has been estimated as 26000 cases (Unnevehr and Grace, 2013). Broader effects including immune suppression and child growth impairment associated with aflatoxin exposure have also been reported (Jiang et al., 2008; Khlangwiset et al., 2011).

1.2.2.1 Effects of aflatoxin exposure on hepatocellular carcinoma in humans

HCC is a disease of global health concerns, which was recognised as the sixth most common cancer worldwide (Okeke et al., 2020). Hepatitis B virus (HBV), hepatitis C virus (HCV) and dietary exposure to aflatoxin are three important risk factors of HCC. Aflatoxin as an additional risk factor alongside with hepatitis B virus (HBV), contributes to an increased risk of HCC (Wu et al., 2009). A study assessed the association between aflatoxin exposure and liver cancer in a population in China found that the relative risk of liver cancer in individuals

with HBV infection but aflatoxin biomarker negative was 4.8, but among the individuals with both HBV infection and aflatoxin positive, the relative risk was increased 12.5 fold to 60.1 (Ross et al., 1992). The evidence supports that aflatoxin is not only a standalone risk factor but also in synergism with HBV infection in the initiation of HCC. Because the high prevalence of dietary exposure to aflatoxin and HBV infection in Africa and South Asia, the synergistic effects of the two risk factors on the causation of HCC have been widely reported in epidemiologic studies in those countries (Qian et al., 1994; Diallo et al., 1995; Turner et al., 2000; Wu et al., 2009).

A systematic review and meta-analysis study summarised 479 studies conducted in China, Taiwan, and sub-Saharan Africa to evaluate the odds ratios (ORs) and population attributable risk (PAR) of aflatoxin-related HCC, it was found that the ORs of HCC was higher in the group with both HBV and aflatoxin positive than it in the group of HBV or aflatoxin alone (73.0 vs 11.3 or 6.37). PAR is an index represents for the number or proportion of disease cases that could be reduced if a risk factor was eliminated in the population. In the meta-analysis study, the PAR of aflatoxin-related HCC is 17%, but is 21% in HBV positive population and 8.8% in HBV negative population, suggesting that reducing aflatoxin could contribute to low incidence of HCC (Liu et al., 2012).

1.2.2.2 Molecular mechanism of aflatoxin-associated liver cancer

The molecular mechanism of aflatoxin-induced hepatocarcinoma has been investigated over many years. The most well-established mechanism of aflatoxin-induced hepatocarcinoma is that the mutagenic DNA adducts formed by aflatoxin exposure could cause genetic changes that lead to cancer ultimately. An earlier human study found G to T substitutions at codon 249 is a hotspot for mutations of p53, a tumour suppressor gene, in liver tumours of HCC patients in South Africa and Qidong, China, with this hotspot being associated with aflatoxin exposure (Bressac, Kew, et al., 1991; Hsu et al., 1991). A larger number of liver tumour specimens (167) were collected to determine the frequency of codon 249 mutation of p53 gene in Africa and Asia, and it was found that more than 50% of patients with high aflatoxin exposure had a hotspot mutation at codon 249 of p53, and the hotspot mutation was strongly associated with dietary aflatoxin intake (Bressac, Puisieux, et al., 1991). Further, several *in vitro* studies investigating the mutational properties of DNA adduct formed by AFB_1 exposure indicated that the predominant mutation caused by the DNA adduct was also G to T transversion, which is similar to the mutation pattern in human liver tumours (Aguilar et al., 1993; Bailey et al., 1996; Mace et al., 1997).

Since the consistent results in both human and *in vitro* studies, AFB_1-DNA adduct caused mutations in p53 could explain the carcinogenicity of aflatoxin B_1.

1.2.2.3 Effects of aflatoxin exposure on child growth impairment

Child growth impairment is a great concern of low-income countries, which will result in severe health complications. Since immature immune system and rapid development at the initial growth stages of children, they are more sensitive to the health challenges, such as environmental exposure to xenobiotics of high toxicity, which would cause growth faltering in children and eventually lead to high incidence of mortality, morbidity, and impaired cognitive development (De Onis et al., 1997). According to the standards of assessing children's growth and development released by the World Health Organization (WHO), three anthropometric indices are used, height-for-age Z-score (HAZ), weight-for-age Z-score (WAZ), and weight-for-height Z-score (WHZ), smaller than minus 2 are defined as stunting, underweight and wasting, respectively (WHO, 2006).

Through measuring the levels of aflatoxin exposure biomarker and analysing the association with anthropometric indices, a number of studies have found that aflatoxin exposure at an early stage of life is related to child growth impairment, especially when the exposure occurs *in utero*, and feeding with breast milk or weaning foods. Turner et al. examined AF-alb levels in maternal blood and measured height and weight in their infants, it was found that higher AF-alb levels in maternal blood had significant association with lower weight and height in the children (p<0.012 and p<0.044, respectively) (Turner et al., 2007). In addition, their multiple regression model predicted that a reduction of AF-alb *in utero* from 110 pg/mg to 10 pg/mg would contribute to an increase of 800 g weight and 2 cm of height in the infants at 12 months age (Turner et al., 2007). AFM_1, a metabolite of AFB_1 but less toxic than AFB_1, in breast milk could also contribute to aflatoxin exposure in early infancy and subsequently cause child growth impairment. A small but significantly negative association between AFM_1 level in breast milk and WAZ and HAZ has been found (p<0.05), indicating that exposure to AFM_1 through breast milk feeding is also associated with child growth faltering (Magoha et al., 2014). Although breast milk feeding shows the risk of aflatoxin-associated child growth impairment, it is still encouraged to prolong breastfeeding period. Because on the one hand, breast milk provides necessary nutrition and immunological substances to child growth and development, on the other hand, evidence shows that fully weaned children

had higher aflatoxin exposure levels compared to exclusively breastfed children (Ezekiel et al., 2022). Approximately two-fold higher AF-alb level have been measured in weaned children than partially or exclusively breastfed children in Benin and Togo, it was positively associated with the frequency of maize consumption after weaning (p<0.01). It was also found that the high AF-alb level was strongly associated with stunting and underweight in children (p=0.0001 and 0.0038, respectively), suggesting weaning status could be an underlying determinant of growth impairment related to aflatoxin exposure (Gong et al., 2003). In Africa, maize and peanuts, as typical foods consumed by children after weaning, are highly susceptible to aflatoxin contamination. There is high prevalence and level of aflatoxin exposure in African children, and more evidence of negative association between aflatoxin exposure and child growth were reported in Africa (Gong et al., 2002; Gong et al., 2004; Turner et al., 2007; Magoha et al., 2014; Watson et al., 2018; Wangia-Dixon et al., 2020).

However, there are some studies that did not find the negative association between aflatoxin exposure and child growth, which might be due to shorter exposure period and lower exposure levels, and variation in measurement methods (Magoha et al., 2016; Mitchell, Hsu, et al., 2017; Mahfuz et al., 2021). Based on the aforementioned studies, it is difficult to determine whether aflatoxin exposure is a causal factor for child growth impairment. More studies are warranted in the field of mechanism elucidation behind the effects of aflatoxin on impaired growth, to better understand the relationship between aflatoxin exposure and child growth.

1.2.2.4 Molecular mechanism of aflatoxin-associated growth impairment

Suggested explanations of potential mechanisms for aflatoxin-associated child growth impairment may include: contribution to enteropathy, immune suppression and/or changed insulin-like growth factor (IGF) axis via liver toxicity (Gong, Turner, et al., 2008). After exposure to a combination of AFB_1 and AFM_1, intestinal barrier dysfunction in ICR mice and reduced intestinal integrity and permeability in Caco-2 cell line were observed, so aflatoxin exposure related intestinal damage may affect nutrition absorption and subsequent growth (Gao et al., 2021). It was also postulated that the immunosuppressive effects of aflatoxin might increase infection risk to various diseases, for example, HIV, diarrhoea, and possibly compromised vaccine efficacy, which might suppress appetite and impair nutrition absorption (Gong, Turner, et al., 2008).

The possibility that changes to the IGF axis may be involved, in the effects of aflatoxin exposure on child growth has been investigated. A study assessed

AF-alb level and the protein levels of IGF1 and IGFBP3 in Kenyan schoolchildren, and regression analysis in the study showed an inverse linear correlation between AF-alb and both IGF1 and IGFBP3. Furthermore, a path analysis indicated that lower expression of IGF1 contributed to 16% of the effects of aflatoxin exposure on child height (Castelino et al., 2015). Similarly, in a study conducted among rural Gambian infants, IGFBP3 level at 12 months of age was negatively associated with Ln AF-alb at 6 months of age (r=-0.12, *p*=0.043), but not IGF1 (Watson et al., 2018). Aflatoxin exposure *in utero* associated with differential DNA methylation for 71 CpG sites was first observed in The Gambian infants aged at 2-8 months old. The differential methylation was observed in growth factor genes (such as: *FGF12* and *IGF1R*) and immune-related genes (such as: *CCL28*, *TLR2*, and *TGFBI*), which implies DNA methylation might be another mechanism explaining aflatoxin-related child stunting (Hernandez-Vargas et al., 2015). In different Gambian infants aged at 24 months from the same population, some differentially methylated positions and regions related to aflatoxin exposure were also found, which affected gene expression of several nearby genes involved in inflammatory, signalling and growth pathways (Ghantous et al., 2021). However, alteration of gene expression of the highlighted genes with differential methylation in the former study was not seen in the infants aged at 24 months. Small changes of DNA methylation level with an average effect size of 1.7% observed in the genes may not contribute to the biological relevance (Hernandez-Vargas et al., 2015). To date, the findings of aflatoxin-associated DNA methylation and altered protein expression in the studies may shed light on aflatoxin-associated impaired child growth, however, a consistent alteration of specific genes affected by aflatoxin exposure has not been determined. Further research on finding a matching pattern between gene expression and DNA methylation from the highlighted pathways would facilitate the understanding of mechanism behind aflatoxin-associated growth impairment, also help to figure out the biological significance of aflatoxin-associated DNA methylation.

1.2.2.5 Effects of aflatoxin exposure on immune modulation in humans

Aflatoxin-related immune modulation may be both innate and adaptive immunity include cell-mediated immunity and humoral immunity, which has been extensively investigated in animal studies (Raisuddin et al., 1993; Marin et al., 2002; Yunus et al., 2011). However, in human studies in terms of the effects of aflatoxin exposure on immune modulation, a limited number of studies

have investigated the association between AF-alb level and immunological parameters. One immune parameter, secretory IgA (sIgA) in saliva was significantly reduced in children with detectable AF-alb than those without detectable levels (50.4 µg/mg protein vs 70.2 µg/mg protein, $p<0.0001$), suggesting a disruption of the mucosal barrier in innate immunity for bacterial and virus defence thereby may increase the susceptibility to infectious disease (Turner et al., 2003). Evidence of depressed immunity with aflatoxin exposure has also been reported in alteration of proportions of some specific cell types involved in cell-mediated immunity. A study shows that significantly reduced percentages of CD3+CD69+ and CD19+CD69+ cells ($p=0.002$ for both) as well as the level of perforin or both perforin and granzyme A in CD8+ T cells ($p=0.012$ for both) were found in participants with high aflatoxin exposure level compared to participants with low aflatoxin exposure levels (Jiang et al., 2005). Furthermore, a negative association between aflatoxin exposure level and the percentages of CD3+CD69+ ($p=0.001$) and CD19+CD69+ ($p=0.032$) was found, which indicates that reduction of those immunological parameters could lead to cellular immunity impairment and increase the susceptibility to infectious disease (Jiang et al., 2005). There is an inconsistency of antibody response induced by aflatoxin between humans and animals. Generally reduced antibody response with aflatoxin exposure level was observed in animals (Azzam and Gabal, 1997; Gabal and Dimitri, 1998; Fernandez et al., 2000). However, more recently, a study conducted in The Gambian infants found that antibody response, involved in humoral immunity, against diphtheria was significantly higher in high aflatoxin exposure group compared to low exposure group at 12 and 24 weeks of age (0.46 IU/ml vs 0.16 IU/ml, $p<0.0001$; 1.46 IU/ml vs 0.96 IU/ml, $p=0.0011$, respectively) (Xu et al., 2021). Turner et al. also found a small positive association between antibody response to pneumococcal serotype and aflatoxin exposure level ($p=0.05$) (Turner et al., 2003). Because of the inconsistent effects of aflatoxin exposure on antibody response in humans and animals, more further studies are required to work in this field to make a conclusion.

Suppressed immune function by aflatoxin exposure may contribute to the infection of other diseases. Aflatoxin exposure related immune suppression contributing to an increased infection risk of human immunodeficiency virus (HIV) disease has been reported. Several studies indicated the coincidence of aflatoxin exposure and HIV disease in African populations, suggesting the effects of aflatoxin exposure on immune suppression may promote the progression of HIV disease. For example, there is evidence to show that there

were significantly lower percentages of CD4+ T regulatory cells (p=0.009) and naive CD4+ T cells (p=0.029), as well as B-cells (p=0.03) in HIV positive participants with high AF-alb level than participants with HIV positive and low AF-alb level (Jiang et al., 2008). Another study also shows that the risk to liver disease and jaundice in HIV positive participants with high aflatoxin exposure was higher compared to HIV positive participants with low aflatoxin exposure (odds ratio=5.47, 95% CI=1.03-22.85) (Jolly et al., 2011).

Therefore, all evidence to date tells us that aflatoxin can disrupt both innate and adaptive immunity to exert immune modulation. Due to some inconsistent and inconclusive findings in the studies discussed above, more studies exploring the effects of aflatoxin exposure on immune response are warranted, particularly on antibody response and non-HIV populations, to determine the roles of aflatoxin exposure in immunosuppression. Besides, exploration of molecular target involved at aflatoxin-influenced immune suppression could provide support to therapy in populations suffering from aflatoxin-related diseases.

1.2.2.6 Mechanism of immunosuppression by aflatoxin

The mechanism of aflatoxin exposure related immune suppression is not fully understood yet, but current state of knowledge proposes that aflatoxin exerts effects on immune system through the disruption in innate immunity, cellular-mediated immunity, and humoral immunity. Components including natural killer (NK) cells and phagocytic leukocytes in response to aflatoxin have been extensively studied in animals and cell models to reflect the effects on innate immunity. Evidence show that decreased NK cell function in mice, compromised phagocytic activity in chickens have been observed after aflatoxin feeding (Michael et al., 1973; Reddy and Sharma, 1989; Mohsenzadeh et al., 2016). The effects of aflatoxin on humoral immunity and cellular-mediated immunity have both been reported, but the findings of aflatoxin suppressed cellular-mediated immunity are more consistent. The inconsistence of the effects of aflatoxin on antibody response is not only in humans as discussed above, but also was observed in animals. Several studies show that low concentration (50 and 200 pb) of aflatoxin treatments in animals failed to cause any effects on antibody formation in ducklings and chicken (Cheng et al., 2001; Oguz et al., 2003). However, more evidence indicated that aflatoxin feeding in various animals decreased the antibody levels against disease (Azzam and Gabal, 1998; Qureshi et al., 1998; Marin et al., 2002; Samuel et al., 2009). It was proposed that higher aflatoxin concentration,

exposure length to aflatoxin and species are considered as factors that responsible for the variation of effects on antibody response (Meissonnier et al., 2006).

Cellular-mediated immunity is mainly through the involvement of lymphocytes (T cells and T cell subsets), macrophages, and the release of cytokines, which is also the main mechanism explaining for the immune suppressive effects of aflatoxin. Compared to humoral immunity, the effects of aflatoxin exposure on cellular-mediated immunity are more consistent in terms of concentrations. Either low or high concentration (300 µg/kg or 600 µg/kg) of aflatoxin exposure shows suppressed effects on cell-mediated immunity in rats (Raisuddin et al., 1993). Zimmermann et al. conducted MTT assay in broiler lymphocytes treated with different concentrations of AFB_1, and found that 10 µg/ml AFB_1 treatment at 48 hours, 10 and 20 µg/ml AFB_1 treatments at 72 hours significantly decreased the cell viability of lymphocytes (Zimmermann et al., 2014). A significant decrease of total lymphocytes and T lymphocytes, as well as a reduction of macrophages capacity were observed in broiler chicks fed with 1mg/kg AFB_1, indicating an impairment in cellular-mediated immunity in aflatoxin-treated chicks (Ghosh et al., 1991).

Interference of cytokines production secreted by lymphocytes and macrophages could affect all kind of immune response mentioned above directly or indirectly, but the changes in cytokines production, such as TNF-α and IL-6, caused by aflatoxin have a high variability. For example, Increased mRNA expression of TNF-α in mouse macrophage cells treated with AFB_1 has been reported, while decreased mRNA expression of TNF- α was observed in AFB_1-fed pigs (Marin et al., 2002; Ma et al., 2021). The examples of conflicting modulation in cytokines by aflatoxin might because of the differences in host response. However, even in the same species, such as piglets, it was found that low dose (140 and 280 ppb) of AFB_1 feeding caused a decreased mRNA expression of TNF- α, while a significant increase of TNF- α was observed in piglets fed with higher AFB_1 (1807 µg/kg) (Marin et al., 2002; Meissonnier et al., 2008). Additionally, decreased mRNA expression of IL-6 and TNF- α was found in broilers fed with 0.6 mg/kg AFB_1, while increased mRNA expression of IL-6 and TNF- α was reported in 0.074 mg/kg AFB_1-fed broilers (Li et al., 2014; Jiang et al., 2015). A biphasic effect of aflatoxin that high and low levels of aflatoxin may have opposite effects could be a reason proposed for the conflicting results in the same species.

Therefore, the available data to date indicated aflatoxin affects both innate and adaptive immune in humans, animals as well as in cell models. Current evidence exists that is inconsistent and inconclusive as discussed above. Briefly, sample size, exposure level and exposure length in human studies, species variation and treatment concentration of aflatoxin in animals and cells may be the reasons for the conflicting results. Hence, the effects of aflatoxin exposure on immune system still needs further investigation, which could provide a better understanding of the molecular mechanism of immunosuppressive effects and offer opportunity to target therapy for aflatoxin-promoted immune diseases.

1.3 Fumonisin

Fumonisin, one of the most economically important fungal toxins, was discovered and characterized after a field outbreak of equine leukoencephalomalacia (ELEM) in South Africa in 1970 (Marasas, 2001). Fumonisin produced by *Fusarium verticillioides* (formerly *F. moniliforme*) and *F. proliferatum* mainly contaminates maize and maize-based foods worldwide, causing huge economic losses and health concerns in humans and animals (Bezuidenhout et al., 1988; Fandohan et al., 2003). Naturally occurring fumonisins are fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3) (Figure 1-3). FB_1 is the most prevalent and toxic form of the fumonisins group, and it is classified as group 2B carcinogen by IARC (IARC, 2002). Although FB_1 is non-genotoxic, dietary exposure to FB_1 has been reported to associated with liver and esophageal cancers, neural tube defects, and growth impairment in humans (Hendricks, 1999; Sun et al., 2007; Chen et al., 2018).

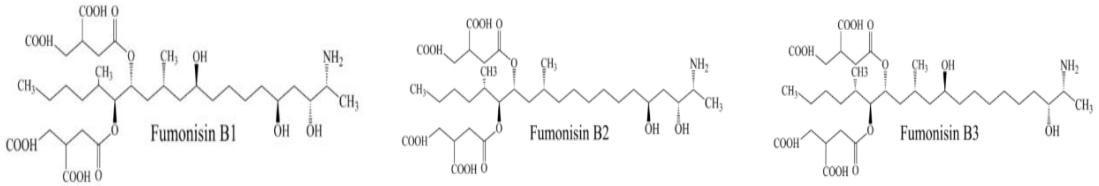


Figure 1-3 Chemical structure of fumonisin B_1, B_2, and B_3 (Kostić et al., 2019).

1.3.1 Absorption and elimination of fumonisin

Although some evidence indicated gene expression of CYP450 genes was induced by fumonisin in animals or cell line, there is little or no direct evidence showing fumonisin is metabolized by CYP enzymes (Chuturgoon et al., 2014; Cao et al., 2020). Fumonisin is poorly absorbed without metabolism by CYP enzymes in the body and rapidly excreted in most species, but small amount of

fumonisin residues can be found in the liver and kidney, which are two major target organs of fumonisin, one possible target organ intestine affected by fumonisin was also reported (Bouhet and Oswald, 2007). After either intravenous or oral administration of FB_1 in cows, at two hours post-dosing no detectable FB_1 was found in plasma, suggesting a negligible absorption of FB_1 (Prelusky et al., 1995). Approximately only 4% absorption of fumonisin B_1 was reported in piglets after oral administration of fungal culture (Fodor et al., 2008). Rats were intragastrically and intravenously administrated with radiolabelled fumonisin B_1 (^{14}C FB_1), and the results showed that 80% and 3% radiolabel were recovered in feces and urine, respectively, after intragastric administration, while around 35% elimination in feces was found in intravenously administrated rats (Norred et al., 1993). Besides, the persistence of ^{14}C FB_1 was found in the liver (up to 0.4% of dose) and kidney (up to 0.1% of dose) of rats 96 hours postdosing, implying fumonisin accumulation in the two major target organs over a prolonged exposure to fumonisin (Norred et al., 1993). Thereby, evidence in different animal species indicated the low bioavailability and rapid elimination of FB_1.

1.3.2 Toxicity mechanism of fumonisin

Fumonisin exposure has been implicated in various toxic effects including neurotoxicity, hepatotoxicity, immunotoxicity and growth retardation in animals and humans (Mathur et al., 2001; Marasas et al., 2004; Taranu et al., 2005; Kimanya et al., 2010; Shirima et al., 2015). Molecular mechanisms of fumonisin toxicity mainly are because of the disruption sphingolipid metabolism, induction of oxidative stress and epigenetic modification (Figure 1-4).

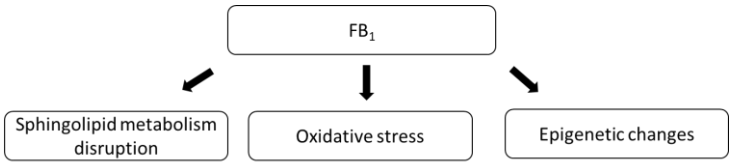


Figure 1-4 Molecular mechanisms of FB_1-induced toxicity.

Disruption of sphingolipid metabolism is recognized as the best-known mechanism of fumonisin toxicity (Figure 1-5). Due to the structural similarity of FB_1 to long-chain base backbones of sphingolipids, FB_1 acts as an inhibitor of ceramide synthase, which is a crucial enzyme to catalyses sphingoid bases and responsible for sphingolipid synthesis (Wang et al., 1991). This inhibition leads to the accumulation of free sphinganine (Sa), inhibition of sphingosine (So) formation, elevation of Sa/So, and disruption of sphingolipid biosynthesis (Wang et al., 1991; Voss et al., 1995; Merrill et al., 2001). Numerous animal

studies have observed increased free sphingoid bases or the elevation of Sa/So together with liver and kidney toxicity after the injection or feeding of FB_1 (Gumprecht et al., 1995; Tsunoda et al., 1998; Enongene et al., 2000; Grenier et al., 2012; Hahn et al., 2015). Since sphingolipids play crucial roles in cell membrane and lipoprotein structure, the disruption of sphingolipid biosynthesis affected cell membrane integrity and resulted in the inhibition of cell growth and ultimate cell apoptosis (Yoo et al., 1996; Gelderblom et al., 2001).

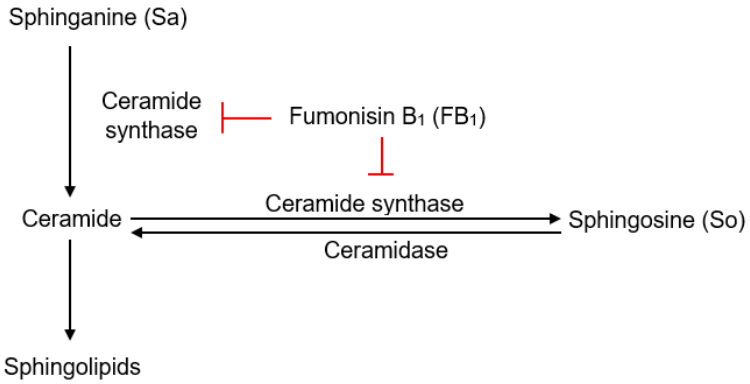


Figure 1-5 Sphingolipid metabolism disruption caused by fumonisin B_1 (FB_1).

The second mechanism of fumonisin toxicity is oxidative stress induced by FB_1, which refers to the over-production of reactive oxygen species (ROS) and the imbalance between free radicals and antioxidant and will lead to cell damage (Liu et al., 2019). Higher production of ROS can cause lipid, protein, and DNA damage (Sharifi-Rad et al., 2020). ROS production with increased lipid peroxidation, necrotic cell death as well as decreased GSH levels were observed in rat and mouse neural cell cultures, indicating oxidative stress might play an important role in the neurotoxicity of FB_1 (Stockmann-Juvala et al., 2004). In human intestinal Caco-2 cells, 10-150 µM FB_1 was used to treat the cells, and it was found an induction of lipid peroxidation thus altering the cell membrane and leading to cell death ultimately (Kouadio et al., 2005). Evidence of nucleic acid and protein changes caused by FB_1 has been reported, where a significant increase of both DNA and protein oxidation was observed in rat spleen mononuclear cells exposed to FB_1 for 48 h (Mary et al., 2012).

Recently, accumulating evidence suggested that epigenetic modification related to FB_1 might prevail in its toxicity mechanism. Epigenetic modification mainly refers to three mechanisms including histone modification, DNA methylation and non-coding RNA regulation (Portela and Esteller, 2010). In rat kidney epithelial cells treated with FB_1, dose-dependent and time-dependent effects of FB_1 on global histone modifications were observed (Sancak and Ozden, 2015). DNA methylation was evident in several cell line studies. Global

DNA methylation was decreased by 200 μM FB_1 treatment in HepG2 cells (Chuturgoon et al., 2014). In rat kidney (NRK-52E) and liver (Clone 9) cells, although not a significant global DNA methylation was observed, CpG promoters of specific genes such as *c-myc* in Clone 9 cells, *VHL* in both cells were shown to be methylated after FB_1 treatments (Demirel et al., 2015a). One microRNA, miR-27b, of small noncoding RNAs was significantly down-regulated thereby in turn up-regulated CYP1B1 in HepG2 cells after FB_1 treatments (Chuturgoon et al., 2014). These studies illustrate fumonisin capability in the alteration of epigenetic events, typically through histone modifications, DNA methylation and non-RNA regulation.

1.3.3 Assessment of fumonisin exposure in humans

Maize production is predominantly from Africa and South Asia, which are susceptible to fumonisin contamination, and populations from those areas living by maize-based foods consumption are at great risk of fumonisin exposure (Erenstein et al., 2022). In early assessment of fumonisin exposure, exposure levels were estimated from food consumption frequency or determined in contaminated food products because of limited availability of validated biomarkers (Torres et al., 2007). Several putative biomarkers have been proposed to measure the levels of fumonisin in various physiological samples such as serum, urine, faeces, hair, and nails. Given the low bioavailability of fumonisin, it poses analytical challenges and requires further validation to serve as biomarkers.

1.3.3.1 Validation of biomarkers of fumonisin exposure

Several putative biomarkers, such as sphingolipid metabolites and free urinary FB_1 (UFB_1), were proposed as potential biomarkers of fumonisin exposure (Shephard et al., 2007). The validation has been conducted through establishing a good correlation between fumonisin-containing food or fumonisin intake and levels of the proposed biomarkers in serum and/or urine samples (Riley et al., 1993; Gong et al., 2008; Voss and Riley, 2013). Correlations between sphingolipid-based biomarkers (particularly Sa, So, and the Sa/So ratio) and fumonisin intake are initially validated in animal studies. Due to the rapid reversibility of Sa and So, the ratio of Sa/So is a preferred putative biomarker. In an early study, a significant dose-dependent and time-dependent increase of the ratio of Sa/So in serum of pigs fed with FB_1 was observed, suggesting the ratio of Sa/So could be a good marker to reflect fumonisin exposure (Riley et al., 1993). Similarly, a significant increase of Sa/So in ducks

fed with 45 mg/kg/day FB_1 after 6 days (Tran et al., 2003). These studies implied that levels of Sa/So could be a sensible, specific, and reliable index of fumonisin exposure in animals. However, a few studies have sought for the validation of Sa/So in human samples, and most of them did not find significant association between the levels of Sa/So and fumonisin exposure (Van der Westhuizen et al., 1999; Solfrizzo et al., 2004; Van der Westhuizen et al., 2010; Xu et al., 2010). The Sa/So ratio in human samples failed to reflect the exposure levels might because the exposure in most populations is not high enough to produce dramatic changes of Sa/So ratio. One study in China found slight increase of urinary Sa/So ratio in the populations frequently consumed homegrown corn with high contamination of FB_1, suggesting the Sa/So ratio may only be used in human studies when fumonisin exposure level is very high (Qiu and Liu, 2001). Therefore, the ratio of Sa/So is a useful biomarker in animal studies, but not viable and sensitive for the assessment of fumonisin exposure in humans.

UFB_1 levels have been measured and validated in several populations with known high exposure levels to fumonisins. In a Mexican population with consumption of maize-based tortillas, and it was found that UFB_1 levels were significantly correlated with maize intake which is commonly contaminated by fumonisins (Gong et al., 2008). The statistically significant correlation between UFB_1 and FB_1 intake was also observed in other human studies, indicating UFB_1 is sensitive and validated as a valuable tool in assessing human exposure to fumonisin (Xu et al., 2010; Van der Westhuizen et al., 2011; Riley et al., 2012; Torres et al., 2014).

1.3.3.2 Fumonisin exposure levels in populations worldwide

Fumonisin commonly contaminates corn and corn-based products worldwide, so dietary exposure to fumonisin has been assessed in different populations based on the biomonitoring of biomarker in urine samples. Table 1-2 summarizes the studies assessing fumonisin exposure levels in populations over the last decades. There is a variation of fumonisin exposure levels among different countries, and higher exposure levels were mainly observed in Africa, Asia, and South America. For example, 1.3, 0.7, and 2.27 ng/ml UFB_1 were reported in the populations from Africa, Asia, and South America, respectively, while 0.004 ng/ml UFB_1 was found in a Swedish population, demonstrating a much lower exposure level of fumonisin in European countries than the level in the countries with warmer climate (Torres et al., 2014; Wallin et al., 2015; Chen et al., 2018; Fan et al., 2019).

Table 1-2 Fumonisin exposure levels in populations worldwide in recent 10 years (2012-2022).

Reference	Country	Age	Number, positive percentage (%)	Biomarkers	[a] Mean; [b] GM (95% CI); [c] Median	Range
Shirima et al., 2013	Tanzania	12-22 months	147 (96%)	UFB_1	[b]167.3 (135.4-206.7) pg/ml	NR
Shephard et al., 2013	Transkei	19-97 years	54 (96%)	UFB_1	[a]0.841±1.06 ng/ml	0.04-4.94 ng/ml
Torres et al., 2014	Guatemala	18-70 years	NR	UFB_1	Jutiapa: [a]2.27 ng/ml Chimaltenango: [a]0.38 ng/ml Escuintla: [a]0.26 ng/ml	NR
Shirima et al., 2015	Tanzania	6-14 months	At recruitment: 157 (98%) 6 months after recruitment: 141 (96%) 12 months after recruitment: 146 (100%)	UFB_1	At recruitment: [b]313.9 (257.4-382.9) pg/ml 6 months after recruitment: [b]167.3 (135.4-206.7) pg/ml 12 months after recruitment: [b]569.5 (464.5-698.2) pg/ml	NR
Wallin et al., 2015	Sweden	NR	252 (6%)	UFB_1	[a]0.004±0.022 ng/ml	NR

Chen et al., 2018	Tanzania	24-36 months	94 (80%)	UFB_1	[a]1.3±0.5 ng/ml	<0.01-16.6 ng/ml
Fan et al., 2019	China	18-66 years	260 (2.7%)	UFB_1	[a]0.699±0.232 µg/l	0.305-0.993 µg/l
Andrews-Trevino et al., 2021	Nepal	18-22 months	683 (100%)	UFB_1	[b]192.07 (163.76-225.28) pg/mg creatinine	6.57-132373.1 pg/mg creatinine
Xia et al., 2022	Pakistan	4-80 years	292 (77%)	UFB_1	[a]17.4±26.8 pg/ml	<0.001-225 pg/ml

UFB_1: urinary fumonisin B_1; CI: confidence interval; NR: not report.

1.3.4 Health effects of fumonisin on animals and humans

Acute and chronic toxicity of fumonisin exposure have been suggested in both animals and humans. Since sufficient evidence of cancer initiation of FB_1 exposure in animals but insufficient evidence of cancer in humans, FB_1 is characterized by IARC as a group 2B possible carcinogen for human (IARC, 2002). The fatal diseases and health effects caused by fumonisin exposure in animals include ELEM, porcine pulmonary oedema syndrome (PPE), hepatotoxic, hepatocarcinogenic and nephrocarcinogenic effects (Šegvić and Pepeljnjak, 2001). In horses, outbreaks of ELEM associated to the consumption of FB_1-contaminated feeds has been reported in several regions, which is lethal and causes the formation of liquefactive necrotic lesions in the cerebrum (Ross et al., 1992; Morgavi and Riley, 2007). In pigs, the indicative clinical signs of FB_1 exposure related PPE disease include the observations of suppressed feed intake, respiratory distress, weakness, and death (WHO, 2000). Further, feeding of purified FB_1 responsible for the occurrence of renal and liver tumors in rodents has been reported, demonstrating FB_1 is a carcinogen in animals (Howard et al., 2001). Currently, there is no direct evidence show that fumonisin causes adverse health effects on humans, but it has been implicated with neural tube defects, oesophageal and liver cancer, and child growth impairment.

1.3.4.1 Association between fumonisin and neural tube defects

Incidence of neural tube defects (NTD) has been reported in populations who consume a large amount of fumonisin-contaminated maize. A study that assessed FB_1 levels in maize in both lowland and highland areas of Guatemala found that there was significantly higher FB_1 in lowland maize than highland maize (Torres et al., 2007). However, due to the frequent transportation of lowland maize to highland, the inhabitants in highland areas of Guatemala were still at great risk of fumonisin exposure (Torres et al., 2007). A retrospective study investigating the incidence of NTD in newborns from Guatemala during the year 2000 found an extremely high prevalence of NTD in some regions, such as in Quetzaltenango, which had the highest frequency of 106 NTD/10000 live births compared to the general American population of ≤3/10000 live births (Marasas et al., 2004). Therefore, it was proposed that high consumption of maize as their staple food is likely to be a risk factor contributing to the high prevalence of NTD. The speculation has been further supported in Mexican-American women who consume large amounts of maize and possibly exposed

to high level of fumonisin, it was found that increased fumonisin exposure levels were associated with the increasing of odds ratios (ORs) for NTD occurrences, apart from in the group with highest fumonisin exposure levels, suggesting that fumonisin exposure increases the risk of NTD, but more evidence of the correlation is required to establish the connection between fumonisin and NTD (Missmer et al., 2006).

1.3.4.2 Association between fumonisin and cancer initiation

There is no direct evidence that FB_1 is a causative factor of cancer initiation in humans, but human oesophageal and liver cancers have been etiologically linked to FB_1 exposure in certain areas of South Africa and Asia. In Transkei, Africa and Henan province, China, the FB_1 contamination levels in corn from high-risk of esophageal cancer (EC) areas are higher than in low-risk areas, such as 4 μg/g vs 54 μg/g FB_1 in mouldy maize in low-risk area and high-risk area, respectively, implying greater FB_1 exposure in humans could be related to the high incidence of esophageal cancer (Rheeder et al., 1992; Yoshizawa et al., 1994). Almost 5-fold higher level of FB_1 in rice from high EC-risk area than it from low EC-risk area was observed (43.8 μg/g vs 8.93 μg/g, respectively, p=0.01), which is also evident there was a significantly positive correlation between FB_1 contamination levels in rice and the risk of EC (Alizadeh et al., 2012). Similarly, in Huaian, an area with highest incidence of EC in China, the FB_1 level in corn samples from this area was significantly higher than the level in another area, Huantai ($p<0.001$). Therefore, those studies suggest a possible role of FB_1 contributing to human esophageal carcinogenesis (Sun et al., 2007).

Only three studies have reported the association between fumonisin and liver cancer in China. A study assessed FB_1 levels in maize samples collected at Haimen (Jiangsu Province, high risk area) and Penlai (Shandong Province, low risk area) in three years from 1993, and FB_1 levels in maize samples harvested in Haimen were approximately 50 folds higher than the levels in Penlai (5.11 vs 0.10 ppm), which speculates that fumonisin might play an important role in the initiation of liver cancer (Ueno et al., 1997). FB_1 contamination levels in Fusui, China, a high-risk area of liver cancer, were also higher than the levels in Huantai, an area with low risk of liver cancer (mean level: 1.27 vs 0.65 mg/kg, respectively) (Sun et al., 2007). However, a case-control study investigating the relationship between FB_1 levels in nails and HCC in two Chinese cohort (Haimen and Linxian) found that there was no statistically significant association between FB_1 and HCC in the two areas (OR=1.10, 95% CI=0.64-1.89 in Haimen and OR=1.47, 95% CI=0.70-3.07 in Linxian), but specifically, a

significant association between FB_1 and HCC among women in Linxian cohort was observed (OR=11.23, 95% CI=1.71-73.80) (Persson et al., 2012). Since the association between FB_1 intake and FB_1 levels in nails was not validated and the significant association between FB_1 and HCC was found in a small sample size of women, this study does not support a correlation between FB_1 and HCC. Although clear evidence of hepatocarcinogenesis of FB_1 in animals and preliminary findings of higher FB_1 levels in humans from high-risk area, the contribution of FB_1 to human liver cancer is not yet confirmed.

1.3.4.3 Association between fumonisin and growth impairment

Decades of animal studies have indicated the association between fumonisin exposure and growth retardation, and limited epidemiological studies also find similar results in humans (Rotter et al., 1996; Gbore et al., 2010; Chen et al., 2018; Yu et al., 2022). The association between fumonisin exposure and child growth retardation was first reported in a Tanzania study in 2010 (Kimanya et al., 2010). Maize samples consumed by the infants in the study were contaminated by fumonisin at levels from 21 to 3201 µg/kg, which significantly exceeded the provisional maximum tolerable daily intake (TDI) of 2 µg/kg bodyweight, and the infants at 12 months age exposed to fumonsin intake above the TDI were significantly shorter and lighter by 1.3 cm and 328 g, respectively (Kimanya et al., 2010). The association was also investigated in a cohort of 166 children recruited at 6-14 months age and followed at 6^{th} and 12^{th} month after the recruitment. UFB_1 concentrations and growth parameter (length-for-age z-scores, LAZ) were assessed at the three time points, and it was found that there was a negative association between UFB_1 concentrations at recruitment and LAZ at 6 months (p=0.016) and 12 months (p=0.014) after recruitment (Shirima et al., 2015). Another study in Tanzanian children also found the similar results, which shows that fumonisin exposure levels were inversely associated with underweight (p=0.0285 with non-detectable samples included and p=0.005 with non-detectable samples excluded) (Chen et al., 2018).

Current evidence of a negative association between fumonisin exposure and child growth was mostly reported in Tanzanian children from rural areas, where there is a high consumption of fumonisin-contaminated maize as staple foods. Recently, a study investigating the association between fumonisin exposure and child growth was also conducted in Nepal. UFB_1 levels were assessed in children at 18-22 months age and child growth outcomes were measured at the age of 24-26 months, which indicated that increased odds of underweight was

found with the increasing of UFB_1 levels (OR=1.09, 95% CI: 1.00, 1.18, *p*=0.043) (Andrews-Trevino et al., 2022).

1.4 Co-exposure to aflatoxin and fumonisin in humans

Co-exposure to multiple mycotoxins is widely reported because of the prevalent co-occurrence of mycotoxins in foods, it is possible that the combination of mycotoxins may have synergistic effects on adverse health (Speijers and Speijers, 2004). Aflatoxin and fumonisin are often found together on food crops such as maize, rice, and wheat flour. Massive studies have reported high contamination levels of aflatoxin and fumonisin in foods, but relatively less studies assessed the co-exposure levels to aflatoxin and fumonisin in populations, only a few studies reported from Africa, Asia, and South America (Li et al., 2001; Sun et al., 2011; Torres et al., 2015). Some epidemiology and animal studies reported that co-exposure to aflatoxin and fumonisin may play a contributory role in growth impairment and carcinogenesis (Gelderblom et al., 2002; Tessari et al., 2006; Shirima et al., 2015).

In table 1-3, the co-exposure levels of aflatoxin and fumonsin in humans and the impacts on health have been summarised. A high prevalence of co-exposure to aflatoxin and fumonisin was found in several studies. Shirima et al found that 82% of children were exposed to both aflatoxin and fumonisin in three regions of Tanzania, with the geometric mean of overall AF-alb and UFB_1 of 12.9 pg/mg and 167.3 pg/ml, respectively, in the three villages (Shirima et al., 2013). In a study in Mexico, 53% of 106 adults had detectable co-exposure to aflatoxin and fumonisin (Elmore et al., 2021). Urine samples from those adults were detected with geometric mean levels of AFM_1 and UFB_1 of 4.3 pg/mg and 50.1 pg/mg creatinine, respectively (Elmore et al., 2021). Differences in the sensitivities of the analytical methods between these studies limit the direct comparison, although the overall aflatoxin and fumonisin exposure in the Tanzanian children were likely higher than that reported in adults in Mexico. Geographic variation in contamination and maize consumption levels are two key factors determining aflatoxin exposure levels. With regarding of varied levels of fumonisin exposure, a few factors, including different food processing methods, lower level of fumonisin contamination in maize, differences in urine excretion by age, all could contribute to the variation of fumonisin exposure between children and adults. Both studies reported high frequency of the exposure to aflatoxin and fumonisin, but the impacts of the co-exposure on children or adults were not investigated.

Table 1-3 Summary of the evidence of co-exposure to aflatoxin and fumonisin and the effects on health.

Reference	Study site	Participants	Exposure levels	Health indicators	Confounders	Main findings
Shirima et al., 2013	Tanzania	148 children aged 12-22 months	AF-alb: [b]12.9 (9.9-16.7) pg/mg UFB_1: [b]167.3 (135.4-206.7) pg/ml	NR	NR	A high prevalence at 82% of the children were exposed to both aflatoxin and fumonisin.
Elmore et al., 2021	Mexico	108 adults	Urinary AFM_1: [a]4.3±3.9 pg/mg creatinine Urinary FB_1: [a]50.1±51.5 pg/mg creatinine	NR	NR	53% of the participants were exposed to both aflatoxin and fumonisin.
Ediage et al., 2013	Cameroon	220 children aged 1-5 years old	Urinary AFM_1: [b]0.33 (0.06-4.7) ng/ml Urinary FB_1: [b]2.96 (0.06-48) ng/ml	HAZ, WAZ, and WHZ	NR	Significant differences between the weaning categories of the children and mean levels of AFM_1 in the urine were observed but were not found between weaning categories and urinary FB_1.

Shirima et al., 2015	Tanzania	166 children aged 6-14 months	AF-alb: [b]4.7 (3.9-5.6) pg/mg at recruitment [b]12.9 (9.9-16.7) pg/mg at the 6th month after recruitment [b]23.5 (19.9-27.7) pg/mg at the 12th month after recruitment UFB_1: [b]313.9 (257.4-382.9) pg/ml at recruitment [b]167.3 (135.4-206.7) pg/ml at the 6th month after recruitment [b]569.5 (464.5-698.2) pg/ml at the 12th month after recruitment	HAZ, WAZ, and WHZ	Village, breastfeeding, maternal education, socioeconomic status, and protein and energy intakes.	There was a significantly negative association between LAZ and the mean level of UFB1 of the three timepoints ($p<0.001$), but the negative association between AF-alb and LAZ was not significant.

Chen et al., 2018	Tanzania	114 children under 36 months	AFB_1-lys: [a]5.1 (3.5-6.6) pg/mg UFB_1: [a]1.3 (0.8-1.8) ng/ml	HAZ, WAZ, and WHZ	NR	UFB_1 level was negatively associated with WAZ (non-detectable samples excluded, p=0.0053; non-detectable samples included, p=0.0285). There was no significantly negative association between AFB_1-lys and HAZ, WAZ, and WHZ.
Tessema et al., 2021	Ethiopia	102 children aged 6-35 months	4-51% children exposed to aflatoxin and 0-11% children exposure to fumonisin.	Biomarkers of inflammation (C-reactive protein, α-1-glycoprotein), protein status (transthyretin, lysine, tryptophan), IGF1, HAZ	Sex, age, time of assessment, intervention arm and inflammation	When all aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1) examined as a group, there was significant association between aflatoxin exposure and each outcome (AGP, CRP, transthyretin, IGF-1, lysine, tryptophan and HAZ, p<0.0001 for each outcome, apart from p=0.007 for transthyretin).

Xue et al., 2019	Huaian, China	190 ESCC cases and 380 participants without ESCC	AFB_1-lys: [c]1.77 pg/mg in ESCC cases [c]1.49 pg/mg in non-ESCC participants UFB_1: [c]176.13 pg/mg creatinine in ESCC cases [c]56.92 pg/mg creatinine in non-ESCC participants	Risk of ESCC	NR	AFB_1-lys and UFB_1 were both significantly higher in cases group compared to non-ESCC group ($p<0.05$ and 0.01, respectively). An increased risk to ESCC was correlated to exposure to both aflatoxin and fumonisin ($p<0.001$ for both).

a: mean; b: geometric mean (95% CI); c: median; HAZ: height-for-age z-score; WAZ: weight-for-age z-score; WHZ: weight-for-height z-score; ESCC: esophageal squamous cell carcinoma; NR: not reported.

1.4.1 Impacts of co-exposure to aflatoxin and fumonisin on growth impairment

Two studies in the Tanzania in 2015 and 2018 not only showed the high prevalence of aflatoxin and fumonisin exposure, but also reported the impacts of aflatoxin and fumonisin exposure on child growth (Shirima et al., 2015; Chen et al., 2018). In 2015, the study in Tanzania found that 99% children were exposed to aflatoxin, with geometric mean levels of AF-alb of 4.7, 12.9, and 23.5 pg/mg albumin at recruitment, the 6th months, and the 12th months after recruitment, respectively (Shirima et al., 2015). One hundred percent of children were exposed to fumonisin, with geometric mean concentrations of UFB_1 of 313.9, 167.3, and 569.5 pg/ml (Shirima et al., 2015). In this study, the negative association between AF-alb and growth did not reach statistical significance. However, the level of UFB_1 were negatively associated with child growth (Shirima et al., 2015). In the study from 2018, 72% children had detectable aflatoxin exposure and 80% had fumonisin exposure. In those children, mean levels of AFB_1-lys and UFB_1 both measured by LC-MS/MS were 5.1 pg/mg and 1.3 ng/ml, and no associations were found between aflatoxin exposure and growth impairment, but a negative association between UFB_1 and underweight was found (Chen et al., 2018). These two studies suggest fumonisin exposure alone or together with aflatoxin may contribute to child growth impairment. Another study in rural Ethiopia investigating the impacts of co-exposure to aflatoxin and fumonsin on child growth found that the exposure prevalence and levels of fumonisin in this population was low and no association between fumonisin exposure and child growth was observed (Tessema et al., 2021). In the study, applying the levels of all aflatoxin groups and the biomarkers of aflatoxin in a joint statistical model suggests that aflatoxin exposure was significantly associated with child growth.

No negative association between aflatoxin exposure and child growth in the two Tanzania studies does not negate the previously established association between aflatoxin exposure and child growth impairment, but may be because aflatoxin exposure levels required to reach a threshold as high to affect child growth. Different biomarkers of aflatoxin measured by different method might also contribute to the different outcome. Shirima et al. (2015) found a mean level of AF-alb of 4.7 pg/mg at recruitment, which is much lower compared to the mean level of AF-alb of 37.4 pg/mg at recruitment in children from Benin, therefore it is possible higher aflatoxin exposure level will cause child growth impairment (Gong et al., 2004). Chen et al. (2018) utilized LC-MS/MS and

measured mean level of AFB_1-lys of 5.1 pg/mg, but it was reported that AF-alb measured by ELISA is approximately three times higher than the level measured by AFB_1-lys by IDMS (Scholl et al., 2006). Assuming the scaling factor of 3 is a reasonable way to compare exposure levels measured by different biomarkers in different methods, AFB_1-lys of 5.1 pg/mg in the study would be equivalent to 15.3 pg/mg AF-alb, which is also lower than the 37.4 pg/mg AF-alb found in the Benin study (Gong et al., 2004). Therefore, it is possible there is a threshold of aflatoxin exposure level to have the negative effects on child growth.

With regarding of the negative association between fumonisin exposure and child growth also need to be interpreted with cautions, because fumonisin is rapidly eliminated from the urine and the UFB_1 levels reflect only very recent exposure (Shephard et al., 1994). In addition, there is no evidence directly showing fumonisin exposure is a causal factor of human chronic diseases. The negative association between fumonisin exposure and child growth observed due to fumonisin exposure alone or affected by simultaneous exposure to aflatoxin is not clear. Hence, the negative association between fumonisin and child growth observed in the two studies needs further research to support the notion and possible mechanism illustrating the association between fumonisin and growth is required.

1.4.2 Impacts of co-exposure to aflatoxin and fumonisin on cancers

Aflatoxin is a group Ⅰ carcinogen associated with risk of hepatocellular carcinoma, whilst fumonisin is a group 2B carcinogen, which has been implicated with the risk for oesophageal carcinoma in the Transkei region of the southern Africa (Rheeder et al., 1992; Ostry et al., 2017; Ramalho et al., 2018). It has been posited that aflatoxin and fumonisin may jointly contribute to aetiologies of human liver and oesophageal cancer, but the evidence is not strong (Sun et al., 2011). As shown in table 1-3, only one epidemiological study quantitatively analysed the biomarkers of aflatoxin and fumonisin in serum and urine samples using HPLC-fluorescence in the Huaian area of China, and found that both aflatoxin and fumonisin exposure levels were significantly higher in cases of esophageal squamous cell carcinoma (ESCC) than the control group without ESCC (p<0.05 and 0.01, respectively), with co-exposure to both aflatoxin and fumonisin being associated with increased risk to ESCC (p<0.001 for both) (Xue et al., 2019).

Scarce evidence from biomarkers-based studies shows the effects of co-exposure to aflatoxin and fumonisin on cancer initiation in humans, but the

potential link of the co-exposure to liver and esophageal cancer has been demonstrated in studies through assessing aflatoxin and fumonisin contamination levels in foods in high-risk areas of cancers in China (Ueno et al., 1997; Li et al., 2001; Sun et al., 2011). Aflatoxin and fumonisin were assessed in 240 corn samples collected from 1993-1995 in Haimen (high-risk area of liver cancer) and Penlai (low-risk area of liver cancer) in China, and evidence shows that there was no significant difference of aflatoxin contamination level in the corn samples between Haimen and Penlai, but fumonisin contamination level in the samples was significantly higher (10 folds and 50 folds in 1993 and 1995, respectively) in the high risk area compared to low risk area, which suggests fumonisin might play an important role in the initiation of liver cancer (Ueno et al., 1997). Similarly in Guangxi, China, both aflatoxin and fumonisin contaminations levels in corns from the high-risk area of primary hepatocellular carcinoma were significantly higher than the levels in low-risk area (aflatoxin: 531 vs 55 μg/kg; fumonisin: 655 vs 170 μg/kg, respectively) (Li et al., 2001). A study conducted in a high-risk area (Huaian, China) and low-risk area (Huantai, China) for oesophageal cancer found that the average daily intake of AFB_1 and FB_1 was significantly higher in Huaian in comparison with Huantai (AFB_1 intake: 1.723 vs 0.397 μg; FB_1 intake: 460.0 vs 92.4 μg) (Sun et al., 2011).

The evidence above implies there may be an association between co-exposure of aflatoxin and fumonisin with increased risk of growth impairment and cancer initiation, due to concerns regarding methodological limitations, more evidence is warranted to further illustrate the interaction between aflatoxin and fumonisin and the effects on human health. According to the studies in table 1-3, most studies had relatively small population sizes, thus may be insufficient to detect the effects of the co-exposure on human health. In future, the effects of co-exposure to aflatoxin and fumonisin on health should be investigated in larger populations that would have more statistical power. In addition, most of the studies did not adjust for other confounders, such as: age, gender, and geographical variables. Since the relationship between the co-exposure and health outcomes has not been established, more studies on biological mechanisms regarding the effects of co-exposure to aflatoxin and fumonisin are prompted.

1.5 The epigenome

Epigenome refers to the complete description of all epigenetic changes across the genome (Bernstein et al., 2007). Historically, the term “Epigenetics”, coined by Conrad Waddington, was defined as “the branch of biology which studies the causal interaction between genes and their products, which bring the phenotype into being” (Waddington, 1942). Epigenetic mechanism influences gene expression pattern that is heritable without causing any changes in DNA sequence (Delcuve et al., 2009). Three key epigenetic mechanisms are involved: DNA methylation, histone modifications, and nucleosome positioning, which are important for gene expression regulation and noncoding RNA expression (Portela and Esteller, 2010). Currently, DNA methylation is one of the best characterized epigenetic mechanisms.

1.5.1 DNA methylation

DNA methylation refers to a methyl group donated by S-adenosylmethionine (SAM) and brought by DNA methyltransferases (DNMTs) to the fifth carbon atom of cytosine within CpG dinucleotides to form 5-methyl cytosine (Figure 1-6), which is heritable and reversible (Smith and Meissner, 2013). In eukaryotes, 60-90% CpG sites are methylated, and most unmethylated CpG sites are clustered in CpG islands of promoter regions of genes (Tucker, 2001). CpG islands are regions distributed in genomic DNA with the features of high content of CG dinucleotide (over 50%) and an observed-to-expected ratio of CpG at least 0.6, ranging from 200 bp to several kilobases and predominantly unmethylated (Gardiner-Garden and Frommer, 1987). Typically, these CpG islands are located in the promoter regions of housekeeping genes and tumour suppressor genes (Bird, 2002).

DNA methylation patterns are properly establish and maintain by DNMTs, which is essential for mammalian development (Robertson, 2005). DNA methylation is mainly catalysed by three DNMTs, DNMT1, DNMT3a, and DNMT3b (Gujar et al., 2019). DNMT 3a and 3b are responsible for establishing a new methylation pattern and known as *de novo* DNMTs (Okano et al., 1999). Inactivation of both DNMT 3a and 3b by gene targeting perturbed *de novo* methylation in mouse embryonic carcinoma cells, it also led to multiple developmental defects, such as: growth impairment, neural tube defects, and death, in mice (Okano et al., 1999). On the other hand, DNMT1 maintains the methylation pattern from parent DNA strand and copy it to the daughter DNA strand during DNA replication, it also has *de novo* DNMT activity with the

cooperation of DNMT3a and 3b (Fatemi et al., 2002; Song et al., 2012). In mice, inactivation of DNMT1 in the thymus caused a significant demethylation, which resulted in an inhibited proliferation of naïve T cells and increased expression of cytokines (Lee et al., 2001).

Alteration of DNA methylation, either demethylation or hypermethylation, affects many normal cellular processes, including genomic imprinting, X-chromosome inactivation, and gene transcription (Paulsen and Ferguson-Smith, 2001). Genomic imprinting is a phenomenon that genes inherited from its parental origin, in which one of the gene copies, either from maternal or paternal allele, is suppressed, and another one is expressed (Moore and Haig, 1991). DNA methylation is a key mechanism responsible for genomic imprinting. A study found that the mice embryos with deficient DNMT activity caused abnormal expression of several imprinted genes such as H19, insulin-like growth factor 2, and its receptor (Li et al., 1993). X-chromosome inactivation is an important biological process in female during embryonic development to avoid having twice as many gene products as males (Lyon, 1961). High methylation preventing transcription was evident in the regulation of inactivating X chromosome, while in cultured cells treated with DNA methylation inhibitor partially reactivated the inactive X chromosome, suggesting the dynamic role of DNA methylation in X chromosome inactivation (Mohandas et al., 1981; Grant and Chapman, 1988). Generally, gene-specific methylation is known to have a negative association with gene expression. A typical example is that hypermethylation of promoter regions of tumour suppressor genes resulted in gene silencing and subsequently lead to cancer initiation and progression (Herman, 1999).

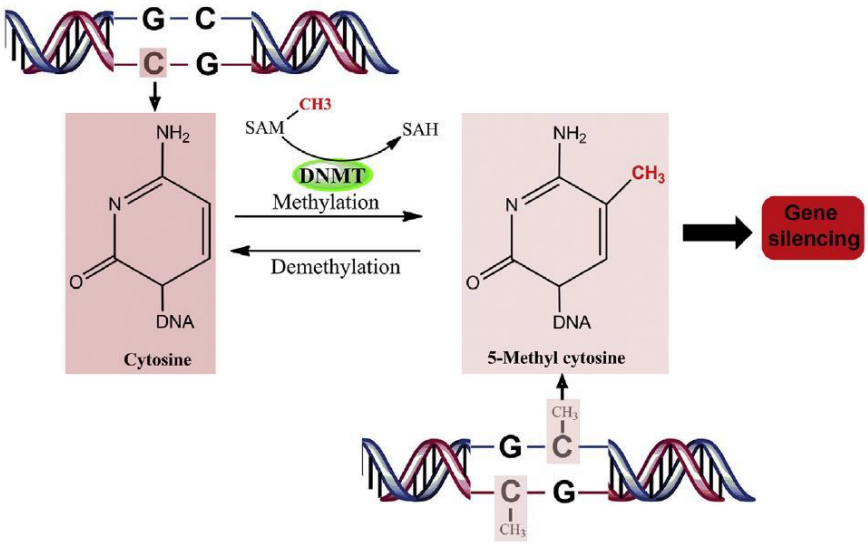


Figure 1-6 The formation of DNA methylation. This diagram illustrates how cytosine in a CpG site is methylated by DNA methyltransferases (DNMTs) to form 5-methyl cytosine (^{5}mC). S-adenosylmethionine (SAM) is used as a methyl donor, and a methyl group (-CH3) is added to the fifth carbon atom of cytosine, which induce hypermethylation and gene silencing (Smith and Meissner, 2013).

1.5.2 DNA methylation related human disease

The importance of DNA methylation is emphasized by the growing evidence found in the association between various human diseases and abnormal DNA methylation alterations. The most well-documented human diseases linked to DNA methylation are cancers and growth failure.

Association between DNA methylation and cancers was first reported in 1983, when the study indicated global hypomethylation were found in human tumour tissues compared to normal counterparts (Feinberg and Vogelstein, 1983). Genomic hypomethylation is reported to cause genomic instability and increased risk of cancers, which was also evident in a case-control study quantifying the percentage of ^{5}mC in leucocyte DNA in both cases of bladder cancer and controls, and it was found that the median ^{5}mC percentage was significantly lower in cases compared to controls (3.03% vs 3.19%, p=0.0002), a contribution of the hypomethylation to risk of bladder cancer also suggested (Moore et al., 2008). However, studies of genome-wide hypomethylation in cancers nowadays have been largely overshadowed by research on gene-specific methylation related cancers. Hypermethylation in tumour suppressor genes has been found almost in every cancer type, causing gene silencing and uncontrollable tumour cell invasion (Battagli et al., 2003; Yang et al., 2003;

Dulaimi et al., 2004; Robertson, 2005; Ovchinnikov et al., 2012; Kim et al., 2019). Effects of gene-specific hypomethylation on cancers also occur. For instance, a gene of melanoma associated antigen family (MAGE), *MAGE-A1*, is often methylated in normal somatic tissues, but it was found less methylated and re-expressed in tumour cells (De Smet et al., 1999; De Smet et al., 2004).

Child growth and development are complex processes that could be influenced by a variety of factors, and DNA methylation has been implicated with poor infant growth. Evidence shows that significantly decreased methylation at the *H19*/*IGF2* imprinting control region of chromosome 11p15.5 (ICR1) in placental samples was associated with intrauterine growth restriction (IUGR) (p<0.001) (Bourque et al., 2010). Further a study also suggested DNA methylation patterns in human placentas were significantly correlated with infant growth (Banister et al., 2011). In patients with Russell-Silver syndrome, which is a disorder of pre- and post-natal growth restriction, methylation of ICR1 and low birth weight were both observed, and the methylation percentage at ICR1 associated with *H19*/*IGF2* was significantly higher in the control groups compared to the percentage in the patients (38.4% vs 31.1%, p<0.0001) (Peñaherrera et al., 2010). Hence, those evidence demonstrates that variations in DNA methylation acts functionally in infant growth.

1.5.3 Environmental exposure and DNA methylation

Environmental exposure is a crucial factor causing DNA methylation, either global DNA methylation or gene-specific methylation, of which, chemical exposure is the most well studied (Martin and Fry, 2018). In white blood samples of 58 women prevalently exposed to chemical contaminants, such as lead, mercury, and bisphenol A, global methylation was determined by measuring methylation levels of *LINE-1* and *Alu* elements, and it was found that there was no difference *of LINE-1* methylation between high exposure groups and low exposure groups for lead, mercury, and bisphenol A, but significantly decreased methylation at the promoters of specific genes, *COL1A2* and *TSP50*, was observed in higher exposure groups for lead and bisphenol A exposure, respectively (Hanna et al., 2012). However, bisphenol A exposure positively associated with global methylation was reported in human placenta (p=0.002), although the association was not found in other tissues, such as liver and kidney, suggesting there might be a tissue-specific DNA methylation patterns (Nahar et al., 2015). Despite findings of the chemical exposure affected DNA methylation are not consistent, the evidence still demonstrate that

environmental exposure can influence methylation in a genome-wide or gene specific manner.

1.5.3.1 Effects of aflatoxin exposure on DNA methylation

As discussed above, aflatoxin exposure contributes to human cancers and child growth impairment. To illustrate the mechanism behind the adverse health effects, DNA methylation alteration has been speculated as an important mechanism having roles in regulating carcinogenicity and toxicity of aflatoxin in the cancer development and child growth. AFB_1 exposure levels were negatively associated with global methylation levels in white blood cells of a Taiwanese cohort free of cancer (Wu et al., 2013). In human tumour samples of another batch of Taiwanese populations, the inverse association between AFB_1 exposure and global methylation was also reported (r=-0.36, p=0.034), suggesting the hypomethylation caused by AFB_1 exposure might have implications in AFB_1 associated HCC development (Zhang et al., 2012).

Extensive studies have investigated the association between aflatoxin exposure and DNA methylation of tumour suppressor genes. Zhang et al. reported high frequencies of methylation in the promoters of two genes, *p16* and *RASSF1A* (85% and 47%, respectively), in 83 HCC tissue samples in Taiwanese, and a significant association between *RASSF1A* methylation levels and the levels of AFB_1-DNA adducts was found in tumour tissues (Zhang et al., 2002). Another cancer related gene, *GSTP1* was also hypermethylated in HCC and similarly there was a statistically association between *GSTP1* promoter methylation and AFB_1-DNA adducts (OR=2.81, 95% CI=1.03-7.70), suggesting aberrant promoter methylation of those tumour suppressor or cancer-related genes is closely related to the hepatocarcinogenesis of AFB_1 (Zhang et al., 2005).

Effects of aflatoxin exposure on DNA methylation in embryonic and early development have been investigated. Aflatoxin exposure levels in pregnant women was associated with DNA methylation of 71 CpG sites in their infants aged 2-8 months old, and the aflatoxin-associated differentially methylated genes were found in growth factor and immune-related genes, such as: *FGF12* and *IGF1R*, *CCL28*, *TLR2* and *TGFBI* (Hernandez-Vargas et al., 2015). A follow up DNA methylation analysis was performed in the same population when the infants were 2 years old, and the association between aflatoxin exposure and differentially methylated CpG sites and regions was found, some of which affected gene expression. Pathway analysis in the study indicated that aflatoxin-associated methylated regions were enriched in inflammatory and

growth pathways (Ghantous et al., 2021). Compared to the former study, only 10% genes located in aflatoxin-associated differential methylation sites and regions were the same in the two studies (Hernandez-Vargas et al., 2015; Ghantous et al., 2021). Although the relevance of biological function of aflatoxin-associated differential DNA methylation is not determined, those studies demonstrated that aflatoxin-associated DNA methylation might be a potential mechanism that affects early development.

1.5.3.2 Effects of fumonisin exposure on DNA methylation

Scarce studies on effects of fumonisin exposure on DNA methylation in humans can be found, current evidence of how fumonisin affects DNA methylation are from cell studies *in vitro*. With respect to fumonisin affected global methylation, in the human intestinal Caco-2 cell line treated with 10 μM and 20 μM FB_1 the percentage of ^{5}mC increased from 4.5% to 9% and 9.5%, respectively (Kouadio et al., 2007). On the contrary, 200 μM FB_1 induced significant global hypomethylation and structural changes of DNA in HepG2 cells, implicating global hypomethylation might be a mechanism contributes to liver carcinogenicity of FB_1 (Chuturgoon et al., 2014). In HEK293 cells, a type of human embryonic kidney cells, it was found global methylation was significantly increased when the cells were treated with 100 μM FB_1 (Sugiyama et al., 2021). Different types of cell line used in the above studies and different doses of FB_1 applied in the cells may lead to the different DNA methylation types. It was suggested doses of FB_1 lower than 50 μM could induce hypermethylation while higher doses of FB_1 over 200 μM might result in hypomethylation, but more studies investigating fumonisin associated DNA methylation in various cell lines are needed (Huang et al., 2019).

Gene-specific methylation related to fumonisin is also only reported in cell line studies and there is a lack of evidence in humans. In rat liver (Clone 9) and kidney (NRK-52E) cells, methylation levels of tumour-related genes, such as: *c-myc* and *VHL*, were tested, and promoter regions of *c-myc* gene were methylated in Clone 9 cells after 10 and 50 μM FB_1 treatments but were unmethylated in NRK-52 cells, while promoter regions of *VHL* were methylated in both cell lines after FB_1 treatments (Demirel et al., 2015b). The aberrant DNA methylation observed in tumour-related genes may cause inactivation of the genes and lead to increasing risk to cancers. Due to the limited evidence showing fumonisin caused DNA methylation alterations, more detailed studies in the effects of fumonisin on DNA methylation are dire need currently.

1.6 Hypothesis

Previous studies have shown that high levels of dietary exposure to aflatoxin and fumonisin have adverse health effects on humans, but the impact of aflatoxin exposure on child growth is conflicting, and the molecular mechanism of their health effects is not fully understood. Previous work from our group found that aflatoxin exposure *in utero* was associated with DNA methylation and gene expression in infants, particular genes involved in immune and growth factors pathways (Hernandez-Vargas et al., 2015). The hypothesis investigated in this study is that aflatoxin exposure contributes to child growth impairment and other adverse health effects. With respect to molecular mechanisms, we propose that gene expression, and DNA methylation, may be altered by aflatoxin and/or fumonisin to affect key pathways involved in growth, immune response, and cancer.

1.7 Aims

Aim 1 (Chapter 3)

1a. To assess aflatoxin exposure levels in children in Africa and South Asia.

1b. To investigate the association between aflatoxin exposure and child growth and child mortality.

Aim 2 (Chapter 4)

2a. To investigate the toxicity pattern of aflatoxin and fumonisin and whether there is a joint toxicity of aflatoxin and fumonisin in combination *in vitro*.

2b. To investigate the effects of aflatoxin and fumonisin on gene expression of genes related to growth factors and immune-related pathways *in vitro*.

2c. To investigate the effects of aflatoxin on DNA methylation of candidate differentially expressed genes *in vitro*.

Aim 3 (Chapter 5)

3a. To investigate the transcriptome profile in response to aflatoxin *in vitro*.

3b. Conduct the functional analysis of the differentially expressed genes in response to aflatoxin *in vitro*, to figure out the associated pathways and diseases.

Aim 4 (Chapter 6)

4a. To investigate the association between aflatoxin exposure and metabolomic *in vivo*.

4b. To investigate the enriched pathways of the metabolites associated to aflatoxin exposure *in vivo*.

Chapter 2 Methods

2.1 Materials and equipment

Aflatoxin B_1 (AFB_1, purity ≥98.0%, Sigma), fumonisin B_1 (FB_1, purity ≥98.0%, Sigma), dimethyl sulfoxide (DMSO, 99.99%, Sigma), phosphate buffered saline (PBS, Merck), acetic acid, ammonium sulphate, BIO-RAD Protein assay dye reagent concentrate, pronase, bovine serum albumin, absolute ethanol, methanol, Sep-pak C18 cartridges, Tween 20, foetal bovine serum, dried skimmed milk powder, goat anti-rabbit IgG peroxidase labelled antibody, 3,3',5,5'-Tetramethylbenzidine (TMB), 1M hydrochloric acid (HCL), 30% hydrogen peroxide, 96 well ELISA microplates (Cat: 655061), 3-(4.5-dimethylathiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), Minimum Essential Medium (MEM), penicillin-streptomycin, trypsin, RNase/DNase free filter tips and microtubes, nuclease-free water, 384-well Roche qPCR plates. Molecular Biology Grade ethanol and isopropanol were purchased from Fisher Scientific (UK). DNA Mini Kit, RNeasy Mini Kit, QuantiTech® Reverse Transcription Kit, and QuantiNova® LNA PCR Panels were purchased from QIAGEN (UK). ReliaPrep™ RNA Cell Miniprep System was purchased from Promega (USA). TaqMan® Reverse Transcription Reagents and SYBR™ Green PCR Master Mix were purchased from Thermo Fisher Scientific (UK).

2.2 Aflatoxin-albumin adduct measurement (ELISA)

Aflatoxin exposure levels in serum samples were measured using the aflatoxin-albumin enzyme-linked immunosorbent assay (ELISA). Before ELISA, aflatoxin-albumin adduct from serum samples were extracted and purified, including the procedures of heat inactivation, albumin extraction, protein quantification, albumin hydrolysis, albumin precipitation, and purification of aflatoxin residues.

2.2.1 Heat inactivation

Serum samples were incubated in a water bath at 56 ℃ for 45 minutes for inactivation of possible virus. Samples were then transferred and stored in a -80 ℃ freezer for further analysis.

2.2.2 Albumin extraction

Each serum aliquot of 250 µl was transferred into a 1.5 ml microtube and 375 µl cold saturated ammonium sulphate was added dropwise. The mixture was vortexed and centrifuged at a speed of 9000 g for 15 minutes at 4 ℃. The

supernatant containing albumin was collected and added into a new 1.5 ml microtube and 50 µl of 1 M acetic acid added, then it was centrifuged at the speed of 9000 g for 15 minutes at 4 ℃. The supernatant was discarded, and the pellet was dissolved in 500 µl PBS.

2.2.3 Protein quantification

The PBS-dissolved samples from albumin extraction were diluted 1:50 in distilled water, and the samples were further diluted 10-fold to make a 1:500 dilution. A stock of 0.1 mg/ml human serum albumin was diluted to a series of standards (2, 5,10, 15, 20, 25, and 30 µg/ml) to make a protein standard curve. 40 µl filtered Bio-Rad reagent was added into each well of a 96-well plate followed by an addition of 160 µl samples/standards into the wells. The plate was immediately read at 620 nm in a Multiskan Go Microplate Spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). Then 2 mg albumin equivalent volume to be used in albumin hydrolysis was calculated in Excel based on the albumin concentrations read.

2.2.4 Albumin hydrolysis

2 mg albumin equivalent volume was taken into a 15 ml falcon tube and mixed with 67 µl pronase (10 mg/ml), then the total volume was adjusted to 0.8 ml with PBS. The mixture was incubated in a water bath at 37 ℃ for 15 hours. Then 100 µl bovine serum albumin (stock: 100 mg/ml) and two-fold volumes (1.8 ml) of cold ethanol were added. The samples were placed in a -20 ℃ freezer for at least two hours. Then the supernatant was removed to new 50 ml falcon tubes after centrifuging the samples at 1000 g for 15 minutes, and diluted with PBS to make a final volume of 30 ml.

2.2.5 Sep-pak C18 purification

After dilution to 30 ml with PBS, the final ethanol concentration in the samples was reduced to 5%, so that the aflatoxin residues can bind to Sep-pak cartridges for purification. The purification was performed with the usage of an eight-channel peristaltic pump (Cole-Parmer, Saint Neots, UK) and Sep-pak C18 cartridges. After washing tubes in the pump with neat methanol, cartridges were attached to the end of the tubes and washed with 5 ml 80% (v/v) methanol, followed by washing with 10 ml distilled water. Then the hydrolysed 30 ml samples were loaded and purified through each tube and cartridge. After that, the cartridge was washed with 5 ml distilled water and 5 ml 5% (v/v) methanol and finally eluted with 5 ml 80% (v/v) methanol to collect the aflatoxin residues

into 15 ml falcon tubes. Samples in the 15 ml falcon tubes were dried in a Speed-vac (Genevac EZ-2 plus, Manchester, UK) overnight and reconstitute in 0.5 ml PBS.

2.2.6 ELISA

2.2.6.1 Preparation of ELISA reagents

Four quality controls were determined before testing samples with ELISA. AF-epoxide was previously synthesized by Dr Gaoyun Chen and stored in the dark in -80 ℃ freezer for future use to make the quality controls. In brief, different volumes of AF-epoxide was used to spike blank human serum to get a series of concentrations, then four controls were accepted when the values lie within the following ranges: <0.7 fmol/25 µl, 0.7-1.5 fmol/25 µl, 1.0-2.5 fmol/25 µl, and 2.5-4.0 fmol/25 µl. Stock of AFB_1-ovalbumin (1 mg/ml) synthesised by reacting AFB_1-8,9-dichloride or AFB_1-8,9-dibromide with ovalbumin, and stock AFB_1-lysine standards (40 pmol) were both synthesised in our laboratory by Dr Gaoyun Chen. Stock of primary aflatoxin antibody was generated by immunisation in New Zealand White rabbits with conjugate which was produced by reacting of AFB_1-Cl_2 with bovine serum albumin by Brigitte Chapot and Chris Wild (Chapot and Wild, 1991). All the stock solutions were stored in -80 ℃ freezer for future use to make working solutions. Secondary stock solution of AFB_1-ovalbumin prepared by 10 folds dilution in PBS and aliquoted to 13 µl (0.1 mg/ml) was stored in -20 ℃ for further 2000 folds dilution of working solution preparation. A set of 8 AFB_1-lysine standards at the final concentration of 0.2, 0.5, 1, 2, 3, 4, 6, and 10 fmol/25 µl was prepared and aliquoted.

2.2.6.2 ELISA measurement

ELISA was performed to determine aflatoxin-albumin adduct (AF-alb) levels as shown in Figure 2-1. In details, each well of high-binding 96-well plates were coated with 2.5 ng AFB_1-ovalbumin in 50 µl PBS, apart from the wells left for the blank group, and the plates were dried in an incubator at 37 ℃ overnight. Then, the coated plates were washed with PBS-0.05% Tween 20 (PBST) buffer for at least five times, followed by an addition of 200 µl 5% (w/v) milk solution in PBS into each well, then the plates were incubated at room temperature for one hour in the dark. During the incubation, primary anti-aflatoxin antibody was diluted with PBS and 0.75% (v/v) foetal bovine serum added, then mixed with PBS, four quality controls, a set of eight standards, and samples at the volume

of 1:1 in microtubes. All mixtures were placed at room temperature and incubated in the dark for 30 minutes before adding to the plates.

After the incubation for 1 hour in milk solution, the milk solution was discarded, and the plates washed with PBST buffer for five times. Then the plates were dried with tissues. 50 µl of each mixture were added into the wells according to the plate layout. Then the plates were covered with plate films and placed in an ELISA plate shaker (iEMS, Thermo Fisher Scientific, Loughborough, UK) at 650 rpm for 90-minute incubation.

After that, the mixture solution was discarded from the plates and the plates washed with PBST buffer for 5 times and dried with tissues. Then 50 µl secondary antibody (goat anti-rabbit IgG labelled with peroxidase) was added into each well. The plates were covered with plate films and placed back into the ELISA plate shaker to incubate at 650 rpm for 90 minutes.

After secondary antibody incubation, the solution was discarded and washed with PBST buffer for 4 times followed by a single distilled water wash. The plates were dried with tissues. Then, 50 µl TMB peroxidase substrate was added into each well. The plates were moved into an incubator at 37 ℃ and incubated for 20 minutes. Then 50 µl of 1 M HCL was added into each well to stop the enzymatic reaction. Lastly, the plates were read immediately in a plate reader at 450 nm to obtain the concentration of AF-alb.

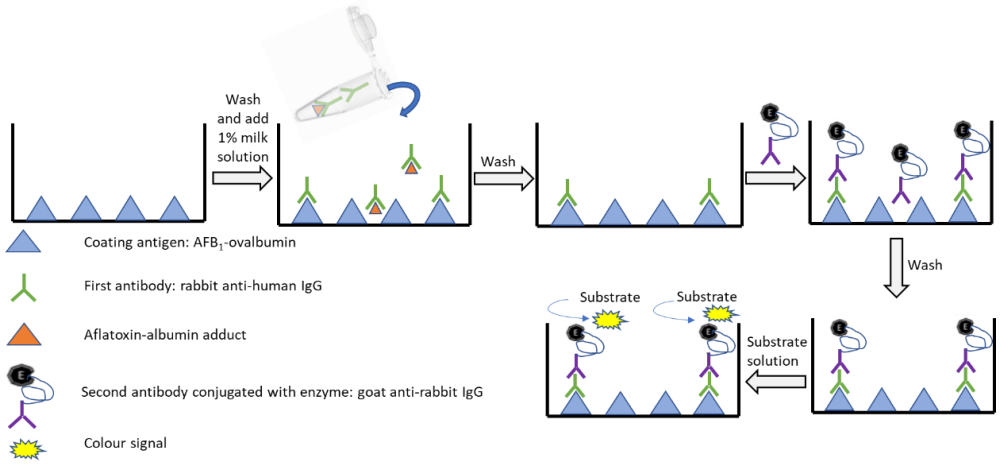


Figure 2-1 Procedures of aflatoxin-albumin enzyme-linked immunosorbent assay (ELISA).

2.2.6.3 Consistency test of ELISA measurement

Before testing the samples in this study, four randomly selected samples (Appendix 2.1) from previous project in our group (ELISA-ready samples) were

analysed by ELISA to check board, which ensures a consistency of the ELISA method. A high consistency was determined by that the coefficient of variation (CV) percentage between the two tests below than 25%. After passing the check board test, all the serum samples were tested by ELISA as above procedures described.

After finishing the assessment of AF-alb in all samples, six serum samples were randomly selected for re-extraction, and they were re-tested by ELISA to verify previous results. A scatterplot was made to illustrate the correlation between the first and the second test (Appendix 2.2).

2.2.7 Data analysis of ELISA

The percentage of inhibition was calculated based on the optical density (OD) based on formula 1, then the corresponding concentrations of samples were determined from a standard curve (Figure 2-2). The standard curve should meet the criteria including shape of sigmoidal, at 20% inhibition the concentration is 0.6-0.8 fmol/25 µl, at 50% inhibition the concentration is 1.8-2.5 fmol/25 µl. After determination of concentrations from the standard curve, the samples concentration was finally transformed to concentration in pg/mg using formula 2.

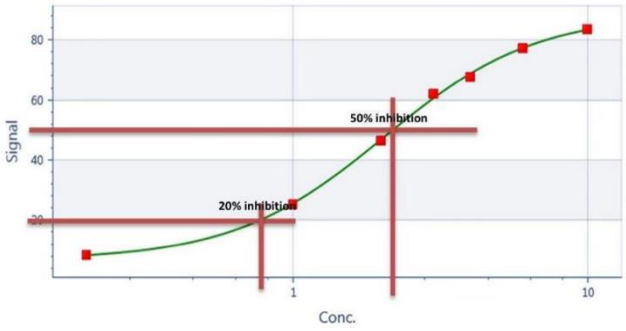


Figure 2-2 Standard curve of ELISA.

$$Inhibition\ percentage = \frac{Control\ mean\ OD - sample\ \ OD}{Control\ mean\ OD} \quad formula\ 1$$

$$mean\ in\ pg/mg = \ mean\ in\ \ fmol/25\ \mu l \times 4.57 \quad formula\ 2$$

In formula 2, 4.57 is derived from the molecular weight of aflatoxin-albumin adduct. For the ELISA method, 2 mg albumin was used, so this formular is only applicable for this standard 2mg method. The limit of detection (LOD) of the

method is 3 pg AF-alb adduct/ mg albumin, so if the samples are below this LOD they were recognised as negative samples and further assigned a value of half LOD as 1.5 pg/mg for the purpose of statistical analysis. Samples over 50% inhibition were diluted to test again to make sure the inhibition percentage falls into the range 20%-50%.

Each batch of samples were tested along with four quality controls in two replicates on at least two different days. Concentrations of the four quality controls should fall into the range as it discussed above. Then both samples and quality controls are acceptable when the coefficient variation percentage (%CV) is less than 10% within a day, as well as below 25% between different days. If the %CV failed to meet the criteria, more repeats of the samples were conducted until the %CV below 10% and 25% within intra- and inter-assay were reached.

2.3 Cell culture

All cell culture procedures were performed in a class II biological laminar airflow cabinet with strict aseptic technique applied in every procedure.

Human hepatocyte line 16 (HHL-16), a non-tumorigenic human liver cell line, was kindly provided by Dr. Arvind H. Patel (MRC Virology Unit, Glasgow) (Clayton et al., 2005). HHL-16 cells were cultured in Gibco Minimum Essentia Media (MEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were grown in an incubator at 37 ℃ with a humidified atmosphere and 5% CO_2 supplied. The cells were passaged every 2 or 3 days when the cell confluency reaches up to 80%. Passage 20-30 were used for all cell experiments.

2.4 Cell treatments

AFB_1 powder was dissolved in sterile-filtered DMSO to form a stock concentration of 20 mg/ml, and the final concentrations of AFB_1 used in other experiments were diluted with cell culture medium. FB_1 powder was dissolved in sterile PBS to give a stock concentration of 10 mg/ml. Three types of treatments were used: individual AFB_1 treatments, individual FB_1 treatments, and combined AFB_1 and FB_1 treatments. In single AFB_1 or FB_1 treatments, various concentrations of AFB_1 (1, 5, 10, 20, 50, and 100 µg/ml) and FB_1 (10, 30, 50, 100, and 150 µg/ml) were used to treat the cells for 24 or 48 hours. In the combination of AFB_1 and FB_1, 1 µg/ml AFB_1 + 10 µg/ml FB_1 (A1_F10), 1 µg/ml AFB_1 + 50 µg/ml FB_1 (A1_F50), 1 µg/ml AFB_1 + 100 µg/ml FB_1 (A1_F100),

5 µg/ml AFB_1 + 100 µg/ml FB_1 (A5_F100), and 10 µg/ml AFB_1 +100 µg/ml FB_1 (A10_F100) were used.

2.5 MTT assay

To assess the impact of AFB_1 and/or FB_1 on cell viability, the MTT assay was used. HHL-16 cells were seeded into 96-well plates at the density of 2×10^4 /well overnight to allow attachment. Various concentrations of single AFB_1 and/or FB_1 treatments (see 2.4) in serum free MEM medium were used to treat cells incubated at 37 ℃ with 5% CO_2 for 24 and 48 hours. Untreated cells were used for the normalization of DMSO and PBS treatments. DMSO and PBS treatments as control groups for normalization of single AFB_1 and FB_1 were used, respectively. After incubation, 10 µl MTT solution (5 mg/ml) was added into each well and incubated at 37 ℃ with 5% CO_2 for 4 hours. Then, 100 µl solubilising solution was added into each well and the plates were placed back into the incubator overnight. Lastly, OD value was measured at 540 nm and 690 nm using a Multiskan Go Microplate Spectrophotometer. The cell viability percentage was calculated as formula 3 shown.

$$\%\ \text{cell viability}=\frac{(A540-A690)\ sample}{(A540-A690)control}\times100 \qquad \text{formula 3}$$

2.6 Interaction analysis

Combined effects were evaluated by combination index (CI) determined in the CompuSyn software (Combosyn Inc., Paramus, NJ, USA) based on the median-effect equation of the mass-action law (Chou and Talalay, 1984):

$$log\frac{fa}{fu}=m\times\log(D)-m\times\log(Dm)$$

Where fa and fu represent affected and unaffected fractions, respectively. D is dose of the toxin, Dm is dose required for median effect. m is the sigmoidicity coefficient, when m>1 stands for sigmoidal, m=1 stands for hyperbolic, and m<1 stands for flat sigmoidal. The linear regression correlation coefficients of the median effect plots were verified and greater than 0.95 (Chou, 2010).

The interaction analysis was conducted by calculating the CI values for the combination of AFB_1 and FB_1 in the software based on Chou-Talalay method (Chou, 2006):

$$Combination\ index\ (CI)=\frac{(D)1}{(Dx)1}+\frac{(D)2}{(Dx)2}$$

Where (Dx) 1 and (Dx) 2 were concentrations of AFB_1 and FB_1 alone to exert x% effect, while (D)1 and (D)2 were concentrations of AFB_1 and FB_1 in combination to exert the same effect. CI<1, =1, and >1 indicate synergistic, additive, and antagonistic effects, respectively.

2.7 QuantiNova® LNA® PCR Panels assay

2.7.1 RNA extraction

After treatment with various concentrations, to collect the cells, the previous media containing AFB_1 and FB_1 were aspirated, and the cells were washed with PBS. Then the cells were trypsinized for 5 minutes, and new media containing FBS was added to stop the trypsinization. The cell pellet was collected after centrifuge and the supernatant was aspirated out prior to the collection.

After harvest of the cells, total RNA from the cells was extracted using RNeasy Mini Kit according to the manufacturer's instructions. Buffer RLT was added to the tubes containing the cell pellet to homogenize the cells, and equal volume of 70% ethanol was added to the homogenized lysate and mix well by pipetting. Then the mixture was transferred to a RNeasy spin column placed in a 2 ml collection tube to centrifuge at 12000 rpm for 1 minute, followed by one Buffer RW1 wash and two-time Buffer RPE washes. After that, the RNeasy spin column was placed in a new collection tube and centrifuged at full speed for 2 minutes to dry the column membrane. Finally, RNA was eluted with 30-50 µl RNase-free water and collected in a new 1.5 ml RNase-free microtube. The concentration and quality of RNA samples were determined by NanoDrop ND-100 Spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). Purified RNA samples were directly used for reverse transcription or stored in -80 ℃ freezer for future usage.

2.7.2 Reverse transcription

After determination of RNA concentrations and quality, 1 µg RNA was used to synthesise cDNA with QuantiTech® Reverse Transcription Kit. 1 µg RNA was mixed with 2 µl gDNA Wipeout Buffer, 1 µl Internal Control RNA, and variable RNase-free water to make up a volume of 14 µl in total. After incubating the mixture at 42 ℃ for 2 minutes, 6 µl Reverse-transcription master mix containing 1 µl Quantiscript Reverse Transcriptase, 4 µl Quantiscript RT Buffer, and 1 µl RT Primer Mix was added into the 14 µl mixture to make a total reaction volume of 20 µl. The reaction was then incubated in a thermal cycler (Applied Biosystems, Thermo Fisher Scientific, Loughborough, UK) as follows: 42 ℃ for

15 minutes, and 95 ℃ for 3 minutes. Then the synthesised cDNA samples were directly used or store in -20 ℃ freezer for further analysis.

2.7.3 Quantitative Real-time PCR

QuantiNova® LNA® PCR Focus Panels were used for the gene expression analysis of human growth factor genes, which contains 84 human growth factor gens (Appendix 2.3), six reference genes, three QuantiNova Internal Controls, and three positive PCR controls. Each 20 µl synthesized cDNA was diluted with 90 µl RNase-free water, and 100 µl was taken out and added with 500 µl 2X QuantiNova SYBR Green PCR Master Mix, and 400 µl RNase-free water to make the qPCR components. 10 µl of the qPCR components was added into each well of the QuantiNova® LNA® PCR Focus Panels. Roche LightCycler 480 (Roche, Welwyn, UK) was used for qPCR running with the following thermal program: 95 ℃ for 2 minutes, followed by 45 cycles of 95 ℃ for 5 seconds, 60 ℃ for 1 minute, then a melting curve was set up as 60 ℃ for 15 seconds and 95 ℃/continuous.

2.7.4 Data analysis

Cycler threshold (Cq) values were uploaded to QIAGEN Web-based data analysis portal (https://geneglobe.qiagen.com/gb/analyze). The average Cq of three times was calculated for gene expression analysis. A heatmap showing the fold changes of gene expression was generated.

2.8 Quantitative Real-time PCR of specific genes

2.8.1 RNA extraction

Cells were harvested with same procedures as it described in 2.7.1. Total RNA from the collected cells were extracted by using ReliaPrep™ RNA Cell Miniprep Kit, and procedures are followed by the manufacturer's instructions. According to the cell number, an appropriate volume of BL+TG buffer (kit provided) was added into the tubes containing cell pellets, then 100% isopropanol was added, followed by vortexing. The lysate was transferred to a spin ReliaPrep™ Minicolumn placed in a collection tube and centrifuged at 12000 g for 1 minute at room temperature. The liquid in the collection tubes was discarded and the ReliaPrep™ Minicolumn was washed with RNA Wash Solution and centrifuged at 12000 g for 1 minute. Then DNase Ⅰ incubation procedure was performed. A mix of 24 µl Yellow Core Buffer, 3 µl 0.09M MnCl2, and 3 µl DNase Ⅰ enzyme was prepared and added into the ReliaPrep™ Minicolumn membrane for each

sample. After incubating the DNase I Mix at room temperature for 15 minutes, Column Wash Solution was added into the column and centrifuged as before. Followed by two times of addition of RNA Wash Solution and centrifuge at 12000 g for 1 minute. The wash solutions were discarded and after centrifuge at the full speed for 2 minutes, the ReliaPrep™ Minicolumn was placed into a new 1.5 ml RNase-free microtube. 30-50 µl RNase-free water was added into the membrane to elute RNA. After a centrifuge, the RNA samples were collected and tested by NanoDrop ND-100 Spectrophotometer. Purified RNA samples were directly used for reverse transcription or stored in -80 ℃ freezer for future use.

2.8.2 Reverse transcription

TaqMan® Reverse Transcription Reagents were used for the cDNA synthesis. 1 µg equivalent volume of RNA samples was calculated and used to prepare each reverse transcription reaction. Other reagents in total of 8.4 µl were added to make the reverse transcription reaction mix, which includes 2 µl of 10×RT Buffer, 1.4 µl of 25 mM $MgCl_2$, and 1 µl of the five components: 10 mM dNTP mix, 100 mM DTT, 20 U/ µl RNase inhibitor, 50 U/ µl MultiScribe RT, and 50 µM Random Hexamers. Finally, variable RNase-free water was added to make the total volume to 20 µl. Then, each reaction mix was incubated in a thermal cycler under the following the conditions: 25 ℃ for 10 minutes, 37 ℃ for 30 minutes, 95 ℃ for 5 minutes, and 4 ℃ hold for indefinitely. At this stage, synthesis of cDNA samples was completed, and the cDNA samples were used for downstream experiments or stored in -20 ℃ freezer for future use.

2.8.3 Quantitative Real-time PCR (RT-qPCR)

The mRNA expression levels of specific genes were analyzed by RT-qPCR with the usage of SYBR™ Green PCR Master Mix to quantify the fluorescence in a LightCycler 480 instrument. For each qPCR reaction of 10 µl, 4 µl (20 ng) of 10-time diluted cDNA was mixed with 5 µl of SYBR™ Green PCR Master Mix, 0.5 µl (500 nM) of the primer pairs (both forward and reverse primer). The 10 µl reaction mix was added into 384-well plate in duplicate. The plate was loaded into LightCycler 480 instrument and run under the thermal program set as: 95℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s, 60 ℃ for 1 min, then 95 ℃ for 5 s, 60 ℃ for 1 min, and 95 ℃/continuous performed for melting curve analyses to monitor and confirm the accuracy of specific amplification. Cycle threshold (Cq) values of all samples were collected and normalized against reference genes beta-actin (ACTB) and glyceraldehyde 3-phosphate

dehydrogenase (GAPDH). Relative gene expression was determined by using the comparative threshold cycle $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.8.4 Primer design and optimization

Primer pairs were designed through NCBI Primer design website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer pairs should meet the following criteria: target amplification length is 70-250 bp with a GC content between 50-60%, length of the primer pairs is 18-24 bases, melting temperature (Tm) of the primer pairs is between 55-65 ℃, and the Tm between the two primers is less than 5 ℃, self-complementary and highly repeated nucleotides are avoided. Candidate primer pairs selected based on the criteria were blasted in NCBI Blast website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure the specificity of target genes. After confirmation that the primer pairs are unique for the genes of interest, primer pairs were ordered and tested for their performance in qPCR.

Primer efficiency was determined from a standard curve. Cq values of primer pairs were obtained in three serially diluted (1:10) cDNA samples synthesised from single RNA sample, and it was plotted as Y axis of the standard curve, while log-transformed cDNA quantity was plotted as X axis. In the plot of linear standard curve, slope, and coefficient of determination (R^2) value of the line were calculated. A good qPCR reaction should have primer efficiency between 90-110%, which corresponds to a slope between -3.1 to -3.6 (Taylor et al., 2010; Sowa et al., 2022). Besides, melting curve of primer pairs should have only single sharp peak, which indicates expected specific product amplified without primer dimer (Taylor et al., 2010). In this study, the primer pairs used and passed both efficiency test and melt temperature analysis were summarized in Table 2-1 with sequences, slope, and R^2. *CYP1A2*, *3A4* and *3A5* lack primer efficiency data because the low abundance of those transcripts in HHL-16 cells, and cDNA samples were not able to be diluted for primer efficiency test of those genes.

Table 2-1 Summary of primers used in qPCR.

Gene name	Accession number	Forward (5’ to 3’)	Reverse (5’ to 3’)	Product size (bp)	Slope of primer efficiency	R^2
CYP1A2	NM_000761.5	CCTTCGCTACCTGCCTAACC	GTCCCGGACACTGTTCTTGT	124	-	-
CYP3A4	NM_017460.6	CACTCACCCTGATGTCCAGC	TAGGTGGGTGGTGCCTTATTG	75	-	-
CYP3A5	NM_000777.5	TCCTCTATCTATATGGGACCCG	AGCACAGGGAGTTGACCTTC	184	-	-
IL6	NM_000600.5	AGGACATGACAACTCATCTC	GGTGCCCATGCTACATTTGCC	90	-3.6	0.999
CCL20	NM_004591.3	CAAGAGTTTGCTCCTGGCTGC	TTGCTTGCTGCTTCTGATTCGC	75	-3.3	0.999
BMP2	NM_001200.4	CTAAGGAGGACGACAGCACC	AAGAAGTCCCCAGCCAAGTG	109	-3.2	0.996
NDP	NM_000266.4	TCTATGCTCTCCCTGCTGGT	GAGGACAGTGCTGAACGACA	225	-3.5	0.985
ACTB	NM_001101.5	CTGAACCCCAAGGCCAAC	AGCCTGGATAGCAACGTACA	87	-3.5	1
GAPDH	NM_002046.7	GAAGGTGAAGGTCGGAGTCAAC	CAGAGTTAAAAGCAGCCCTGGT	71	-3.5	0.999

2.9 DNA methylation analysis

2.9.1 DNA extraction

DNA from HHL-16 cells was extracted by using DNA Mini Kit. Cells were collected as the procedures described in 2.7.1. The cell pellet was resuspended with 200 µl PBS. Then, 20 µl proteinase K and 200 µl Buffer AL was added into the cell pellet. After vortex for 30 seconds, the samples were incubated in a heat block at 56 ℃ for 10 minutes. 200 µl absolute ethanol was added into the samples and mixed well by vortex. The mixture was transferred to the Mini spin column placed in a 2 ml collection tube and centrifuged at 8000 rpm for 1 minute. After discarding the solution in the collection tube, 500 µl Buffer AW1 was added into the Mini spin column and centrifuged at 8000 rpm for 1 minute. Then 500 µl Buffer AW2 was added and centrifuged at 14000 rpm for 3 minutes. The Mini spin column was placed in a new 2 ml collection tube and centrifuged at 14000 rpm for 1 minute to dry the column membrane. Finally, the Mini spin column was placed in a new 1.5 ml nuclease-free microtube and added with nuclease-free water, and DNA samples were eluted and collected after a centrifuge at 8000 rpm for 1 minute. The quality and concentrations of DNA samples were quantified by NanoDrop ND-100 Spectrophotometer. Purified DNA samples were kept in -20 ℃ for further analysis.

2.9.2 DNA methylation sequencing

DNA methylation analysis at the promoter regions of interested genes was performed by the Genome Centre, Barts and The London, Queen Mary University of London, after receiving the extracted DNA samples by our lab. The DNA samples were tested for a quality control, and only those that passed the test were used for the next step. Primer pairs were designed to cover the areas of interest genes. After PCR optimization of the primer pairs, bisulfite conversion of controls and samples were performed. Then samples were amplified by PCR, and the products were pooled and cleaned after a gel electrophoresis, then the samples were barcoded. The barcoded samples were diluted to 4.1 nM and loaded onto MiSeq for sequencing. Finally, Bismark analysis on raw data was performed to get methylation levels of specific loci. A report of methylation levels of each specific loci was provided by the Genome Centre, Barts and The London, Queen Mary University of London.

2.10 RNA-Sequencing analysis

RNA extraction was conducted as for the procedures described in 2.8.1. Then the RNA samples were shipped to Novogene Co., Ltd (Cambridge, UK) for RNA-Sequencing analysis. Briefly, quality a control test was performed in the RNA samples, which qualified for sequencing only those samples with the RNA integrity number (RIN) over 4. After the confirmation that all the RNA samples passed the quality control test, a poly-A enriched RNA library was constructed and mRNA-sequencing with 30 million reads of paired end reads of 150 bp in each direction for each sample was performed. Finally, after data quality control, a bioinformatics analysis was carried out and completed by Novogene Co., Ltd.

Chapter 3 Impacts of aflatoxin exposure on children from Africa and South Asia

3.1 Introduction

Severe malnutrition is a huge health burden in Africa and South Asia that contributes to child morbidity and mortality. Malnutrition is commonly assessed and indicated by the anthropometric indices reflecting child growth (Kwena et al., 2003; Wang et al., 2009; Habyarimana, 2016). High prevalence of malnutrition is found in Africa and South Asia (Kramer and Allen, 2015). Malnourished children under the age of 5 are at greater risk of death, with evidence showing that more than 10 million children died each year, and it was found 42 countries account for the 90% of the death in children younger than 5 years old, and malnutrition was suggested as an underlying risk factor contributing to child deaths (Black et al., 2003; Collins et al., 2006). Although the worldwide number of child deaths in 2019 has been reduced by 59% since 1990, Africa and South Asia account for over 80% of child mortality in children under five in 2019 (Sharrow et al., 2022).

Exposure to naturally occurring aflatoxin may also contribute to poor growth of children and lead to severe health complications. In resource-limited areas, such as Africa and South Asia, maize and peanuts are susceptible to aflatoxin contaminations, which results in a great threat to health in the populations who consume maize and peanuts as staple foods (Waenlor and Wiwanitkit, 2003; Kana et al., 2013). Dietary exposure to aflatoxin in African children has been found to start *in utero* and persist in early life (Castelino et al., 2014). A number of studies found pregnant women exposed to aflatoxin may lead to aflatoxin exposure of the foetus in the intrauterine environment. For example, 100% of maternal blood samples and 48.5% of cord blood samples had detectable AF-alb in a Gambian population (Turner et al., 2007). Gong et al. (2003) found AF-alb levels in children aged 1-3 years old in Benin and Togo increased with increasing age, and were also significantly correlated with weaning status (p=0.0001) (Gong et al., 2003). Such exposure *in utero* and during early child development, raises the risks to health complications related to aflatoxin.

Aflatoxin is a known group I carcinogen, and dietary exposure to aflatoxin leads to liver cancer, and is implicated with immune suppression (Wild et al., 2015; Gong et al., 2016b). Recently, there is growing interest on the impact of aflatoxin exposure on child growth impairment. An inverse association between aflatoxin exposure and child growth was found in children in Benin and Togo, first in a cross-sectional study, then a follow-up cohort study (Gong et al., 2002; Gong et al., 2003). However, the causal relationship between aflatoxin exposure and child growth impairment has not been determined and there is

some conflicting evidence of the effects of aflatoxin exposure on child growth. Compared to the studies in Benin and Togo (Gong et al., 2002; Gong et al., 2003; Gong et al., 2004), aflatoxin exposure levels in children were lower in some studies that did not find the inverse association between aflatoxin exposure and child growth, suggesting there might be a threshold of aflatoxin exposure level to affect child growth (Mitchell, Riley, et al., 2017; Passarelli et al., 2020).

Poor growth in childhood contributes to the high rate of mortality in children in Africa and South Asia (Kimani and Gatimu, 2022). Both regions have a high prevalence of child malnutrition (Gulati, 2010). Children under the age of 5 with severe malnutrition were found to have increased risk of death, and suffering from malnutrition at the early stage would lead to reduced lifespan (Djoumessi, 2022). Risk factors of child mortality are complex, including undernutrition, hygiene conditions, access to healthcare, socioeconomic status, and infectious diseases (Rutstein, 2000). Although guidance and recommendations for child care are advised, these are often not fully implemented and the mortality rate in Africa and South Asia remains unacceptably high (Nemetchek et al., 2018). A prospective cohort study conducted by the Childhood Acute Illness and Nutrition (CHAIN) Network recruited 3101 acutely ill children in Africa and South Asia, which found over 10% deaths among the children during admission in hospital or post-discharge (The Childhood Acute Illness and Nutrition (CHAIN) Network, 2022). Therefore, identification of the underlying risk factors of child mortality is needed, which may provide opportunity to better intervention implementation to improve child health.

This chapter of the study aimed to investigate the characteristic of aflatoxin exposure levels in malnourished children in Africa and South Asia, and to understand the association between aflatoxin exposure and child growth, as well as to explore whether aflatoxin exposure is an underlying risk factor to child mortality.

3.2 Methods

3.2.1 Study subjects

Due to time and resource limit, this current study is a pilot study of the CHAIN Network (https://chainnetwork.org/) Nested Case-Cohort Study (CNCC), and detailed information of the CHAIN Network protocol has been published (Childhood Acute Illness and Nutrition Network, 2019). Ethical approval was obtained from the Oxford Tropical Research Ethics Committee, and the

institutional review boards of all partner sites, with the trial registration number of NCT03208725 (Childhood Acute Illness and Nutrition Network, 2019). Written informed consent was received from all the participants. In the CHAIN Network, 3101 children aged 2 to 24 months from nine sites in six countries were recruited in Africa and South Asia from the year of 2016 to 2019 (Figure 3-1) when the children were admitted to hospitals due to acute illness. Details of the nine sites included three sites (Kilifi, Mbagathi, and Migori) in Kenya, one site (Kampala) in Uganda, one site (Blantyre) in Malawi, and one site (Banfora) in Burkina Faso in the continent of Africa, and in South Asia, one site (Karachi) in Pakistan, and two sites (Dhaka and Matlab) in Bangladesh were included. The enrolment started at the hospitalisation and the children were followed up for the post-discharge of 45, 90, and 180 days. During the hospitalisation and post-discharge, 350 children died. The primary question of the study is to identify risk factors contributing to the deaths.

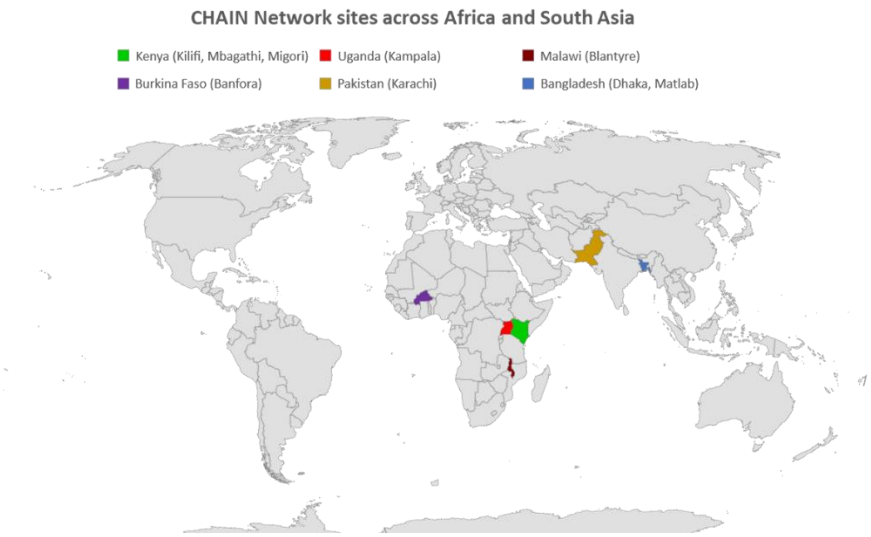


Figure 3-1 CHAIN Network sites of the enrolled children in Africa and South Asia.

The CNCC was specifically designed to explore the biological mechanisms and risk factors for death in the acutely ill children (Figure 3-2). A sub-cohort of 24% of children from the CHAIN Network (3101 children) cohort was randomly selected. The sub-cohort consisted of 658 survivors (sub-cohort with no failure), and 109 deaths (sub-cohort who failed). Then, an addition of remaining death cases (n=241, non-sub-cohort cases) was added into the 24% random sub-cohort to give the total death cases of 350. In addition, a random selection of 30 community participants for each site (n=270), who live in the same community as the hospitalised children and without known HIV or tuberculosis and no recent hospital admission before the enrolment, was added. In the

CNCC study, clinical, demographic, and anthropometry were assessed. With regards to the biological mechanism and risk factors investigation, including serum lipidomics, serum metabolomics, serum micronutrients, plasma proteomics, enteric pathogens, and serum aflatoxins were assessed by a range of laboratories (Njunge et al., 2022).

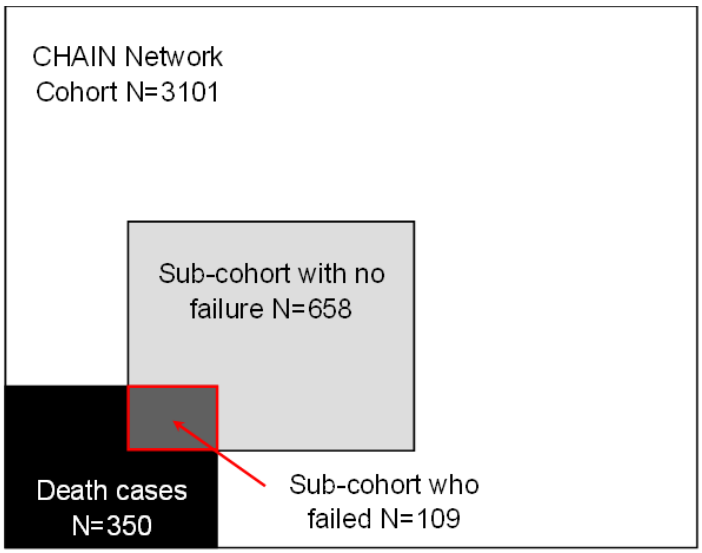


Figure 3-2 Cohort selection from the CHAIN Network for the CHAIN Nested Case-Cohort study (Njunge et al., 2022).

In this pilot study, 470 serum samples, collected at the admission timepoint, from the children (2 to 24 months) recruited in the CNCC study were available for aflatoxin exposure biomarker analysis. Among the samples, 125 serum samples were excluded because the samples were clotted, and albumin could not be extracted from these samples, so the remaining 345 samples were analysed for aflatoxin-albumin adduct biomarker (AF-alb).

3.2.2 Aflatoxin-albumin adduct measurement

AF-alb levels were assessed in 345 serum samples of the acutely ill children aged at 2-24 months from the CNCC study, using the enzyme-linked immunosorbent assay (ELISA) as is described in chapter 2.2 (Chapot and Wild, 1991, with minor modifications).

3.2.3 Data collection

All the clinical, demographic, anthropometric, and outcome data were recorded and collected by the CHAIN Network consortium following standard protocols. In brief, demographic information and anthropometric data were recorded in

case report form (CRF) before the beginning of the study at all sites after the completion of standardised training. Child death cases in hospitals were also recorded by trained study clinicians in the CRF, which were identified based on the standard WHO methods (The Childhood Acute Illness and Nutrition (CHAIN) Network, 2022). In the event of death in hospitals, a designated member from the study team conducted and completed a standard mortality audit questionnaire.

3.2.4 Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics 27 (version 27, IBM, Armonk, New York). Distribution of AF-alb levels were left-skewed, therefore the data was log transformed prior to further data analysis. Geometric means were presented in the results. AF-alb levels lower than LOD (3 pg/mg albumin) were assigned a value of ½ LOD (1.5 pg/mg albumin) for further statistical analysis. Difference of AF-alb levels across the nine sites and in the children from different age groups was tested by One-Way ANOVA analysis. Difference of AF-alb levels between two continents, Africa and South Asia, as well as between children with alive or dead outcome was analysed by Student's t-test. Correlation between AF-alb levels and anthropometric indices was tested by Pearson correlation analysis. Partial correlation test controlling for age, study sites, and sex was also used to analyse the association between AF-alb and anthropometric indices. A binary logistic regression analysis was used to determine whether aflatoxin exposure is a risk factor for child mortality. In all the analysis mentioned, $p<0.05$ was considered to show statistically significant difference. After statistical analysis in SPSS, all figures were generated using GraphPad Prism (Version 7.04, Dotmatics, California, USA).

3.3 Results

3.3.1 Characteristic of AF-alb levels in the enrolled children

In total, 345 serum samples of the children aged at 2-24 months from the CNCC study in Africa and South Asia were assessed. Among them, 62% (n=214) of samples tested positive for AF-alb (over LOD), and 38% (n=131) were non-detectable. AF-alb levels ranged from 1.5 to 1371.9 pg/mg, with a mean level of 24.9 pg/mg. The geometric mean of the AF-alb levels in those children was also calculated, which was 5.4 pg/mg (Table 3-1).

Table 3-1 Descriptive data of overall AF-alb levels in the enrolled children.

Descriptive data of AF-alb levels in the children	
Positive samples (N, %)	214, 62
AF-alb non-detectable samples (N, %)	131, 38
Mean (pg/mg)	24.9
Median (pg/mg)	4.4
Skewness	9.5
Minimum (pg/mg)	1.5
Maximum (pg/mg)	1371.9
Geometric mean (95% CI) (pg/mg)	5.4 (4.7, 6.3)

AF-alb: aflatoxin albumin adduct. CI: confidence interval. Samples below the limit of detection (LOD) of 3 pg/mg were given a value of 1.5 pg/mg and regarded as AF-alb non-detectable samples. AF-alb levels over LOD were positive samples.

3.3.2 Comparison of AF-alb levels in different age groups

Children in the study were divided into three groups based on their age < 6 months old, 6 to 12 months old, and 12 to 24 months old. Geometric mean AF-alb levels was 3.1, 4.5, and 8.7 pg/mg in the children younger than 6 months old, the group of 6 to 11 months old, and 12 to 24 months group, respectively, so there were increased AF-alb levels with increasing age. One-Way ANOVA analysis showed that there was a significant increase of AF-alb levels with age group increase (Figure 3-3).

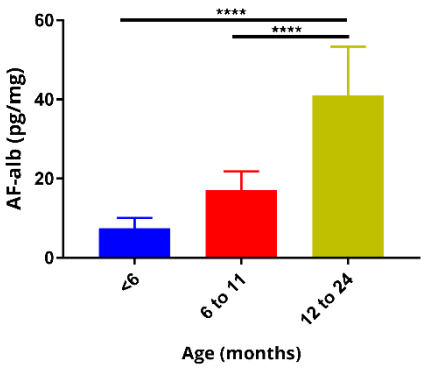


Figure 3-3 AF-alb levels in the enrolled children of different age groups. Increased AF-alb levels with the increasing age of children were observed. Natural log transformed AF-alb was used in One-Way ANOVA test for statistical analysis. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

3.3.3 Comparison of AF-alb levels in different sites

The children in Africa had a higher geometric mean level of AF-alb compared to the children from South Asia (7.5 vs 2.4 pg/mg), which was a statistically significant difference (Figure 3-4). In each site of the two continents, AF-alb positive sample numbers, positive percentage, geometric mean levels, and significant difference of each site were summarized (Table 3-2). Among the sites, Kampala had the highest geometric mean level of AF-alb at 28.5 pg/mg compared to other eight sites. Even in the same continent of Africa, AF-alb levels were varied, and the AF-alb levels at Kampala had a significant difference compared to the levels at other African sites (Figure 3-5).

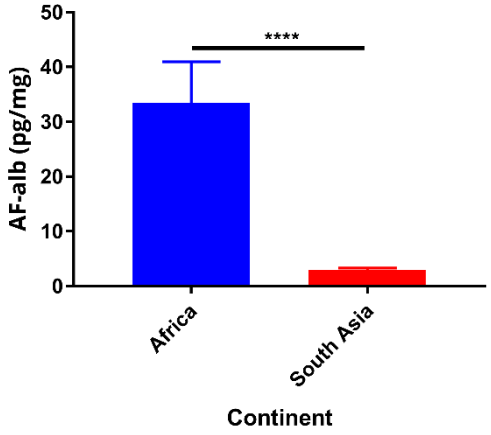


Figure 3-4 Comparison of AF-alb levels between children in Africa and South Asia. There was a significant difference of AF-alb in children from Africa and South Asia. Natural log transformed AF-alb was used in Student's t test for statistical analysis. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Table 3-2 Descriptive data of aflatoxin exposure in different sites.

Sites (City)	N (%)	N of positive samples (%)	Geometric mean (95% CI) of AF-alb (pg/mg)
D: Kampala (Uganda)	19 (5.6)	17 (89.5)	28.5 (11.9-68.0) [A, b, c, E, f, g, h, i]
A: Kilifi (Kenya)	34 (9.9)	31 (91.1)	12.4 (8.2-18.9) [b, c, D, f, g, h]
E: Blantyre (Malawi)	22 (6.4)	19 (86.4)	12.2 (6.6-22.2) [b, c, D, f, g, h]
I: Banfora (Burkina Faso)	58 (17.0)	42 (72.4)	8.8 (5.8-13.5) [b, c, d, f, g, h]
C: Migori (Kenya)	70 (20.5)	40 (57.1)	4.3 (3.3-5.8) [a, d, e, G, h, i]
B: Mbagathi (Kenya)	41 (12.0)	24 (58.5)	4.1 (2.8-5.9) [a, d, e, h, i]
F: Karachi (Pakistan)	20 (5.8)	9 (45)	2.9 (1.9-4.2) [a, d, e, i]
G: Dhaka (Bangladesh)	50 (14.6)	24 (48)	2.6 (2.2-3.0) [a, C, d, e, i]
H: Matlab (Bangladesh)	28 (8.2)	5 (17.9)	1.8 (1.5-2.1) [a, b, c, d, e, G, i]

Each site has been assigned a letter from A to I in alphabetical order (A: Kilifi; B: Mbagathi; C: Migori; D: Kamapala; E: Blantyre; F: Karachi; G: Dhaka; H: Matlab; I: Banfora). Significant difference among different site groups is denoted as letters as superscript. Upper-case letters indicate results significant at the 0.05 level and lower-case indicate results significant at the 0.001 level.

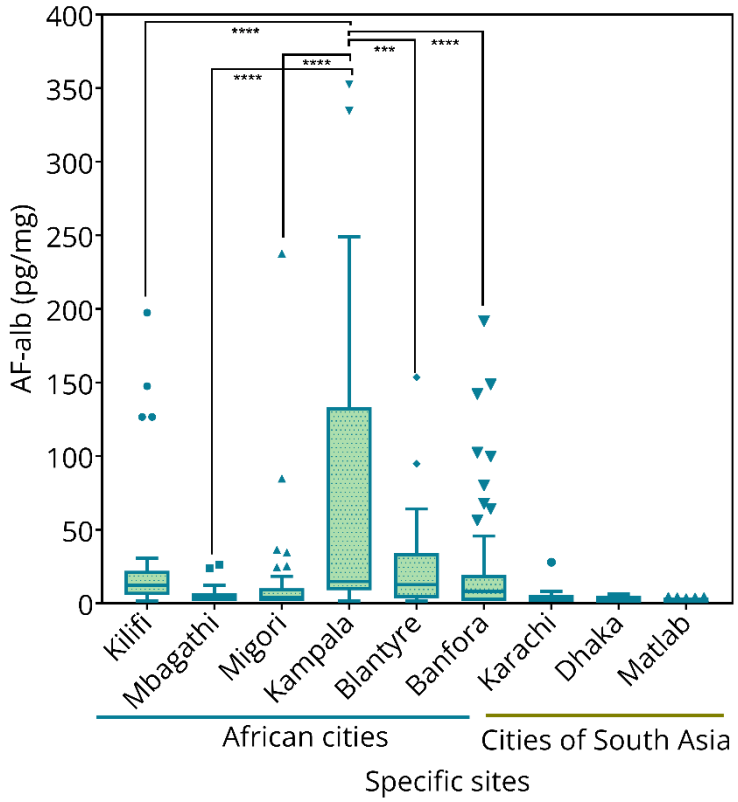


Figure 3-5 Boxplot describing the distribution of AF-alb levels in children from specific sites. Three outliers of AF-alb over 400 pg/mg were excluded. Natural log transformed AF-alb was used in One-Way ANOVA test for statistical analysis. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

3.3.4 Association between AF-alb and anthropometric indices

Three growth indices HAZ, WAZ, and WHZ were calculated based on the height and weight of the children measured at admission timepoint. CHAIN populations were assessed for Z score calculation (Njunge et al., 2022), which was also utilised in this study. Linear Pearson correlation between natural log transformed AF-alb levels and anthropometric indices was performed. Natural log transformed AF-alb levels were negatively correlated with the three anthropometric indices, but only the correlation with HAZ reached statistically significant level (r=-0.109, p=0.046). After adjustment for age, study sites, and sex in partial correlation test, there was significant inverse associations between natural log transformed AF-alb levels and HAZ, WAZ, and WHZ (r=-0.108, p=0.049; r=-0127, p=0.020; r=-0.108, p=0.047, respectively) (Figure 3-6).

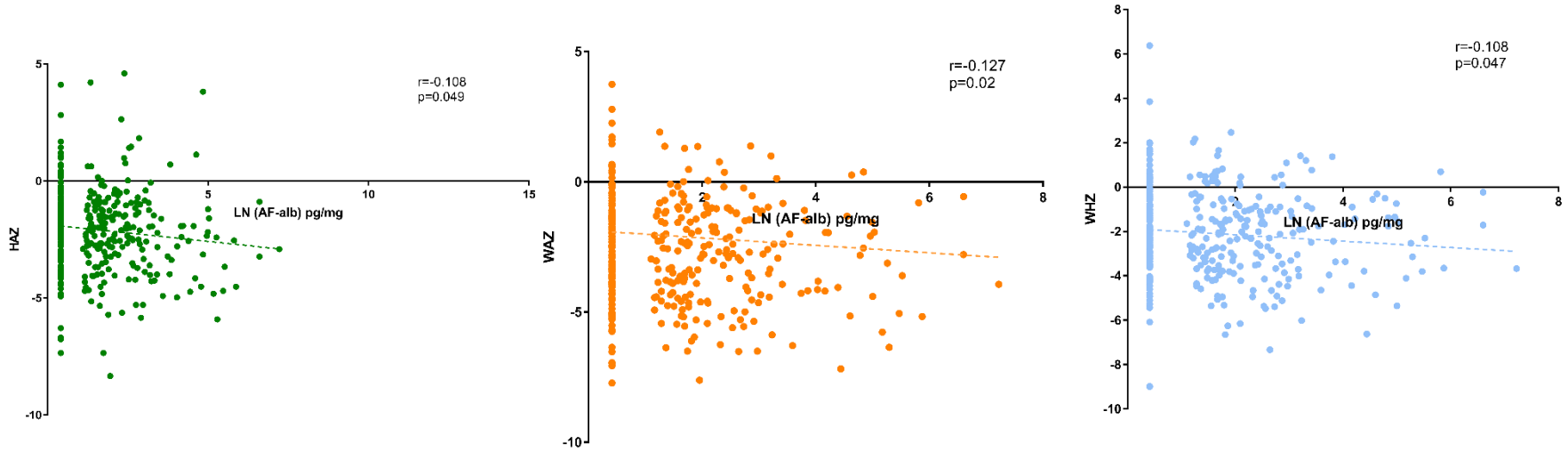


Figure 3-6 Correlation between natural log transformed AF-alb levels and anthropometric indices. HAZ: height-for-age z-score, WAZ: weight-for-age z-score, WHZ: weight-for-height z-score. There was significantly negative association between LN (AF-alb) levels and HAZ, WAZ, and WHZ after the adjustment of children's age, study sites, and sex.

3.3.5 Association between aflatoxin exposure and child mortality

Deaths of the children were recorded during the admission, hospitalisation, and until 6 months after discharge. AF-alb levels were assessed and compared in children from both “alive” and “dead” groups. Dead children had significantly higher AF-alb geometric mean levels than those alive, 7.3 *vs* 4.9 pg/mg, respectively (Figure 3-7). To determine whether aflatoxin exposure is a contributing factor to child death, a binary logistic regression analysis was performed. Children’s survival outcome (alive or dead), a categorical variable was used as dependent variable. AF-alb (natural log transformed), a continuous variable was used as one of the possible factors to process the binary logistic regression analysis.

The results indicated that with one unit increase of natural log transformed AF-alb, the likelihood of child death increased by 20.3% (p=0.04). After adjustment of age, the logistic regression analysis showed that with one unit increasing of natural log transformed AF-alb, the likelihood of child death increased by 26.0% (p=0.018) (Table 3-3).

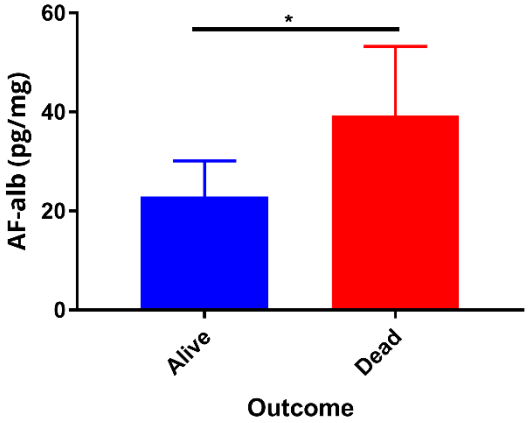


Figure 3-7 Comparison of AF-alb levels in the children with different survival outcome. A significant difference of AF-alb in children with alive and dead outcome was observed. Natural log transformed AF-alb was used in Student’s t test for statistical analysis. *p<0.05.

Table 3-3 Logistic regression analysis of the risk of aflatoxin exposure to child death.

Factors	Regression coefficient	Odds ratio (95% CI)	P value
AF-alb	0.185	1.203 (1.009-1.434)	0.04*
AF-alb[a]	0.231	1.260 (1.040-1.526)	0.018*

Logistic analysis was conducted to determine the factors associated with children's survival condition based on the survival outcome during admission. a: adjusted with children's age.

3.4 Discussion

The present study assessed aflatoxin exposure levels in acutely ill children admitted to hospitals in Africa and South Asia. We found aflatoxin exposure levels varied by site and children's age, and there was a negative association between aflatoxin exposure and child growth. This is also the first study to investigate the contribution of aflatoxin exposure to child death in severely malnourished children in Africa and South Asia, with aflatoxin exposure a potential risk factor contributing to child deaths. These findings indicated that aflatoxin exposure has important implications for child health effects, which reinforces the need for interventions to reduce and prevent aflatoxin exposure in children, especially for optimizing child development in resource-limited settings.

In this study, high prevalence (62%) and high concentration of AF-alb (geometric mean: 5.4 pg/mg) in the children were observed, and geometric mean level of AF-alb in Africa was notable higher than South Asia (7.5 vs 2.4 pg/mg), reinforcing the evidence that aflatoxin is a particular public health issue in Africa. Aflatoxin exposure variation has been reported to be related to factors of environment, dietary habits, and agricultural practices (Cotty and Jaime-Garcia, 2007; Benkerroum, 2020). A temperature range from 25 to 30 ℃ is a favourable environment for *Aspergillus* growth, and aflatoxin contamination in foods in the areas with higher humidity are higher than the levels in areas with drier climate (Gnonlonfin et al., 2013). Such climate conditions are more prevalent in many parts of Africa compared to South Asia. In those areas, maize and groundnut, which are also more highly produced and frequently consumed as staple foods in Africa than South Asia, are easily susceptible to aflatoxin contamination (Wu et al., 2011). In addition, farming practices, such as poor storage of foods and limited access to pesticides and fungicides, contribute to high aflatoxin exposure in Africa (Hell et al., 2000). Therefore, multiple factors taken together explain why the aflatoxin exposure levels in African sites are higher than Asian sites.

Even within Africa, we found the concentration of AF-alb had significant geographical variation. Children at Kampala (Uganda) had the highest geometric mean AF-alb level of 28.5 pg/mg, which might be because of the favourable climatic conditions provided to fungi growth during crop cultivation and storage in Kampala, where annual rainfall is over 1200 mm (Kaaya et al., 2006; Asiki et al., 2014). Food sources, including maize, sorghum, peanuts, and cassava, easily contaminated by aflatoxin and regularly consumed in Ugandan

diets contributed to high prevalence of aflatoxin exposure in Uganda (Omara et al., 2020). AF-alb levels (geometric mean: 12.2 pg/mg) assessed in Malawi in this study is concordant with the levels reported in previous studies, in which mean concentration of AFB_1-lys was reported to be 20.5 pg/mg in Malawi (Seetha et al., 2018). Geometric mean of AF-alb levels at two sites in Kenya in this study were similar, at 4.3 and 4.1 pg/mg at Migori and Mbagathi, respectively. The aflatoxin exposure levels in Kenya in this study are significantly lower than the levels reported in Kenya before 2015, especially lower than the levels reported in 2004, 2005, and 2010, when aflatoxin outbreaks occurred, and it was reported that AF-alb level reached as strikingly high as 539.7 pg/mg in children in 2004 (Gong et al., 2012). After outbreaks of aflatoxicosis in Kenya during these periods of high aflatoxin contamination, the Kenyan government launched a series of regulations to control and reduce aflatoxin in foods (Omara et al., 2021). For instance, limit of aflatoxin in foods for human consumption in Kenya has been set as 10 ppb, which mitigates the dietary exposure to aflatoxin in populations to some extent (Sirma et al., 2018). However, a lack of rigorous implementation of the regulations still causes a high exposure to aflatoxin in inhabitants of some areas of Kenya. Thus, aflatoxin exposure levels found in our data at each site are consistent with the levels previously reported, and the geographic variation of aflatoxin exposure levels is also validated in this study.

Increased aflatoxin exposure levels with increasing child age were observed in this study, which has been reported previously in Gambia and Tanzania (Shirima et al., 2015; Watson et al., 2018) Aflatoxin is known to cross the placental barrier and exposure to aflatoxin can start *in utero* (Partanen et al., 2010). Generally, after birth, infants are breastfed or fed with weaning foods, then gradually change to consume the same family foods as adults. Breastmilk is known to have some aflatoxin contamination, particularly the much less toxic AFM_1, therefore, breastmilk is also a source of dietary exposure to infants, but at lower levels (Tomerak et al., 2011). Six months of exclusive breastfeeding in infants are recommended by the WHO (World Health Organization, 2001), therefore in the context, the aflatoxin exposure levels in the children younger than 6 months assessed in this study might come from breastfeeding. During the breastfeeding period, weaning practice is a determinant factor that affects aflatoxin exposure in infants. As it was discussed above (in chapter 1, section 1.2.1.5), Gong et al. showed that the infants who consumed weaning foods, such as thin porridge made with maize, had twofold higher aflatoxin exposure levels compared to the breastfed children, therefore appropriate extension of

breastfeeding is helpful to reduce aflatoxin exposure (Gong et al., 2003). Although breastmilk does have the possibility to be contaminated with AFM_1, the toxicity of AFM_1 is 10 times lower than AFB_1, so extended breastfeeding is still advocated to avoid high aflatoxin exposure in children (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2020). After getting older, children gradually change to eat family foods, such as: maize and peanut, so have a greater chance to consume aflatoxin-contaminated foods, resulting in an increased amount of aflatoxin exposure. Thus, diet transformation of children probably explains the finding that aflatoxin exposure levels increase with increasing age.

As discussed in the introduction section, some earlier studies determined high aflatoxin exposure is associated with child growth impairment, but the negative association between aflatoxin and child growth was not seen in some studies. In the current study, notable negative association between AF-alb levels and the three anthropometric indices was determined after adjusting for confounders, which strengthens the evidence that aflatoxin exposure adversely affects child growth. The possible explanations of the conflicting results might be that there is a threshold of aflatoxin exposure to affect child growth and a relationship to the age of children. With regard to aflatoxin exposure levels, geometric mean of AF-alb in earlier studies in Benin and Togo were higher compared to the levels in the current study (32.8 pg/mg vs 5.4 pg/mg, respectively) (Gong et al., 2002). However, compared to some previous studies from other areas that also determined the negative association, aflatoxin exposure levels in our study have similarity to the levels previously reported (Turner et al., 2007; McMillan et al., 2018; Alamu et al., 2020). For example, similar aflatoxin exposure levels of 4.97 and 6.51 pg/mg were found in children aged 6 to 24 months at two sites of Zambia, in which significantly inverse association between aflatoxin exposure and child growth was reported (Alamu et al., 2020). There are much lower aflatoxin exposure levels in the studies that did not find a significant association between aflatoxin exposure and child growth impairment compared to the studies mentioned above (Mitchell, Hsu, et al., 2017; Andrews-Trevino et al., 2019; Passarelli et al., 2020; Mahfuz et al., 2021). For instance, one of the studies found a geometric mean AFB_1-lys of 1.52 pg/mg in pregnant women did not affect infant growth (Passarelli et al., 2020). Based on current evidence, it is concluded that presumably relative high aflatoxin exposure levels contribute to the growth impairment.

Another possibility of the inconclusive findings is that the effects of aflatoxin exposure on child growth are less profound when the children are getting older.

Similar to the current study, the majority studies that found the negative association were conducted in children of approximate 24 months old (Shouman et al., 2012; Watson et al., 2018; Lauer et al., 2019; Tesfamariam et al., 2022). In a study investigating the association between aflatoxin exposure and child growth in children aged 1-11 years old in Pakistan, although a high geometric mean of AFB_1-lys of 11.27 pg/mg was found, there was not a significant association between aflatoxin exposure and the growth parameters in a fixed effect model, implying age might play a confounding role to determine the association between aflatoxin exposure and child growth (Ashraf et al., 2022).

Although the adverse health effects of aflatoxin on child growth was found either in this study or previous studies, it is more complex to establish a causality between aflatoxin exposure and child growth. Compared to the highly significant association that was observed in the early studies in Benin and Togo (Gong et al., 2002; Gong et al., 2004), the inverse association found in our study and most of other studies was more moderate (Turner et al., 2007; Magoha et al., 2014; Kiarie et al., 2016; Watson et al., 2018; Alamu et al., 2020). Besides, most of the studies are cross-sectional rather than longitudinal, but two longitudinal studies in Benin and The Gambia supported that aflatoxin exposure significantly restricted child growth (Gong et al., 2004; Watson et al., 2018), for example, 1.7 cm reduction of height over 8-month follow-up was reported (Gong et al., 2004). Lastly, complex confounders also contribute to a difficulty to conclude the causality relationship. For example, in a prospective cohort study in rural Ethiopia, AFB_1-lys was quantified in maternal serum and fetal weight was measured by three rounds of ultrasound, it was found that fetal exposed to aflatoxin had significantly lower rate of weight gain compared to the unexposed group after the adjustment of potential confounders (β_{adj}=-0.92 centiles/week, 95% CI: -1.77, -0.06 centiles /week, p=0.037) (Tesfamariam et al., 2022). Therefore, the existence of potential confounders, such as age, geographical site, infections and health conditions during gestation, as well as socioeconomic status also make it difficult to establish the causal relationship (Leroy et al., 2015; Passarelli et al., 2020).

Although the causal relationship between aflatoxin exposure and child growth impairment cannot be determined according to current evidence, the findings in this study that aflatoxin exposure inversely associated with the three anthropometric indices strengthens the evidence of adverse effects of aflatoxin on child growth. The growing evidence that aflatoxin exposure is associated

with growth impairment emphasizes the importance to seek strategies to reduce dietary exposure to aflatoxin during child development.

Notably, the present study is the first study to indicate that there may be an increased risk of child mortality associated with aflatoxin exposure, in the acutely ill children. Owing to efficient policy implementation and healthcare improvements, the mortality rate of children under 5 in Africa has declined from the peak (over 78.1 deaths per 1000 live births) in year 2003 to around 40-50 deaths per 1000 live births after the year 2008 (Bamford et al., 2018). However, this mortality rate remains unacceptable and more non-natural causes, congenital disorders and non-communicable diseases contributing to the proportion of death has been reported (Bamford et al., 2018). Although there are limitations to the current study, such as socioeconomic status as a confounding factor, the finding that aflatoxin exposure was associated with increased risk of death, together with the association with impaired growth, provides further impetus for targeting aflatoxin exposure to improve child health. Previous studies have illustrated that malnutrition is implicated in approximately one in three child deaths (Bain et al., 2013). Due to the correlation between aflatoxin exposure and child growth impairment, it is reasonable that aflatoxin exposure is implicated with child deaths in the acutely ill children. Therefore, to better delineate the effects of aflatoxin exposure on child mortality, further tailored studies avoiding the confounding factors are required, particularly there is a need for mechanistic studies.

This study was subject to several limitations. This study was only a cross-sectional pilot study. Here we only assessed aflatoxin exposure levels at the timepoint of admission, and aflatoxin exposure levels in the children at the follow-up timepoints were not determined in the pilot study. Besides, additional data such as: socioeconomic, food consumption, infection, and clinical symptoms were not analysed in this study.

However, there were also some notable strengths. First, the children enrolled in this study had geographical diversity with a large sample size, and comprehensive data collection of the children was performed by well-trained CHAIN study staff, which ensured a high quality and completeness of data. Second, we were blinded to the outcomes and data analysis process when the serum samples were tested, so possible bias during the exposure assessment was avoided. Lastly, there was a high prevalence of aflatoxin exposure and stunting in the cohort and mortality data was recorded, which provided an

opportunity to illustrate the association between aflatoxin exposure and child growth as well as mortality.

In conclusion, aflatoxin exposure levels in children in Africa and South Asia varied by geographic factor and age. Aflatoxin exposure levels in African children were higher than South Asian children. In Africa, children at Kampala had the highest aflatoxin exposure levels. There was an increased aflatoxin exposure level with increasing age. A significant but moderate association between aflatoxin exposure and child growth impairment was observed after the adjustment of age, study sites, and sex. Aflatoxin exposure as a risk factor contributing to child mortality was also found in this study.

Chapter 4 Alteration of gene expression and DNA methylation in growth factors and immune-related genes in liver cells exposed to aflatoxin and fumonisin

4.1 Introduction

Epidemiological studies have reported that aflatoxin exposure can cause liver cancer and be associated with immune suppression and child growth impairment (Wild et al., 2015). Although there is no direct evidence showing the causal relationship between fumonisin exposure and human diseases, fumonisin exposure implicated with esophageal and liver cancers, and child growth impairment has been posited (Sun et al., 2007; Kimanya et al., 2010; Wang et al., 2014). Joint exposure to both aflatoxin and fumonisin may increase the risks to esophageal and liver cancers, as well as child growth impairment (Li et al., 2001; Sun et al., 2011; Shirima et al., 2015; Chen et al., 2018; Xue et al., 2019). Because such epidemiological studies are limited in that most of them are cross-sectional and without controlling for possible cofactors, such as socioeconomic status, health conditions, and the presence of other environmental exposures, firm conclusions about co-exposure to aflatoxin and fumonisin and the link to greater adverse health effects cannot be drawn. However, the evidence suggests that aflatoxin and fumonisin may at least contribute to cancer and child growth impairment, and more studies into the molecular mechanism of co-exposure to aflatoxin and fumonsin are required to support or refute this hypothesis.

As both aflatoxin and fumonisin carry a number of health risks for humans, the high prevalence of co-occurrence of aflatoxin and fumonisin in foods is of concern, because co-exposure to mycotoxins may have synergistic or additive effects (Kpodo et al., 2000; Shirima et al., 2013). Co-occurrence of aflatoxin and fumonisin was the most observed mixture in approximately 30%, 80%, and 50% of the studies from Africa, Asia, and South America, respectively (Smith et al., 2016). Evidence in various animal studies confirms that co-exposure to aflatoxin and fumonisin had synergistic or additive effects on liver damage and inhibition of weight gain (Carlson et al., 2001; Orsi et al., 2007; Qian et al., 2016). In a molecular epidemiology study in Tanzania, a high prevalence of the co-exposure in children suggested that fumonisin exposure alone or together with aflatoxin may contribute to child growth impairment (Shirima et al., 2015; Chen et al., 2018). Moreover, it has been posited that aflatoxin and fumonisin exposure may jointly contribute to aetiologies of chronic diseases in humans (Sun et al., 2011). One study in the Huaian area of China found that aflatoxin and fumonisin exposure levels were significantly higher in ESCC cases than the control populations, with co-exposure to higher levels of aflatoxin and fumonisin being associated with increased risk of ESCC (Xue et al., 2019).

Mechanistic investigation of aflatoxin toxicity is well-established in animals and humans. AFB_1 is metabolized by several major CYP enzymes in the liver to form highly reactive AFB_1-*exo*-8,9-epoxide (Wild and Turner, 2002). The epoxide reacts with DNA to primarily form mutagenic AFB_1-N^7 adducts, which are mutagenic due to a frequent mutation of G to T during DNA replication (Bailey et al., 1996). The mutation is identical to the predominant mutation found in human liver tumours associated with dietary exposure to aflatoxin. In the HCC samples, a hotspot of the mutation (G to T transversion) at codon 249 of p53, a tumour suppressor gene, was strongly associated with dietary exposure to aflatoxin (Bressac et al., 1991). Therefore, the mutagenic property of AFB_1 is believed to be a reason for aflatoxin carcinogenicity.

Recent evidence reported that dietary exposure to aflatoxin was associated with differential DNA methylation in growth factor genes and immune-related genes in Gambian children, which may be one possible mechanism of aflatoxin-related growth impairment (Hernandez-Vargas et al., 2015; Ghantous et al., 2021). With respect to a specific immune-related pathway, *IL6/STAT3* pathway, has been proposed to be possibly affected by aflatoxin from an earlier study in our lab, on the basis of the finding that significantly up-regulation of *STAT3* in Gambian infants was associated with higher aflatoxin exposure in the mothers during pregnancy (Castelino, 2013). Therefore, gene expression and DNA methylation in the pathways affected by aflatoxin are one of the interests in this study.

Mechanistic investigation of fumonisin toxicity is relatively less extensive. To our best knowledge, it has been postulated that because of the structural similarity to the long-chain (sphingoid) base backbones of sphingolipids, fumonisin exerts toxicity through the inhibition of ceramide synthase (Wang et al., 1991). Thereby, fumonisin disrupts sphingolipid metabolism, consequently affecting diverse cellular activities, which might contribute to the toxicity of fumonisin (Marasas et al., 2004). However, the ability to inhibit ceramide synthase may not fully explain the fumonisin toxicity, especially carcinogenicity. Other toxicological mechanisms, such as: oxidative stress, apoptosis, affected cell proliferation and the alteration of cytokine expression, were also reported in previous studies (Lim et al., 1996; Jones et al., 2001; Taranu et al., 2005; Kouadio et al., 2005). Recently, a major trend studying the impacts of fumonisin on DNA methylation also indicated that fumonisin affected DNA methylation was observed in various cell lines (Mobio et al., 2000; Kouadio et al., 2007; Demirel et al., 2015). Since DNA methylation is widely involved in

carcinogenesis (Counts and Goodman, 1995), fumonisin-altered DNA methylation might explain or contribute to the carcinogenicity of fumonisin.

Due to the possibility that there could be an additive or synergistic effect of co-exposure to aflatoxin and fumonisin on cancer initiation and growth impairment, it is important to investigate and clarify the molecular mechanism of aflatoxin and fumonisin toxicity. In this study we have used an immortalised liver cell line model to investigate the joint effects of aflatoxin and fumonisin, particularly focusing on the effects on gene expression and DNA methylation. The objectives of this study are to determine: (a) the potential ability of FB_1 to modulate AFB_1-induced hepatoxicity; (b) the effects of single AFB_1/FB_1 on gene expression of genes involved in growth factors and immune-related pathways; (c) the effects of combined AFB_1 and FB_1 on gene expression of candidate genes screened from growth factors and immune-related pathways; (d) the effects of AFB_1 on DNA methylation of some differentially expressed genes found in response to AFB_1 treatments.

4.2 Methods

4.2.1 Cell maintenance

HHL-16 (Human hepatocyte line 16) is a non-tumorigenic human liver cell line, which is derived from primary human hepatocytes obtained from a healthy liver(Clayton et al., 2005). The cell line is immortalised by the infection of a retrovirus vector LXSN16E6E7 expressing human papillomavirus type 16 (HPV16) E6 and E7 oncoproteins (Clayton et al., 2005). The HHL-16 cells were cultured in appropriate vessels as described in Chapter 2, Section 2.3.

4.2.2 Cytotoxicity test

Various concentrations of AFB_1 (1, 5, 10, 20, 50, and 100 µg/ml), FB_1 (10, 30, 50, 100, and 150 µg/ml), and combined AFB_1 and FB_1 (A1_F10, A1_F50, A1_F100, A5_F100, and A10_F100) as described in Chapter 2, Section 2.4 were used to treat HHL-16 cells for 24 and 48 hours. Because AFB_1 stock (20 mg/ml) was dissolved in DMSO, diluted DMSO treatments (D1, D5, D10, D20, D50, and D100) were used to normalize AFB_1 treatments, which is equivalent to the DMSO concentration in the corresponding AFB_1 treatment. Diluted PBS treatment in cell culture media (equivalent to the PBS concentration in 150 µg/ml FB_1 treatment) was used to normalize FB_1 treatments. Then the cytotoxicity of AFB_1 and/or FB_1 was assessed by MTT assay as described in Chapter 2, Section 2.5. The combined effects of AFB_1 and FB_1 were

determined by the combination index analysis as described in Chapter 2, Section 2.6.

4.2.3 Human Growth Factors QuantiNova® LNA® PCR Panels assay

1 and 10 µg/ml AFB_1 treatments, 50 µg/ml FB_1 treatment, and the combination treatment of AFB_1 and FB_1 (A1_F50) were used to treat HHL-16 cells for 24 hours. Then RNA samples were extracted in the treated cells as described in Chapter 2, Section 2.7.1. Following, cDNA samples were synthesised with the extracted RNA by reverse transcription as described in Chapter 2, Section 2.7.2. To evaluate the effects of single AFB_1 and FB_1 treatments, as well as combined AFB_1 and FB_1 treatment on gene expression of the Human Growth Factors QuantiNova® LNA® PCR Panels (QIAGEN), quantitative real-time PCR was used to detect the gene expression levels as described in Chapter 2, Section 2.7.3.

4.2.4 Gene expression analysis of specific genes by RT-qPCR

To validate the results obtained from the PCR Panels assay, various concentrations of AFB_1 (1, 5, 10, and 20 µg/ml), and FB_1 (10, 50, and 100 µg/ml), and combined AFB_1 and FB_1 (A1_F50 and A5_F100) were used to treat HHL-16 cells for 24 hours for gene expression analysis of candidate genes (*IL6*, *CCL20*, *BMP2*, and *NDP*). After the treatments, the treated cells were harvested for RNA extraction as described in Chapter 2, Section 2.8.1. cDNA samples were synthesised as described in Chapter 2, Section 2.8.2. Gene expression analysis of genes of interest was performed as described in Chapter 2, Section 2.8.3 by using the self-designed primers validated as it described in Chapter 2, Section 2.8.4.

4.2.5 DNA methylation analysis

DNA samples from 20 µg/ml AFB_1 treatment and DMSO treatment on HHL-16 cells, and from untreated HHL-16 cells, for 24 hours were extracted as described in Chapter 2, Section 2.9.1. DNA methylation levels at the promoter regions of two interested genes (*IL6* and *CCL20*) were measured by the Genome Centre, Queen Mary University of London, Blizard Institute, London as it described in Chapter 2, Section 2.9.2. The principle of DNA methylation analysis using bisulfite pyrosequencing is indicated as Figure 4-1 shown. The principle of pyrosequencing is indicated in Figure 4-2 (Rybicka et al., 2016). One area of interest for the assessment of DNA methylation levels was located in the *IL6* gene promoter region and contained 18 CpG sites in a 1203 bp region

from 22726063 to 22727265 on chromosome 7. A second area of interest was located in the *CCL20* gene promoter region and contained 21 CpG sites in a 2144 bp region from 227811861 to 227814004 at chromosome 2. The interested CpG sites and sequence of *IL6* and *CCL20* were presented in Appendix 4.1.

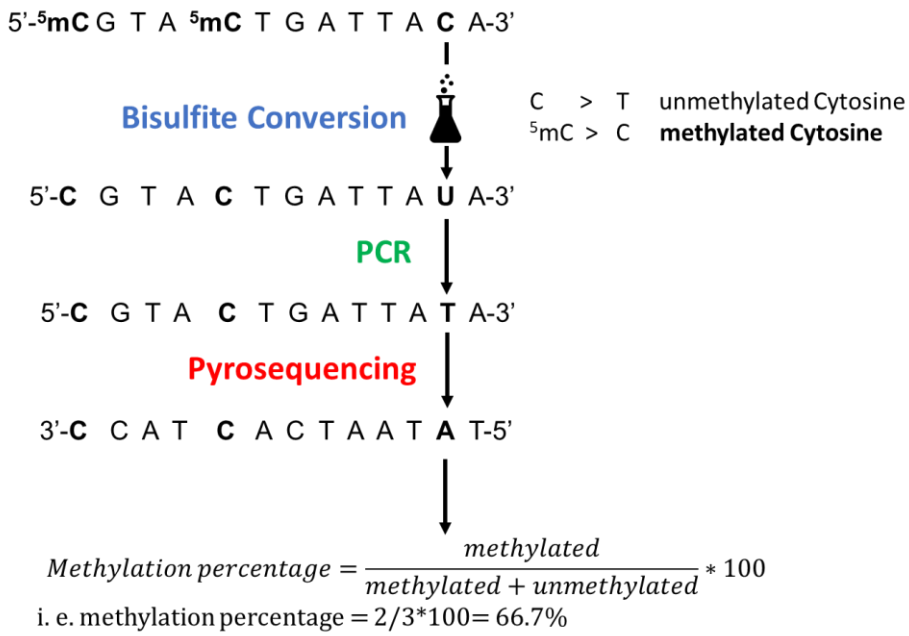


Figure 4-1 Principle of DNA methylation analysis by bisulfite conversion. PCR: polymerase chain reaction. Original DNA samples are treated with bisulfite to convert unmethylated cytosine (C) to adenine (T), while methylated cytosine (^{5}mC) kept as cytosine (C). Then bisulfite converted DNA samples will be amplified by polymerase chain reaction (PCR). After PCR amplification, methylated cytosine (^{5}mC) remains as cytosine (C), while the bisufite converted unmethylated cytosine (C) to adenine (T) are amplified as uracil (U). Then the amplified products will be used as templates in pyrosequencing, which is based on "sequencing by synthesis" principle. The sequence of complementary strand will be detected and after aligning it with original DNA sequence, methylated cytosine and unmethylated cytosine can be distinguished. Finally, methylation percentage will be calculated as shown by the equation in the figure.

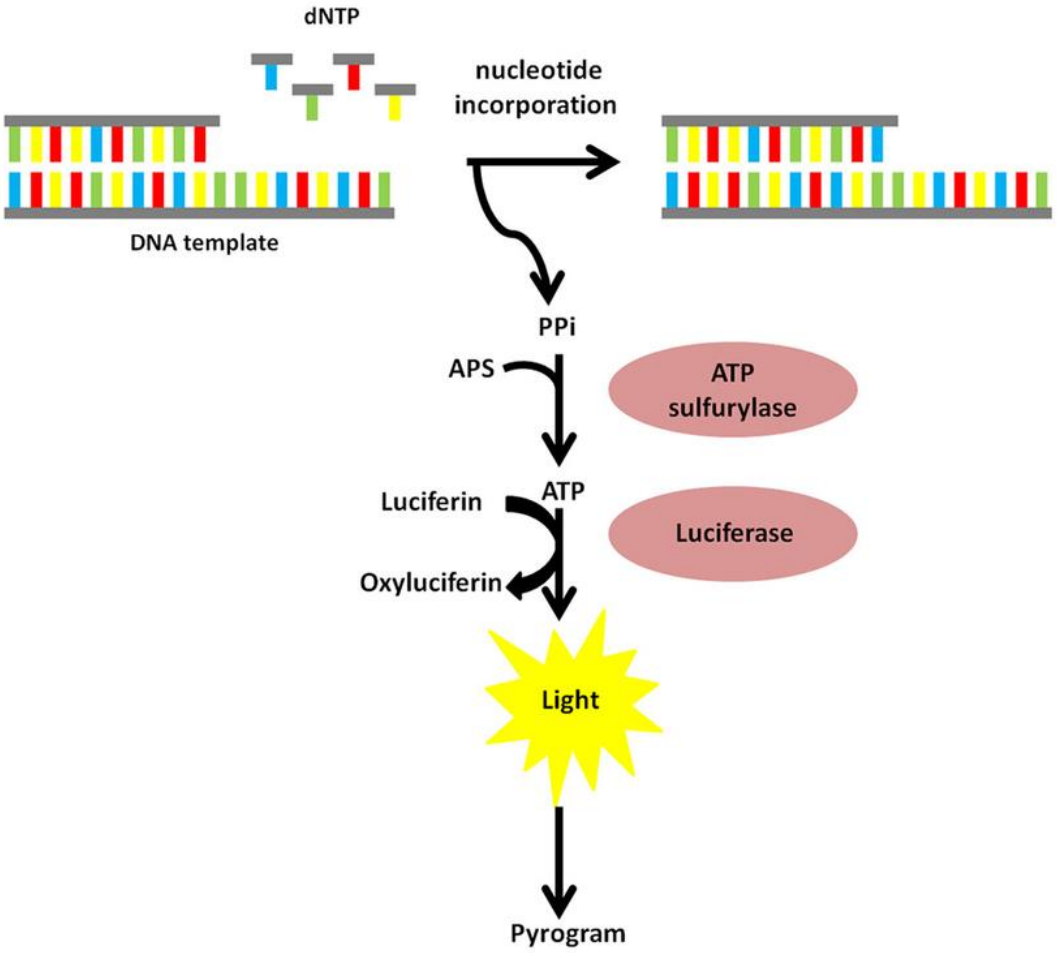


Figure 4-2 Principal of pyrosequencing. dNTP: deoxyribonucleotide triphosphates; PPi: pyrophosphate; adenosine 5´ phosphosulfate: APS; Pyrosequencing is a method detecting DNA methylation levels based on "sequencing by synthesis". During the synthesis of new DNA strands, each time one of the four dNTPs is added to the reaction. A successful incorporation of complementary dNTP releases PPi. Other dNTP failed to incorporation are degraded by apyrase. Released PPi with the presence of APS, and catalysed by ATP sulfurylase, leads to the production of ATP. Followed, ATP drives luciferase to transform luciferin to oxyluciferin and this reaction generates a visible light signal, which is detected by a charge coupled device (CCD) camera and seen as a peak in the analysis software, Pyrogram. The height of each peak indicates the proportion of the incorporated nucleotides number (Rybicka et al., 2016).

4.2.6 Statistical analysis

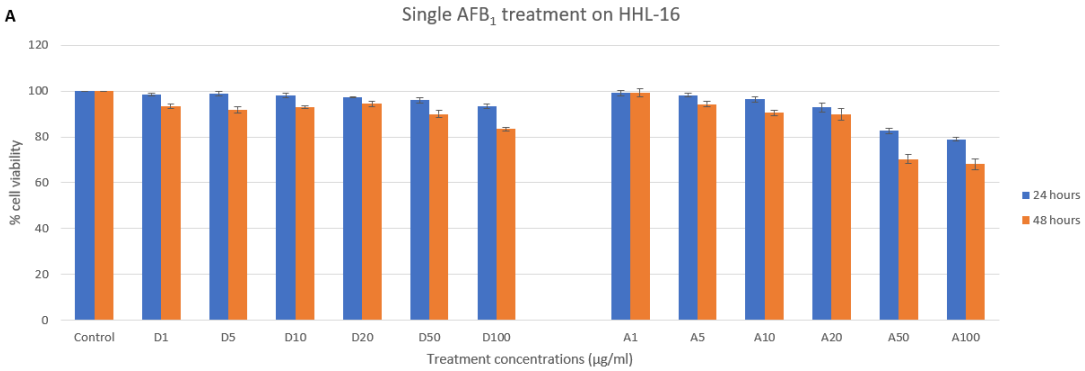
Results were presented as mean ± SEM unless otherwise stated. Significance comparison among groups were conducted by using one-way ANOVA. Data was processed and analysed in GraphPad Prism. GraphPad Prism or Excel were used to present the data graphically. $p<0.05$ was considered as statistically significant difference between groups. In gene expression analysis by RT-qPCR, *GAPDH* and *ACTB* were used as reference genes for

normalization, and relative gene expression analysis was performed based on the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.3 Results

4.3.1 Effects of single AFB_1 or FB_1 treatments on cell viability

To investigate the toxicity pattern of AFB_1 and FB_1 on HHL-16 cells, cell viability of HHL-16 cells was determined by MTT assay after treatments with individual AFB_1 or FB_1 for 24 and 48 hours (Figure 4-3). Either with the increasing of AFB_1 concentration from 1 to 100 µg/ml or treatment time from 24 to 48 hours, the cell viability was decreased, indicating a dose-dependent and time-dependent toxicity of AFB_1 in HHL-16 cells (Figure 4-3A). With respect to the toxicity of FB_1, decreased cell viability with the increasing of concentration and time was also observed, so there was also a dose-dependent and time-dependent manner of FB_1 toxicity in HHL-16 cells (Figure 4-3B).



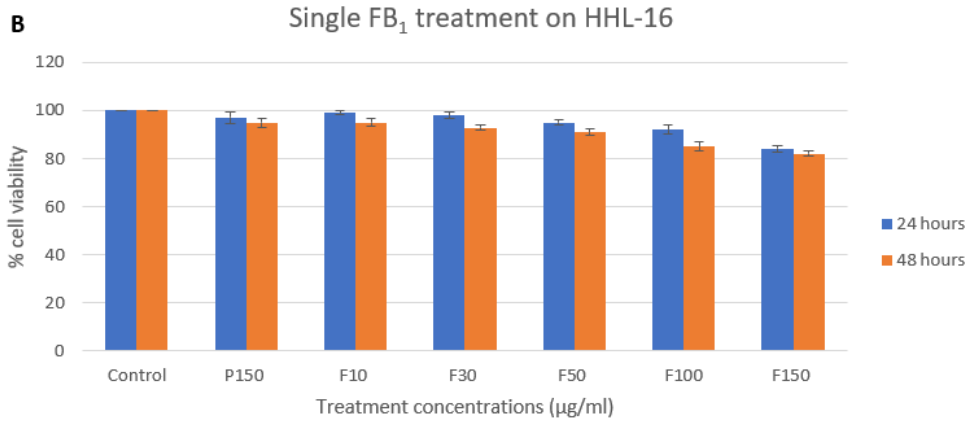


Figure 4-3 Single AFB_1 **(A)** or FB_1 **(B)** treatments on HHL-16 cells for 24 and 48 hours. Control: untreated cells; D: diluted DMSO treatments (equivalent to the concentration in corresponding AFB_1 treatments); P: PBS treatment. F: FB_1 treatments. DMSO and PBS treatments were normalized against the control group. AFB_1 treatments were normalized against corresponding DMSO treatments (i.e. A1 against D1). FB_1 treatments were normalized against PBS treatment. With the increasing of either AFB_1/FB_1 concentrations or treated time, the cell viability was decreased, so there were dose-dependent and time-dependent cytotoxicity of both AFB_1 and FB_1 in HHL-16 cells. Data are presented as mean ± SEM, and the results were repeated three times.

4.3.2 Effects of combined AFB_1 and FB_1 treatments on cell viability

To determine the combined effects of AFB_1 and FB_1, different combinations of AFB_1 and FB_1 were used to treat HHL-16 cells for 24 and 48 hours. The observed combined effect was displayed (Figure 4-4) as the percentage of reduced cell viability caused by AFB_1 and FB_1 treatments. Expected additive effect of AFB_1 and FB_1 was calculated from the sum of the reduced cell viability caused by individual AFB_1 and individual FB_1. The result showed that the observed combined effects of AFB_1 and FB_1 caused more reduction of cell viability compared to the expected additive effects of them, so there was a synergistic effect of AFB_1 and FB_1 on HHL-16 cells (Figure 4-4). With the increasing of combined concentrations or treatment time, more reduction of cell viability was caused, so the combined effect of AFB_1 and FB_1 toxicity was dose-dependent and time-dependent manner in HHL-16 cells.

In addition, a computer program, CompuSyn, was used to calculate the CI values in AFB_1 and FB_1 combination (Chou and Talalay, 1984; Chou, 2006). As the result indicated that the CI values of the 5 combinations of AFB_1 and FB_1 all lower than 1 (Table 4-1 and 2), confirming a synergistic interaction of the combination of AFB_1 and FB_1.

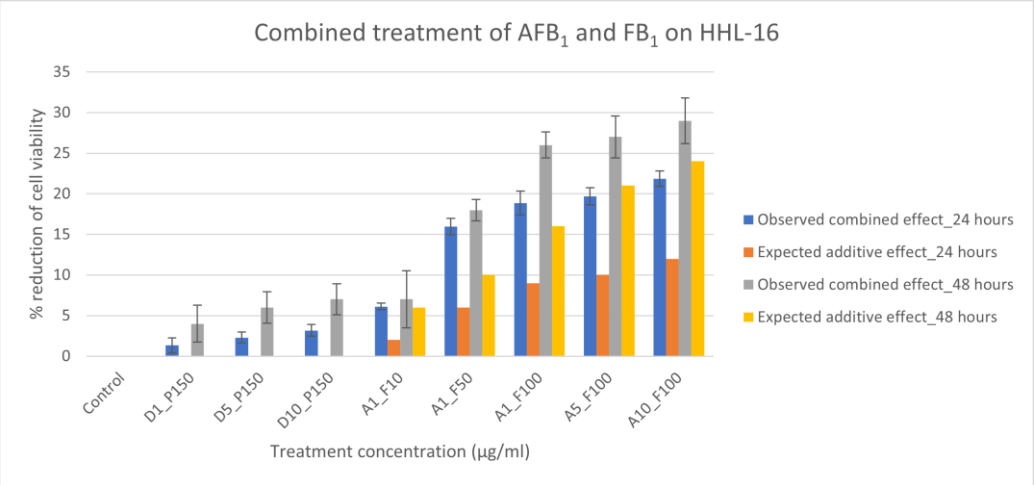


Figure 4-4 Combined treatments of AFB_1 and FB_1 on HHL-16 cells for 24 and 48 hours. Control: untreated cells; D: DMSO treatment; P: PBS treatment; A: AFB_1 treatment; F: FB_1 treatment; Combined PBS and DMSO treatment were normalized against control groups. Combined AFB_1 and FB_1 treatments were normalized against corresponding combined treatment of DMSO and PBS. Expected additive effect: the sum of the toxic effect of single AFB_1 and FB_1. Combined AFB_1 and FB_1 treatment has a synergistic effect on HHL-16 cells 24 and 48 hours post the treatment, it is both dose-dependent and time-dependent manner. Data was presented as mean ± SEM, and the results were repeated three times.

Table 4-1 Combination index calculated by CompuSyn in the combined AFB_1 and FB_1 treatments on HHL-16 cells for 24 hours.

Mycotoxin	**Combination ratio**	**24 hours treatment**	
		CI	DRI
AFB_1_1 µg/ml	1:10	0.21	16.6
FB_1_10 µg/ml			6.5
AFB_1_1 µg/ml	1:50	0.29	72.1
FB_1_50 µg/ml			3.6
AFB_1_1 µg/ml	1:100	0.47	95.4
FB_1_100 µg/ml			2.2
AFB_1_5 µg/ml	1:20	0.48	20.8
FB_1_100 µg/ml			2.3
AFB_1_10 µg/ml	1:10	0.47	12.2
FB_1_100 µg/ml			2.6

Table 4-2 Combination index calculated by CompuSyn in the combined AFB_1 and FB_1 treatments on HHL-16 cells for 48 hours.

Mycotoxin	**Combination ratio**	**48 hours treatment**	
		CI	DRI
AFB_1_1 µg/ml	1:10	0.53	8.7
FB_1_10 µg/ml			2.4
AFB_1_1 µg/ml	1:50	0.33	31.2
FB_1_50 µg/ml			3.4
AFB_1_1 µg/ml	1:100	0.27	54.7
FB_1_100 µg/ml			4.0
AFB_1_5 µg/ml	1:20	0.31	11.6
FB_1_100 µg/ml			4.4
AFB_1_10 µg/ml	1:10	0.34	6.6
FB_1_100 µg/ml			5.3

4.3.3 mRNA expression of cytochrome P450 (CYP450) genes

In humans, aflatoxin is mainly metabolized by CYP450 enzymes to produce toxic metabolites, so to investigate whether AFB_1 is metabolized in the cell line, gene expression of several key CYP genes including *CYP1A2*, *3A4*, and *3A5* was determined in HHL-16 cells after AFB_1 treatments. The qPCR result (Figure 4-5) shows that the maximal level of gene expression fold change was observed in *CYP3A4* gene. Significantly increased expression of *CYP3A4* (5.76 folds, p=0.0027) and *3A5* (5.09 folds, p=0.0037) were observed in the cells after 20 µg/ml AFB_1 treatment compared to the control groups. Although gene expression of *CYP1A2* was increased after AFB_1 treatments, the change was not significant.

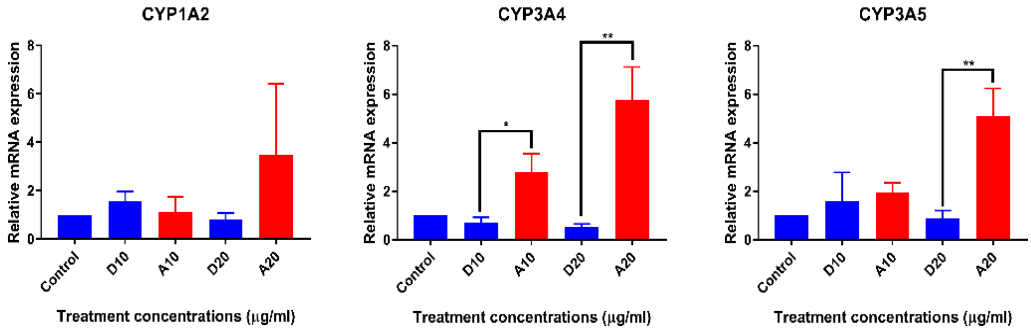


Figure 4-5 Gene expression of cytochrome P450 (CYP450) genes in HHL-16 cells exposed to AFB_1 for 24 hours. Control: untreated cells; D: DMSO treatment; A: aflatoxin treatment. ACTB: Beta-actin, a reference gene used for the normalization to *CYP1A2*, *CYP3A4* and *CYP3A5*. A significant increase of *CYP3A4* and *CYP3A5* were observed in HHL-16 cells after 20 µg/ml AFB_1 treatment for 24 hours. Although there is an increased expression of *CYP1A2*, it did not reach significant level.

4.3.4 Gene expression analysis in Growth Factors Pathway in HHL-16 cells after individual AFB_1 or FB_1 treatment

To validate the hypothesis that gene expression of human growth factors could be altered by AFB_1 or FB_1 treatments in the cells, human growth factors pathway-focused gene expression analysis was performed with the usage of QuantiNova® LNA® PCR Panels in HHL-16 cells after individual AFB_1 or FB_1 treatment. Figure 4-6 shows the heat map of gene expression fold change of several selected genes in HHL-16 cells after individual 10 µg/ml AFB_1 or 50 µg/ml FB_1 treatment, the full data was presented in Appendix 4.2. The significant differentially expressed genes were identified by fold change greater than 2-fold with *p* value less than 0.05. Six genes including *BMP2*, *CXCL1*, *HBEGF*, *NGF*, *NDP*, and *NRG2* were significantly differentially expressed in HHL-16 cells after 10 µg/ml AFB_1 treatment for 24 hours compared to control group. In addition, the AFB_1 treatment showed that the most significantly up-regulated of *BMP2* (3.53-fold, p=0.0014) and down-regulated of *NDP* (3.44-fold, p=0.01) were observed. However, there was no notable gene expression changes in HHL-16 cells after 50 µg/ml FB_1 for 24 hours.

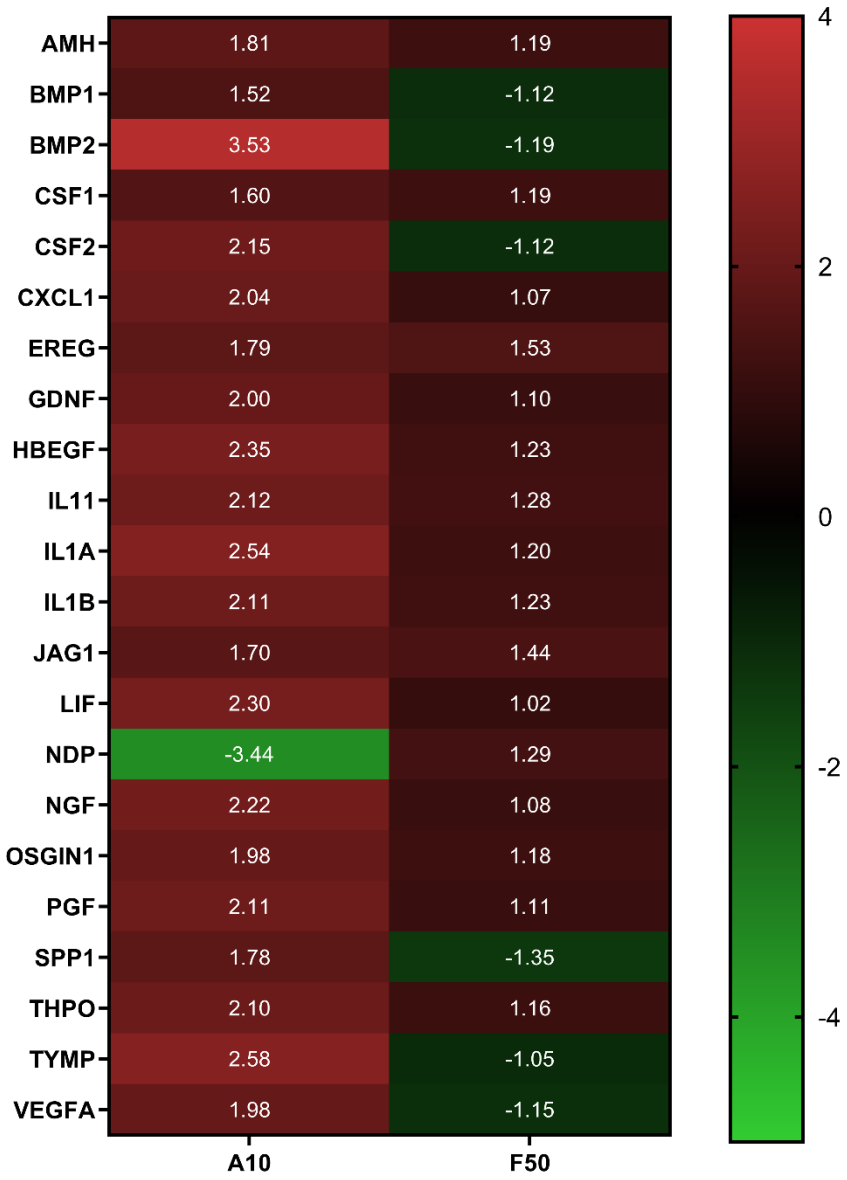


Figure 4-6 Heatmap of gene expression fold change of several selected genes in growth factors pathway in HHL-16 cells after individual 10 µg/ml AFB_1 or 50 µg/ml FB_1 treatment for 24 hours. A: AFB_1 treatment; F: FB_1 treatment. The colours represent the up-regulated (red) and down-regulated (green) genes compared to its control group.

4.3.5 Gene expression analysis in Growth Factors Pathway in HHL-16 cells after combined AFB_1 and FB_1 treatment

With regard to the gene expression changes of growth factors pathway in HHL-16 cells after combined AFB_1 and FB_1 treatment, the full data was presented in Appendix 4.3. Most of genes showed no significant change after the combined 1 µg/ml AFB_1 and 50 µg/ml FB_1 (A1_F50) treatment compared to control group. However, the gene expression of several genes, such as: *CSF2*, *GDNF*, *JAG1*,

PTN, *TGFB1*, and *VEGFA*, was decreased by higher than 1.5-fold though did not reach 2-fold change (Figure 4-7).

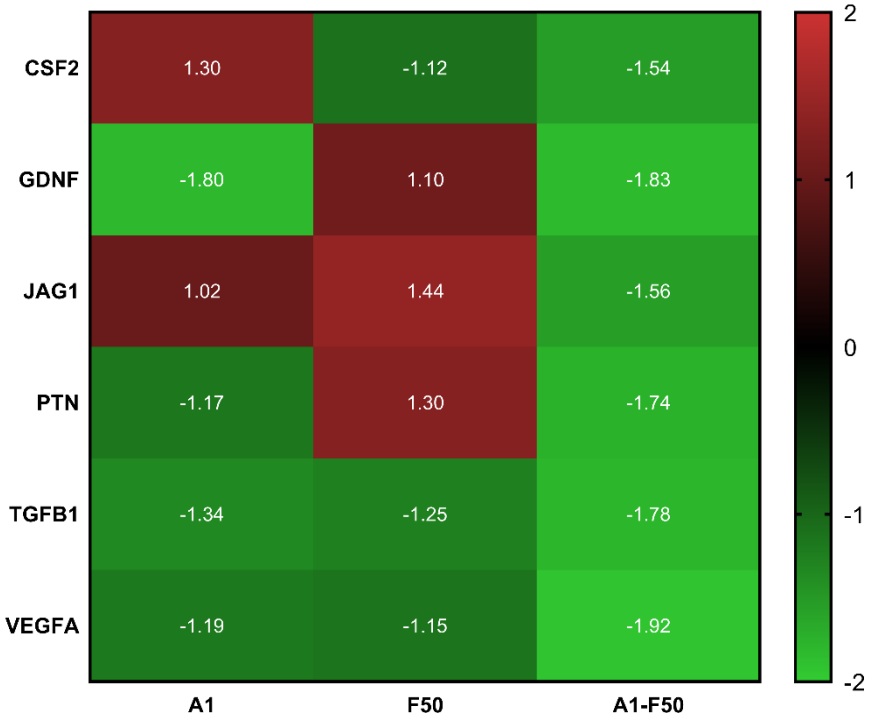


Figure 4-7 Heat map of gene expression fold change of several selected genes in growth factors pathway in HHL-16 cells after combined treatment of 1 µg/ml AFB_1 and 50 µg/ml FB_1 for 24 hours. The colours represent the up-regulated (red) and down-regulated (green) genes.

4.3.6 Gene expression analysis of selected genes by RT-qPCR in HHL-16 cells after single AFB_1 or FB_1 treatments

The highest differentially expressed genes *BMP2* and *NDP* observed in the gene expression analysis of growth factors pathway in HHL-16 cells were selected as genes of interest for further measurement. Previous data in our lab found that two immune-related genes, *IL6* and *CCL20*, were differentially expressed in HHL-16 cells after single AFB_1 treatments (Castelino, 2013; Xu, 2020), therefore the two genes were selected as quality controls to ensure result consistency, and also were measured as gene of interest for further investigation. Hence, gene expression of the four genes, *BMP2*, *NDP*, *IL6*, and *CCL20*, were separately measured by RT-qPCR in HHL-16 cells after AFB_1 treatments of various concentrations (1, 5, 10, and 20 µg/ml). As shown in Figure 4-8, the results were consistent with the results of the growth factors pathway screening, as well as the previous results in HHL-16 cells. mRNA expression of *IL6*, *CCL20*, and *BMP2* were significantly increased and *NDP*

was significantly decreased after AFB_1 treatments, which were dose-dependent effects.

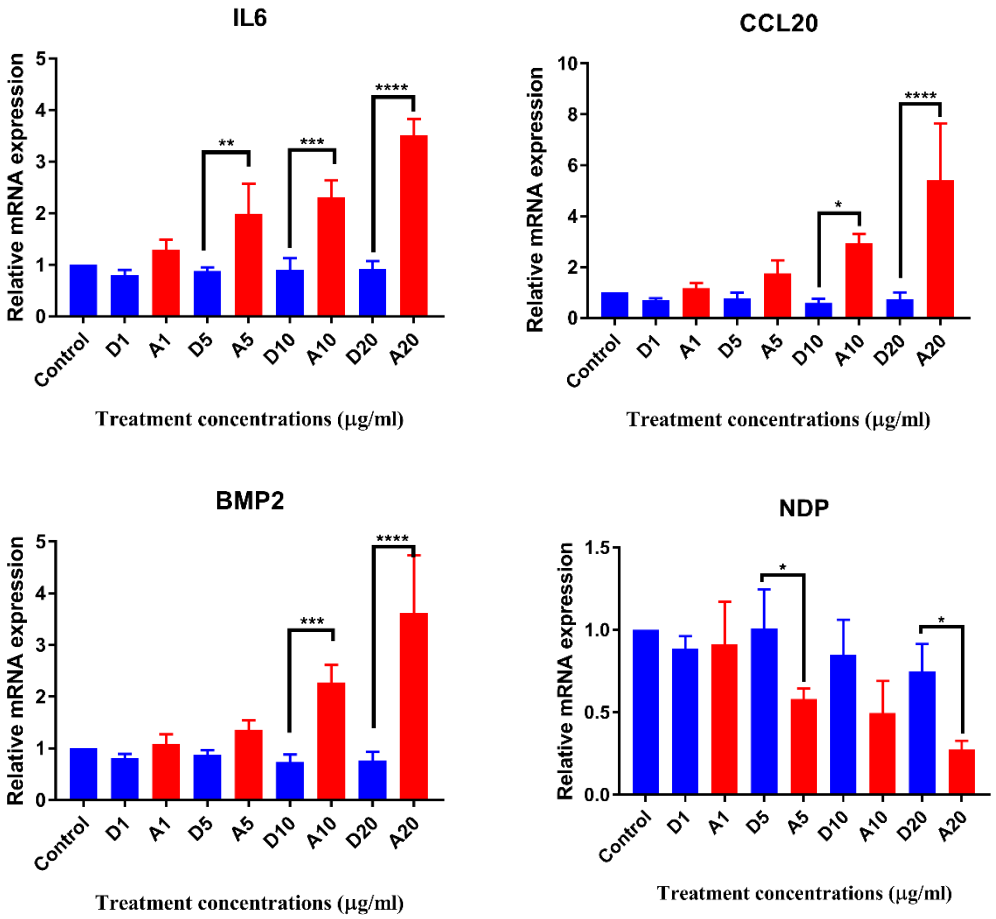


Figure 4-8 Gene expression analysis of the candidate genes involved in immune and growth factors pathways in HHL-16 cells after 1, 5, 10, and 20 μg/ml AFB_1 treatments for 24 hours. Control: untreated cells; D: DMSO treatments; A: AFB_1 treatments. *GAPDH* and *ACTB*, two reference genes were used to normalize the gene expression of *IL6*, *CCL20*, *BMP2*, and *NDP*. Dose-dependent increasing of *IL6*, *CCL20*, and *BMP2* was observed, and dose-dependent decreasing of *NDP* was found in HHL-16 cells after AFB_1 treatments.

mRNA expression levels of the four genes were also measured in HHL-16 cells after single FB_1 treatments with more concentrations (10, 50, and 100 μg/ml) used. In HHL-16 cells after single FB_1 treatments for 24 hours, the results (Figure 4-9) showed an increased trend of mRNA levels of *IL6* and *NDP*, while a decreased trend of mRNA levels of *CCL20*. However, the changes of mRNA levels of the three genes did not reach statistical significance. mRNA level of

BMP2 in HHL-16 cells after FB_1 treatments for 24 hours did not change compared to the control group.

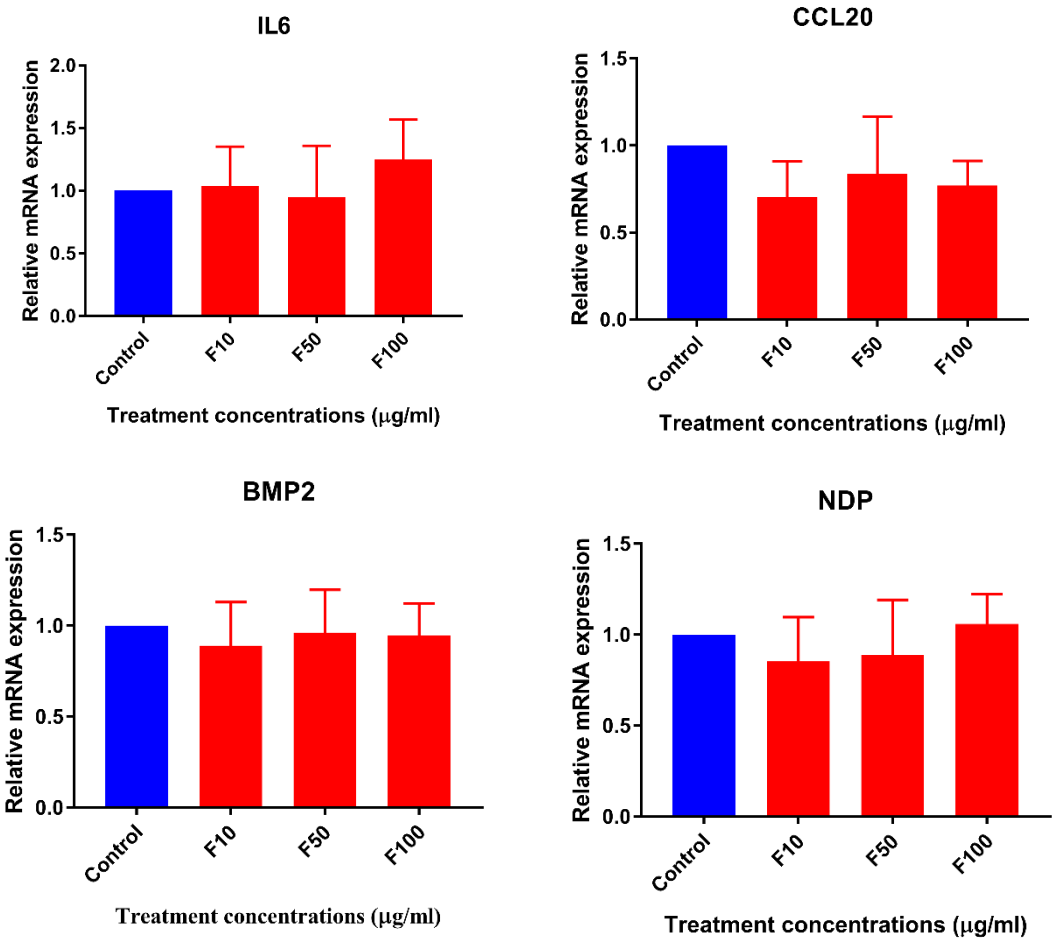


Figure 4-9 Gene expression analysis of the candidate genes involved in immune and growth factors pathways in HHL-16 cells after FB_1 treatments for 24 hours. Control: untreated cells. F: FB_1 treatments. *GAPDH* and *ACTB*, two reference genes were used to normalize the gene expression of *IL6*, *CCL20*, *BMP2*, and *NDP*. No significantly differential gene expression of *IL6*, *CCL20*, *BMP2*, and *NDP* in HHL-16 cells after 10, 50, and 100 µg/ml FB_1 for 24 hours.

4.3.7 Gene expression analysis of selected genes by RT-qPCR in HHL-16 cells after combined AFB_1 and FB_1 treatments

To test the combined effects of AFB_1 and FB_1 on gene expression of the four candidate genes, mRNA levels of the four genes were measured in HHL-16 cells after combined AFB_1 and FB_1 treatments (A1_F50 and A5_F100) for 24 hours. The results (Figure 4-10) indicated that *IL6* gene expression level was significantly higher in the combined treatment of 5 µg/ml AFB_1 and 100 µg/ml FB_1 than the individual 100 µg/ml FB_1 treatment, also with a tendency to be

higher than 5 µg/ml AFB_1 treatment alone. Although the gene expression fold change of *BMP2* in combined treatment of 5 µg/ml AFB_1 and 100 µg/ml FB_1 did not reach 2-fold, it was found that the mRNA level of *BMP2* was significantly higher in the combined treatment than individual 100 µg/ml FB_1 treatment. However, there was no significant difference of mRNA levels of *CCL20* and *NDP* genes after the combined treatments of AFB_1 and FB_1 compared to the individual AFB_1 or FB_1 treatments.

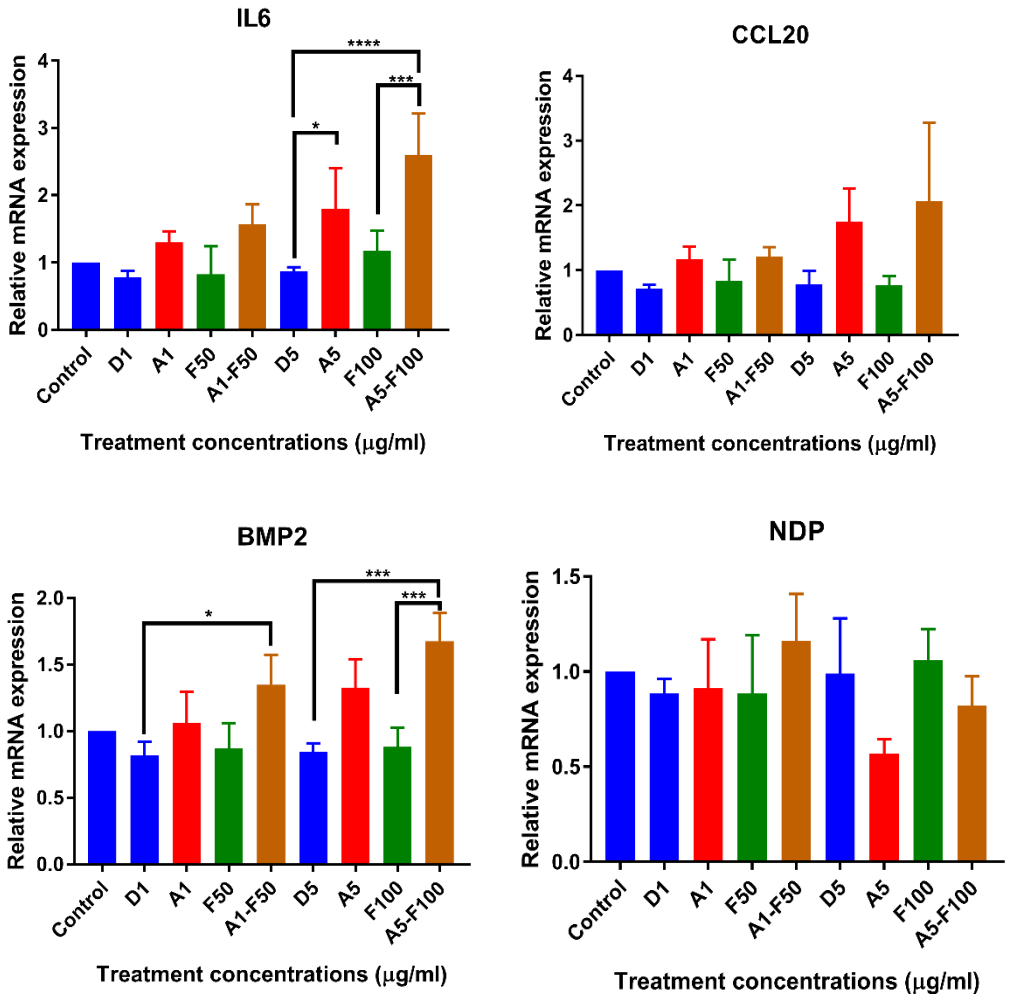


Figure 4-10 Gene expression analysis of the candidate genes involved in immune and growth factors pathways in HHL-16 cells after combined AFB_1 and FB_1 treatments for 24 hours. Control: untreated cells. D: DMSO treatments. A: AFB_1 treatments. F: FB_1 treatments. *GAPDH* and *ACTB*, two reference genes were used to normalize the gene expression of *IL6*, *CCL20*, *BMP2* and *NDP*. Blue, red, green, and brown columns represent the control, single AFB_1, single FB_1, and combined AFB_1 and FB_1 treatment, respectively. *IL6* and *BMP2* expression levels were significantly higher after the combined treatment of 5 µg/ml AFB_1 and 100 µg/ml FB_1 than 100 µg /ml FB_1 treatment alone, also with a tendency to be higher than 5 µg/ml AFB_1 treatment alone.

4.3.8 DNA methylation analysis of *IL6* and *CCL20* in HHL-16 cells after single AFB_1 treatment

To explore whether the gene expression changes of *IL6* and *CCL20* is a cause of DNA methylation, DNA methylation levels at the areas of interest of *IL6* and *CCL20* in HHL-16 cells after 20 µg/ml AFB_1 treatment for 24 hours, including the promoter regions and parts of the areas near the transcription start site, has been analysed by the Genome Centre, Barts and The London, Queen Mary University of London. Results (Figure 4-11) demonstrated that there was significant difference in DNA methylation between position 1-7 and position 8-18 in *IL6* in HHL-16 cells, but there is no significant difference of each CpG sites after 20 µg/ml AFB_1 treatment for 24 hours compared to the control group (D20 treatment).

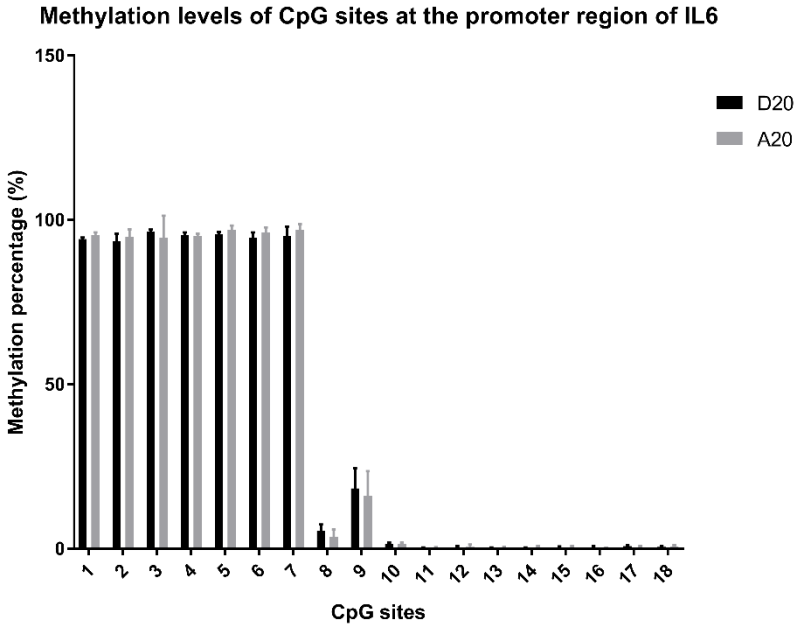


Figure 4-11 DNA methylation analysis at promoter regions of *IL6* in HHL-16 cells after 20 µg/ml AFB_1 treatment for 24 hours. D: DMSO treatment. A: AFB_1 treatment. The DNA methylation levels of 18 CpG sites were analysed in the *IL6* promoter in total. Data presented as mean ± SD with three biological replicates.

With respect to the methylation levels in *CCL20*, 21 CpG sites were analysed within the promoter regions and parts areas around its transcription start site. As the results (Figure 4-12) indicated that there was variation of methylation levels across the CpG sites, and there was a decreased methylation level in position 21 after AFB_1 treatment compared to the control treatment (D20). Due

to methylation levels in two replicates not determined in position 21 in the DMSO treatment, it was not possible to perform a statistical difference analysis between AFB_1 treatment. However, because there is no difference between untreated cells and the DMSO treatment (Figure 4-13), an imputed mean value for the DMSO treatment was calculated based on the addition of the methylation levels in position 21 from the untreated cells and the DMSO treatment. Then a statistical comparison to AFB_1 treatment was performed based on the imputed data. The results showed that it was a significant decrease of methylation level at position 21 after 20 µg/ml AFB_1 treatment on HHL-16 cells for 24 hours compared to the DMSO treatment (p=0.02) (Figure 4-12).

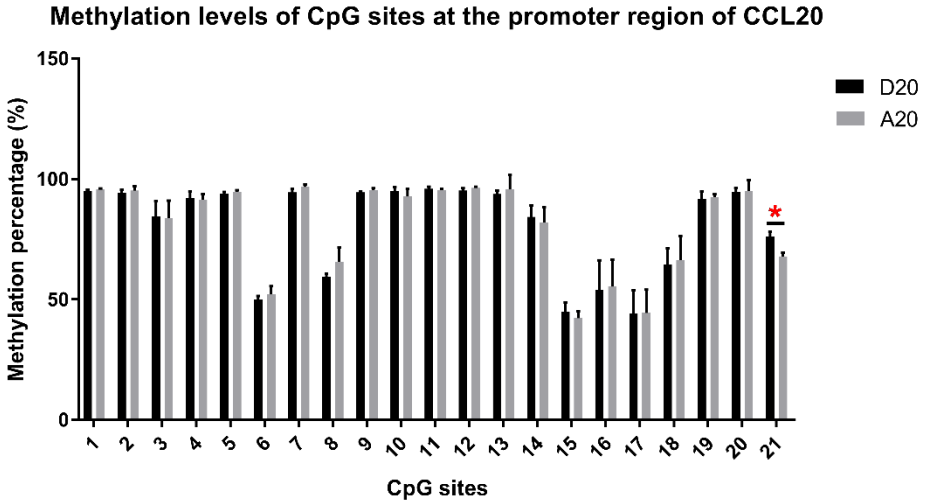


Figure 4-12 DNA methylation analysis at the promoter region of *CCL20* in HHL-16 cells after 20 µg/ml AFB_1 treatment for 24 hours. D: DMSO treatment. A: AFB_1 treatment. Totally, DNA methylation levels of 21 CpG sites around the promoter regions of *CCL20* were analysed. Data presented as mean ± SD with three biological replicates.

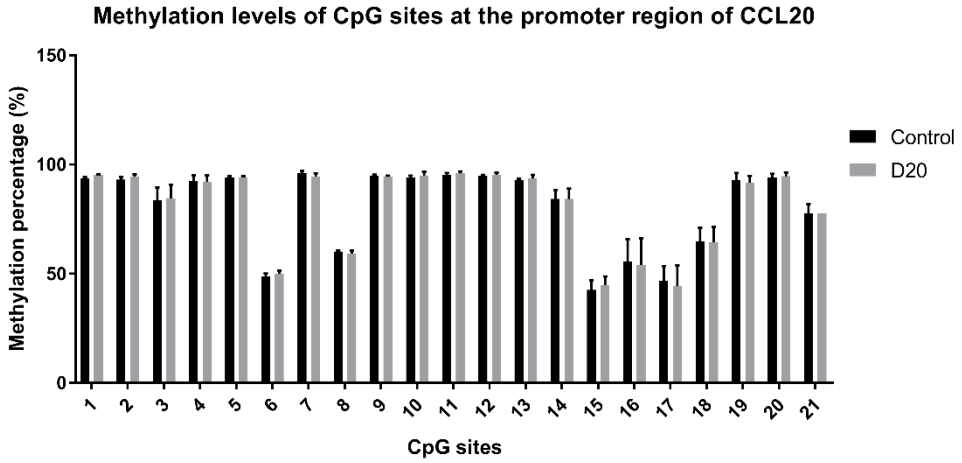


Figure 4-13 Comparison of DNA methylation levels at analysed sites of *CCL20* between control and DMSO treatments in HHL-16 cells treatment for 24 hours. Control: untreated cells. D: DMSO treatment. Data presented as mean ± SD with three biological replicates, apart from only one biological replicate determined for the last position (21) in D20 treatment.

4.4 Discussion

The main findings from this study were: 1) single AFB_1 or FB_1 treatments had dose-dependent and time-dependent toxicity on HHL-16 cells; 2) combined AFB_1 and FB_1 treatments on HHL-16 cells had synergistic toxic effect; 3) compared to individual AFB_1 or FB_1 treatments, combined AFB_1 and FB_1 treatments led to higher mRNA expression of *IL6* and *BMP2* in HHL-16 cells, and it reached significant difference compared to FB_1 treatment alone. 4) DNA methylation reduction at one CpG site of *CCL20* with slightly statistical significance was found in HHL-16 cells after AFB_1 treatment for 24 hours.

To date most mycotoxin toxicity studies in human cell lines has utilised HepG2 cells, a human liver carcinoma cell line. Here, we have used a novel non-tumorigenic cell line, HHL-16, which has been immortalised from the primary hepatocytes with the infection of retrovirus vector expressing HPV 16 E6/E7 oncoproteins (Clayton et al., 2005). Therefore, the use of this cell line avoids the interference of cancer-related changes on gene expression.

The dose-dependent toxicity pattern of either AFB_1 or FB_1 observed in the present study was in line with previous MTT results published for HepG2 cells (Chuturgoon et al., 2015; Zhang et al., 2021). Comparison at 20% inhibition of cell viability shows that AFB_1 or FB_1 toxicity is more cytotoxic in HepG2 cells than in HHL-16 cells. Approximately 5 µg/ml AFB_1 caused 20% reduction of cell

viability in HepG2 cells was reported, while in HHL-16 cells around 50 µg/ml AFB_1 was required to reach the same level of toxicity effect (Liu et al., 2012). The concentration of FB_1 resulting in 20% cell viability inhibition was around 70 µg/ml reported in HepG2 cells, while was 150 µg/ml observed in this study in HHL-16 cells (Chuturgoon et al., 2015). Thereby, these comparisons highlight there might be different susceptibilities to AFB_1 or FB_1 in different cell lines.

Combined effects of AFB_1 and FB_1 on cytotoxicity have been explored in various cell lines, varied from additive and synergistic, which might be associated with the factors of various methods applied for cytotoxicity test, different binary concentrations used, and cell specificity. Additive effects of 1.28 µg/ml AFB_1 and 8 µg/ml FB_1 on cell viability reduction were found in a bovine kidney cell line (MDBK), but the inhibition effect on cell viability was more pronounced after the binary treatment when it was tested by MTT assay than neutral red assay (19% vs 6% inhibition) (Clarke et al., 2014). Synergistic effects on cytotoxicity of combined AFB_1 and FB_1 treatments at higher concentrations in HepG2 cells were observed in several studies (Du et al., 2017; Mao et al., 2023), for example, 8 µg/ml AFB_1 and 160 µg/ml FB_1 treatment on HepG2 cells caused a significant loss of cell viability compared to individual AFB_1 or FB_1 treatment (Chen et al., 2023). Besides, in a rat spleen mononuclear cell line (SMC), 6 µg/ml AFB_1 and 7 µg/ml FB_1 did not cause a significant difference of cell viability, but when the concentration increased by 2-fold to 12 µg/ml AFB_1 and 14 µg/ml FB_1, the cell viability was significantly decreased (Mary et al., 2012). Therefore, evidence mentioned above also stresses the importance of cell viability measurement methods, treatment concentration, and cell specificity on combined cytotoxicity effects. In the current study, the synergistic cytotoxicity of AFB_1 and FB_1 treatments found in HHL-16 cells is in line with, and strengthens the previous evidence as discussed above. Therefore, the findings highlight the potential hazard from combined contamination of crops with these two mycotoxins.

Gene expression of *IL6*, *CCL20*, *BMP2* were significantly increased in HHL-16 cells after AFB_1 treatments for 24 hours, while *NDP* gene expression level was significantly decreased. *IL6* is regarded as both pro-inflammatory and anti-inflammatory cytokine with pleiotropic functions, which is not only involved in immune system, but also involved in regenerative process and metabolism regulation, as well as bone homeostasis (Scheller et al., 2011). Generally, up-regulation of *IL6* was proposed to be related to an activated immune response to stress such as infections, inflammation, and injuries, while down-regulated *IL6* could be a reason of noticeable immune suppression (Tanaka et al., 2014).

For instance, decreased gene expression of *IL6* was observed in piglets fed with co-contaminated diet of mycotoxins, and reduction of both IgG response and lymphocytes activation were observed, so down-regulated *IL6* was speculated to be a likely reason for the impaired immune response (Grenier et al., 2011). On the contrary, increased *IL6* gene expression has been previously reported in broilers and pigs fed with AFB_1-contaminated diet, and it was suggested to be related with tissue lesions observed (Meissonnier et al., 2008; Ma et al., 2015). Another study found that increased production of *IL6* was correlated with liver lesions in rats fed with an intermittent dosing regimen of AFB_1 (Hinton et al., 2003). Therefore, the observation of increased mRNA level of *IL6* in HHL-16 cells in this study may at least support the hypothesis that inflammatory response is associated with the hepatoxicity of AFB_1. Increased expression of *CCL20*, which we found here in HHL-16 cells, has also been seen in chickens administrated with AFB_1 (Guo et al., 2022). *CCL20*, a chemokine, can be induced by the combination of IL6 and its soluble receptor (sIL-6R) (Meares et al., 2012). In astrocytes, it was found that *IL6* combined to sIL-6R significantly increased expression of *CCL20* through the activation of *STAT3* and binding of phosphorylated STAT3 to the promoter of *CCL20* (Meares et al., 2012). Elevated CCL20 production and its receptor CCR6 has been related to the progression of a variety of human cancers, including liver cancer, colorectal cancer, breast cancer and kidney cancer, also indirectly modulates the function of immune cells in response to inflammatory diseases (Ghadjar et al., 2009; Kadomoto et al., 2020). Therefore, the increased expression of *IL6* and *CCL20* in response to AFB_1 may play a role in AFB_1-related inflammatory diseases and cancers.

Our study is the first to find aberrant expression of *BMP2* and *NDP* in hepatocytes cell line after AFB_1 treatments. Evidence indicated that *BMP2* plays roles in bone formation and has effects on downstream growth factors (Feng et al., 2010; Nakamura et al., 2023), and recent evidence found that *BMP2* is implicated with various cancers (Wu et al., 2022; Zhang et al., 2022; Zırh et al., 2022; Jiang et al., 2023). For instance, increased gene expression of BMP2 has been reported in human osteoblasts treated with cadmium and in patients with non-alcoholic fatty liver disease (Bodo et al., 2010; Marañón et al., 2022). Typically, with respect to the functions of *BMP2* in the liver, it was indicated that *BMP2* plays a dynamic role in liver physiology. Decreased expression of *BMP2* after partial hepatectomy in rats was found during the process of liver regeneration, which deduced that there might be a negative association between *BMP2* expression and hepatocyte proliferation (Xu et al.,

2006). However, increased gene expression of *BMP2* was observed during alcohol-induced fibrosis in rats, and in non-alcoholic liver disease (NAFLD) patients, as well as in hepatocellular carcinoma (Marañón et al., 2022; Hong et al., 2023; Jiang et al., 2023). Therefore, the up-regulated level of *BMP2* found in HHL-16 cells after AFB_1 treatments might indicate a novel mechanism in aflatoxin-induced chronic liver injury.

Another differentially expressed gene, *NDP*, is a potent upstream mediator of Notch signalling, which is a highly conserved pathway crucial in development and implicated in malignant transformation (Bray, 2006; El-Sehemy et al., 2020). Suppressed *NDP* gene expression may affect downstream growth and development and contribute to disease onset. Therefore, the aberrant gene expression changes of the four genes that we observed in HHL-16 cells following AFB_1 may reflect the impact of aflatoxin on liver injury, immune modulation, growth failure, and cancers could be at least in part be due to disruption of cytokines, chemokine, and growth factors, although the effects of the altered gene expression on specific pathway connections to modulate cell response is not known. Due to the pleiotropic features of genes, it is difficult to figure out the in-depth pathway connections via the several differentially expressed genes, investigation on a whole genome perspective need to be performed to test the hypothesis.

We did not find any significant effects of individual FB_1 on gene expression of members of the human growth factors pathway and the two immune-related genes (*IL6* and *CCL20*) in HHL-16 cells, although previous reports have shown effects of FB_1 on such pathways. In rats fed with 250 mg/kg FB_1, overexpression of transforming growth factor beta 1 (TGF-β1) was found in the rat liver with fibrosis, which suggests that the significant apoptosis and fibrosis induced by FB_1 in rats could be explained by the aberrant expression of TGF-β1 (Lemmer et al., 1999). Significantly increased expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2) were found in mice intraperitoneally administrated with 4.5 mg/kg FB_1 (Sozmen et al., 2014). Similar alteration in gene expression of immune-related genes was also widely reported in various animals or animal cell lines after FB_1 treatment (Taranu et al., 2005; Marin et al., 2006; Cheng et al., 2006; Zhu and Wang, 2022). Particularly in piglets, different gene expression changes of cytokines in intestine and lung in response to FB_1 were indicated, and the evidence showed that cytokines expressed differently might depend on the organ (Halloy et al., 2005; Bouhet et al., 2006). It was demonstrated that pigs, or laboratory rodents are considered as more sensitive to fumonisins (Bolger, 2001; Voss et al.,

2007), so it can be inferred that there was no significant difference in gene expression in HHL-16 cells in response to FB_1 might because of the species difference of susceptibility to fumonisins. Similar to the findings in HHL-16 cells treated with FB_1, no significant difference of the mRNA levels of *IL-2* and *IFN γ* was also reported in a human lymphoblastoid Jurkat T cell line after FB_1 treatments (Luongo et al., 2006). Therefore, the discrepancies between previous evidence and gene expression data in HHL-16 in response to FB_1 may be explained by the species-specific difference and specificity of cell lines.

Despite there being no significant changes in gene expression induced by FB_1 on its own, the combined treatment induced higher mRNA levels of *IL6* and *BMP2* in HHL-16 cells compared to FB_1 treatment alone, also with a trend to be higher than AFB_1 treatment alone , suggesting a synergistical effect of AFB_1 and FB_1 on the transcription of *IL6* and *BMP2*. It should be noted that although gene expression changes of *IL6* and *BMP2* in the cells after single FB_1 treatment did not reach significant difference compared to the control group, it still induced gene expression of *IL6* after 100 µg/ml FB_1 treatment, albeit at a much lower potency than AFB_1. Synergistic induction of *IL6* could be a reason for more cell apoptosis caused by the mixture of mycotoxins, which will contribute to more inflammation response (Mao et al., 2023). Besides, IL6 has been shown to enforce proliferation and anti-apoptotic effects in tumour cells to promote tumour progression (Waldner et al., 2012). The evidence of elevated *IL6* expression in our study might shed light on the mechanism of higher incidence of cancers reported in animals and humans exposed to both AFB_1 and FB_1.

BMP2 is a growth factor and plays an important role in bone formation, but recent studies found that BMP2 may also play a role in carcinoma progression. Significantly higher *BMP2* expression was found in positive tumour budding group than negative tumour budding group in human hepatocellular carcinoma (Jiang et al., 2023). In addition, exogenous BMP2 injected into mice enhanced liver cancer growth through the activation of myeloid-derived suppressor cells to secret more IL6, which could promote cell proliferation of liver cancer cells (Wu et al., 2020). Uncontrolled cell proliferation in healthy cells is a hallmark of the transformation to cancer cells, eventually contributing to cancer progression (Gold, 1999). In this study, although *BMP2* gene expression was not induced by FB_1, the mixture of AFB_1 and FB_1 induced higher *BMP2* gene expression compared to AFB_1 or FB_1 treatment alone, which might be because of in the presence of AFB_1, more FB_1 was absorbed in the cells (Yu et al., 2020). On the other hand, the increased *BMP2* in the combined treatments of AFB_1 and FB_1,

may also indicate that FB_1 might have the potential to increase the carcinogenicity of AFB_1. Therefore, it can be deduced that the combination of AFB_1 and FB_1 would cause more inflammatory response and the carcinogenicity of AFB_1 could be enhanced by the presence of FB_1 through the activation of *IL6* and *BMP2* related signalling pathways. However, it is necessary to point out that analysis *in vitro* could not represent the full biological effects of *IL6* and *BMP2 in vivo*, as the interaction between them to promote cancer is more complex *in vivo* due to the coordination among different organs. Further studies *in vivo* to replicate the results in this study are warranted.

Previous evidence suggested that decreased DNA methylation level at a specific CpG site of the promoter region of *IL6* was related to its increased mRNA level (Nile et al., 2008). In this study, we also assessed the methylation status of 18 CpG sites around the transcription start site of *IL6* in HHL-16 cells after AFB_1 treatment. The methylation pattern of *IL6* was consistent with the previous evidence (Nile et al., 2008), which showed significant difference of methylation levels among the CpG sites. Especially there were much higher methylation levels at the first seven CpG sites of *IL6*, while other 11 CpG sites had significantly lower methylation levels. However, no significant difference of methylation levels at each CpG sites was found between the control group and AFB_1 treatment group. In contrast, in a study of lung tumours, significantly higher mRNA level of *IL6* was observed in adjacent non-tumorous lung tissues than lung tumour tissues, and there was a slightly significant inverse association between DNA methylation and mRNA level of *IL6* in the tumour tissues (Spearman r=-0.49, p=0.02) but not the non-tumorous tissues (Tekpli et al., 2013). Therefore, here no significant difference of DNA methylation of *IL6* was observed in HHL-16 cells did not weaken the evidence of aflatoxin-caused DNA methylation, there might be a possibility of DNA methylation changes of *IL6* when the cells exposed to AFB_1 for a longer time to cause a transformation to cancer cells. Besides, we propose there might be different epigenetic mechanisms responsible for the gene expression changes of *IL6* in non-tumour and tumour cells. For example, histone modification at *IL6* promoter region significantly increased *IL6* was also reported in endothelial cells (Shamloul et al., 2021). Other explanations for the observed up-regulation of *IL6* in HHL-16 cells are proposed to relate with the binding of some transcription factors, which could enhance the expression of *IL6*. Several studies support the evidence that activation of NF-kB, one of the well-known transcription factors, contributed to the increased expression of *IL6* in cells, suggesting a possible mechanism for

IL6 gene expression change (Kopp and Ghosh, 1995; Chauhan et al., 1996; Golovine et al., 2008).

Concerning DNA methylation of *CCL20* in HHL-16 cells after AFB_1 treatment, we found a significant difference of decreased DNA methylation at a CpG site near the transcription start site compared to the control group, which might be responsible for the up-regulation of *CCL20* in HHL-16 cells after AFB_1 treatments. Evidence indicated that elevated *CCL20* was associated with promoter hypomethylation in esophageal cancer, which promotes cancer progression and immune disorder (Nan et al., 2021). Recently, promoter hypomethylation of *CCL20* causing increased mRNA level of *CCL20* was also indicated in esophageal adenocarcinoma, and the methylation patterns was correlated with esophageal status, which suggested that *CCL20* could be a novel biomarker for early detection of esophageal cancer (Maity et al., 2022). Therefore, the hypomethylation change and overexpression of *CCL20* observed in HHL-16 cells after AFB_1 treatment strengthen the evidence of aflatoxin-caused DNA methylation. However, since the statistical significance analysis was completed in subject to an imputed mean DNA methylation of the control group, more DNA methylation analysis on *CCL20* in other cell lines treated with AFB_1 as well as in animals or populations with AFB_1 exposure are required for further validation.

In summary, the present results indicate that AFB_1 and FB_1, either alone or in a combination, had dose-dependent and time-dependent toxicity in HHL-16 cells. Combination of AFB_1 and FB_1 showed a synergistic toxicity in the cells. AFB_1 increased transcription of *IL6*, *CCL20*, *BMP2* and decreased *NDP* gene expression level. Combined treatment with AFB_1 and FB_1 showed a synergistic induction of mRNA expression levels of *IL6* and *BMP2*, suggesting that the presence of FB_1 may increase the cytotoxicity of AFB_1 through increasing the inflammatory response and disrupting the cell growth balance. A slightly significant difference of DNA methylation at a specific CpG site of *CCL20* was observed in HHL-16 cells after AFB_1 treatment. More attention needs to be paid to the co-occurrence of aflatoxin and fumonisin in nature, and further studies *in vivo* should be conducted to confirm the findings obtained in this study.

Chapter 5 Transcriptome profiling of human hepatocyte cell line HHL-16 in response to aflatoxin B_1

5.1 Introduction

As previously discussed, dietary exposure to aflatoxin can cause acute aflatoxicosis, liver cancer, and be implicated with immune suppression and growth impairment (Wild et al., 2015; Gong et al., 2016a). In the pilot study of CNCC study in Chapter 3, a high prevalence of aflatoxin exposure in the children has been observed, and it was found that aflatoxin exposure was inversely associated with child growth, and it also was a possible risk factor contributing to child mortality. Due to the adverse health effect of aflatoxin exposure in humans, the mechanism of aflatoxin toxicity was investigated in Chapter 4. We observed that aflatoxin treatments in a human hepatocyte cell line caused gene expression changes of several genes (*IL6*, *CCL20*, *BMP2*, and *NDP*) that play roles in pathways of inflammatory response, growth, and cancer. However, due to the pleiotropy of those genes, specific affected pathways by aflatoxin are not fully understood. Therefore, in this study, a whole genome approach analysing the transcriptome changes in HHL-16 cells after aflatoxin treatment was adopted, with the aim to find more significantly affected genes and pathways and better understand aflatoxin-related health effects.

With the recent development of deep-sequencing technologies, the accessibility of RNA-Seq has been applied to transcriptome profiling. Understanding of the transcriptome helps to find the functional elements of the genome and reveal the molecular constituents of cells, as well as to figure out the molecular mechanisms of development and disease (Wang et al., 2009). RNA-Seq has been used to assess the effects of aflatoxin on transcriptome changes in various animals and *in vitro* studies (Merrick et al., 2013; Zhang et al., 2016; Monson et al., 2016; Ma et al., 2021; Wu et al., 2021). For example, hepatic transcriptome changes in ducklings administrated with AFB_1 showed that differentially expressed genes (DEGs) involved in phase I metabolism, phase II detoxification, carcinogenesis, and fatty acid metabolism (Zhang et al., 2016). In addition, pathways including activation of cell cycle, cellular growth and proliferation, DNA replication and repair, lipid metabolism, and amino acid metabolism have been significantly enriched in the pathway analysis of differentially expressed genes in HepaRG cells with AFB_1 treatment (Tryndyak et al., 2018). However, there are some inconsistencies of the enriched pathways found in different animals or cell lines, and more evidence of

transcriptome changes caused by aflatoxin is required. Moreover, most of the current evidence of the effects of aflatoxin on transcriptome profile in cell lines are based on cancer-derived cells. Systematic measurement of gene expression in non-cancer cells treated with AFB_1 has not been explored.

Therefore, in this study, RNA-Seq method was used to analyse the alteration of hepatic transcriptome in HHL-16 cells (non-cancer cells) after AFB_1 treatment, which will provide a better understanding and more evidence of aflatoxin-induced health effects.

5.2 Methods

5.2.1 Cell maintenance and treatments

HHL-16 cells were cultured in appropriate flasks or plates as described in Chapter 2, Section 2.3. The cells were treated with 20 µg/ml AFB_1, and its control treatment, 1:1000 diluted DMSO for 24 hours, which is equivalent to the concentration in 20 µg/ml AFB_1 treatment. Each treatment had three technical repeats, i.e., three wells with the same treatment in a 6-well plate. In addition, three biological repeats were performed.

5.2.2 RNA extraction

After incubation for the designated time, HHL-16 cells were harvested as described in Chapter 2, Section 2.7.1, and RNA extraction from the cells was performed as described in Chapter 2, Section 2.8.1. The RNA samples were shipped with dry ice to Novogene Co., Ltd for RNA-Sequencing.

5.2.3 RNA-Seq

RNA-Sequencing was performed in Novogene Co., Ltd through Illumina platforms, which is based on a paired-end 150 bp sequencing strategy, and it was followed with the steps as shown in Figure 5-1.

With respect to sample quality control, concentrations and quality of the RNA, samples were tested by Agilent 5400. Only the samples meeting the requirement of both amount and quality were used for sequencing, which requires the amount of RNA samples to be at least over 200 ng and the RNA Integrity Number (RIN) to be at least over 4 (scale from 1-10, 10 is the best quality).

To get purified messenger RNA (mRNA), Poly-T oligo-attached magnetic beads were used in the total RNA samples to capture Poly-A enriched mRNA.

Then the purified mRNA samples were fragmented, and cDNA samples were synthesised by reverse transcription to complete library construction.

After finishing the library preparation, a step of library quality control was performed. The libraries were checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution test. Only quantified libraries were pooled and sequenced by using Illumina NovaSeq platform to generate paired-end reads of 150 bp length.

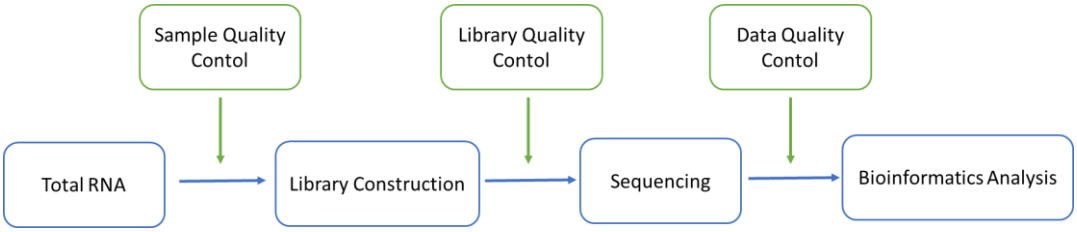


Figure 5-1 Procedures of RNA-Sequencing.

5.2.4 Data analysis

Bioinformatic analysis of RNA-Seq data was carried out as shown in the figure (Figure 5-2).

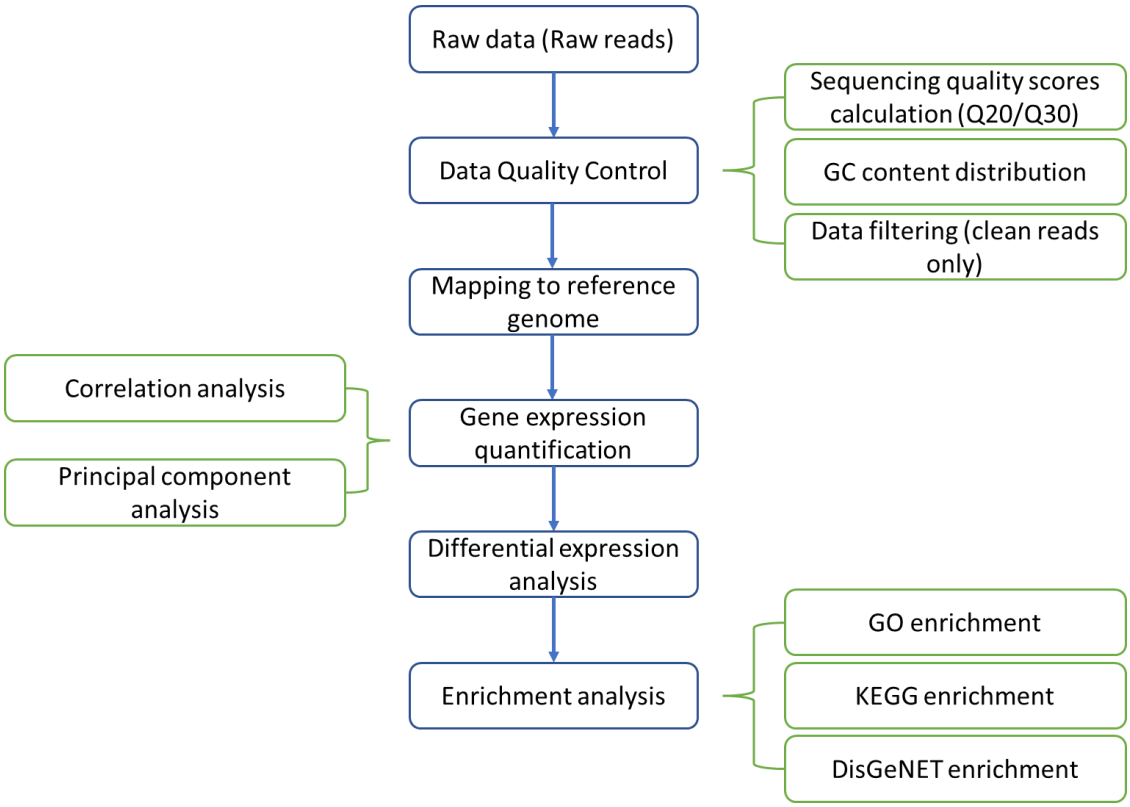


Figure 5-2 Technology flow of bioinformatic analysis of RNA-Seq.

5.2.4.1 Data quality control

Data quality control of raw data (raw reads) was carried out before bioinformatics analysis. Raw reads were inputted to fastp software (Chen et al., 2018), which obtained clean reads from the raw reads and removed reads containing adapter, undefined reads, and reads of low quality. Meanwhile, two sequencing quality scores, Q20 and Q30, which represent for a probability of incorrect base calling of 1 in 100 (99%), and 1 in 1000 (99.9%), respectively, as well as GC content of the clean reads were calculated.

5.2.4.2 Mapping to reference genome

Then the clean reads were aligned to human reference genome, and the reads numbers mapped to each gene were counted. Gene expression quantification was estimated based on the count of sequencing mapped to the genome, because the number of read counts is proportional to gene expression level.

5.2.4.3 Quantification of gene expression level

Gene expression levels were normalized by using FPKM (Fragments Per Kilobase of transcript per Million base pairs sequenced) method, which is a normalization method based on gene length and sequencing depth (Mortazavi et al., 2008). Total counts in a sample were added up and divided by 1,000,000 (per million) as a scale factor. Then the counts for each gene in the sample were divided by the scaling factor, which was normalized to sequencing depth. Followed divided by the gene length to get FPKM.

5.2.4.4 Differential expression analysis

Differentially expressed genes were determined by using DESeq2 software (Love et al., 2014), which differentiate differentially expressed genes (DEGs) based on the raw count numbers. DESeq2 automatically performs a normalization based on built-in standardization algorithm where geometric mean of raw counts of each gene across all samples is calculated. The raw counts for a gene in each sample is divided by the geometric mean. The median of these ratios in a sample is the size factor for the sample. Finally, raw counts of each gene in each sample were normalized by the size factor (median ratio). Besides, DESeq2 used a model based on negative binomial distribution for significant test. In addition, adjusted p value (padj) was calculated based on Benjamini and Hochberg's method to control for false discovery rate (Hochberg

and Benjamini, 1990). Genes in agreement with the screening threshold, |log2(FoldChange)| ≥1 and padj ≤ 0.05, were assigned as significant DEGs.

5.2.4.5 Enrichment analysis of differentially expressed genes

Gene Ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment, and DisGeNET enrichment of the differentially expressed genes were performed by a clusterProfiler R package. Padj smaller than 0.05 was considered as significantly enriched by the differentially expressed genes.

5.3 Results

5.3.1 Alignment of sequencing reads

Concentrations and quality of total RNA samples from HHL-16 cells treated with AFB_1 and its control group were tested for sample quality control, all the RNA samples passed the sample quality control, having a concentration of at least 200 ng/μl and the best integrity value of 10 (Appendix 5.1). All RNAs that passed these criteria were used for sequencing, and a total of raw reads approximately ranged from 61 million to 81 million of 150bp-paired ends, with high quality metrics (Q30) were collected. After data filtering of reads with adapter, undetermined reads, and low-quality reads, there were a minimum of 61 million reads in each of the six samples, and over 96% reads in each sample were successfully mapped after the alignment to human reference genome (Homo sapiens: GRCh38/hg38) (Table 5-1).

Table 5-1 Summary of RNA-Seq datasets in HHL-16 cells.

Sample	Raw reads	Clean reads	Q20	Q30	GC %	Total map (%)
D20_1	62197290	61235132	97.45	92.97	49.76	58871277(96.14)
D20_2	78716718	76914754	97.46	93.04	49.98	74008832(96.22)
D20_3	81508632	80514018	97.59	93.32	49.59	77674229(96.47)
A20_1	81284790	80136996	97.28	92.69	49.83	77047567(96.14)
A20_2	79376720	78113318	97.43	93.02	50.06	75257819(96.34)
A20_3	82993838	81694306	97.59	93.2	49.6	78994023(96.69)

D20: 1 in 1000 diluted DMSO treatment (equivalent to the concentration in 20 μg/ml AFB_1 treatment) in HHL-16 cells for 24 hours. A20: 20 μg/ml AFB_1 treatment.

5.3.2 Differential gene expression analysis

Gene expression levels (FPKM) in each sample were calculated, and correlation analysis and principal component analysis were performed. The results showed that there was a good correlation among the three biological replicates, and the principal component analysis (PCA) plot indicated a clear separation between AFB_1 treated group and its control group (Appendix 5.2). In the DEGs analysis, the volcano map showed that there were 280 up-regulated and 296 down-regulated genes in HHL-16 cells after 20 µg/ml AFB_1 treatment were found (Figure 5-3). The 20 most up-regulated and down-regulated genes were summarised (Table 5-2). Noteworthy, the four differentially expressed genes (*IL6*, *CCL20*, *BMP2*, and *NDP*) found in Chapter 4, Section 4.3.6, Figure 4-8, is consistent with the RNA-Seq result in this chapter. Therefore, in the RNA-Seq results, *IL6*, *CCL20*, *BMP2* were also significantly up-regulated in HHL-16 cells after 20 µg/ml AFB_1 treatment for 24 hours with Log2FoldChange of 1.91, 3.35 and 1.31 (padj=2.18E-23, 0.0001, and 1.33E-13), respectively, and it was also confirmed that *NDP* was significantly down-regulated with Log2FoldChange of -2.37 (padj=0.0002). Notably, apart from gene expression of *CYP1A2*, *3A4*, and *3A5* tested in Chapter 4, Section 4.3.3, Figure 4-5, the RNA-Seq data indicated that another gene encoding a CYP enzyme, *CYP1A1*, was also significantly up-regulated (Log2FoldChange=1.82, padj=1.00E-12) in HHL-16 cells after the AFB_1 treatment.

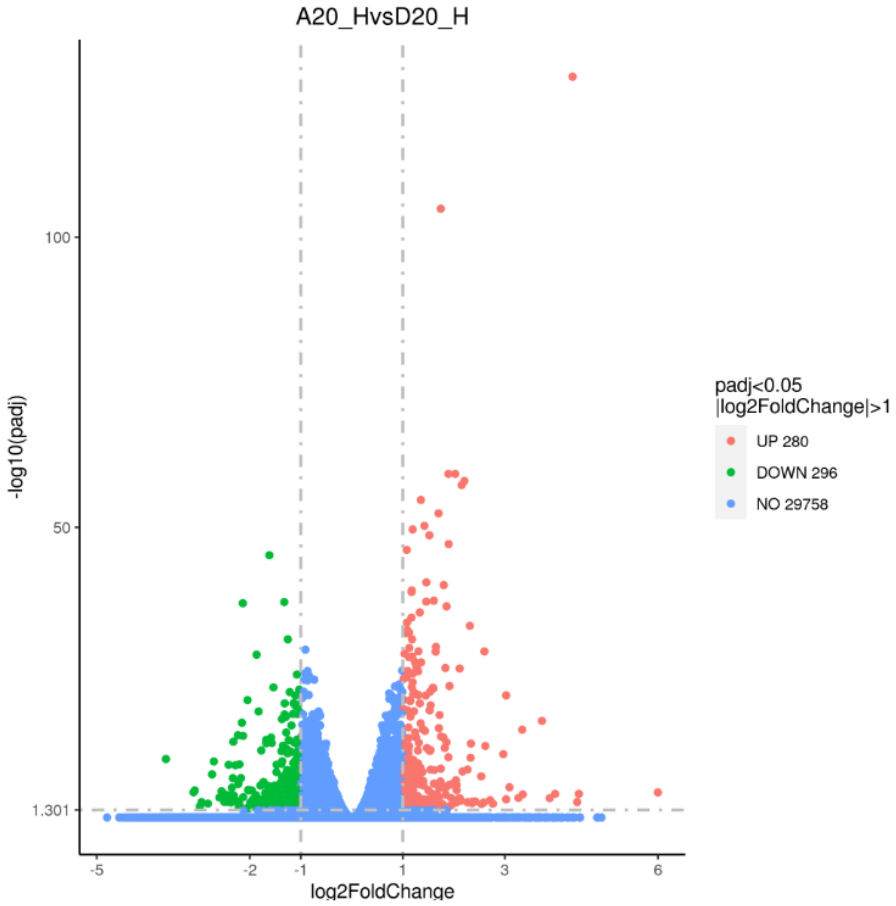


Figure 5-3 Volcano map of differentially expressed genes in HHL-16 cells after 20 μg/ml AFB_1 treatment for 24 hours.

Table 5-2 Summary of 20 the most up-regulated genes in HHL-16 cells after 20 μg/ml AFB_1 treatment for 24 hours.

No.	Gene name	Log2FoldChange	Padj	Gene description
1	*CALCA*	6.00	4.69E-05	Calcitonin related polypeptide alpha
2	*TUBB2BP1*	4.45	8.36E-05	Tubulin beta 2B class IIb pseudogene 1
3	*AC022217.2*	4.42	0.002	Novel transcript
4	*IL24*	4.33	2.14E-128	Interleukin 24
5	*RHCG*	3.99	8.45E-05	Rh family C glycoprotein
6	*AC022217.1*	3.88	0.0005	Family with sequence similarity 58, member A (FAM58A) pseudogene
7	*C11orf96*	3.73	2.22E-17	Chromosome 11 open reading frame 96
8	*CCL20*	3.35	0.0001	C-C motif chemokine ligand 20
9	*CXCL8*	3.34	7.23E-16	C-X-C motif chemokine ligand 8
10	*AL596223.1*	3.27	0.0004	Uncharacterized LOC101928994
11	*ARC*	3.09	6.17E-06	Activity regulated cytoskeleton associated protein
12	*NPPC*	3.02	0.0007	Natriuretic peptide C
13	*HAP1*	3.02	8.97E-22	Huntingtin associated protein 1
14	*DMBT1*	2.97	1.22E-11	Deleted in malignant brain tumors 1

No.	Gene name	Log2FoldChange	Padj	Gene description
15	*AC003092.1*	2.77	0.004	Novel transcript
16	*SPINK1*	2.71	0.001	Serine peptidase inhibitor, Kazal type 1
17	*NR4A3*	2.62	4.64E-13	Nuclear receptor subfamily 4 group A member 3
18	*UBE2QL1*	2.60	2.39E-29	Ubiquitin conjugating enzyme E2 Q family like 1
19	*ZNF341-AS1*	2.58	0.003	ZNF341 antisense RNA 1
20	*IL1RL1*	2.54	0.005	interleukin 1 receptor like 1

Table 5-3 Summary of 20 the most down-regulated genes in HHL-16 cells after 20 μg/ml AFB_1 treatment for 24 hours.

No.	Gene name	Log2FoldChange	Padj	Gene description
1	*NLGN1*	-3.64	8.36E-11	Neuroligin 1
2	*LINGO2*	-3.10	4.48E-05	Leucine rich repeat and Ig domain containing 2
3	*AC110373.1*	-3.08	2.19E-05	Glycerol kinase 2 pseudogene
4	*BMS1P7*	-2.98	0.01	BMS1, ribosome biogenesis factor pseudogene 7
5	*BNC2-AS1*	-2.95	0.002	BNC2 antisense RNA 1
6	*TMEM178B*	-2.82	0.003	Transmembrane protein 178B
7	*GRIP1*	-2.74	3.69E-08	Glutamate receptor interacting protein 1
8	*PAPPA2*	-2.70	2.20E-10	Pappalysin 2
9	*KCNIP1*	-2.60	0.0004	Potassium voltage-gated channel interacting protein 1
10	*PDGFD*	-2.55	2.92E-05	Platelet derived growth factor D
11	*PRKCQ*	-2.50	0.002	Protein kinase C theta
12	*EDN2*	-2.49	0.0002	Endothelin 2
13	*ENOX1*	-2.46	0.0001	Ecto-NOX disulfide-thiol exchanger 1
14	*TNFSF18*	-2.45	0.0006	TNF superfamily member 18

No.	Gene name	Log2FoldChange	Padj	Gene description
15	*PCDHB13*	-2.45	0.002	Protocadherin beta 13
16	*PCDH18*	-2.45	0.0003	Protocadherin 18
17	*MDGA2*	-2.41	8.19E-10	MAM domain containing glycosylphosphatidylinositol anchor 2
18	*GNG7*	-2.40	0.0003	G protein subunit gamma 7
19	*PCDHB14*	-2.37	0.003	Protocadherin beta 14
20	*NDP*	-2.36	0.0002	Norrin cystine knot growth factor

5.3.3 GO enrichment analysis of the differentially expressed genes

Totally, 576 significantly differentially expressed genes in HHL-16 cells in response to AFB_1 were used for GO enrichment analysis to identify the gene properties from three aspects, biological process (BP), cellular component (CC), and molecular function (MF). The top 10 enriched GO terms for each category are shown in Figure 5-4. Within the biological process category, 10 of the most significantly enriched terms were listed in the figure, which included: regulation of signalling receptor activity, regulation of cation channel activity, and response to molecule of bacterial origin. In cellular component category, the most abundant groups were synaptic membrane, postsynapse, and postsynaptic membrane. In the molecular function category, totally 6 significantly enriched terms were found, which included receptor ligand activity, cytokine activity, receptor regulator activity, acetylgalactosaminytransferase activity, growth factor activity, and cytokine receptor binding (Figure 5-4).

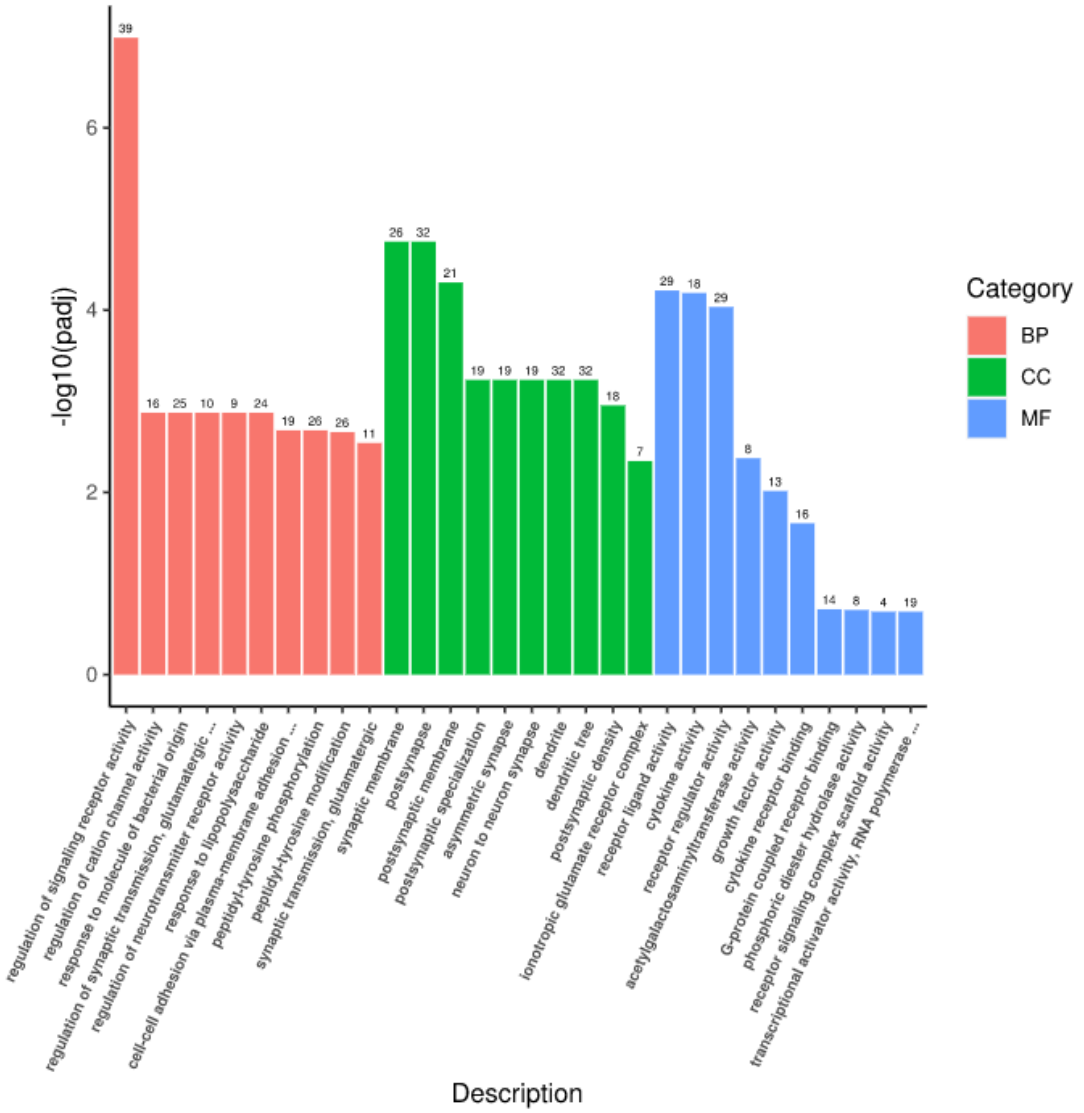


Figure 5-4 Histogram of GO enrichment analysis of the differentially expressed genes in HHL-16 cells in response to the AFB_1 treatment. BP: biological process; CC: cellular component; MF: molecular function. The most significant 10 GO Terms for each category were selected for display. If the significant GO Terms of each category were less than 10, the other terms followed by that not significant would also be drawn and shown in the figure to make it to 10 GO terms displayed.

5.3.4 KEGG enrichment analysis of the differentially expressed genes

To find the significantly enriched pathways related to the DEGs, KEGG enrichment analysis was carried out. The top 20 enriched KEGG pathways were selected as shown in Figure 5-5. The KEGG enrichment analysis indicated that 7 pathways were significantly enriched, including cytokine-cytokine receptor interaction, NF-kappa B signalling pathway, TNF signalling

pathway, IL-17 signalling pathway, Amoebiasis, MAPK signalling pathway, and Lipid and atherosclerosis (Figure 5-5). The detailed information of significant levels, up-regulated, and down-regulated genes involved in the 7 significantly enriched pathways was summarised (Table 5-4).

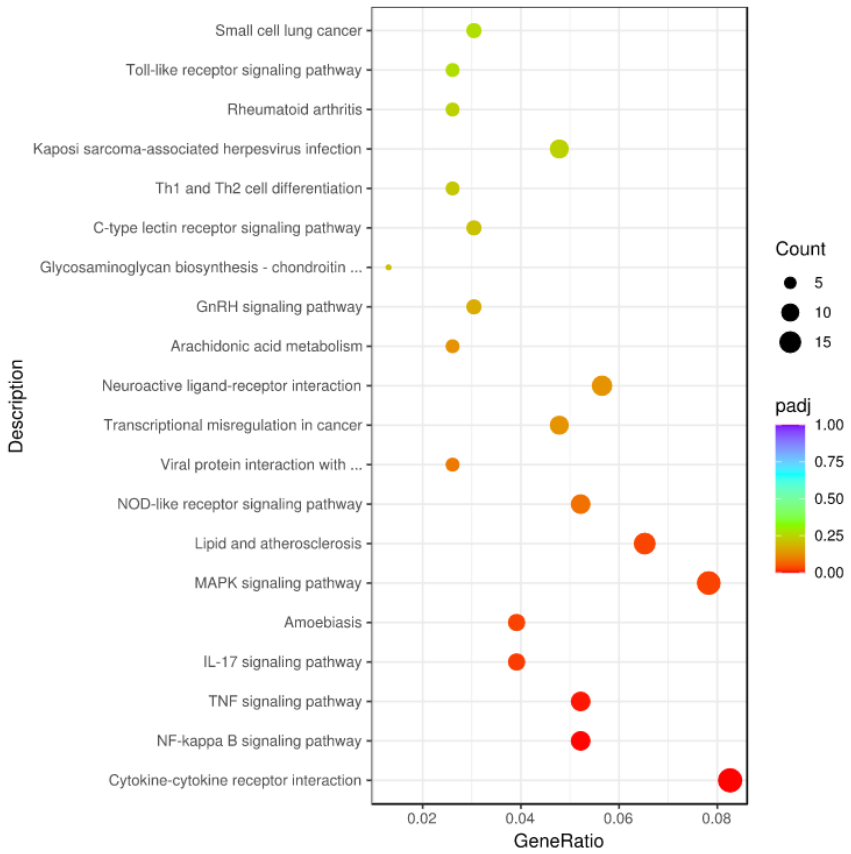


Figure 5-5 Scatter plot of KEGG enrichment analysis of the differentially expressed genes in HHL-16 cells in response to the AFB_1 treatment. The size of a point represents the gene numbers that annotated to the specific KEGG pathway. The colour from purple to red represents the significant levels of enriched pathways.

Table 5-4 Summary of significant levels, up-regulated and down-regulated genes in the significantly enriched pathways from KEGG analysis.

Significantly enriched pathways	**Padj**	**Up-regulated genes**	**Down-regulated genes**
Cytokine-cytokine receptor interaction	0.0003	*IL24, IL11, IL6, LIF, CXCL8, BMP2, CXCL2, TNFSF15, NGFR, CXCL3, IL1RN, IL10RA, IL1RL1, TNFRSF9, CSF2*	*INHBB, IL17RE, TNFSF18, TNFSF4*
NF-kappa B signalling pathway	0.0007	*RELB, GADD45A, NFKB2, TNFAIP3, TRAF1, BIRC3, CXCL8, CXCL2, PTGS2, CXCL3*	*PRKCQ, CD14*
TNF signalling pathway	0.005	*TNFAIP3, IRF1, IL6, TRAF1, LIF, BIRC3, CXCL2, PTGS2, CXCL3, CSF2*	*MAP2K6, MAPK10*
IL-17 signaling pathway	0.02	*TNFAIP3, IL6, CXCL8, CXCL2, PTGS2, CXCL3, CSF2*	*IL17RE, MAPK10*
Amoebiasis	0.02	*TNFAIP3, IL6, CXCL8, CXCL2, PTGS2, CXCL3, CSF2*	*PLCB4, COL1A1, LAMA2, CD14*
MAPK signalling pathway	0.02	*DDIT3, RELB, GADD45A, NFKB2, AREG, PLA2G4C, DUSP2, NGFR, SCAT8, DUSP5, HSPA6, MAPK8IP2*	*PDGFD, CACNG4, MAP2K6, CD14, DUSP9, MAPK10*
Lipid and atherosclerosis	0.03	*DDIT3, ERN1, IL6, CXCL8, IRF7, CYP1A1, CXCL2, CXCL3, HSPA6,*	*PLCB4, CAMK2A, MAP2K6, CD14, MAPK10*

5.3.5 DisGeNET enrichment analysis of the differentially expressed genes

Significantly associated human diseases to the DEGs in HHL-16 cells in response to the AFB_1 treatment were also discovered by using the DisGeNET database. In total, 47 significantly enriched terms of human diseases were found (Appendix), 20 of the most significantly enriched diseases were displayed in the figure (Figure 5-6). The results indicated that the most associated diseases included inflammation, some types of vascular diseases (i.e. myocardial ischemia), musculoskeletal diseases, premature birth, fetal diseases, susceptibility to infection (i.e. helicobacter pylori infection), and cancer (i.e. primary effusion lymphoma).

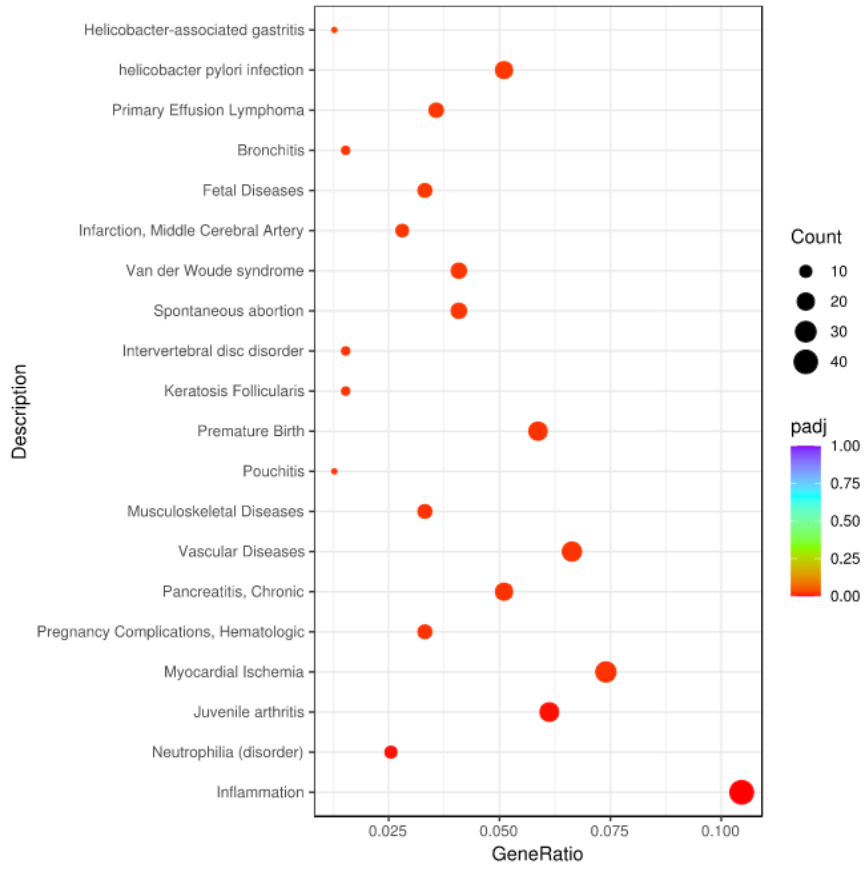


Figure 5-6 Scatter plot of DisGeNET enrichment analysis of the differentially expressed genes in HHL-16 cells in response to the AFB_1 treatment. The top 20 significantly enriched human diseases terms associated to the DEGs were displayed. The size of a point represents the gene numbers that annotated to the specific human disease. The colour from purple to red represents the significant levels of the enrichment.

5.4 Discussion

In this study, we have characterised the transcriptome profile of HHL-16 cells in response to AFB_1 treatment, and the main findings of the study were: 1) 576 significant DEGs were found in HHL-16 cells after 20 µg/ml AFB_1 treatment for 24 hours; 2) 7 significantly enriched pathways associated to the DEGs were discovered, including cytokine-cytokine receptor interaction, NF-kappa B signalling pathway, TNF signalling pathway, IL-17 signalling pathway, Amoebiasis, MAPK signalling pathway, and Lipid and atherosclerosis; 3) significantly enriched human diseases related to the DEGs consisted of the examples of inflammation, early development disease, and cancer. Therefore, in this study, we concluded that AFB_1 modulates the expression of genes related to the pathways that have crucial roles in inflammatory response, growth, and cancers.

To our knowledge, this is the first study to analyse the transcriptome changes in a non-tumorigenic hepatocyte cell line in response to AFB_1. Compared to traditional microarray-based methods, RNA-Seq has several advantages on the analysis of transcriptome changes, which is it is more quantitatively accurate, has much lower limit of detection, and the ability to detect novel transcripts (Mantione et al., 2014; Rai et al., 2018). In this study, apart from the annotated transcripts, some novel transcripts were found in HHL-16 cells after 20 µg/ml AFB_1 treatment for 24 hours, such as: AC245041.2, AC007249.1, and AC007249.2, etc. Additionally, 576 significantly DEGs were found, and the differentially expressed patterns of the four DEGs (*IL6*, *CCL20*, *BMP2*, and *NDP*) measured by RT-qPCR in Chapter 4, Section 4.3.6, Figure 4-8, are consistent with the results measured by RNA-Seq method, indicating the RNA-Seq results are reliable and reproducible. Therefore, to ensure a consistent result of gene expression, it is important to use separate RT-qPCR to validate RNA-Seq results, which has been an empirical practice that widely adopted in various RNA-Seq studies (Liu et al., 2018; Zhang et al., 2018; Reddy et al., 2018; Ma et al., 2021).

GO terms associated with the significant DEGs in HHL-16 cells were divided into three groups: biological process, cellular component, and molecular function. In the group of molecular function, annotated molecular functions of genes in pathways such as: cytokine activity, cytokine receptor binding, and growth factor activity were found to be significantly enriched that related to the DEGs. The GO term, cytokine activity, significantly enriched in macrophages after AFB_1 treatment for 24 hours was also observed (Zhang et al., 2023).

Another GO term, cytokine receptor binding, was reported in human lymphoblastic T cells treated with AFB_1 (Frangiamone et al., 2023). The enriched GO term, growth factor activity, seems to be the first found in this study in terms of aflatoxin caused DEGs, and not reported in other studies investigating transcriptome changes in animals or cell lines exposed to aflatoxin. However, significantly enriched growth factor activity was reported in several human cancer tissues (Rahman et al., 2019; Wang et al., 2020; Zhang et al., 2020; Hong et al., 2021; Ouyang et al., 2022). Therefore, significant DEGs in HHL-16 cells in response to AFB_1 enriched in the molecular functions of inflammatory and growth factor-related terms, is in agreement with previous evidence that aflatoxin affects genes involved in immune and growth factors pathways (Yarru et al., 2009).

Interestingly, some neuro-relevant GO terms such as: synaptic membrane, postsynapse, and postsynaptic membrane, were observed at significant levels of enrichment in HHL-16 cells after the AFB_1 treatment. The odd results led to a speculation of whether neuro characteristics expressed in this cell line. Identification of gene expression of tissue-specific genes (gene markers) is used to characterise cell lines (Qiu et al., 2021). Several neuro-specific or biased gene markers including *MOG*, *GRIN2B*, and *GAD2* were validated with high gene expression in different types of neuronal cells (Brenner et al., 1994; Iida and Kozasa, 2004; Kodama et al., 2012), however, all those neuronal gene markers were not expressed (FPKM=0) in HHL-16 cells (Appendix 5.3), so it is less likely to be neuro-specific cell types existed in HHL-16 cells. To further investigate explanations for the result, a summary of DEGs enriched in neuro-related GO terms was listed (Appendix 5.4), and after blast analysis of those genes in NCBI gene database (https://www.ncbi.nlm.nih.gov/gene), it was found that the majority of the listed DEGs are highly expressed in the brain, but also expressed in gall bladder or the liver with relatively lower level expression. For example, one of the listed DEGs in HHL-16 cells, *MAP2* (microtubule associated protein 2), is particularly expressed in brain (RPKM=82.4) and expressed in gall bladder (RPKM=1.9) as the NCBI RNA-Seq database indicated (Fagerberg et al., 2014). According to the published data of HHL-16 cells, it was confirmed that both biliary and hepatocyte characteristics are displayed in the cells (Clayton et al., 2005), so it provides conceivable evidence that we found lower level expression of those listed DEGs from the RNA-Seq data in HHL-16 cells. Besides, neuro-related GO terms found in hepatocytes treated with AFB_1 were also reported in a bovine fetal hepatocyte-derived cell line (BFH12) as well as human hepatocellular carcinoma cell line (PLC/PRF-5)

(Pauletto et al., 2020; Zhu et al., 2021). Since the listed DEGs are abundantly expressed in neuronal cells or tissue in GO enrichment analysis, it is more likely for those genes to be annotated as neuro cell components, therefore homology-based interference is possibly the reason for the findings of neuro-related GO terms in HHL-16 cells. This issue found in current study indicated a drawback of bioinformatic analysis of RNA-Seq, it is important to eliminate and avoid the crosstalk among DEGs when performing GO enrichment analysis. However, to ensure the integrity of the results here, those listed DEGs with low transcripts were not filtered out for further enrichment analysis.

In KEGG enrichment analysis of DEGs in HHL-16 cells after the AFB_1 treatment, the most significantly enriched pathways were cytokine-cytokine receptor interaction, NF-kappa B signalling pathway, and TNF signalling pathway. The set of DEGs were most significantly involved in cytokine-related pathway, such as: up-regulation of *IL24*, *IL11*, *IL6*, *LIF*, *CXCL8* and *IL1R,* and down-regulation of *INHBB*, *IL17RE*, *TNFSF18*, and *TNFSF4*. An imbalanced expression of cytokines was extensively reported in immune-related organs of animals or cell lines in response to AFB_1, which indicated an inflammatory response and affected immune function (Meissonnier et al., 2008; Bruneau et al., 2012; Qian et al., 2014; Jiang et al., 2015; Iori et al., 2022). Thereby, in view of the interference of cytokine expression in HHL-16 cells caused by AFB_1, at least it is possible to predict that an inflammatory response could be activated in response to the stimulus of AFB_1.

In this study, DEGs enriched in NF-kB pathway included upregulation of *RELB*, *NFKB2*, *GADD45A*, *TNFAIP3*, and *BIRC3*, among others, and downregulation of *PRKCD* and *CD14*. NF-kB pathway is a well-known pathway implicated in various cancers (Chen et al., 2002; Li et al., 2010; Hassanzadeh, 2011), which might be because of the involvement of many transcription factors in the pathway that can be commonly induced by different cellular stimulus to regulate immune and inflammatory response to protect cells from apoptosis (Yamamoto and Gaynor, 2001; Dolcet et al., 2005). Evidence indicated that NF-kB can be activated both by pro-inflammatory cytokines and members of TNF signalling pathway (Zinatizadeh et al., 2021). Excessive production of IL-17A, IL-21, IL-22, TNF-α, and lL-6 were observed in mouse of colorectal cancers, and it was associated with increased activation of STAT3/NF-kB pathway (De Simone et al., 2015). Thereby, we propose up-regulation of several pro-inflammatory cytokines, such as: *IL6*, may potentially activate the NF-kB pathway. In the TNF signalling pathways, up-regulated genes including *TNFAIP3*, *IRF1* and *TRAF1*, were observed in present study. It was evident that TNFAIP3 is an inhibitor of

NF-kB pathway (Boone et al., 2004; Schioppa et al., 2022), while stimulatory effect of TRAF1 on NF-kB was found (Cabal-Hierro et al., 2014), therefore, taken together, the evidence implies a dynamic modulation in terms of NF-kB pathway.

It was reported that in normal cells, NF-kB becomes transiently activated in response to cellular stimuli, then return the inactive status after regulatory mechanisms (Dolcet et al., 2005). However, in tumour cells, multiple mechanism may cause impaired modulation of NF-kB, leading to a continuously activation of the pathway, which facilitates the proliferation and progression of tumour cells and finally contribute to cancers (Kojima et al., 2004; Ogunwobi et al., 2019). Therefore, in this study, according to the affected pathways: cytokine-related, TNF, and NF-kB pathways in HHL-16 cells by AFB_1, together with previous evidence abovementioned, the potential mechanism we could propose is that AFB_1 can cause an inflammatory response, which might further activate NF-kB to regulate genes in response to the stress caused by AFB_1. Meanwhile, TNF signalling pathway has duplex modulation of NF-kB activation via both enhancing activation and suppressing activation. Once the modulation balance of NF-kB is disrupted in a long term under the effects of consistent stimulation of AFB_1, eventually the cells will be driven to an unlimited proliferation and progression of cancer.

In addition, evidence indicated that chronic inflammation was linked to growth impairment by causing disruption of GH-IGF1 axis as well as the link to insulin resistance (Cirillo et al., 2018). Over-expressed IL6 in transgenic mice showed chronic inflammation, which was associated with decreased IGF1 level in serum and caused growth impairment (De Benedetti et al., 1997). However, in HHL-16 cells, we did not observe differentially expressed genes in GH-IGF1 axis. After a deep investigation into RNA-Seq data of gene expression levels (FPKM) of GH-IGF1 axis, we found that genes involved in the GH-IGF1 axis including *GH1*, *GH2*, *GHR*, *IGF1*, and *IGF2* were not expressed in HHL-16 cells, which limited to validate whether increased IL6 lead to abnormalities to GH-IGF axis in the cells after AFB_1 treatment. Generally, IGF1 is predominantly produced by the liver (Kineman et al., 2018). One reason for the absence of GH-IGF1 axis in HHL-16 cells may because the expression of genes in the axis were altered when the cell line was immortalised. Therefore, the effects of IL6 on GH-IGF1 axis are worthy to be investigated in other cell lines or animal models exposed to aflatoxin.

In summary, in AFB_1 treated HHL-16 cells, over 500 DEGs were found, and the most significantly involved pathways of the DEGs were cytokine-related, TNF, and NF-kB pathways. Therefore, it is at least conceivable to hypothesize that AFB_1 modulates the expression of gene sets implicated in inflammatory, growth factors, and cancer, through the interactions among cytokine-related/TNF/NF-kB pathways.

Chapter 6 Metabolomic changes due to aflatoxin exposure in children from Africa and South Asia

6.1 Introduction

Aflatoxin exposure has been reported to cause acute aflatoxicosis, liver cancer, and implicated with immune suppression and child growth impairment (Wild et al., 2015; Gong et al., 2016a). With respect to the mechanistic investigation of aflatoxin toxicity in Chapter 4 and 5 in a hepatocyte cell line (HHL-16), it was found that aflatoxin can affect expression of a number of genes with a diverse range of functions, including genes associated with the inflammatory response, growth factors, and cancer. And aflatoxin caused epigenetic changes may contribute to the changes in gene expression. However, the pathways affected by aflatoxin in the cells may have limitations to be interpreted and applied to *in vivo*. Therefore, current techniques using the integration of multi-omics can facilitate the identification of more specific biomarkers that indicate systematic response to endogenous or exogenous changes were applied (Nicholson and Wilson, 2003).

Omics integration-based strategies including transcriptomic, metabolomic, proteomic, and lipidomic analysis provides new insight into the molecular mechanism of aflatoxins. Aflatoxins have been observed to affect the transcription of genes involved in the inflammatory response in several studies that have been performed in animals and cell lines (Ma et al., 2021; Chao et al., 2022; Xu et al., 2022). For examples, the dysregulation of amino acid metabolism was reported in AFB_1-treated dairy cows, goats, and a human hepatocyte cell line (Hep3B) (Wang et al., 2019; Wang et al., 2021; Shi et al., 2022). Alteration of lipid metabolism was also found in rats exposed to AFB_1 (Rotimi et al., 2017). According to the evidence found in these, aflatoxin can affect transcriptome, metabolome, proteome, and lipidome. However, because of the limited access to human samples, current interests of using omics-technique to study aflatoxin toxicity in cell lines or animal models take precedence over human studies. Therefore, the opportunity to perform the multi-omics analysis in human samples may help to better understand the health effects of aflatoxin on humans.

In the CNCC study described in Chapter 3, apart from the 345 tested samples, another batch of serum samples of acutely ill children from the CNCC study were received and tested for aflatoxin exposure levels by Dr. Lei Xia in our lab. Serum metabolomics, serum lipidomics, and plasma proteomics analysis were performed by the CHAIN consortium collaborated labs (Njunge et al., 2022). With the access to the three omics datasets together with data of aflatoxin exposure levels in those children, we have an opportunity to analyse the

alteration of key metabolomic, lipidomic, and proteomic parameters in the children due to aflatoxin exposure. These experiments might help us to find targeted metabolites, lipids, and proteins to prevent aflatoxin-related diseases, and may provide potential targets for the therapeutic intervention.

This chapter of the study aimed to investigate the metabolomic alterations due to aflatoxin exposure in the acutely ill children in Africa and South Asia, to find the associated metabolites to aflatoxin exposure, and to investigate enriched pathways of the associated metabolites.

6.2 Methods

6.2.1 Study subjects

Participants in this study were the same as the children involved in the CNCC study (Njunge et al., 2022), which was originally recruited in the CHAIN study (The Childhood Acute Illness and Nutrition (CHAIN) Network, 2022). Detailed information of the participants and the ethical approval were described in Chapter 3, Section 3.2.1. The number of analysed participants in the current study were less than the CNCC study, because only the 24% random sub-cohort of the CNCC study were used, and participants lacking enough serum volume for aflatoxin exposure assessment, or without metabolites data were excluded. Finally, a total of 883 participants with available data of both aflatoxin exposure levels and metabolites levels were included.

6.2.2 Aflatoxin-albumin adduct measurement

AF-alb levels in the second batch serum samples of the acutely ill children from the CNCC study were assessed in our lab by Dr. Lei Xia, using the ELISA method as described in Chapter 2.2 (Chapot and Wild, 1991, with minor modifications) .

6.2.3 Serum metabolomics analysis

150 metabolites levels in serum samples of the children were quantified by University of Alberta, using LC-MS/MS assay following the method as published (Njunge et al., 2022). Non-detectable samples were assigned a value of half LOD for analyses. LOD values for each metabolite are summarised in Appendix 6.1.

6.2.4 Data collection

Demographic, strata data based on malnutrition status, aflatoxin exposure levels, and metabolite levels of the analysed children at the admission timepoint were collected. In detail, the enrolled children in this study were grouped into three strata based on the mid-upper arm circumference (MUAC): 1) non-wasted (NW): MUAC ≥ 12.5 cm for age ≥ 6 months or MUAC ≥ 12 cm for age < 6 months; 2) moderately wasted (MW): MUAC 11.5-12.5 cm for age ≥ 6 months or MUAC 11-12 cm for age < 6 months; 3) severely wasted (SW): MUAC <11.5cm for age ≥ 6 months or MUAC <11cm for age < 6 months (Njunge et al., 2022). Another two groups of children with oedema and community children were classified into oedema (EM) and community participants (CP) groups, respectively (Njunge et al., 2022). Variables of children's gender (male or female), age (2-24 months), MUAC, continent (Africa or South Asia), oedema (with oedema or not), parttype (community participants or not), and site (9 sites in Africa and South Asia), and sample batch of metabolites testing (three batches), and AF-alb and 150 metabolites levels were collected in those children.

6.2.5 Analysis of associated metabolites to AF-alb

150 metabolites (Appendix 6.2) and 20 biologically relevant metabolite class and ratios were analysed in both Boruta and Negative Binominal Generalised Linear Mixed Models (NB GLMM) to find the associated metabolites to AF-alb by processing the analysis in R Studio (Version 4.6.5). First, Boruta modelling was performed, which is based on a feature selection algorithm, and it uses a random forest classifier to select the relevant features and remove un-useful features (Kursa and Rudnicki, 2010). With detailed process of Boruta modelling (Figure 6-1), first the 150 metabolites and 20 metabolite class and ratios as original features will be shadowed, then the copied features will be shuffled to remove their correlation between the dependent variable (AF-alb). Then the original features (150 metabolites+20 metabolite class and ratios) were combined with the copied features (shadowed 150 metabolites) to run through a random forest classifier on the combined dataset and perform a variable importance measurement (Z score calculation). Finally, the feature importance was determined as "Rejected", "Tentative" and "Confirmed" based on the maximum threshold of the shadow feature (highlighted in red font) after the maximum runs (500 runs in this study) as custom set. Sometimes, after the maximum run, the importance of a few metabolites cannot be determined, which come out to be the decision of "Tentative". Unimportant metabolites lower

than the maximum threshold of shadow feature were determined as “Rejected” and original metabolites over the maximum threshold of feature shadow had the decision of “Confirmed” as important. Metabolites with the decision of “Tentative” and “Confirmed” were regarded as important metabolite hits for further analysis.

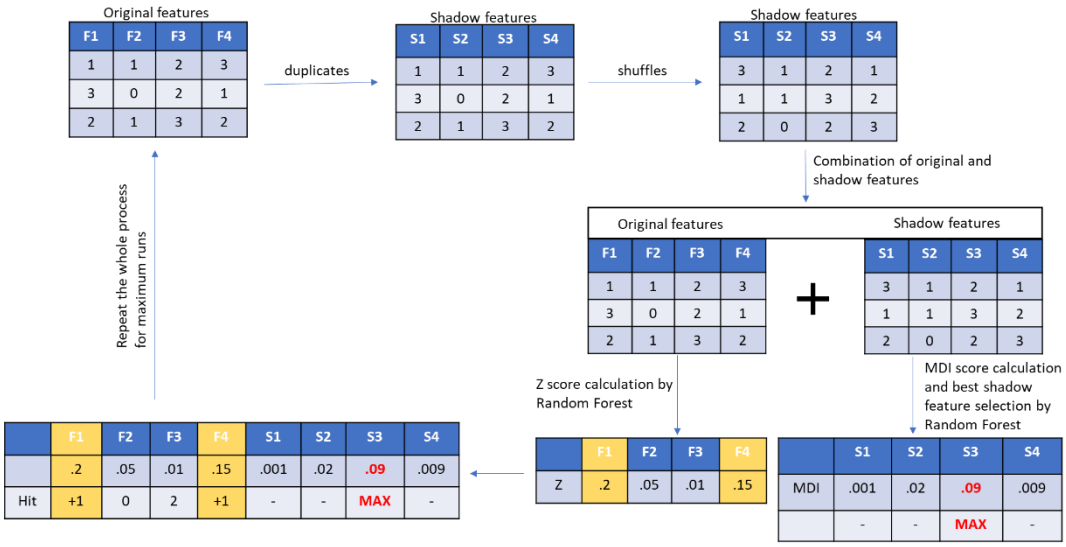


Figure 6-1 Process of Boruta modelling. MDI: Mean Decrease Impurity.

NB GLMM was also used to examine the associated metabolites to AF-alb, which can examine the estimates of the fixed effects and random effects and perform the statistical significance level. Before testing all metabolites in NB GLMM, two fit models were tested to choose a better fitted model. The first fit model was AF-alb as dependent variable, 150 metabolites and 20 metabolite class and ratios as independent variable, adjusted with the variables of MUAC, sex, oedema, Africa, age, sample batch of metabolites testing as fixed effects, and site as random effect. The second fit model was adjusted with the variables of MUAC, oedema, Africa, age, and parttype as fixed effects, and site as random effect. Selection of better fitted model was carried out based on the value of Akaike information criterion (AIC) measured by out of sample deviance, which is an estimator of prediction error (Hewson, 2016). Therefore, the two fit models tested with smaller AIC value was regarded as better fitted model, and it was selected for the analysis to find associated metabolites. Significantly associated metabolites were determined by adjusted *p* value smaller than 0.05.

6.2.6 Enrichment and pathway analysis of the associated metabolites to AF-alb

To explore the pathway involvement of the associated metabolites analysed from Boruta and NB GLMM modelling, pathway analysis of the candidate

metabolites were performed in the Pathway Analysis module on the website of MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/MetaboAnalyst/ModuleView.xhtml), which integrates pathway enrichment and topology analysis (Xia and Wishart, 2016). A list of 46 candidate metabolites in a total were used in the pathway analysis. The concentration table of each candidate metabolite in the 883 children was split into two groups: AF-alb positive and negative groups, and was uploaded into MetaboAnalyst. A total of 0.1% missing values for metabolite concentrations were replaced by 1/5 of minimum positive values of their corresponding variables by default. Then the dataset was proceeded to a name standardization from the conversion of metabolite names to KEGG ID (Appendix 6.3). Data was log base 10 transformed and standardized by auto-scaling. Global test as enrichment method and relative-betweeness centrality as topology analysis were performed in the selected Homo sapiens KEGG pathway library. All tested compounds were used as reference metabolome. Pathways that were significantly affected were found based on the criteria: FDR (adjusted *p* value using False Discovery Rate) smaller than 0.05 and pathway impact over 0.1.

6.3 Results

6.3.1 Characteristics of the enrolled children in the study

Table 6-1 summarises the characteristics of the enrolled children in this study. In the enrolled 883 children, 436 children (49%) with a median age of 9.2 months (IQR 5.8-14) had non-detectable AF-alb, and 447 children (51%) with a median age of 13 months (IQR 8.3-18) had detectable AF-alb, and the median AF-alb was 8.0 pg/mg (IQR 4.4-20) in the aflatoxin exposure positive children. Non-wasted children, moderately wasted children, and community participants had more AF-alb negative samples than AF-alb positive samples (neg vs pos: 51% vs 49%, 54% vs 46%, 56% vs 44%, respectively), while severely wasted children and children with oedema had higher percentage of AF-alb positive samples than the percentage of AF-alb negative samples (neg vs pos: 47% vs 53%, and 22% vs 78%, respectively). In Africa, more AF-alb positive samples (387, 70%) were observed in comparison to AF-alb negative samples. Children in the age groups younger than 12 months (< 6 months and 6-12 months) had more AF-alb negative samples, while higher percentage of AF-alb positive samples (neg vs pos: 40% vs 60%) was found in the age group over 12 months. Besides, in the AF-alb positive group, with the increasing of age group, the

percentage of AF-alb positive samples was gradually increased from 35% to 47% and finally reached to 60%, from the age groups of younger than 6 months to 6-12 months, and over 12 months, respectively.

Table 6-1 Characteristics of the enrolled children.

Characteristic	Aflatoxin Exposure	
	neg, N = 436[1]	**pos**, N = 447[1]
Sex, male	252 (49%)	262 (51%)
Group		
NW, n=241	122 (51%)	119 (49%)
MW, n=167	90 (54%)	77 (46%)
SW, n=180	85 (47%)	95 (53%)
EM, n=74	16 (22%)	58 (78%)
CP, n=221	123 (56%)	98 (44%)
Africa, n=552	165 (30%)	387 (70%)
Age, months	9.2 (5.8, 14)	13 (8.3, 18)
Age group		
≥12 months	162 (40%)	247 (60%)
6-12 months	161 (53%)	140 (47%)
< 6 months	113 (65%)	60 (35%)
AF-alb (pg/mg)	-	8.0 (4.4, 20)

[1] Median (IQR) or Frequency (%)

Data was presented as median (IQR). Neg: negative; pos: positive; NW: non-wasted; MW: moderately wasted; SW: severely wasted; EM: children with oedema; CP: community participants. IQR: Interquartile range.

6.3.2 Associated metabolites to AF-alb in Boruta modelling

In total, 150 metabolites were quantified by the University of Alberta group involved in CHAIN, and the levels of each metabolite were summarised (Appendix 6.2). The total metabolites and 20 biologically relevant metabolite class and ratios were analysed in the Boruta model to find the associated metabolites to aflatoxin exposure. After maximum run of 500 times and based on the mean of importance, 20 metabolite hits with the decision of either “Tentative” (yellow boxes) or “Confirmed” (green boxes) (Appendix 6.4) were determined as important metabolites (Figure 6-2), which included 15 metabolites (i.e. sm.oh.c14.1, tyrosine, serine, and lysopc.a.c16.0) and 5 metabolite class including ketogenic acids, essential amino acids, total amino acids, glucogenic amino acids, and urea cycle amino acids.

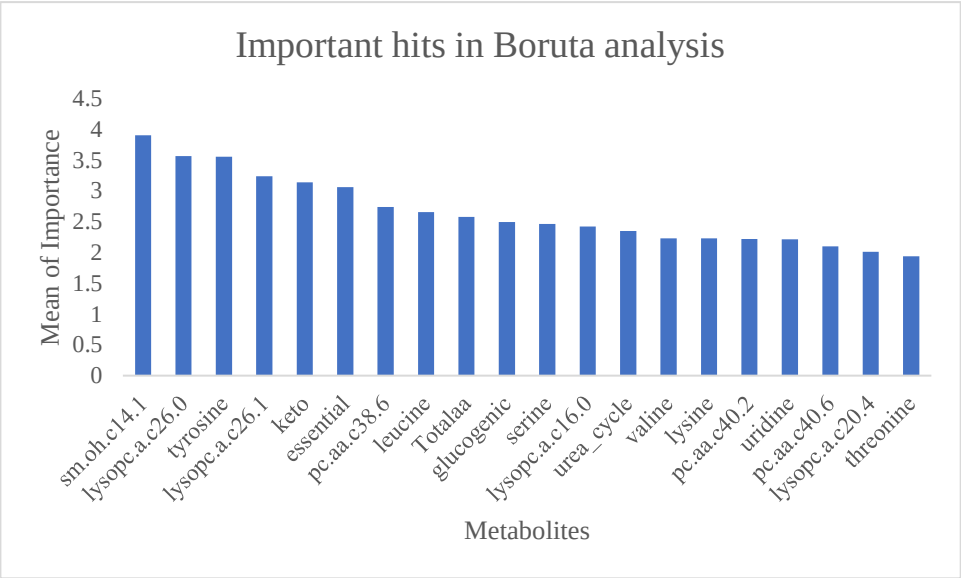


Figure 6-2 The 20 most important metabolite hits in Boruta model. Keto: leucine and lysine; Essential: a combination of essential amino acids including phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine; Totalaa: total amino acids with the combination of arginine, methionine, valine, alanine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, citrulline, ornithine, cystathionine and homocysteine; Glucogenic: glucogenic acids with a combination of alanine, arginine, asparagine, aspartic, glutamic, glutamine, glycine, histidine, methionine, proline, serine, valine, cystathionine, and homocysteine; Urea_cycle: urea cycle amino acids with a combination of ornithine, arginine, citrulline, and asparagine.

6.3.3 Associated metabolites to AF-alb in NB GLMM model

In the NB GLMM analysis, AF-alb was selected as dependent variable, all metabolites or metabolite class and ratios were selected as independent variables, and were adjusted with the variables of MUAC, oedema, Africa, age, and parttype as fixed effects, and site were selected as random effect. The results indicated that 22 metabolites such as: tryptophan, sm.c18.0, and lysine, and 7 metabolite class and ratios were significantly associated with aflatoxin exposure (Table 6-2). Most of them had negative coefficient to AF-alb, apart from the ratio of citrulline and ornithine, and the ratio of citrulline and arginine.

Table 6-2 Significantly associated metabolites and metabolite class and ratios to AF-alb.

Metabolites/class and ratios	FDR_ p value	Coefficient	Metabolites/class and ratios	FDR_ p value	Coefficient
tryptophan	9.84E-05	-0.0221	lysopc.a.c18.0	0.0184	-0.0392
sm.c18.0	0.000119	-0.0458	pc.aa.c32.2	0.0184	-0.29
lysopc.a.c20.4	0.000158	-0.154	pc.aa.c38.0	0.0198	-0.173
pc.aa.c38.6	0.000158	-0.0104	sm.c20.2	0.0198	-2.4
sm.c16.0	0.000269	-0.0117	ornithine	0.0203	-0.0104
lysopc.a.c28.0	0.00124	-3.93	keto	0.0239	-0.00304
lysopc.a.c14.0	0.00241	-0.185	tyr_phe	0.0258	-0.481
lysopc.a.c16.0	0.00241	-0.0142	threonine	0.026	-0.00445
spermi_putres	0.00445	-0.0361	urea_cycle	0.0364	-0.00391
pc.aa.c40.6	0.00587	-0.0249	arginine	0.0433	-0.0093

(Continued Table 6-2) Significantly associated metabolites and metabolite class and ratios to AF-alb.

Metabolites/class and ratios	FDR_ p value	Coefficient	Metabolites/class and ratios	FDR_ p value	Coefficient
lysine	0.0159	-0.00475	pc.ae.c40.6	0.0433	-0.151
pc.aa.c36.6	0.0159	-0.873	citruline_orn	0.0433	1.19
sm.oh.c14.1	0.0159	-0.216	citruline_arg	0.0442	2.26
sm.oh.c16.1	0.0159	-0.313	orn_putr	0.0454	-0.000157
sm.c18.1	0.0159	-0.1			

FDR: False discovery rate; spermi_putres: the ratio of spermidine and putrescine; keto: ketogenic acids including leucine and lysine; tyr_phe: the ratio of tyrosine and phenylalanine; urea_cycle: urea cycle amino acids including ornithine, arginine, citrulline, and asparagine; citrulline_orn: the ratio of citrulline and ornithine; citrulline_arg: the ratio of citrulline and arginine; orn_putr: the ratio of ornithine and putrescine.

6.3.4 Pathway analysis of the candidate metabolites associated with AF-alb

To find the functional pathways of the associated metabolites, concentrations of a list of candidate metabolites in all children analysed from Boruta, and NB GLMM analysis was input into MetaboAnalyst. In the pathway analysis, it was found aflatoxin-associated metabolites were significantly enriched in 14 pathways (Figure 6-3), the impact size of each pathway was also summarised (Table 6-3). The most significantly enriched pathways included pathways such as: arginine and proline metabolism, glutathione metabolism, and D-Glutamine and D-glutamate metabolism. Among the 14 significantly affected pathways, phenylalanine, tyrosine and tryptophan biosynthesis, alanine, aspartate and glutamate metabolism, and D-glutamine and D-glutamate metabolism were the most affected with the top three of impact value.

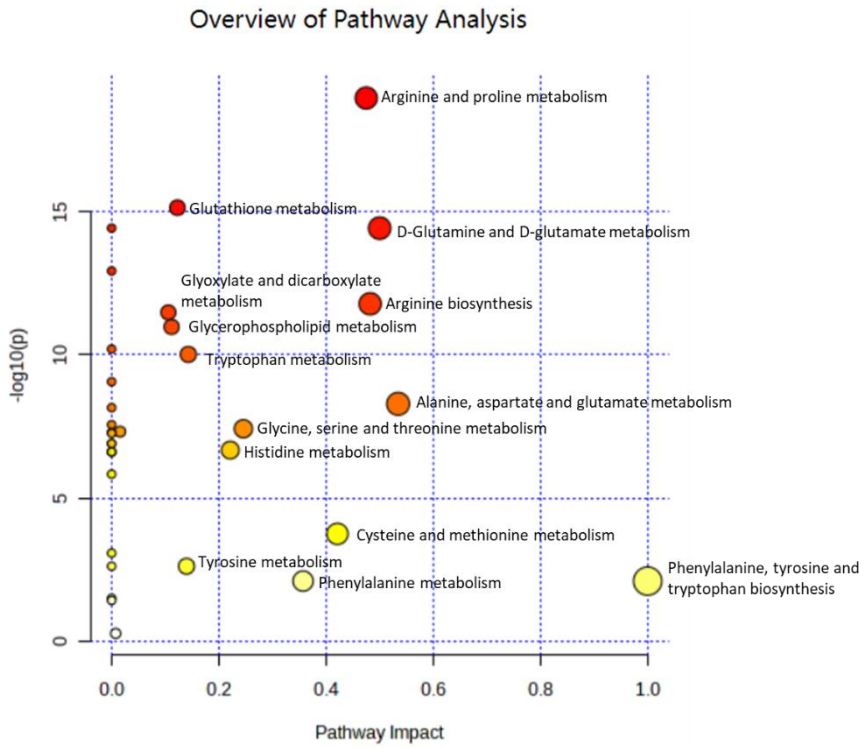


Figure 6-3 KEGG enrichment analysis of candidate metabolites associated to aflatoxin exposure. In total, 14 labelled pathways were significantly enriched with adjusted p value (FDR) smaller than 0.05 and impact effect over 0.1. The circle colour from yellow to red indicated increased significant level. The pathways with larger circle sizes have the higher impact values.

Table 6-3 Significantly affected KEGG pathways of the associated metabolites to aflatoxin exposure.

Pathway name	Match status	FDR_*P* value	Impact
Arginine and proline metabolism	6/12	3.60E-18	0.47
Glutathione metabolism	5/6	1.22E-14	0.12
D-Glutamine and D-glutamate metabolism	2/3	3.16E-14	0.5
Arginine biosynthesis	6/10	9.26E-12	0.48
Glyoxylate and dicarboxylate metabolism	3/5	1.59E-11	0.11
Glycerophospholipid metabolism	2/4	4.44E-11	0.11
Tryptophan metabolism	1/5	3.25E-10	0.14
Alanine, aspartate and glutamate metabolism	5/11	1.44E-8	0.53
Glycine, serine and threonine metabolism	3/9	8.57E-8	0.25
Histidine metabolism	3/4	3.41E-7	0.22
Cysteine and methionine metabolism	3/5	2.32E-4	0.42
Tyrosine metabolism	1/5	0.0028	0.14
Phenylalanine, tyrosine and tryptophan biosynthesis	2/2	0.0087	1.0
Phenylalanine metabolism	2/3	0.0087	0.36

6.4 Discussion

This study is aimed to find the metabolites that are associated with aflatoxin exposure in the African and South Asian children recruited in the CNCC study. To explore the functional roles of the aflatoxin-associated metabolites, as well as to shed light onto the health effects of aflatoxin from the aspect of metabolome alteration, concentrations of 150 metabolites and AF-alb in the children determined by University of Alberta and our lab, respectively, were collected. With the application of Boruta and NB GLMM analysis, several metabolites that were associated to aflatoxin exposure were found and

regarded as candidate metabolites, which were selected for a pathway analysis in MetaboAnalyst 5.0. In this study, 14 significantly enriched pathways of the candidate metabolites were found. Among them, the most significant 3 pathways were arginine and proline metabolism, glutathione metabolism, and D-glutamine and D-glutamate metabolism. Additionally, the top 3 pathways with greatest impact effect were phenylalanine, tyrosine and tryptophan biosynthesis, alanine, aspartate and glutamate metabolism, and D-glutamine and D-glutamate metabolism. Alterations of the 6 listed pathways mentioned above were also observed in a HBV expressing Hep3B cell line treated with AFB_1 (Wang et al., 2021), indicating consistent results between the current study (*in vivo*) and the *in vitro* study.

Alteration of arginine and proline metabolism was observed in our study. Similar findings have also been reported in other studies. Arginine, and proline metabolism which are gut-microbiota dependent metabolic pathways, were significantly disrupted by exposure to AFB_1 in rats (Zhou et al., 2019). Arginine is synthesised from three precursors, glutamine, glutamate, and proline through the involvement of pyrroline-5-carboxylate (P5C) in the small intestine to form citrulline, which is further converted to arginine in the kidney in adults (Bertolo and Burrin, 2008). However, unlike in adults, in neonates, arginine is synthesised from only proline completely in the small intestine in neonates because of weak P5C synthase activity (Bertolo and Burrin, 2008). In neonatal pigs intravenous infusion with an AA solution (containing proline but no glutamine, citrulline or arginine), caused arginine deficiency and led to hyperammonemia and death, while enteral feeding of the AA solution in the neonatal pigs ameliorated arginine deficiency, so the evidence emphasised the important roles of the small intestine in the synthesis from proline to arginine (Brunton et al., 1999). Additionally, arginine has important roles in foetal growth (Wu et al., 2009). Supplementation of L-Arginine combined with routine therapy for 7 days to pregnant women whose foetuses with intrauterine growth retardation at gestation week 33 significantly increased mean birth weight compared to the routine therapy alone group at gestation of week 39 (2971.7 vs 2794.4 g, $p<0.05$) (Xiao and Li, 2005). Therefore, we infer that abnormality of arginine and proline metabolism caused by aflatoxin exposure found in the children in this study might partly contribute to their poor growth.

Glutathione is well-known for the functions of preventing oxidation stress and detoxification of xenobiotic and endogenous compounds (Pizzorno, 2014). Glutathione is the most abundant antioxidant in living cells that acts as free radical scavenger through enzymatic or non-enzymatic reactions, and evidence

of the disruption of glutathione levels due to oxidative stress caused by various stimuli were widely reported (Rahman and MacNee, 2000; Fang et al., 2002; Jozefczak et al., 2012; Rotimi et al., 2019). For example, increased glutathione levels were observed in rats in response to AFB_1 caused oxidative stress (Zhang et al., 2011). On the contrary, near complete depletion of glutathione was observed in mice with excessive oxidative stress because of a co-exposure to AFB_1 and ethanol simultaneously (Adedara et al., 2010). Therefore, the evidence indicates that there is a dynamic balance of glutathione levels between its synthesis and gradually depletion against oxidative stress. Generally, glutathione is mainly synthesized *de novo* from cysteine, glutamate, and glycine, and the levels are reduced when it is utilized to remove free radicals or other reactive oxygen species (Wu et al., 2004). AFB_1-8,9-epoxide, the active metabolite of AFB_1, can be conjugated to glutathione to form mercapturates to detoxify AFB_1 metabolised products (Hayes et al., 1991). Thereby, when glutathione is depleted, it can potentially raise the risks from aflatoxin exposure. It was evident that in extrahepatic tissues of rats fed with AFB_1 and low protein diet there was a significant induction of oxidative stress, reduction in body weight gain and glutathione, which suggested that sufficient protein supplementation is important to ensure glutathione levels to exert normal functions (Rotimi et al., 2018). Therefore, it is important to maintain the homeostasis of glutathione, abnormality of glutathione contributes to oxidative stress, which is implicated with various diseases, such as: inflammation, liver disease, cancer, and kwashiorkor (Wu et al., 2004). In the current study, aflatoxin exposure may have caused the alterations of glutathione metabolism may be because of oxidative stress effect caused by aflatoxin and insufficient protein in those malnourished children, and the disruption of glutathione metabolism may be one of the mechanisms responsible for the explanation of adverse health effects caused by aflatoxin.

Disruption of phenylalanine, tyrosine and tryptophan biosynthesis was also observed in this study in the acutely ill children. Phenylalanine, tyrosine, and tryptophan are three aromatic amino acids play roles in protein synthesis (Parthasarathy et al., 2018). Protein deficiency is closely associated with child growth impairment (Semba, 2016). Significantly lower levels of phenylalanine and tryptophan were observed in stunted children compared to non-stunted children, indicating the importance of adequate intake of the essential amino acids to improve child growth (Semba et al., 2016). Evidence indicated that dietary supplementation of phenylalanine, tyrosine, and tryptophan to severe malnourished children at the baseline for 10 days significantly increased protein

synthesis (Hsu et al., 2014). Therefore, the effects of aflatoxin exposure on phenylalanine, tyrosine and tryptophan biosynthesis found in the present study might contribute to protein deficiency and finally lead to stunted growth, which could be part of the mechanism of aflatoxin-associated child growth impairment.

In addition, a recent study indicated that human gut microbiota generated aromatic amino acids (phenylalanine, tryptophan, and tyrosine) affect intestinal permeability and immune response in mice (Dodd et al., 2017). In the study, a gut symbiont *Clostridium sporogenes* producing the three aromatic amino acids was genetically manipulated to cause the loss of the production of phenylalanine, tryptophan, and tyrosine, and colonization of the mutant bacteria into gnotobiotic mice lead to an activation of immune response and significantly increased intestinal permeability (Dodd et al., 2017). Increased intestinal permeability has important roles in development of several diseases, including inflammation and immune-related diseases, diabetes, and susceptibility to infectious and xenobiotic agents (De-Souza and Greene, 2005; Arrieta et al., 2006). A recent study found that aflatoxin exposure levels assessed from aflatoxin-contaminated maize in a small cohort of 35 children were significantly associated with dysbiotic intestinal microbiome and stunting in the children, suggesting aflatoxin exposure related microbial dysbiosis may contribute to child growth impairment (Voth-Gaeddert et al., 2019). Therefore, the abnormal changes of the three aromatic amino acids observed in this study together with previous evidence imply that there might be interactions between metabolites and microbiome due to aflatoxin exposure. It is therefore worth to carry out integrate investigation of the effects of aflatoxin on both metabolites and microbiome, to shed light into the mechanism of aflatoxin-associated child growth impairment.

It was also found that glutamine and glutamate metabolism was significantly affected by aflatoxin exposure in the children in this study. Glutamine is a precursor of glutamate, which is also a substrate for the synthesis of glutamine due to an interconversion between glutamine and glutamate (Tapiero et al., 2002). Glutamine and glutamate have multiple roles in protein synthesis and muscle growth (Newsholme et al., 2003). They are also substrates to produce arginine, proline, and glutathione, and as fuel sources for the gut (Newsholme et al., 2003). With respect to the link between glutamate and glutathione, it is possible that aflatoxin influenced glutamate may affect glutathione synthesis, and lead to more oxidative stress in the body and impaired detoxification of aflatoxin metabolites. Since evidence indicated that premature infants are particularly susceptible to oxidative stress (Ozsurekci and Aykac, 2016),

children might be at greater risks to oxidative stress-related diseases in terms of aflatoxin exposure. Because of the discussion at the beginning of this chapter, arginine and proline are regarded as essential for child growth, abnormal glutamate metabolism might indirectly contribute to impaired growth through interrupting the synthesis of arginine and proline. In addition, previous evidence also indicates that glutamine and glutamate were associated to neonatal growth through its functions on the intestine. There is evidence showing that dietary glutamate was preferred to be oxidized and utilised by the intestine mucosa over glucose for energy production in growing piglets, which strengthen the importance of enteral glutamate levels (Reeds et al., 2000). A study indicated that administration of glutamine for one week to lipopolysaccharide-challenged piglets protected them against intestinal injury, and increased body weight (Haynes et al., 2009). Additionally, a recent study reported that supplementation of 2% glutamate to young pigs with mycotoxin-contaminated feeds significantly alleviate the abnormalities of intestinal structure caused by mycotoxin, which might potentially improve growth performance, because poor growth is related to intestinal injuries and poor absorption of nutrients in impaired intestine caused by mycotoxins (Duan et al., 2014). Therefore, from the findings of abnormality of glutamine and glutamate levels associated to aflatoxin exposure in the children in this study, and previous evidence showing their crucial roles in growth, we could hypothesize that aflatoxin exposure disrupted glutamine and glutamate metabolism lead to child growth impairment through two aspects: 1) interference of metabolites synthesis that have roles in growth and antioxidation; 2) effects on the intestinal structure to potentially affect microbiome and nutrients absorption.

In conclusion, in this study, affected pathways of aflatoxin exposure associated metabolites play important roles in oxidative stress-related diseases, protein synthesis, and growth of the children. Besides, since aflatoxin associated metabolites found in this study also have potential implications with respect to their effects on the intestine and microbiome, a few important questions in terms of the interactions among candidate metabolites, the intestine, and microbiome, remain to be addressed to better illustrate aflatoxin effects on child growth. Further investigation based on an integrated analysis of metabolome and microbiome in response to aflatoxin exposure is warranted.

Chapter 7 Overall discussion and conclusion

7.1 Overall discussion

This thesis has assessed aflatoxin exposure levels by measuring the biomarker, AF-alb adduct in the serum samples of acutely ill children exposed to aflatoxin in Africa and South Asia, and analysed the associations between aflatoxin exposure and child growth, as well as child mortality (Chapter 3). Following on, the potential mechanisms of aflatoxin health effects were explored *in vitro*, especially from the effects on gene expression (pathways and transcriptome) and DNA methylation (Chapter 4 and 5). In consideration of high prevalence of co-exposure to aflatoxin and fumonisin in nature, *in vitro* toxicity assessment and possible mechanisms related to gene expression alterations of co-exposure to aflatoxin and fumonisin were also investigated (Chapter 4). Further, to shed new insights on the mechanism of aflatoxin health effects, metabolomic changes due to aflatoxin exposure in the African and South Asian children were studied (Chapter 6).

In the pilot study of CNCC study in Chapter 3, a high prevalence (62%) of aflatoxin exposure with geometric mean of 5.4 pg/mg was found in the acutely ill children in Africa and South Asia. Geographical variations of aflatoxin exposure levels were found in those children. It is believed that aflatoxin-producing fungi favour an environment of high temperature and humidity to produce more aflatoxin (Gnonlonfin et al., 2013). Maize and peanuts as staple crops in Africa are more susceptible to aflatoxin contamination than rice as staple crop in Asia, therefore, with the possibility to more aflatoxin contamination in foods, inhabitants in Africa tend to have higher aflatoxin exposure levels. In this study, children were exposed to aflatoxin from an early age (< 6 months), and we found increased aflatoxin exposure levels with increasing age. Evidence indicates that aflatoxin can cross the placental barrier (Partanen et al., 2010), so there is a risk for infants to aflatoxin exposure starting from *in utero* if the pregnant women were exposed to aflatoxin. After the birth, breastfeeding containing AFM_1 is also a source of aflatoxin exposure, but it is considered as approximately 10 times less toxic compared to AFB_1 exposure based on animal studies (Cullen et al., 1987; (JECFA), 2017). Previous evidence indicated that weaning practice is an important factor contributing to the age variations of aflatoxin exposure levels (Gong et al., 2003). Because dietary exposure to aflatoxin from foods is the major route of aflatoxin exposure, and after weaning, infants or children will have more chance to eat

contaminated foods, such as: maize, maize-based foods, and peanuts, which is a likely reason for the increased exposure levels with increasing age.

Since there are conflicting findings of the associations between aflatoxin exposure and child growth, the association was also investigated in the pilot study (Chapter 3). Consistent with the early study that reported that aflatoxin exposure is associated with child growth impairment in Benin and Togo (Gong et al., 2002), negative associations between aflatoxin exposure and anthropometric indices were also found in current study. Although the aflatoxin exposure levels in the current study are lower than the levels reported in the Benin and Togo study, it is comparable to the levels reported in several recent studies (McMillan et al., 2018; Alamu et al., 2020), which also determined the inverse correlation between aflatoxin exposure and child growth. Some studies did not find the negative association, possibly due to even lower aflatoxin exposure levels or older age of children when linear growth is slower than above studies (Andrews-Trevino et al., 2019; Passarelli et al., 2020; Mahfuz et al., 2021; Ashraf et al., 2022), therefore we propose that relative high aflatoxin exposure levels and earlier exposure to aflatoxin have more profound effects on child growth. Evidence also emphasised that the first 1000 days from conception to 2-year-old are important period for the effects of aflatoxin exposure on child growth (Groopman et al., 2014; Gong et al., 2016). Therefore, prevention strategies to reduce aflatoxin exposure could be made to improve the aspects of environmental control, breastfeeding, and weaning practice.

The potential roles of aflatoxin exposure on mortality are of important public health implication. Previous evidence indicated that AFB_1 contributed to poultry mortality, for example, Increased concentration of AFB_1 treated diets caused an elevated mortality in young broilers (Cravens et al., 2013). The novel finding of this pilot study (Chapter 3) is that aflatoxin exposure was a risk factor contributing to child mortality in acutely ill children. Although policy and healthcare improvement to reduce child mortality has been advocated, the child mortality rate is still unacceptably high in some resource-limited areas, such as Africa and South Asia. Causes of childhood mortality in low- or middle- income countries are complex and usually confounded by many factors, such as: socioeconomic status, infection, health status, nutritional status, and clinical symptoms. However, not all those factors were analysed as confounding factors in this study when determining the association between aflatoxin exposure and child mortality in this pilot study. Previous evidence showed that malnutrition contributed to around one third of child deaths (Bain et al., 2013). Since the recruited children in this plot study had a high proportion of severe

malnutrition conditions, and due to the correlation between aflatoxin exposure and child growth impairment determined, it is plausible that aflatoxin exposure is implicated with child mortality in the severely malnourished children. To sum up the findings of the pilot study in African and South Asian children, aflatoxin exposure levels have geographic and age variations in the children, and it is inversely associated with child growth, and is a possible risk factor contributing to child mortality in acutely ill children. Therefore, it is necessary to promote efficient interventions to prevent aflatoxin exposure, especially for the improvement of growth, development, and survival of children in resource-limited settings. To demonstrate the association between aflatoxin exposure and child mortality, larger studies with consideration of important confounding factors are necessary.

To investigate the toxicity and mechanism of aflatoxin-associated health effects singly and in combination with fumonisin, another mycotoxin simultaneously contaminating foods in nature, an experiment was carried out using an *in vitro* model (Chapter 4 and 5). Previous evidence in our lab found that aflatoxin affects DNA methylation and gene expression of growth factors, and immune-related genes in Gambian children (Castelino, 2013; Hernandez-Vargas et al., 2015; Ghantous et al., 2021), which may provide a new insight of the mechanism to explain aflatoxin health effects. Therefore, gene expression and DNA methylation alterations due to aflatoxin on its own or with fumonisin, were investigated in a human hepatocyte cell line (HHL-16). In the *in vitro* study (Chapter 4 and 5), effects of aflatoxin on gene expression were initially analysed in a human growth factors pathway and immune-related genes, then the impacts of aflatoxin on transcriptome of HHL-16 cells were investigated. Dose-dependent up-regulation of *IL6*, *CCL20*, *BMP2* and down-regulation of *NDP* were observed in HHL-16 cells after AFB_1 treatments for 24 hours. Aberrant gene expression of the four genes in response to aflatoxin in the literature is varied, which may depend on the specificity of cell lines or animals and different doses applied (Jiang et al., 2015; Ma et al., 2015). The known susceptibility difference to aflatoxin among different species (Monson et al., 2015) could be one of the reasons responsible for the different gene expression alteration patterns. Different concentrations of aflatoxin used in the treatments might also affect varied gene expression pattern. For example, down-regulated levels of IL6 in broilers exposed to 0.6 mg/kg AFB_1, while up-regulated levels of IL6 in broilers exposed to 70 µg/kg AFB_1-contained diet were found (Jiang et al., 2015; Ma et al., 2015), indicating there might be a exposure concentration-based variation driven gene expression difference. Since *IL6*, *CCL20*, *BMP2*,

and *NDP* found in this study have roles in inflammatory response, immune modulation, growth, and cancers (Bray, 2006; Ghadjar et al., 2009; Tanaka et al., 2014; Kadomoto et al., 2020), the interference of gene expression of the four genes caused by AFB_1 in HHL-16 cells may partly explain the impacts of aflatoxin on liver toxicity, immune modulation, growth failure, and cancers. However, due to multiple roles of the genes in different pathways, it is difficult to draw a conclusion of aflatoxin health effects from a broader aspect of pathway connections.

Thereby, in the *in vitro* study in Chapter 5, an in-depth transcriptome sequencing in HHL-16 cells after 20 µg/ml AFB_1 treatment for 24 hours was performed and over 500 DEGs were found. Notably, the four DEGs (*IL6*, *CCL20*, *BMP2*, and *NDP*) found from the aforementioned pathway analysis were also observed in RNA-Seq results, indicating a good consistency between the different methods. Further, it was found that the DEGs were significantly enriched in the pathways such as: cytokine-cytokine receptor interaction, NF-kB signalling pathway and TNF signalling pathway. Cytokines are important to maintain normal immune function and control inflammation (Kany et al., 2019), so the aberrant expression of various cytokines observed in this study at least indicated an inflammatory response activated by AFB_1, which is the same conclusion reached from both the pathway analysis in Chapter 4 and the transcriptome analysis in Chapter 5. Under a condition of cellular stress, the NF-kB pathway can be transiently activated by both proinflammatory cytokines and the members of the TNF pathway (Lin and Karin, 2007; Zinatizadeh et al., 2021). Imbalance of NF-kB can lead to uncontrolled proliferation of cells and finally contribute to cancers (Kojima et al., 2004; Ogunwobi et al., 2019). Therefore, according to the findings from the effects of AFB_1 on gene expression in HHL-16 cells, we hypothesize that AFB_1 modulates the expression of gene sets that have roles in inflammation, growth factors, and cancers through the interactions among cytokine-related/TNF/NF-kB pathways.

Since there is a growing interest in aflatoxin-affected DNA methylation, DNA methylation of *IL6* and *CCL20* in HHL-16 cells after 20 µg/ml AFB_1 treatment for 24 hours was also analysed (Chapter 4). Although no significant change of DNA methylation of *IL6* was found, it is worth to highlight that AFB_1 caused a significant hypomethylation of a CpG site near the transcription start site of *CCL20*. Therefore, we speculate hypomethylation at the CpG site of *CCL20* might be responsible for the up-regulated gene expression of *CCL20* in HHL-16 cells after AFB_1 treatment. It is evident that hypomethylation at promoter region and increased mRNA levels of *CCL20* were found in esophageal

adenocarcinoma patients, showing the possibility of using *CCL20* as a biomarker for esophageal cancer (Maity et al., 2022). Thus, the finding of aberrant DNA methylation and gene expression of *CCL20* in HHL-16 cells in response to AFB_1 strengthens the evidence of aflatoxin-affected DNA methylation, also suggests the potential application of *CCL20* as a novel biomarker of cancers due to aflatoxin exposure. However, since the statistical analysis of DNA methylation at the CpG site of *CCL20* was subjected to an imputed mean level of DNA methylation in the control group, the effects of AFB_1 on DNA methylation of *CCL20* around its promoter region merit further investigation in other cell lines or animals to validate.

Given the high prevalence of co-exposure to aflatoxin and fumonisin previously reported (Shirima et al., 2015), it is also our interest to explore their combined toxicity in this study. In the *in vitro* study (Chapter 4), synergistic effects of AFB_1 and FB_1 on toxicity and gene expression were observed. According to evidence in the literature, varied effects of aflatoxin and fumonisin on the inhibition of cell viability of various cells from additive to synergistic have been reported (McKean et al., 2006; Clarke et al., 2014; Chen et al., 2023). The varied effects of combined aflatoxin and fumonisin treatments on cells may be related to the factors of different methods and various concentrations applied to measure cell viability, and cell specificity. In this study, AFB_1 and FB_1 in combination caused more reduction of cell viability of HHL-16 cells compared to the single treatments, which indicate the possible synergistic cytotoxicity of aflatoxin and fumonisin and highlights the importance to reduce the potential hazard of the combined contamination of the two mycotoxins in crops. Synergistic effects on the induction of *IL6* and *BMP2* gene expression levels in HHL-16 cells after combined AFB_1 and FB_1 treatment were also observed in the current study. As previous evidence indicated that in the presence of AFB_1, FB_1 may be more readily absorbed by the cells (Yu et al., 2020), here we propose that may be more cellular stress caused by the combined treatment, and which might be the possible reasons for the synergistic effects on gene expression modulation by AFB_1 and FB_1 found in this study. Since IL6 and BMP2 have roles in carcinoma progression (Wu et al., 2020; Jiang et al., 2023), it is plausible to deduce that the combination of AFB_1 and FB_1 would cause more inflammatory response and potentially increase the carcinogenicity of AFB_1 with the presence of FB_1 through the enhanced activation of *IL6* and *BMP2*. However, because the *in vitro* study may not fully represent the biological functions *in vivo*, it is worth to replicate the results *in vivo* studies.

With the opportunity provided by CHAIN study, metabolomics alteration due to aflatoxin exposure in the African and South Asian children was analysed (Chapter 6). In the study, affected metabolites by aflatoxin exposure were significantly enriched in the pathways including arginine and proline metabolism, glutathione metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, and glutamate-related metabolism. Evidence found that arginine, proline, and glutamate have crucial roles in fetal growth, and supplementation of them improved the growth performance (Xiao and Li, 2005; Duan et al., 2014). Besides, glutamate is reported to have various roles, such as: as fuel source of intestine to affect intestinal structure and potentially affect microbiome as well as nutrients absorption, as substrates for the synthesis of arginine, proline, and glutathione (Newsholme et al., 2003). Glutathione is known as antioxidation and detoxification of xenobiotic and endogenous compounds (Pizzorno, 2014). Abnormal metabolism of glutathione caused by aflatoxin exposure in the children in this study may contribute to higher risk to oxidative stress-related diseases, such as: inflammation, liver disease, cancers, and kwashiorkor. In addition, disruption of phenylalanine, tyrosine and tryptophan biosynthesis in the children exposed to aflatoxin was also observed in this study. Prior evidence indicated that phenylalanine, tyrosine and tryptophan are important for protein synthesis, and insufficiency of protein could lead to child growth impairment (Semba et al., 2016; Parthasarathy et al., 2018). Therefore, the disruptions of the enriched pathways of aflatoxin-association metabolites in the children might be the underlying mechanisms responsible for child growth impairment and child mortality.

Therefore, in this thesis, we found aflatoxin exposure is prevalent in African and South Asian children, and aflatoxin exposure contributes to child growth impairment and child mortality. Further *in vitro* and *in vivo* mechanism investigation demonstrate that aflatoxin can cause the disruption of gene expression, DNA methylation, and metabolite levels, which have important roles in inflammatory, growth, and cancer pathways. Additionally, in the presence of fumonisin, there are possibly synergistic effects of aflatoxin and fumonisin on cytotoxicity and gene modulation. Therefore, the findings in this study may contribute to a better understanding of aflatoxin health effects and highlight the importance to avoid co-exposure to aflatoxin and fumonisin.

7.2 Limitations and future works

1. In the pilot study in Chapter 3, it was found that aflatoxin exposure levels varied by children’s age and geographic factors, and aflatoxin exposure is

associated with child growth impairment and a potential risk factor to child mortality. However, the pilot study is subject to some limitations. First, the pilot study is an observational study. Aflatoxin exposure levels were only assessed in the children at the admission timepoint, it was not assessed at discharge timepoint in the pilot study. In addition, it is important to take a good consideration of the underlying confounding factors, such as: socioeconomic, food consumption, infection status, and clinical symptoms. Notably, some of the children in the pilot study are malnourished children, so the malnutrition status could be an important confounding factor contributing to child growth impairment and child mortality. Aflatoxin exposure as a risk factor to child mortality was found in the pilot study, but it is difficult to figure out the reasons, whether is the sole effect of aflatoxin on mortality or because of the implication with malnutrition and subsequently contribute to mortality. During the assessment stage, we were blind to the samples, although it provides a strength of unbiased assessment, it also brings some limitations into the pilot study, which result in limited access to the full information and data of those children. Under this condition, the potential confounding factors discussed as above were not applicable to analysed during the analysis.

Therefore, based on the findings and limitations from the pilot study, some future directions for the full cohort study of CNCC are proposed as below: A) assess aflatoxin exposure levels in the children also at the discharge timepoint, and carry out the association analysis of aflatoxin exposure levels between admission and discharge timepoints, which will be helpful to explore the longitudinal effects of aflatoxin on those children; B) take the confounding factors (malnutrition status, age, sites, and socioeconomic status) into consideration when perform the analysis of aflatoxin effects on child growth and mortality; C) compare the results of the full cohort and this pilot study after adjustment analysis of the confounding factors, which will validate the findings from this pilot study, also give more confidence of the findings.

2. In the *in vitro* study (Chapter 4 and 5), it was aimed to figure out molecular mechanism of aflatoxin and health effects. From the specific PCR Panels pathway analysis in Chapter 4, it was found that aflatoxin can cause a disruption of gene expression and DNA methylation of genes that have important roles in inflammatory, growth, and cancer pathways. But RNA-

Seq analysis of whole transcriptome in HHL-16 cells after aflatoxin treatment in Chapter 5 did not find growth related pathway, only inflammatory and cancer pathways were found. After deep exploration of the RNA-Seq data, a limitation of the cell line HHL-16 was found. GH-IGF1 axis may not expressed or at very low transcript levels limits to find more growth-related DEGs, which further hinder the enrichment analysis to find associated growth pathways and diseases. Moreover, due to the limited time and the consideration of cost-effective, combined effects of aflatoxin and fumonisin were only carried out in some specific genes rather than the whole transcriptome in the cells. Lastly, the individual cell line (HHL-16) may not fully represent biological functions of different tissues working together in the body.

Therefore, there are several points can be improved for future work in HHL-16 cell line to investigate mechanism of mycotoxin effects on growth and combined effects of aflatoxin and fumonisin. A) to generate some over-expression vectors to induce higher expression of GH-IGF1 axis in HHL-16 cells, then carry out further investigation after confirming induced expression of GH-IGF1; B) perform the same experiments in more cell lines or animals to validate the results obtained from this study and supplement the evidence, typically from the gene expression and DNA methylation aspects; C) studies investigating effects of aflatoxin and/or fumonisin on gene expression and DNA methylation in populations are also warranted, which will be helpful to draw a stronger conclusion; D) to better illustrate effects of aflatoxin and fumonisin on transcriptome, whole transcriptome analysis in the cells after aflatoxin and/or fumonisin treatments merits further investigation.

3. In the *in vivo* study in Chapter 6, it was found that aflatoxin exposure affected metabolites have roles in oxidative stress-related diseases, protein synthesis, and growth. However, one of the limitations is that metabolites may be affected by several factors in the reality. For example, metabolites can also be affected by other environmental exposures (i.e. air pollution and chemical exposure), and unhealthy lifestyles (i.e. alcohol consumption and smoke), and dietary pattern etc. Since those factors were not analysed in this *in vivo* study in Chapter 6, further studies investigating aflatoxin effects on metabolomic alteration should consider the confounding factors as many as possible to better illustrate the mechanism.

Thereby, for further investigation of aflatoxin effects on metabolome, my proposed future work would be as follows: A) during the analysis to find associated metabolites, more different models can be used, such as PCA and OPLS-DA. B) validate current results in different populations and with the consideration of collecting data of the confounding factors discussed above. C) investigate the effects of aflatoxin on metabolome in different cell lines or animals, and compare the results both *in vitro* and *in vivo*, to get rid of the confounding factors effects, which might provide a better understanding of aflatoxin effects on metabolome. D) a comprehensive multi-omics strategy-based human epidemiological study would provide a more in-depth and broader understanding of aflatoxin health effects. E) based on the metabolomic results observed in this study, an integrated analysis of metabolome and microbiome in the children is suggested, which might illustrate novel mechanisms of aflatoxin effects.

7.3 Conclusions

The aflatoxin exposure levels have been determined in the acutely ill children from Africa and South Asia, and the levels have geographical and age variations. Aflatoxin exposure levels in the children are associated with child growth impairment, and aflatoxin exposure is a risk factor to increase child mortality. The mechanism investigation of aflatoxin effects on gene expression and DNA methylation indicated that aflatoxin affects the expression of genes that play roles in inflammatory, growth factors, and cancers through the interactions among cytokine-related/TNF/NF-kB pathways. Aflatoxin can cause a hypomethylation of *CCL20*. There are synergistic effects of aflatoxin and fumonisin co-exposure on cytotoxicity and gene expression modulations *in vitro*. Furthermore, in the metabolomic analysis, aflatoxin exposure can affect several metabolite levels, which are significantly enriched in the pathways having important roles in oxidative stress-related diseases, protein synthesis, and growth.

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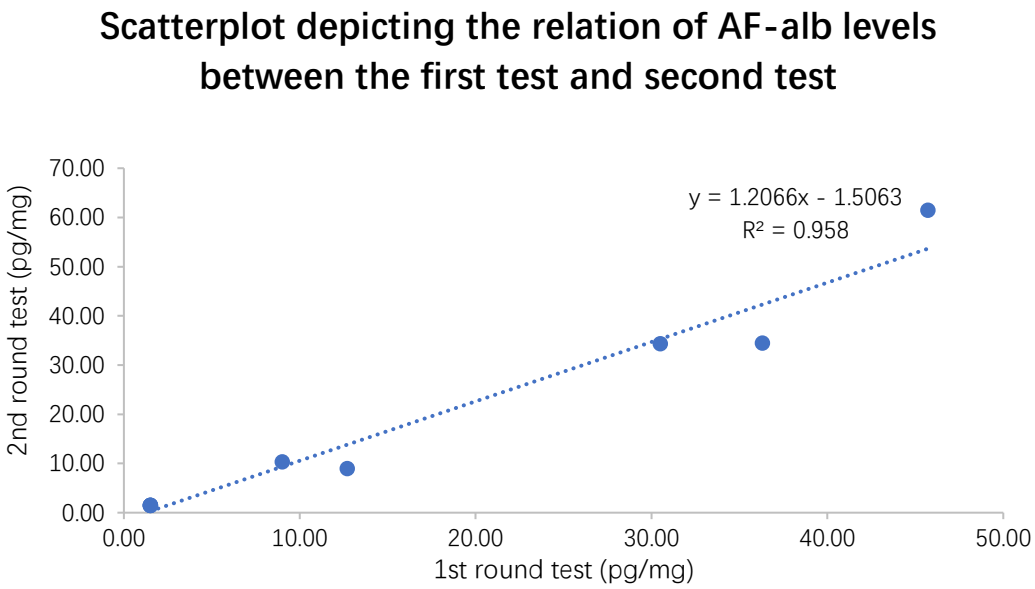
Appendices

Appendix 2.1 Testing ELISA ready samples from previous Tanzania study in our lab to check board.

ELISA ready samples	AF-alb previously reported (pg/mg)	AF-alb tested in this study (1st) (pg/mg)	AF-alb tested in this study (2nd) (pg/mg)	Average of AF-alb	SD	CV%
A_1000f	2.76	2.88	2.77	2.79	0.05	2
B_1000f	1.52	1.75	1.30	1.52	0.00	0
C_50f	1.3	0.89	1.09	1.15	0.22	19
D_50f	0.42	0.53	0.35	0.43	0.01	3

ELISA ready samples: four samples were selected from previous Tanzania study in our lab. f: folds dilution. SD: standard deviation. CV: coefficient of variation.



Appendix 2.2 Scatterplot of AF-alb levels between two-round tests.

Appendix 2.3 QuantiNova® LNA® PCR Focus Panels.

Position	Assay	Name	Symbol	Ensembl	Description
A01	SBH1219737	ENST00000221496.4	AMH	ENSG00000104899	anti-Mullerian hormone Source HGNC Symbol Acc HGNC 464
A02	SBH0006040	ENST00000525528.1	BDNF	ENSG00000176697	brain derived neurotrophic factor Source HGNC Symbol Acc HGNC 1033
A03	SBH1219801	ENST00000354870.5	BMP1	ENSG00000168487	bone morphogenetic protein 1 Source HGNC Symbol Acc HGNC 1067
A04	SBH0038117	ENST00000295379.2	BMP10	ENSG00000163217	bone morphogenetic protein 10 Source HGNC Symbol Acc HGNC 20869
A05	SBH1219802	ENST00000378827.5	BMP2	ENSG00000125845	bone morphogenetic protein 2 Source HGNC Symbol Acc HGNC 1069
A06	SBH1219803	ENST00000282701.3	BMP3	ENSG00000152785	bone morphogenetic protein 3 Source HGNC Symbol Acc HGNC 1070
A07	SBH0613995	ENST00000417573.5	BMP4	ENSG00000125378	bone morphogenetic protein 4 Source HGNC Symbol Acc HGNC 1071
A08	SBH1219804	ENST00000370830.4	BMP5	ENSG00000112175	bone morphogenetic protein 5 Source HGNC Symbol Acc HGNC 1072
A09	SBH1219805	ENST00000283147.7	BMP6	ENSG00000153162	bone morphogenetic protein 6 Source HGNC Symbol Acc HGNC 1073
A10	SBH1219806	ENST00000450594.6	BMP7	ENSG00000101144	bone morphogenetic protein 7 Source HGNC Symbol Acc HGNC 1074
A11	SBH0222101	ENST00000372827.8	BMP8B	ENSG00000116985	bone morphogenetic protein 8b Source HGNC Symbol Acc HGNC 1075
A12	SBH0130317	ENST00000399837.8	ADA2	ENSG00000093072	adenosine deaminase 2 Source HGNC Symbol Acc HGNC 1839
B01	SBH0003745	ENST00000221804.5	CLC	ENSG00000105205	Charcot-Leyden crystal galectin Source HGNC Symbol Acc HGNC 2014
B02	SBH1219913	ENST00000420111.6	CSF1	ENSG00000184371	colony stimulating factor 1 Source HGNC Symbol Acc HGNC 2432
B03	SBH1219914	ENST00000296871.4	CSF2	ENSG00000164400	colony stimulating factor 2 Source HGNC Symbol Acc HGNC 2434
B04	SBH0378721	ENST00000225474.6	CSF3	ENSG00000108342	colony stimulating factor 3 Source HGNC Symbol Acc HGNC 2438
B05	SBH0436458	ENST00000610462.1	CSPG5	ENSG00000114646	chondroitin sulfate proteoglycan 5 Source HGNC Symbol Acc HGNC 2467
B06	SBH0404660	ENST00000395761.3	CXCL1	ENSG00000163739	C-X-C motif chemokine ligand 1 Source HGNC Symbol Acc HGNC 4602
B07	SBH0194476	ENST00000373970.4	DKK1	ENSG00000107984	dickkopf WNT signaling pathway inhibitor 1 Source HGNC Symbol Acc HGNC 2891
B08	SBH0331848	ENST00000503921.5	ERAP1	ENSG00000164307	endoplasmic reticulum aminopeptidase 1 Source HGNC Symbol Acc HGNC 18173
B09	SBH0074479	ENST00000244869.3	EREG	ENSG00000124882	epiregulin Source HGNC Symbol Acc HGNC 3443
B10	SBH0534985	ENST00000612	258.4	FGF1	fibroblast growth factor 1 Source HGNC Symbol Acc HGNC 3665
B11	SBH0204430	ENST00000575235.5	FGF11	ENSG00000161958	fibroblast growth factor 11 Source HGNC Symbol Acc HGNC 3667
B12	SBH0028571	ENST00000441825.8	FGF13	ENSG00000129682	fibroblast growth factor 13 Source HGNC Symbol Acc HGNC 3670
C01	SBH0177601	ENST00000376143.4	FGF14	ENSG00000102466	fibroblast growth factor 14 Source HGNC Symbol Acc HGNC 3671
C02	SBH0253385	ENST00000518533.5	FGF17	ENSG00000158815	fibroblast growth factor 17 Source HGNC Symbol Acc HGNC 3673
C03	SBH0058115	ENST00000294312.4	FGF19	ENSG00000162344	fibroblast growth factor 19 Source HGNC Symbol Acc HGNC 3675
C04	SBH1220000	ENST00000264498.7	FGF2	ENSG00000138685	fibroblast growth factor 2 Source HGNC Symbol Acc HGNC 3676
C05	SBH0597208	ENST00000215530.6	FGF22	ENSG00000070388	fibroblast growth factor 22 Source HGNC Symbol Acc HGNC 3679
C06	SBH0347299	ENST00000237837.1	FGF23	ENSG00000118972	fibroblast growth factor 23 Source HGNC Symbol Acc HGNC 3680
C07	SBH0225015	ENST00000312465.11	FGF5	ENSG00000138675	fibroblast growth factor 5 Source HGNC Symbol Acc HGNC 3683
C08	SBH0454239	ENST00000228837.2	FGF6	ENSG00000111241	fibroblast growth factor 6 Source HGNC Symbol Acc HGNC 3684
C09	SBH0051743	ENST00000560979.1	FGF7	ENSG00000140285	fibroblast growth factor 7 Source HGNC Symbol Acc HGNC 3685
C10	SBH0087968	ENST00000461657.1	FGF9	ENSG00000102678	fibroblast growth factor 9 Source HGNC Symbol Acc HGNC 3687
C11	SBH1220001	ENST00000297904.4	VEGFD	ENSG00000165197	vascular endothelial growth factor D Source HGNC Symbol Acc HGNC 3708
C12	SBH0309229	ENST00000580279.2	GDF10	ENSG00000266524	growth differentiation factor 10 Source HGNC Symbol Acc HGNC 4215
D01	SBH0256075	ENST00000257868.9	GDF11	ENSG00000135414	growth differentiation factor 11 Source HGNC Symbol Acc HGNC 4216
D02	SBH0310916	ENST00000502572.1	GDNF	ENSG00000168621	glial cell derived neurotrophic factor Source HGNC Symbol Acc HGNC 4232
D03	SBH1220031	ENST00000644934.1	GPI	ENSG00000105220	glucose-6-phosphate isomerase Source HGNC Symbol Acc HGNC 4458
D04	SBH0028682	ENST00000230990.7	HBEGF	ENSG00000113070	heparin binding EGF like growth factor Source HGNC Symbol Acc HGNC 3059
D05	SBH1220091	ENST00000337514.10	IGF1	ENSG00000017427	insulin like growth factor 1 Source HGNC Symbol Acc HGNC 5464
D06	SBH0264962	ENST00000418738.2	IGF2	ENSG00000167244	insulin like growth factor 2 Source HGNC Symbol Acc HGNC 5466
D07	SBH1220095	ENST00000423557.1	IL10	ENSG00000136634	interleukin 10 Source HGNC Symbol Acc HGNC 5962
D08	SBH1220097	ENST00000585513.1	IL11	ENSG00000095752	interleukin 11 Source HGNC Symbol Acc HGNC 5966
D09	SBH1220099	ENST00000231228.2	IL12B	ENSG00000113302	interleukin 12B Source HGNC Symbol Acc HGNC 5970
D10	SBH1220103	ENST00000524595.5	IL18	ENSG00000150782	interleukin 18 Source HGNC Symbol Acc HGNC 5986
D11	SBH0663647	ENST00000263339.3	IL1A	ENSG00000115008	interleukin 1 alpha Source HGNC Symbol Acc HGNC 5991
D12	SBH0079231	ENST00000263341.6	IL1B	ENSG00000125538	interleukin 1 beta Source HGNC Symbol Acc HGNC 5992
E01	SBH0225582	ENST00000226730.4	IL2	ENSG00000109471	interleukin 2 Source HGNC Symbol Acc HGNC 6001
E02	SBH0584080	ENST00000296870.2	IL3	ENSG00000164399	interleukin 3 Source HGNC Symbol Acc HGNC 6011
E03	SBH1220109	ENST00000350025.2	IL4	ENSG00000113520	interleukin 4 Source HGNC Symbol Acc HGNC 6014
E04	SBH1220115	ENST00000243786.3	INHA	ENSG00000123999	inhibin subunit alpha Source HGNC Symbol Acc HGNC 6065
E05	SBH1220116	ENST00000242208.5	INHBA	ENSG00000122641	inhibin subunit beta A Source HGNC Symbol Acc HGNC 6066
E06	SBH1220117	ENST00000295228.4	INHBB	ENSG00000163083	inhibin subunit beta B Source HGNC Symbol Acc HGNC 6067
E07	SBH0407654	ENST00000254958.10	JAG1	ENSG00000101384	jagged 1 Source HGNC Symbol Acc HGNC 6188
E08	SBH0627052	ENST00000546616.1	JAG2	ENSG00000184916	jagged 2 Source HGNC Symbol Acc HGNC 6189
E09	SBH1220168	ENST00000272134.5	LEFTY1	ENSG00000243709	left-right determination factor 1 Source HGNC Symbol Acc HGNC 6552
E10	SBH1221132	ENST00000366820.10	LEFTY2	ENSG00000143768	left-right determination factor 2 Source HGNC Symbol Acc HGNC 3122
E11	SBH1220172	ENST00000249075.4	LIF	ENSG00000128342	LIF, interleukin 6 family cytokine Source HGNC Symbol Acc HGNC 6596
E12	SBH0371009	ENST00000243562.13	LTBP4	ENSG00000090006	latent transforming growth factor beta binding protein 4 Source HGNC Symbol Acc HGNC 6717
F01	SBH0046663	ENST00000395566.8	MDK	ENSG00000110492	midkine Source HGNC Symbol Acc HGNC 6972
F02	SBH0389766	ENST00000260950.4	MSTN	ENSG00000138379	myostatin Source HGNC Symbol Acc HGNC 4223
F03	SBH0209223	ENST00000470584.1	NDP	ENSG00000124479	NDP, norrin cystine knot growth factor Source HGNC Symbol Acc HGNC 7678
F04	SBH0318562	ENST00000369512.2	NGF	ENSG00000134259	nerve growth factor Source HGNC Symbol Acc HGNC 7808
F05	SBH0463463	ENST00000287139.7	NODAL	ENSG00000156574	nodal growth differentiation factor Source HGNC Symbol Acc HGNC 7865
F06	SBH0274670	ENST00000652592.1	NRG1	ENSG00000157168	neuregulin 1 Source HGNC Symbol Acc HGNC 7997
F07	SBH0471786	ENST00000361474.6	NRG2	ENSG00000158458	neuregulin 2 Source HGNC Symbol Acc HGNC 7998
F08	SBH0290718	ENST00000545131.5	NRG3	ENSG00000185737	neuregulin 3 Source HGNC Symbol Acc HGNC 7999
F09	SBH0148171	ENST00000303212.2	NRTN	ENSG00000171119	neurturin Source HGNC Symbol Acc HGNC 8007
F10	SBH0012802	ENST00000543548.1	NTF3	ENSG00000185652	neurotrophin 3 Source HGNC Symbol Acc HGNC 8023
F11	SBH0048137	ENST00000565123.5	OSGIN1	ENSG00000140961	oxidative stress induced growth inhibitor 1 Source HGNC Symbol Acc HGNC 30093
F12	SBH0518114	ENST00000506880.5	PDGFC	ENSG00000145431	platelet derived growth factor C Source HGNC Symbol Acc HGNC 8801
G01	SBH1220303	ENST00000238607.10	PGF	ENSG00000119630	placental growth factor Source HGNC Symbol Acc HGNC 8893
G02	SBH0231621	ENST00000597721.1	PSPN	ENSG00000125650	persephin Source HGNC Symbol Acc HGNC 9579
G03	SBH0080420	ENST00000348225.6	PTN	ENSG00000105894	pleiotrophin Source HGNC Symbol Acc HGNC 9630
G04	SBH0646520	ENST00000453443.5	SLCO1A2	ENSG00000084453	solute carrier organic anion transporter family member 1A2 Source HGNC Symbol Acc HGNC 10956
G05	SBH0180162	ENST00000237	623.11	SPP1	secreted phosphoprotein 1 Source HGNC Symbol Acc HGNC 11255
G06	SBH0577809	ENST00000471721.1	TDGF1	ENSG00000241186	teratocarcinoma-derived growth factor 1 Source HGNC Symbol Acc HGNC 11701
G07	SBH1220443	ENST00000598	758.5	TGFB1	transforming growth factor beta 1 Source NCBI gene Acc 7040
G08	SBH0321723	ENST00000647395.1	THPO	ENSG00000090534	thrombopoietin Source HGNC Symbol Acc HGNC 11795
G09	SBH0332854	ENST00000587758.5	TNNT1	ENSG00000105048	troponin T1, slow skeletal type Source HGNC Symbol Acc HGNC 11948
G10	SBH1220500	ENST00000395681.6	TYMP	ENSG00000025708	thymidine phosphorylase Source HGNC Symbol Acc HGNC 3148
G11	SBH0420322	ENST00000425836.6	VEGFA	ENSG00000112715	vascular endothelial growth factor A Source HGNC Symbol Acc HGNC 12680
G12	SBH1220517	ENST00000618562.2	VEGFC	ENSG00000150630	vascular endothelial growth factor C Source HGNC Symbol Acc HGNC 12682
H01	SBH1220543	ENST00000646664.1	ACTB	ENSG00000075624	actin beta Source HGNC Symbol Acc HGNC 132
H02	SBH1220550	ENST00000558401.6	B2M	ENSG00000166710	beta-2-microglobulin Source HGNC Symbol Acc HGNC 914
H03	SBH1220545	ENST00000396861.5	GAPDH	ENSG00000111640	glyceraldehyde-3-phosphate dehydrogenase Source HGNC Symbol Acc HGNC 4141
H04	SBH1220546	ENST00000298556.8	HPRT1	ENSG00000165704	hypoxanthine phosphoribosyltransferase 1 Source HGNC Symbol Acc HGNC 5157
H05	SBH1220553	ENST00000546989.5	RPLP0	ENSG00000089157	ribosomal protein lateral stalk subunit P0 Source HGNC Symbol Acc HGNC 10371
H06	SBH1218553	Sybr_HGDC	HGDC	Sybr_HGDC	Human Genomic DNA Contamination
H07	SBH1218551	Sybr_QIC	QIC	Sybr_QIC	QuantiNova Internal Control
H08	SBH1218551	Sybr_QIC	QIC	Sybr_QIC	QuantiNova Internal Control
H09	SBH1218551	Sybr_QIC	QIC	Sybr_QIC	QuantiNova Internal Control
H10	SBH1218550	Sybr_PPC	PPC	Sybr_PPC	Positive PCR Control
H11	SBH1218550	Sybr_PPC	PPC	Sybr_PPC	Positive PCR Control
H12	SBH1218550	Sybr_PPC	PPC	Sybr_PPC	Positive PCR Control

Gene 1: *IL6* (chr7: 22726063 to 22727265).

>chr7:22726063-22727265

TCTGAACCAGCTTGACCCAATAAGAAATTCTTGGGTGCCGACGCGGAAG
CAGATTCAGAGCCTAGAGCCGTGCCTGCGTCCGTAGTTTCCTTCTAGCT
TCTTTTGATTTCAAATCAAGACTTACAGGGAGAGGGAGCGATAAACACAA
ACTCTGCAAGATGCCACAAGGTCCTCCTTTGACATCCCCAACAAAGAGG
TGAGTAGTATTCTCCCCCTTTCTGCCCTGAACCAAGTGGGCTTCAGTAAT
TTCAGGGCTCCAGGAGACCTGGGGCCCATGCAGGTGCCCCAGTGAAAC
AGTGGTGAAGAGACTCAGTGGCAATGGGGAGAGCACTGGCAGCACAAG
GCAAACCTCTGGCACAGAGAGCAAAGTCCTCACTGGGAGGATTCCCAAG
GGGTCACTTGGGAGAGGGCAGGGCAGCAGCCAACCTCCTCTAAGTGGG
CTGAAGCAGGTGAAGAAAGTGGCAGAAGCCACGCGGTGGCAAAAAGGA
GTCACACACTCCACCTGGAGACGCCTTGAAGTAACTGCACGAAATTTGA
GGATGGCCAGGCAGTTCTACAACAGCCGCTCACAGGGAGAGCCAGAAC
ACAGAAGAACTCAGATGACTGGTAGTATTACCTTCTTCATAATCCCAGGC
TTGGGGGGCTGCGATGGAGTCAGAGGAAACTCAGTTCAGAACATCTTTG
GTTTTTACAAATACAAATTAACTGGAACGCTAAATTCTAGCCTGTTAATCT
GGTCACTGAAAAAAAATTTTTTTTTTTTCAAAAAACATAGCTTTAGCTTATT
TTTTTTCTCTTTGTAAAACTTCGTGCATGACTTCAGCTTTACTCTTTGTCAA
GACATGCCAAAGTGCTGAGTCACTAATAAAAGAAAAAAAGAAAGTAAAGG
AAGAGTGGTTCTGCTTCTTAGCGCTAGCCTCAATGACGACCTAAGCTGC
ACTTTTCCCCCTAGTTGTGTCTTGCCATGCTAAAGGACGTCACATTGCAC
AATCTTAATAAGGTTTCCAATCAGCCCCACCCGCTCTGGCCCCACCCTCA
CCCTCCAACAAAGATTTATCAAATGTGGGATTTTCCCATGAGTCTCAATAT
TAGAGTCTCAACCCCCAATAAATATAGGACTGGAGATGTCTGAGGCTCAT
TCTGCCCTCGAGCCCACCGGGAACGAAAGAGAAGCTCTATCTCCCCTCC
AGGAGCCCAGCTATG

Gene 2: *CCL20* (chr2: 227811861 to 227814004).

>chr2: 227811861-227814004

GCGTGATCTCGGCTCGCCGTAACTTCTGCTTCCTGGGCTCATGCGATTC
TCCTGCCTCAGCCTCCCAAGTAGCTGGGATTACAGGCATGGACTACCAC
ACCCGGCTAATATTTGTATTTTTAGTAGAGACAGGGTTTCACTATGTTGG
CCAGTCTGGTCTCGAACTCCTGACCTCAGGCAGTCCACCCACCTCGGCC
TCCCAAAATGCTAGGATTACAGGCATGAGCCACCACTCCTGGCCAGACA
CATTGGTTATTTAGGAATATGTGGTTTCCTTTCTGTCTGTTACTGATTTCT
GATTTAATTCCATTGTGGTTTGAATGTATACTTTATGTGATTTCAGTCGGT
TTACATTTACTGAGACTTGTTCCATAACCTACCATATGCTTTATCCTAGAG

AACATTCCATGTGCAACTATTCTGTTGATGTTGGATAGAGTAGTCCTTCG
ATGCCTGCTAAGTCTATTTGGCTTATAGTGTTGTTCAAGTCTTCTATTTTC
TTGTGGATCTTCTGCCTAAATTTTATTAATTAGTGAAAATGTGGTACCAAA
GCTGTCAACTTTTATTGTTAAATTTCCTATTCTGCTGGGCACGGTGGCTC
ATGCCTGTAATGAGGTCAGGAGTTCAGCCTGGGCAACATGGTGAAACCA
CGTCTCTACTAAAAATACAAAAATTCGCTGGGCGTGGTGGCACGTGCCT
GTAGTCCCAGCTACTCAGGAGGCTGAGGCAGGCGAATCGCTTGAACCC
AGGAGGCGGAGGTTGTAGTGAGCCGAGATGGTGCCACTGCACTCCAGC
CTGGGCAACAAGAGCAAAATTCTGTCTCAAAAGAAATTATAATAATAAAAT
AAAATAAAATTTCCTATTCTTCCCTTCAATTCTGTCAGTTTTTGCTTTCTGT
AGTTTGGGGTGCTACTGTTAGGTATATATATGGTTATAGGTTTTACATCTT
CCCAATGGGTTAATTTTGTGTTACCATGAAAGTCCCTTTTTATTTCCGGTA
ACATTAAAAAAAAATCTATTTTGTCTGATATAGGCATCACCAACTCCAGTT
CTCTTTTGTTTACTGTTTGGTATACCTTTCTTTTGATCCTTTTACTTTCAAC
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TATTACATTATCATCATTAGTACTGTTATTTGACATTTGCTGTGCTGACTA
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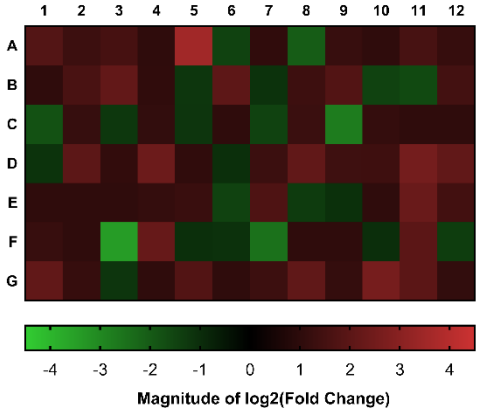
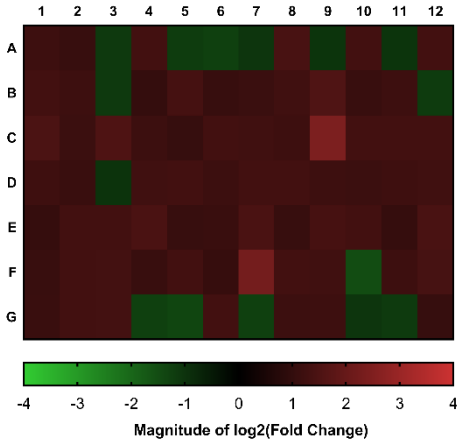
GTTATATTAGTACATCATCATGGAGAGTTAAAGGTAGGTAAGGATTATTTT
CTGAACTGCAATATTGATTAAAGCCATGTGAATGTATAAGATTCTTAGAAG
AGTTGACATTAAATCAAGGTGAAGCTGAGGTTTGAGCCTTACTTAAAGGC
TGATATTTTCCACTCTAACTGCGGACAGTACTGTAGCACTGTTATAGTAC
CTGCTCTGAATGTTAGTCTAGCAACTCAGGGTCTTCTTCATGACAGCTGA
ACCTCAACCATGTGATGGTAAATGTGTAGCAGAGTATGCCTGGCATCCC
ACCTGCTCCTCCTCCCCCTCCTCCTTGACTGGTTCTGGAAAGCAAATAG
GGTGTAACAATAGGAGTTCTGGAATGTTCCTGTGTGGGGCTGACCTTTG
TATCGCTGTTAATCCTCTATTTTCAGACACAAAAATGATTAAGTTAAAACT
GGATGAAAGTCTTTTCTGGGTCACAGGGCTGAGCTGCTTTTGCTCTTTGC
AAATACAAAGAATTTAACAGGATTCTCCCCTTCTCAACTTCCTGTCCCCC
ACCCTGACCTTCGCACCTTCCCAATATGAGGAAAAAGCAGGAAGTTTTCC
TTGCGGGTTTTTTTTATGATGACATGATGGGGCCAGTTGATCAATGGGGA
AAACCCCATGTGGCAACACGCCTTCTGTGTACATTCCCAATATTTGCTAT
AAATAGGGCCATCCCAGGCTGCTGTCAGAATATAACAGCACTCCCAAAG
AACTGGGTACTCAACACTGAGCAGATCTGTTCTTTGAGCTAAAAACCATG
TGCTGTACCAAGAGTTTGCTCCTGGCTGCTTTGATGTCAGTGCTGCTACT
CCACCTCTGCGGCGAATCAGAAGGTAAGTGTCGCTCTTTC

Appendix 4.1 Interested CpG sites (highlighted in yellow background) of *IL6* (18 CpG sites) and *CCL20* (21 CpG sites). Transcription start site is highlighted in green background.

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AMH / 1.81 / B	BDNF / 1.3	BMP1 / 1.52	BMP10 / 1.01 / B	BMP2 / 3.53	BMP3 / -1.45 / B	BMP4 / 1.04	BMP5 / -2.03 / B	BMP6 / 1.19	BMP7 / 1.01 / C	BMP8B / 1.54	ADA2 / 1.17 / B
B	CLC / 1.01 / C	CSF1 / 1.6	CSF2 / 2.15	CSF3 / 1.05 / B	CSPG5 / -1.19	CXCL1 / 2.04	DKK1 / -1.1	ERAP1 / 1.32	EREG / 1.79	FGF1 / -1.46	FGF11 / -1.61 / B	FGF13 / 1.47 / B
C	FGF14 / -1.78 / B	FGF17 / 1.18 / B	FGF19 / -1.22 / B	FGF2 / 1.09	FGF22 / -1.18 / B	FGF23 / 1.01 / C	FGF5 / -1.45	FGF6 / 1.27 / B	FGF7 / -2.73 / B	FGF9 / 1.14 / B	VEGFD / 1.01 / C	GDF10 / 1.01 / C
D	GDF11 / -1.14	GDNF / 2.0	GPI / 1.08	HBEGF / 2.35	IGF1 / 1.04 / B	IGF2 / -1.04	IL10 / 1.28 / B	IL11 / 2.12	IL12B / 1.36 / B	IL18 / 1.34	IL1A / 2.54 / B	IL1B / 2.11
E	IL2 / 1.01 / C	IL3 / 1.01 / C	IL4 / 1.01 / C	INHA / 1.13	INHBA / 1.25	INHBB / -1.46	JAG1 / 1.7	JAG2 / -1.3	LEFTY1 / -1.07 / B	LEFTY2 / 1.01 / C	LIF / 2.3	LTBP4 / 1.43
F	MDK / 1.2	MSTN / 1.01 / C	NDP / -3.44	NGF / 2.22	NODAL / -1.04 / B	NRG1 / -1.08	NRG2 / -2.5	NRG3 / 1.01 / C	NRTN / 1.02	NTF3 / -1.01 / B	OSGIN1 / 1.98	PDGFC / -1.34
G	PGF / 2.11	PSPN / 1.17	PTN / -1.18	SLCO1A2 / 1.01 / C	SPP1 / 1.78 / A	TDGF1 / 1.01 / C	TGFB1 / 1.31	THPO / 2.1 / B	TNNT1 / 1.13	TYMP / 2.58	VEGFA / 1.98	VEGFC / 1.09

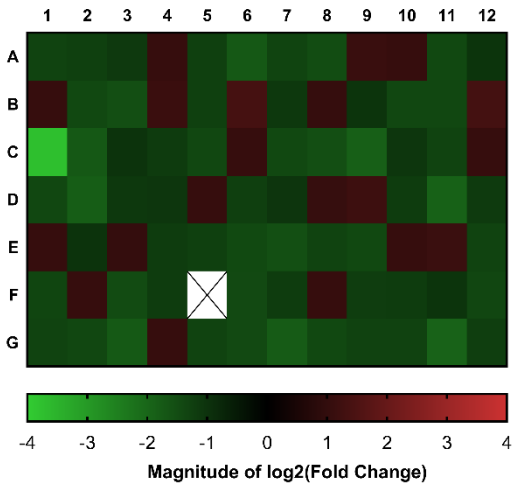


Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AMH / 1.19 / B	BDNF / 1.06	BMP1 / -1.12	BMP10 / 1.27 / C	BMP2 / -1.19	BMP3 / -1.26 / B	BMP4 / -1.05	BMP5 / 1.39 / B	BMP6 / -1.03	BMP7 / 1.27 / C	BMP8B / -1.03	ADA2 / 1.27 / B
B	CLC / 1.27 / C	CSF1 / 1.19	CSF2 / -1.12	CSF3 / 1.0 / B	CSPG5 / 1.34	CXCL1 / 1.07 / B	DKK1 / 1.13	ERAP1 / 1.24	EREG / 1.53	FGF1 / 1.09	FGF11 / 1.19 / B	FGF13 / -1.16 / B
C	FGF14 / 1.46 / B	FGF17 / 1.14 / B	FGF19 / 1.51 / B	FGF2 / 1.17	FGF22 / 1.05 / B	FGF23 / 1.27 / C	FGF5 / 1.24	FGF6 / 1.18 / B	FGF7 / 2.41 / B	FGF9 / 1.27 / C	VEGFD / 1.27 / C	GDF10 / 1.27 / C
D	GDF11 / 1.2	GDNF / 1.1	GPI / -1.01	HBEGF / 1.23	IGF1 / 1.27 / C	IGF2 / 1.16	IL10 / 1.27 / C	IL11 / 1.28	IL12B / 1.18 / B	IL18 / 1.15	IL1A / 1.2 / B	IL1B / 1.23
E	IL2 / 1.02 / B	IL3 / 1.27 / C	IL4 / 1.27 / C	INHA / 1.43	INHBA / 1.07	INHBB / 1.1	JAG1 / 1.44	JAG2 / 1.07	LEFTY1 / 1.35 / B	LEFTY2 / 1.27 / C	LIF / 1.02	LTBP4 / 1.41
F	MDK / 1.08	MSTN / 1.27 / C	NDP / 1.29	NGF / 1.08	NODAL / 1.27 / C	NRG1 / 1.04	NRG2 / 2.25	NRG3 / 1.27 / C	NRTN / 1.23	NTF3 / -1.49 / B	OSGIN1 / 1.18	PDGFC / 1.36
G	PGF / 1.11	PSPN / 1.27	PTN / 1.3	SLCO1A2 / -1.26 / B	SPP1 / -1.35 / A	TDGF1 / 1.27 / C	TGFB1 / -1.25	THPO / 1.16 / B	TNNT1 / 1.19	TYMP / -1.05	VEGFA / -1.15	VEGFC / 1.05



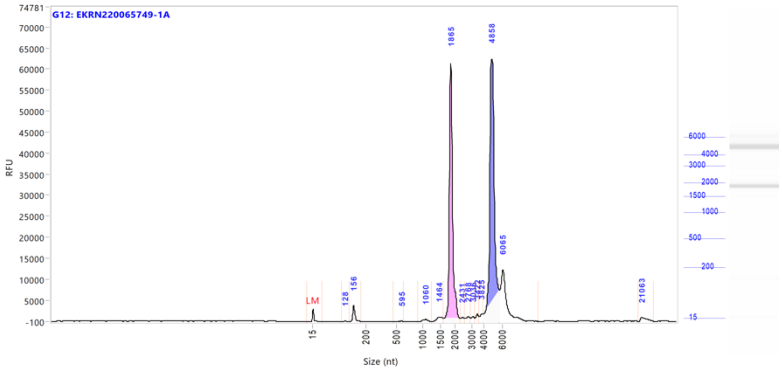
Appendix 4.2 Gene expression fold change in human growth factors pathway in HHL-16 cells after 10 µg/ml AFB_1 treatment (above figures) or 50 µg/ml FB_1 treatment (below figures) for 24 hours in three replicates.

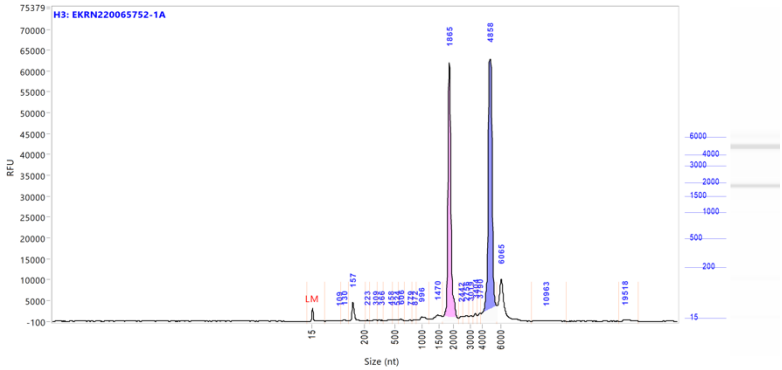
Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AMH / -1.31 / B	BDNF / -1.25	BMP1 / -1.12	BMP10 / 1.04 / C	BMP2 / -1.26	BMP3 / -1.73 / B	BMP4 / -1.34	BMP5 / -1.5 / B	BMP6 / 1.11	BMP7 / 1.04 / C	BMP8B / -1.42	ADA2 / -1.03 / B
B	CLC / 1.04 / C	CSF1 / -1.42	CSF2 / -1.54	CSF3 / 1.12 / B	CSPG5 / -1.26	CXCL1 / 1.34 / B	DKK1 / -1.11	ERAP1 / 1.03	EREG / -1.03	FGF1 / -1.39	FGF11 / -1.39 / B	FGF13 / 1.31 / B
C	FGF14 / -3.73 / B	FGF17 / -1.72 / B	FGF19 / -1.0 / B	FGF2 / -1.17	FGF22 / -1.4 / B	FGF23 / 1.04 / C	FGF5 / -1.42	FGF6 / -1.53 / B	FGF7 / -1.86 / B	FGF9 / -1.07 / B	VEGFD / -1.32 / B	GDF10 / 1.04 / C
D	GDF11 / -1.39	GDNF / -1.83	GPI / -1.11	HBEGF / -1.06	IGF1 / 1.04 / C	IGF2 / -1.24	IL10 / -1.04 / B	IL11 / 1.05	IL12B / 1.18 / B	IL18 / -1.18	IL1A / -1.9 / B	IL1B / -1.14
E	IL2 / 1.04 / C	IL3 / -1.0 / B	IL4 / 1.01 / B	INHA / -1.19	INHBA / -1.25	INHBB / -1.43	JAG1 / -1.56	JAG2 / -1.31	LEFTY1 / -1.41 / B	LEFTY2 / 1.04 / C	LIF / 1.13	LTBP4 / -1.31
F	MDK / -1.35	MSTN / 1.04 / C	NDP / -1.48	NGF / -1.19	NODAL / 25.86 / B	NRG1 / -1.44	NRG2 / -1.18	NRG3 / 1.04 / C	NRTN / -1.2	NTF3 / -1.18 / B	OSGIN1 / -1.03	PDGFC / -1.37
G	PGF / -1.31	PSPN / -1.39	PTN / -1.74	SLCO1A2 / 1.04 / C	SPP1 / -1.31 / A	TDGF1 / -1.44 / B	TGFB1 / -1.78	THPO / -1.42 / B	TNNT1 / -1.29	TYMP / -1.29	VEGFA / -1.92	VEGFC / -1.21



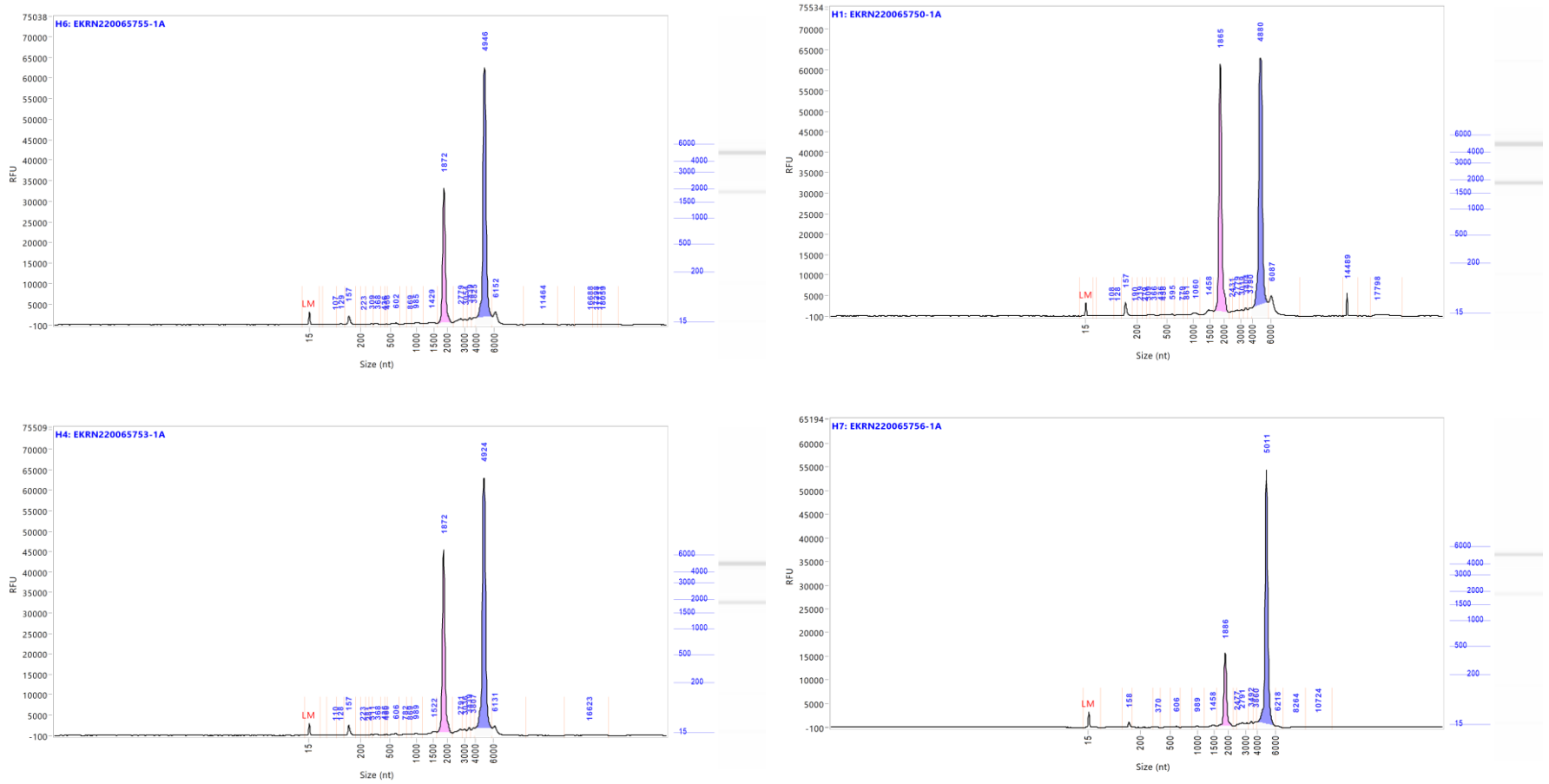
Appendix 4.3 Gene expression fold change in human growth factors pathway in HHL-16 cells after combined treatment of 1 µg/ml AFB_1 and 50 µg/ml FB_1 for 24 hours in three replicates. One extreme outlier of F5 with great variation was excluded.

Sample	Nucleic acid ID	Concentration (ng/μl)	Integrity value	Sample QC Results
D20_1	EKRN220065749-1A	794.00	10	Pass
D20_2	EKRN220065752-1A	638.00	10	Pass
D20_3	EKRN220065755-1A	401.00	10	Pass
A20_1	EKRN220065750-1A	602.00	10	Pass
A20_2	EKRN220065753-1A	489.00	10	Pass
A20_3	EKRN220065756-1A	246.00	10	Pass

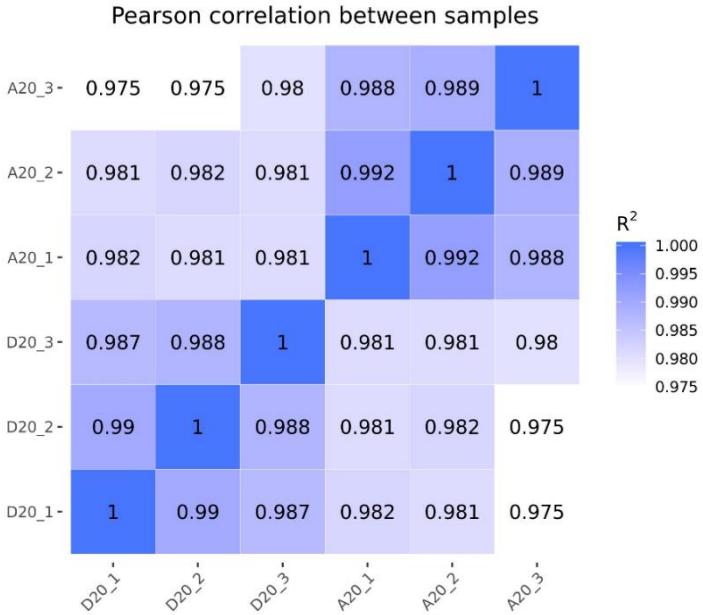


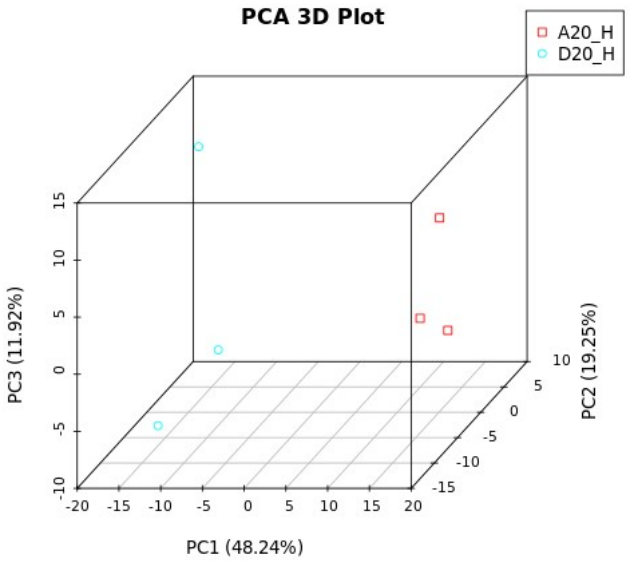


Appendix 5.1 Quality control test of samples for RNA-seq analysis.



(Continued) Appendix 5.1 Quality control test of samples for RNA-seq analysis.





Appendix 5.2 Correlation analysis and principal component analysis among RNA-seq samples in HHL-16 cells after 20 μg/ml AFB_1 treatment for 24 hours.

Appendix 5.3 FPKM value of several neuronal gene markers in HHL-16 cells.

Neuro gene markers	RPKM in NCBI database	FRPKM in HHL-16 cells
MOG	52.4	0, not expressed
GRIN2B	5.9	0, not expressed
GAD2	7.7	0, not expressed

Appendix 5.4 Summary of DEGs in enriched neuro-related GO terms.

DEGs	Brain_RPKM_NCBI	Gall bladder_RPKM_NCBI	Liver_RPKM_NCBI	FPKM_HHL-16
SHANK2	4.2	0.2	0.7	1.1
NR1D1	21	5.6	3.7	6.6
CPEB4	15	8.1	12.6	3.4
ANK2	29	4.5	No	0.6
KCNMA1	4.4	2.3	0.3	1.2
DISC1	1.2	0.9	0.2	0.3
GPHN	6.1	2.6	6.5	2.4
NLGN1	1.8	0.2	No	0.2
PLCB4	1.2	1.8	No	21.1
GRIP1	0.7	0.3	0.03	0.3
MAP2	82.9	1.9	No	1
ANK1	1.1	0.3	No	1.5
MAGI2	2.5	0.3	0.07	0.2
ADORA1	15.42	0.4	No	1.4
GRIK4	2.8	0.07	No	0.5
ARC	5.4	1.4	0.2	0.05
CAMK2A	111.5	No	No	0.3
CACNG4	19.3	0.3	No	0.5

LZTS1	3.1	1.6	0.08	0.4
DNM3	4.4	0.9	0.06	0.1
CHRM4	-	-	-	0.5
PTK2B	18.7	6.3	1.7	0.3
MAPK8IP2	21.5	No	No	0.3
KCTD16	4.6	0.11	No	0.1
CHRM3	3	0.15	0.1	0.1
SHISA9	3.5	0.03	0.1	0.1
LAMA2	0.9	7.9	1.1	0.2
PALMD	3	8.9	13.6	0.2
GABRA5	10.4	0.2	No	0.6
SRCIN1	9	No	0.2	0.1
LRRK2	2.7	5.2	2	0.1
SNAP25	433.5	No	No	1.8

Appendix 6.1 LOD values of each metabolite.

Metabolites	LODs(uM)
Creatinine	0.145
Glycine	0.859
Alanine	0.341
Serine	0.0424
Proline	0.136
Valine	0.161
Threonine	0.185
Taurine	0.0346
Putrescine	0.0075
trans-Hydroxyproline	0.0566
Leucine	0.652
Isoleucine	0.0525
Asparagine	0.0598
Aspartic acid	0.968
Glutamine	0.266
Glutamic acid	0.255
Methionine	0.0291
Histidine	0.114
alpha-Aminoadipic acid	0.00769
Phenylalanine	0.0147
Methionine-sulfoxide	0.0875
Arginine	0.11
Acetyl-ornithine	0.0105
Citrulline	0.75
Serotonin	0.00579
Tyrosine	0.0102
Asymmetric dimethylarginine	0.0115
total dimethylarginine	0.00898
Tryptophan	0.0599
Kynurenine	0.0164
Carnosine	0.00742
Ornithine	0.289
Lysine	0.155
Spermidine	0.00954
Spermine	0.0568
Sarcosine	0.00667
Creatine	0.18
Betaine	1.23
Choline	0.294
Trimethylamine N-oxide	0.266
Methylhistidine	0.00708
Homocysteine	0.248
Hypoxanthine	0.128
Ethanolamine	0.111
Cystathionine	0.0196
N-acetylpetrescine	0.00761
Homocitrulline	0.00427
Urea	1.28
Hydroxy-lysine	0.0386
alpha-aminobutyric acid	0.0121
gamma-aminobutyric acid	0.00689
Lactic acid	0.454
beta-Hydroxybutyric acid	0.111
alpha-Ketoglutaric acid	0.0183
Citric acid	0.175
Butyric acid	0.00909
Propionic acid	0.0127
HPHPA	0.00477
p-Hydroxyhippuric acid	0.00807
Succinic acid	0.0169
Fumaric acid	0.0472
Pyruvic acid	0.045
Isobutyric acid	0.00498
Hippuric acid	0.00705

(Continued) Appendix 6.1 LOD values of each metabolite.

Methylmalonic acid	0.00099
Homovanillic acid	0.000418
Indole acetic acid	0.0101
Uric acid	1.14
5-Hydroxy Indoleacetic acid	0.00728
p-Hydroxyphenylacetic acid	0.00433
Valeric acid	0.0234
Uridine	0.123
Xanthine	0.0111
Guanidineacetic acid	0.0953
Acetyl-lysine	0.00914
Glucose	22.5
LysoPC a C14:0	0.194
LysoPC a C16:1	0.0191
LysoPC a C16:0	0.0474
LysoPC a C17:0	0.152
LysoPC a C18:2	0.0258
LysoPC a C18:1	0.0541
LysoPC a C18:0	0.274
LysoPC a C20:4	0.0179
LysoPC a C20:3	0.0303

LysoPC a C24:0	0.128
LysoPC a C26:1	0.032
LysoPC a C26:0	0.0269
LysoPC a C28:1	0.017
LysoPC a C28:0	0.0765
SM(OH) C14:1	0.015
SM C16:1	0.0597
SM C16:0	0.26
SM(OH) C16:1	0.127
SM C18:1	0.0634
PC aa C32:2	0.0652
SM C18:0	0.0909
SM C20:2	0.0113
PC ae C36:0	0.0194
PC aa C36:6	0.156
PC aa C36:0	0.0898
SM(OH) C22:2	0.0251
SM(OH) C22:1	0.0257
PC aa C38:6	0.0365
PC aa C38:0	0.0511
PC ae C40:6	0.0686

SM(OH) C24:1	0.145
PC aa C40:6	0.0586
PC aa C40:2	0.012
PC aa C40:1	0.125
C0	0.222
C2	0.135
C3:1	0.0256
C3	0.0824
C4:1	0.024
C4	0.0486
C3OH	0.0337
C5:1	0.0305
C5	0.0256
C4OH	0.0237
C6:1	0.0247
C6	0.0929
C5OH	0.0341
C5:1DC	0.025
C5DC	0.0191
C8	0.0316
C5MDC	0.0218

(Continued) Appendix 6.1 LOD values of each metabolite.

C9	0.011
C7DC	0.02
C10:2	0.049
C10:1	0.118
C10	0.0585
C12:1	0.0786
C12	0.0443
C14:2	0.0519
C14:1	0.0716
C14	0.032
C12DC	0.0148
C14:2OH	0.0542
C14:1OH	0.056
C16:2	0.0463
C16:1	0.0201
C16	0.0265
C16:2OH	0.0368
C16:1OH	0.0491
C16OH	0.0426
C18:2	0.0581
C18:1	0.0411
C18	0.0237
C18:1OH	0.0568

Appendix 6.2 Metabolites levels in the children grouped into AF-alb negative and positive groups.

Characteristic	**neg**, N = 436[1]	**pos**, N = 447[1]
Amino acids and derivatives (µM)		
met_creatinine	21 (17, 26)	22 (18, 28)
met_glycine	174 (143, 217)	171 (132, 221)
met_alanine	256 (186, 352)	233 (170, 334)
met_serine	125 (99, 152)	116 (90, 146)
met_proline	189 (144, 239)	172 (130, 230)
met_valine	131 (106, 162)	123 (92, 158)
met_threonine	70 (50, 97)	58 (39, 82)
met_taurine	66 (43, 102)	70 (43, 108)
met_putrescine	0.11 (0.07, 0.15)	0.11 (0.07, 0.16)
met_trans.OHproline	14 (10, 20)	13 (8.7, 18)
met_leucine	58 (45, 74)	52 (37, 72)
met_isoleucine	29 (22, 37)	25 (17, 33)
met_asparagine	31 (25, 39)	33 (25, 42)
met_aspartic.ac	17 (12, 24)	16 (11, 22)
met_glutamine	404 (316, 486)	352 (262, 456)
met_glutamic.ac	110 (76, 153)	87 (57, 118)
met_methionine	18 (13, 23)	16 (11, 22)
met_histidine	52 (42, 62)	52 (42, 63)
met_a.aminoadipic.ac	0.56 (0.36, 0.88)	0.48 (0.30, 0.78)
met_phenylalanine	59 (49, 76)	61 (48, 85)
met_methionine.sulfoxide	1.4 (1.0, 2.2)	1.2 (0.92, 1.8)
met_arginine	64 (51, 82)	57 (43, 73)

(Continued) Appendix 6.2 Metabolites levels in the children grouped into AF-alb negative and positive groups.

met_acetyl.ornithine	0.39 (0.17, 0.91)	0.25 (0.13, 0.52)
met_citrulline	9.9 (6.4, 15)	9.7 (6.3, 15)
met_serotonin	1.6 (0.84, 2.7)	1.3 (0.38, 2.6)
met_tyrosine	51 (40, 67)	49 (34, 68)
met_asymmetric.dimethylarginine	0.55 (0.41, 0.68)	0.51 (0.39, 0.64)
met_total.dimethylarginine	1.7 (1.4, 2.0)	1.6 (1.4, 2.0)
met_tryptophan	33 (22, 50)	29 (12, 41)
met_kynurenine	4.2 (3.0, 5.7)	3.3 (2.1, 5.1)
met_carnosine	0.14 (0.00, 0.57)	0.17 (0.00, 0.67)
met_ornithine	41 (28, 57)	35 (22, 50)
met_lysine	93 (71, 120)	77 (57, 105)
met_spermidine	0.14 (0.10, 0.20)	0.18 (0.12, 0.30)
met_spermine	0.19 (0.16, 0.25)	0.23 (0.17, 0.33)
met_sarcosine	1.4 (0.78, 2.2)	1.0 (0.59, 1.8)
met_creatine	33 (23, 46)	44 (30, 66)
met_bine	58 (36, 89)	72 (45, 117)
met_choline	13 (10, 18)	12 (9.4, 16)
met_trimethylamine.n.oxide	1.5 (1.2, 3.2)	2.7 (1.4, 4.9)
met_methylhistidine	2.8 (2.3, 3.7)	3.0 (2.2, 4.0)
met_homocysteine	10 (9.1, 12)	10 (9.1, 12)
met_hypoxanthine	30 (20, 41)	25 (16, 39)

(Continued) Appendix 6.2 Metabolites levels in the children grouped into AF-alb negative and positive groups.

met_ethanolamine	7.9 (5.5, 12)	11 (7.2, 19)
met_cystathionine	0.53 (0.50, 0.57)	0.53 (0.51, 0.58)
met_n.acetylpetrescine	0.10 (0.07, 0.13)	0.12 (0.09, 0.17)
met_homocitrulline	0.25 (0.16, 0.39)	0.24 (0.14, 0.37)
met_urea	2,155 (1,388, 3,265)	2,090 (1,440, 3,495)
met_OH.lysine	0.37 (0.25, 0.54)	0.41 (0.27, 0.56)
met_a.aminobutyric.ac	6.6 (4.2, 11)	6.6 (4.1, 10)
met_g.aminobutyric.ac	0.11 (0.06, 0.20)	0.09 (0.05, 0.18)
met_lactic.ac	2,425 (1,790, 3,450)	2,735 (2,122, 4,030)
met_b.OHbutyric.ac	587 (117, 1,642)	526 (117, 1,485)
met_a.ketoglutaric.ac	8.8 (6.7, 12)	9.5 (7.1, 14)
met_citric.ac	97 (80, 121)	93 (77, 114)
met_butyric.ac	0.78 (0.63, 1.0)	0.93 (0.71, 1.2)
met_propionic.ac	2.0 (1.5, 2.6)	1.9 (1.3, 2.7)
met_hphpa	0.01 (0.01, 0.01)	0.01 (0.01, 0.02)
met_p.OHhippuric.ac	0.05 (0.04, 0.11)	0.06 (0.04, 0.11)
met_succinic.ac	1.9 (1.4, 2.6)	2.1 (1.6, 3.1)
met_fumaric.ac	1.2 (0.92, 1.7)	1.6 (1.1, 2.4)
met_pyruvic.ac	54 (38, 74)	71 (52, 90)
met_isobutyric.ac	1.6 (1.3, 2.4)	1.8 (1.5, 2.2)
met_hippuric.ac	0.29 (0.13, 0.86)	0.55 (0.22, 1.7)

(Continued) Appendix 6.2 Metabolites levels in the children grouped into AF-alb negative and positive groups.

met_methylmalonic.ac	0.15 (0.09, 0.26)	0.15 (0.10, 0.26)
met_homovanillic.ac	0.03 (0.02, 0.04)	0.03 (0.02, 0.06)
met_indole.acetic.ac	0.66 (0.37, 1.2)	0.69 (0.39, 1.3)
met_uric.ac	276 (209, 377)	280 (217, 370)
met_OH5.indoleacetic.ac	0.05 (0.03, 0.07)	0.05 (0.04, 0.07)
met_p.OHphenylacetic.ac	0.21 (0.08, 0.61)	0.30 (0.12, 0.75)
met_valeric.ac	0.60 (0.50, 0.74)	0.66 (0.55, 0.83)
met_uridine	5.4 (4.6, 6.9)	5.3 (4.1, 7.0)
met_xanthine	3.9 (2.7, 5.5)	3.8 (2.3, 5.5)
met_guanidineacetic.ac	0.71 (0.49, 1.1)	0.76 (0.49, 1.1)
met_acetyl.lysine	0.16 (0.10, 0.25)	0.17 (0.10, 0.28)
Carnitines (μM)		
met_c0	21 (15, 31)	23 (16, 30)
met_c2	12 (7.5, 20)	13 (7.3, 22)
met_c3.1	0.02 (0.01, 0.02)	0.02 (0.01, 0.02)
met_c3	0.25 (0.17, 0.36)	0.26 (0.19, 0.37)
met_c4.1	0.02 (0.02, 0.03)	0.03 (0.02, 0.03)
met_c4	0.14 (0.11, 0.20)	0.14 (0.11, 0.20)
met_c3oh	0.03 (0.03, 0.04)	0.03 (0.03, 0.04)
met_c5.1	0.02 (0.02, 0.02)	0.02 (0.01, 0.02)
met_c5	0.08 (0.06, 0.12)	0.08 (0.05, 0.11)
met_c4oh	0.11 (0.06, 0.21)	0.12 (0.06, 0.23)
met_c6.1	0.03 (0.02, 0.03)	0.03 (0.02, 0.03)
met_c6	0.09 (0.07, 0.12)	0.10 (0.07, 0.13)

(Continued) Appendix 6.2 Metabolites levels in the children grouped into AF-alb negative and positive groups.

met_c5oh	0.03 (0.03, 0.04)	0.03 (0.03, 0.04)
met_c5.1dc	0.02 (0.02, 0.03)	0.02 (0.02, 0.03)
met_c5dc	0.03 (0.02, 0.03)	0.03 (0.02, 0.03)
met_c8	0.11 (0.07, 0.15)	0.12 (0.08, 0.17)
met_c5mdc	0.03 (0.03, 0.04)	0.03 (0.02, 0.03)
met_c9	0.01 (0.01, 0.02)	0.01 (0.01, 0.01)
met_c7dc	0.04 (0.03, 0.05)	0.03 (0.03, 0.05)
met_c10.2	0.04 (0.03, 0.05)	0.03 (0.03, 0.04)
met_c10.1	0.25 (0.21, 0.30)	0.24 (0.20, 0.29)
met_c10	0.25 (0.18, 0.36)	0.26 (0.18, 0.37)
met_c12.1	0.13 (0.09, 0.17)	0.12 (0.10, 0.16)
met_c12	0.11 (0.08, 0.16)	0.09 (0.06, 0.14)
met_c14.2	0.04 (0.03, 0.06)	0.03 (0.02, 0.05)
met_c14.1	0.09 (0.06, 0.14)	0.08 (0.05, 0.13)
met_c14	0.07 (0.05, 0.09)	0.06 (0.04, 0.09)
met_c12dc	0.01 (0.01, 0.01)	0.01 (0.01, 0.01)
met_c14.2oh	0.02 (0.01, 0.02)	0.01 (0.01, 0.02)
met_c14.1oh	0.02 (0.02, 0.03)	0.02 (0.02, 0.03)
met_c16.2	0.01 (0.01, 0.02)	0.01 (0.01, 0.02)
met_c16.1	0.05 (0.04, 0.06)	0.04 (0.03, 0.06)
met_c16	0.15 (0.12, 0.19)	0.14 (0.10, 0.19)
met_c16.2oh	0.01 (0.01, 0.01)	0.01 (0.01, 0.01)
met_c16.1oh	0.02 (0.01, 0.02)	0.02 (0.01, 0.02)
met_c16oh	0.01 (0.01, 0.01)	0.01 (0.01, 0.01)

(Continued) Appendix 6.2 Metabolites levels in the children grouped into AF-alb negative and positive groups.

met_c18.2	0.04 (0.03, 0.06)	0.04 (0.03, 0.05)
met_c18.1	0.11 (0.08, 0.15)	0.11 (0.08, 0.15)
met_c18	0.04 (0.03, 0.05)	0.04 (0.03, 0.06)
met_c18.1oh	0.01 (0.01, 0.02)	0.01 (0.01, 0.02)
LysoPC (μM)		
met_lysopc.a.c14.0	1.3 (0.89, 2.0)	1.2 (0.84, 2.0)
met_lysopc.a.c16.1	0.66 (0.40, 0.93)	0.54 (0.34, 0.88)
met_lysopc.a.c16.0	34 (22, 48)	26 (15, 41)
met_lysopc.a.c17.0	0.33 (0.20, 0.52)	0.29 (0.17, 0.45)
met_lysopc.a.c18.2	11 (5.5, 20)	7.6 (3.3, 14)
met_lysopc.a.c18.1	7.6 (4.3, 11)	6.2 (3.2, 11)
met_lysopc.a.c18.0	8.6 (5.0, 14)	6.8 (3.8, 12)
met_lysopc.a.c20.4	3.0 (1.9, 4.7)	2.2 (1.3, 3.8)
met_lysopc.a.c20.3	2.0 (1.5, 2.6)	1.7 (1.3, 2.3)
met_lysopc.a.c24.0	0.11 (0.09, 0.15)	0.12 (0.09, 0.15)
met_lysopc.a.c26.1	0.04 (0.03, 0.06)	0.04 (0.03, 0.05)
met_lysopc.a.c26.0	0.40 (0.30, 0.55)	0.38 (0.30, 0.49)
met_lysopc.a.c28.1	0.15 (0.11, 0.21)	0.15 (0.12, 0.19)
met_lysopc.a.c28.0	0.15 (0.11, 0.20)	0.13 (0.10, 0.17)
PC (μM)		
met_pc.aa.c32.2	1.9 (1.6, 2.3)	1.9 (1.5, 2.4)
met_pc.ae.c36.0	0.73 (0.59, 0.90)	0.75 (0.59, 0.96)
met_pc.aa.c36.6	0.36 (0.25, 0.55)	0.29 (0.20, 0.43)
met_pc.aa.c36.0	4.7 (3.8, 5.7)	4.5 (3.6, 5.9)

(Continued) Appendix 6.2 Metabolites levels in the children grouped into AF-alb negative and positive groups.

met_pc.aa.c38.6	53 (38, 72)	46 (30, 65)
met_pc.aa.c38.0	2.7 (2.1, 3.4)	2.3 (1.7, 3.2)
met_pc.ae.c40.6	3.2 (2.4, 3.9)	2.8 (2.1, 3.6)
met_pc.aa.c40.6	18 (13, 24)	15 (10, 22)
met_pc.aa.c40.2	0.16 (0.13, 0.21)	0.15 (0.11, 0.20)
met_pc.aa.c40.1	0.19 (0.15, 0.24)	0.18 (0.13, 0.23)
Sphingomyelin (μM)		
met_sm.oh.c14.1	2.5 (1.9, 3.1)	1.9 (1.5, 2.7)
met_sm.c16.1	9.5 (7.8, 12)	8.7 (7.2, 11)
met_sm.c16.0	87 (73, 102)	82 (66, 99)
met_sm.oh.c16.1	1.6 (1.3, 2.1)	1.5 (1.1, 1.9)
met_sm.c18.1	6.0 (4.7, 7.3)	5.2 (4.2, 6.5)
met_sm.c18.0	19 (15, 24)	17 (13, 21)
met_sm.c20.2	0.19 (0.14, 0.24)	0.16 (0.12, 0.22)
met_sm.oh.c22.2	3.8 (3.0, 4.9)	3.4 (2.5, 4.3)
met_sm.oh.c22.1	5.2 (4.1, 6.6)	4.6 (3.1, 6.1)
met_sm.oh.c24.1	0.87 (0.67, 1.1)	0.74 (0.56, 0.96)
Sugars (μM)		
met_glucose	4,593 (4,013, 5,312)	4,886 (4,135, 5,804)
Class and Ratios (μM)		
met_AAA	204 (172, 239)	200 (162, 246)
met_BCAA	217 (175, 270)	200 (147, 264)
met_BCmet_AAA	1.0 (0.90, 1.2)	0.99 (0.82, 1.2)
met_fischer	1.9 (1.5, 2.2)	1.7 (1.4, 2.1)

(Continued) Appendix 6.2 Metabolites levels in the children grouped into AF-alb negative and positive groups.

met_essential	565 (462, 670)	528 (415, 637)
met_glucogenic	1,688 (1,400, 1,926)	1,502 (1,218, 1,851)
met_keto	152 (120, 191)	135 (102, 170)
met_kyn_trp	0.14 (0.08, 0.21)	0.14 (0.09, 0.23)
met_orn_arg	0.61 (0.47, 0.80)	0.60 (0.45, 0.78)
met_orn_putr	412 (230, 686)	326 (186, 563)
met_sero_trp	0.05 (0.02, 0.09)	0.06 (0.02, 0.12)
met_spermi_putres	1.4 (0.85, 2.7)	1.8 (1.1, 3.1)
met_spermine_spermidine	1.4 (1.1, 1.9)	1.3 (0.97, 1.8)
met_tyr_phe	0.86 (0.58, 1.1)	0.75 (0.44, 1.1)
met_totalDMA_arg	0.03 (0.02, 0.03)	0.03 (0.02, 0.04)
met_citruline_orn	0.25 (0.18, 0.35)	0.30 (0.20, 0.41)
met_citruline_arg	0.16 (0.11, 0.22)	0.17 (0.12, 0.25)
met_c2_c0	0.56 (0.29, 1.1)	0.54 (0.29, 1.2)
met_Totalaa	2,140 (1,800, 2,485)	1,914 (1,575, 2,345)
met_urea_cycle	150 (122, 188)	139 (105, 177)

[1] Median (IQR) or Frequency (%)

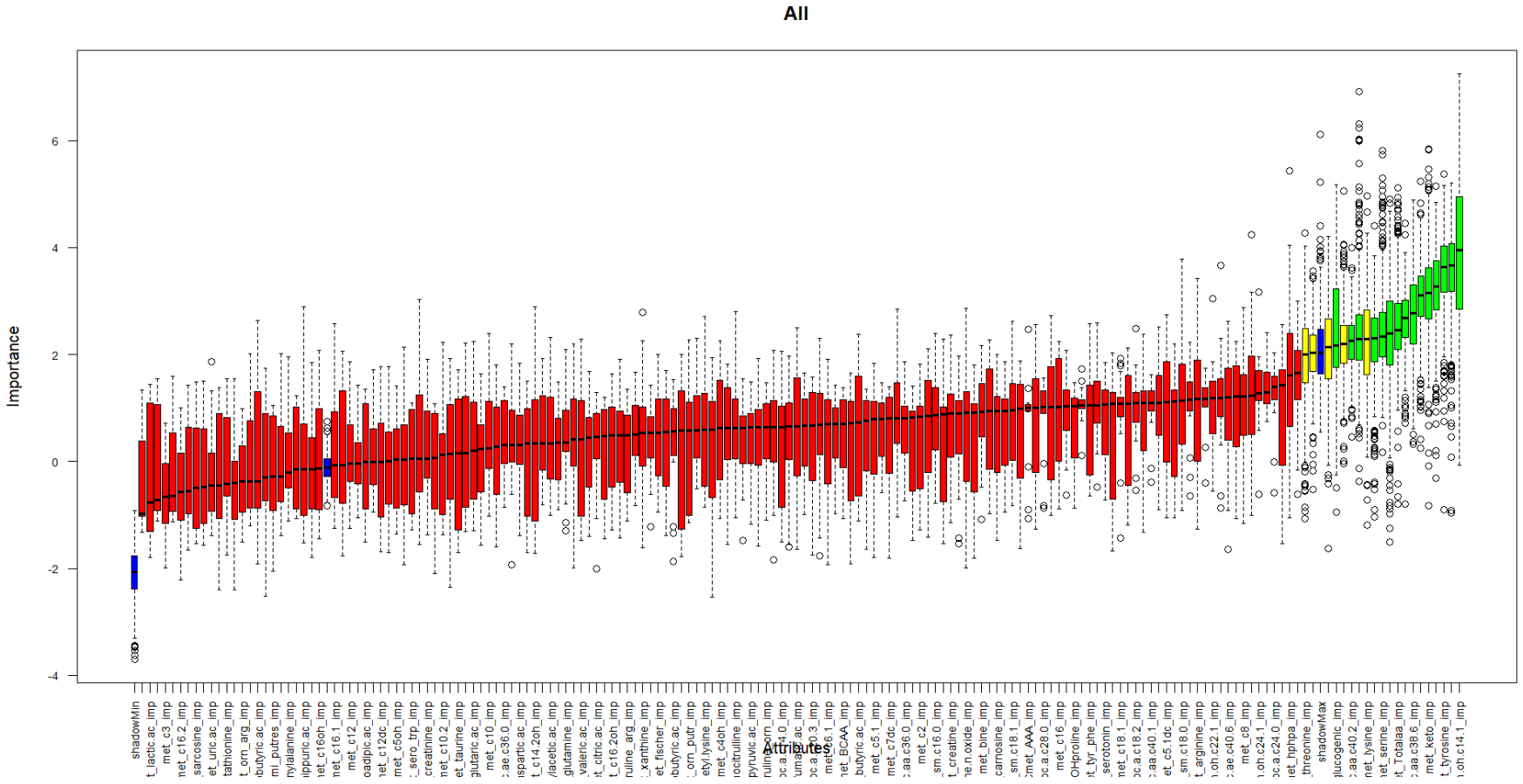
Appendix 6.3 KEGG ID of candidate metabolites and the ratios.

Candidate metabolites and calculated ratios	**Common name**	**Mapped KEGG ID**
Sm.oh.c14.1	SM(d18:0/14:1(9Z)(OH))	C00550
Lysopc.a.c26.0	lysoPC(26:0)	-
Tyrosine	L-Tyrosine	C00082
Lysopc.a.c26.1	lysoPC(26:1(5Z))	-
Pc.aa.c38.6	PC(18:2(9Z,12Z)/20:4(5Z,8Z,11Z,14Z))	C00157
Leucine	L-Leucine	C00123
Serine	L-Serine	C00065
Lysopc.a.c16.0	LysoPC(16:0)	C04230
Valine	L-Valine	C00183
Lysine	L-Lysine	C00047
Pc.aa.c40.2	PC(20:1(11Z)/20:1(11Z))	C00157
Uridine	Uridine	C00299
Pc.aa.c40.6	PC(18:1(9Z)/22:5(4Z,7Z,10Z,13Z,16Z))	C00157
Lysopc.a.c20.4	LysoPC(20:4(5Z,8Z,11Z,14Z))	C04230

Threonine	L-Threonine	C00188
Phenylalanine	L-Phenylalanine	C00079
Tryptophan	L-Tryptophan	C00078
Methionine	L-Methionine	C00073
Isoleucine	L-Isoleucine	C00407
Histidine	L-Histidine	C00135
Alanine	L-Alanine	C00041
Arginine	L-Arginine	C00062
Asparagine	L-Asparagine	C00152
Aspartic.ac	L-Aspartic acid	C00049
Glutamic.ac	L-Glutamic acid	C00025
Glutamine	L-Glutamine	C00064
Glycine	Glycine	C00037
Proline	L-Proline	C00148
Cystathionine	L-Cystathionine	C02291
Homocysteine	Homocysteine	C00155
Ornithine	Ornithine	C00077

Citrulline	Citrulline	C00327
Sm.c18.0	SM(d18:1/18:0)	C00550
Sm.c16.0	SM(d18:1/16:0)	C00550
Lysopc.a.c28.0	lysoPC(28:0)	-
Lysopc.a.c14.0	LysoPC(14:0)	C04230
Pc.aa.c36.6	PC(22:5(4Z,7Z,10Z,13Z,16Z)/14:1(9Z))	C00157
Sm.oh.c16.1	SM OH C16.1	-
Sm.c18.1	SM(d18:1/18:1(9Z))	C00550
Lysopc.a.c18.0	LysoPC(18:0)	C04230
Pc.aa.c32.2	PC(16:1(9Z)/16:1(9Z))	C00157
Pc.aa.c38.0	PC(16:0/22:0)	C00157
Sm.c20.2	SM(d18:0/20:2(11Z,14Z))	C00550
Pc.ae.c40.6	PC(o-18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	-
Spermidine	Spermidine	C00315
Putrescine	Putrescine	C00134
Ratio_Keto	Total ketogenic amino acids (leucine and lysine)	

Ratio_essential	Total essential amino acids (phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine)
Ratio_totalaa	Total amino acids (arginine, methionine, valine, alanine, asparagine, aspartic ac, glutamic ac, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, citrulline, ornithine, cystathionine, and homocysteine)
Ratio_glucogenic	Total glucogenic amino acids (alanine, arginine, asparagine, aspartic, glutamic, glutamine, glycine, histidine, methionine, proline, serine, valine, cystathionine, and homocysteine)
Ratio_urea_cycle	Total urea cycle amino acids (citrulline, ornithine, arginine, citrulline, and asparagine)
Ratio_spermi_putres	Spermidine to Putrescine ratio
Ratio_tyr_phe	Tyrosine to Phenylalanine ratio
Ratio_citruline_orn	Citrulline to Ornithine ratio
Ratio_citruline_arg	Citrulline to Arginine ratio
Ratio_orn_putr	Ornithine to Putrescine ratio



Appendix 6.4 Result of Boruta modelling indicating important metabolites associated with aflatoxin exposure.