Impact of aflatoxin on health in Sub-Saharan Africa

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The 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

Aflatoxins (AFs) are one of the major types of mycotoxins and are common contaminants of crops such as maize and groundnuts, especially in tropical and sub-tropical areas. Suitable climate for fungal growth, subsistence farming practices and dietary reliance on staple crops contribute to the chronic and high exposure to AFs in populations in sub-Saharan Africa. AFs have been identified as carcinogens. However, the impact on growth and immune functions have been less investigated in humans. In this work, aflatoxin exposure of populations from Gambia, Malawi and Tanzania was assessed using the aflatoxin-albumin (AF-alb) biomarker, in relation to various health outcomes. Very high levels of AF-alb were measured in blood samples from cases from the aflatoxicosis outbreak in Tanzania in 2016, which caused 20 deaths. Cases were also found to have abnormal level in liver and kidney function indicators. The Gambia study provided a novel evidence of aflatoxin effect on both innate and adaptive immune function in children through examining the association between aflatoxin exposure and thymus growth and antibody response to vaccination. A significant inverse association was determined in AF-alb level and thymus growth, especially during the first eight weeks of infancy. A potential synergistic effect of aflatoxin on antibody response to diphtheria vaccine was also found in these children. Aflatoxin may also cause damage to the gastro-intestinal tract. In the Malawi study, an association between aflatoxin exposure and environmental enteropathy has been identified. High levels of AF-alb were associated with increase in the expression of immune related genes. The potential molecular mechanism of AFs effect on immune function was examined *in vitro* using three cellular models. Significant stimulation of IL-6/STAT3 pathway by AFs have been determined in peripheral blood lymphocytes,

Jurkat T cells and a human hepatocyte line HHL-16 at both mRNA and protein level. Overall, this study provides evidence that exposure to AFs in children could retard thymus growth, modulate antibody response to vaccination and immune related genes expression. The IL-6/STAT3 pathway also identified as a potential signalling pathway that AFs can modulate immune system through regulate this pathway and further investigation *in vivo* will be needed.

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Abbreviations

AAT	α-1 antitrypsin
Ab	Antibody
AF-alb	Aflatoxin-albumin adduct
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AF-lys	Aflatoxin-lysine
AFM_1	Aflatoxin M_1
AFs	Aflatoxins
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CCL20	Chemokine (C-C motif) ligand 20
CP	Calprotectin
C_t	Cycle threshold
CV	Coefficient of variation
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DTP	Diphtheria, tetanus, pertussis
EDTA	Ethylenediaminetetraacetic acid
EE	Environmental enteropathy
EndoCab	Endotoxin core antibody
FB_1	Fumonisin B_1
FeFol	Iron-folate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM	Geometric mean
HAZ	Height-for-age Z-scores
HBV	Hepatitis B virus

HC	Head circumference
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HHL-16	Human hepatocyte line 16
HPLC	High-performance liquid chromatography
IC30	30% maximal inhibitory concentration
IFNγ	Interferon-gamma
IGF1	Insulin-like growth factor 1
IGFBP3	Insulin Like Growth Factor Binding Protein 3
IL-1	Interlukin-1
IL-10	Interlukin-10
IL-2	Interlukin-2
IL-2R	Interlukin-2 receptor
IL-4	Interlukin-4
IL-6	Interlukin-6
IL-8	Interlukin-8
IQR	Inter quartile range
LC-MS/MS	Liquid chromatography-mass spectrometry
LD50	Median lethal dose
LNS	Lipid-based nutritional supplementation
LPS	Lipopolysaccharide
MMN	Multiple micronutrients
MPO	Myeloperoxidase
MTL	Maximum tolerated levels
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-dipenyltetrazolium bromide solution
MUAC	Mid-upper arm circumference
MyD88	Myeloid differentiation primary response 88
NK cell	Natural killer cell
NO	Nitric oxide
OD	Optical density
OR	Odds Ratio
PAR	Population attributable risk
PBLs	Peripheral blood lymphocytes

PBMCs	Peripheral blood mononuclear cells
PE	Protein-energy
P-STAT3	Phosphorylated-STAT3
RIA	Radio-immunoassay
SDS	Sodium dodecyl sulphate
STAT	Signal transducer and activator of transcription
STAT3	Signal transducer and activator of transcription 3
TI	Thymic index
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF-α	Tumour necrosis factor-α
TNF-β	Tumour necrosis factor-β
WAZ	Weight-for-age Z-scores
WHZ	Weight-for-height Z-scores

Chapter 1: Introduction

1.1 History of Aflatoxins

Mycotoxins are secondary metabolites of various species of fungi which can contaminate crops. Aflatoxin as one of the most toxic type of mycotoxin has been the cause of extensive concern. Aflatoxin was first identified as the toxin responsible for turkey X disease, which killed approximately one million turkeys in England in 1960 (Blount, 1961). After the disease was shown to result from groundnuts imported from Brazil that were contaminated with a species of the fungus *Aspergillus flavus*, the toxic metabolite was named "aflatoxin". Subsequently, four aflatoxins (AFs) were identified based on fluorescence properties under ultraviolet light, with two fluorescing under blue light (425 nm) named aflatoxin B_1 and B_2 (AFB_1 and AFB_2), and another two fluorescing under yellow-green light (450 nm) named aflatoxin G_1 and G_2 (AFG_1 and AFG_2) (Nesbitt *et al.*, 1962). The chemical structure of aflatoxin B and G was first illustrated by Asao *et al.* (1963), as follows (Figure 1):

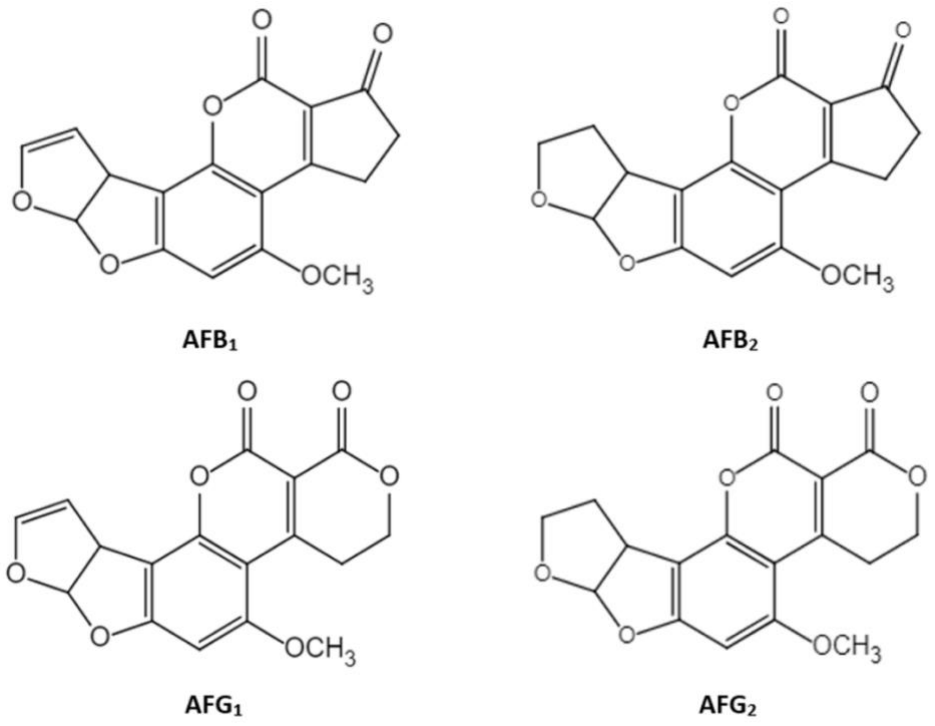


Figure 1 Chemical structures of aflatoxin B (B_1 and B_2) and G (G_1 and G_2)

The genus *Aspergillus* was first catalogued and given the name by an Italian biologist *Pier Antonio Micheli* in 1729. The word "*Aspergillus*" was decided by the shape of the fungus under microscope which is like the holy water sprinkler called an aspergill. Although there are hundreds of species of *Aspergillus*, AFs are mainly produced by *A. flavus* and *A. parasiticus* (Pitt, 2000). Both fungal growth and aflatoxin production are influenced by climate, environmental temperature and humidity, as well as by the interaction of different strains of fungi. It is notable that aflatoxin contamination is widespread in tropical agroecological zones as the high temperature and humidity is favourable to fungal growth. Maximal growth of *A. flavus* has been reported to occur in the temperature range 29 to 35 °C, while the maximal production of aflatoxins occurs at 24 °C (Schindler *et al.*, 1967). In addition, the production of different types of aflatoxin also varied in different species. Ninety percent of isolated *A. flavus* can produce AFB_1, and approximately 70% of strains can produce both AFB_1 and AFB_2 (Fakruddin *et al.*, 2015). On the whole, the production of AFB_1 (45-95%) was significantly higher than the other three aflatoxins, and the production of AFB_2 and AFG_2 were less than 20% of total AFs (Fakruddin *et al.*, 2015). Aflatoxin is used as the generic name to include mixtures of the different aflatoxins, as whilst aflatoxin B_1 is the most common and most toxic, most natural contaminations involve mixtures of different aflatoxins. The toxicity of the main types of aflatoxins has been determined in the order of $AFB_1 > AFG_1 > AFB_2 > AFG_2$ (Wogan & Newberne, 1971).

1.2 Aflatoxin contamination in food and feed crops

1.2.1 Aflatoxin contamination in foods around the world

The aflatoxin contamination problem has drawn global attention since the 1960's. Soon after AFs were identified, contamination problems were found to be severe in tropical and sub-tropical climates. Asian countries such as China, Malaysia, and Philippines were all determined to have high levels of AFs in crops such as maize and groundnuts. In 1960s, an aflatoxicosis outbreak occurred in Malaysian pig farms due to the consumption of aflatoxin contaminated peanut feeds (Lim, 1964). A few years later, another outbreak of aflatoxin poisoning in poultry in the United States was reported with levels of AFs determined to be more than 5000 µg/kg in corn-based feeds and ranging from 60 to 15,000 µg/kg in other feed samples (Smith *et al.*, 1976). In Africa, high concentrations of AFs were also reported in groundnuts (600 to 1100 µg/kg) and maize (100 to 200 pg/mg) in Nigeria in the 1970s (Nwokolo & Okonkwo, 1978). Even European countries such as France, Denmark, Sweden, Norway and Finland were found to have high incidence and high level of AFs in feeds in some crops which were imported from Asian or African countries (Scott, 1978).

An annual worldwide mycotoxin survey program, which is aiming to evaluate the mycotoxins contamination in feeds and feed ingredients around the world, started from 2004 with data reported from several countries so far (Binder *et al.*, 2007; Griessler *et al.*, 2010; Rodrigues *et al.*, 2011; Rodrigues & Naehrer, 2012; Streit *et al.*, 2013; Schatzmayr & Streit, 2013). A recently published review summarised the mycotoxin contamination levels in feeds and feed ingredients from 2004 to 2013 (Murugesan *et al.*, 2015). There have been a total of 15,614 samples in which AFs were determined during the decade, with 4,230 (27%) of the samples being positive with an average of 13 µg/kg and the maximum level of AFs at 6323 µg/kg (Murugesan *et al.*, 2015). The

mycotoxin contamination in agricultural commodities during a recent five year period (2013-2017) were determined by Biomin company with the highest positive percentage of AFs samples being from Asia (38%) and South America (23%), but the maximum level of AFs (491 µg/kg) were reduced when compared with the previous decade (Taschl, 2018).

With the enactment of regulations to limit contamination levels, the AFs contamination problem improved in developed countries (EFSA, 2013, 2017), but is still severe in many low and middle income countries. A recent study conducted in the United Kingdom collected 26 groundnut samples and six peanut butter samples from local retailers (Oplatowska-Stachowiak *et al.*, 2016). In only two samples were the AFs concentration determined to be higher than the limit of detection (LOD) of the ELISA kit used, and the level of AFs in these two samples were lower than 0.5 µg/kg, which is well below the maximum allowed (see section 1.2.2). However, in African countries, due to the climate conditions and economic challenges, the AF contamination problem remains very serious (Falade, 2019). This study is focused on exposure of AFs in African populations.

1.2.2 Regulations to limit aflatoxin levels in food and feed

In 2003, the Food and Agriculture Organization (FAO) updated the worldwide regulations for mycotoxins in food and feed. Since the first setting of limits for mycotoxins in the 1960s, about 100 countries have set up such regulations. The maximum tolerated levels (MTL) of AFs in food, animal feed and milk in several developed and developing countries and some African countries were reported by FAO (2004).

According to the regulations, the lowest MTL of aflatoxin in groundnuts in the European Union is 2 µg/kg for AFB_1 and 4 µg/kg for total aflatoxins. In most countries

the limit of aflatoxins (AFB_1 or total AFs) in foods range from 5 to 20 μg/kg. While the highest limit of total AFs in foods were in India and Malaysia with 30 and 35 μg/kg, respectively. The limit for AFM_1 in milk set as 0.05 μg/kg in most countries.

1.2.3 Aflatoxin contamination in foods and feeds in Africa

When compared with other types of mycotoxins, AFs show a high incidence (43.8%) in foodstuffs in African countries (Darwish *et al.*, 2014). Geographical variations, seasonal change, climate differentiation, social economic conditions and regulations enforcement could all influence the levels of aflatoxin contamination. The toxigenic fungi species, such as *A. flavus*, favour growth in high temperature and humid environment. This resulted in higher risk of fungal contamination in countries located in tropical and subtropical zone.

Africa has been identified as the region with the most severe AFs contamination in crops. In addition, maize and peanuts, which are two of the most susceptible crops for AFs, are widely consumed in Africa as daily staple foods. Table 1 summarizes the AFs contamination levels in foods in African countries during the recent decade. It is obvious that the concentration of AFs in most food samples are significantly higher than the regulated MTL of AFs in foods (5 μg/kg for AFB_1 and 20 μg/kg for total AFs).

Table 1 Aflatoxins contamination in foods in African countries during the recent 10 years (2010—2020).

Country	Type of AFs reported	Type of food	Mean ± SD/ *GM (95% CI)/ †Median (IQR) µg/kg	Range µg/kg	Reference
Northern Africa					
Ethiopia	Total AFs	Stored & market groundnuts		15 – 11,900	(Chala *et al.*, 2013)
Ethiopia	Total AFs	Complementary foods	0.3-9.9		(Ayelign *et al.*, 2018)
Ethiopia	AFB_1	Maize	9.3	0.3-513	(Getachew *et al.*, 2018)
Ethiopia	AFB_1	Sorghum		0.03-33.1	(Taye *et al.*, 2016)
Sudan	AFB_1	Stored groundnuts kernels a. Mayo city b. Umbaddah city c. AI-Helalia city d. AI-Managel city		 a. 17.57 – 67.33 b. 44.89 – 404.0 c. 36.7 – 101.0 d. 25.25 – 80.8	(Bakhiet & Musa, 2011)
Sudan	AFB_1	Groundnut oil Sunflower oil		0.5-70 0.7-35	(Mariod & Idris, 2015)

Algeria	AFB_1	Unshelled peanuts Shelled peanuts Almonds Dried figs		0.53-46.8 0.08-175 0 0.22-83.4	(Ait Mimoune *et al.*, 2018)
Tunisia	AFB_1	Sorghum		0.03-31.7	(Lahouar *et al.*, 2018)
Southern Africa					
Malawi	AFB_1	Groundnuts Maize Sorghum	7.92† 7.22† 10.42†		(Anitha *et al.*, 2019)
Malawi	Total AFs	Households maize	8.3 ± 8.2	0.7-140	(Mwalwayo & Thole, 2016)
Malawi	Total AFs	Market maize		0.3 – 381.9	(Matumba *et al.*, 2014)
Malawi	AFB_1	Stored groundnuts a. Farm house b. Local market c. Warehouse d. Local shops e. Super market		 a. 0 – 2197 b. 0 – 1643 c. 0 – 804 d. 0 – 594 e. 0 – 367	(Monyo *et al.*, 2012)

		f. others		0 – 471	
Malawi	AFB_1	Groundnuts			(Waliyar *et al.*, 2010)
		a. Unshelled		a. 0 – 3871	
		b. Shelled		b. 0 – 2273	
		c. Powder		c. 0 – 653	
		d. Peanut butter		d. 5.3 – 543.6	
		e. Roasted		e. 0 – 367	
		f. Peanut based Ready to Use Food products		f. 2.8 – 57.8	
Malawi	AFB_1	Groundnuts		0 – 3871	(Waliyar *et al.*, 2013)
		Maize		0 - 1335	
Malawi	AFB_1	Groundnuts	52.4 ± 103.2	0-868	(Seetha *et al.*, 2018)
		Maize	16.3 ± 23.6	0-91	
South Africa	Total AFs	Groundnuts		2.1–73.5	(Kamika *et al.*, 2014)
South Africa	AFB_1	Maize			(Mngqawa *et al.*, 2016)
		2011		1-149	
		2012		1-144	
Zimbabwe	AFB_1	Maize		0-11.0	(Hove *et al.*, 2016)

	AFB_2			0-3.0	
	AFG_1			0-4.0	
Zimbabwe	AFB_1	Maize		0.75-26.6	(Murashiki *et al.*, 2017)
Eastern Africa					
Burundi	Total AFs	Cassava	14.95 ± 1.23		(Udomkun *et al.*, 2018)
		Maize	11.21 ± 1.49		
		Sorghum	12.23 ± 0.52		
		Beans	11.52 ± 1.03		
		Soybean	9.54 ± 0.30		
		Groundnut	7.00 ± 1.00		
Uganda	Total AFs	Groundnuts	11.5 ± 0.43		(Kitya *et al.*, 2010)
		Foods	15.7 ± 4.9	0-55	
Uganda	AFB_1	Markets groundnuts	180.7 ± 51	0-849	(Baluka *et al.*, 2017)
	Total AFs		103.1 ± 36.6	0-540	
Uganda	Total AFs	Cereal grains	4.3 (0-9.2) †		(Echodu *et al.*, 2018)
Uganda	Total AFs	Maize	23.5	0-3760	(Sserumaga *et al.*, 2020)
Uganda	Total AFs	Maize flour	7.6 ± 2.3		(Wacoo *et al.*, 2018)

Tanzania	Total AFs	Maize	70 ± 231 65.0 (2-1081)*	1-1081	(Kamala, 2016)
Tanzania	AFB_1	Maize porridge a. Nyabula b. Kikelelwa c. Kigwa	 a. 4.5 b. 5.8 c. 4.7	 0.15-27.6 0.2-34.5 0.2-25.8	(Geary *et al.*, 2016)
Tanzania	AFB_1	Fresh kernels/grains Stored kernels/grains	28.7 116.0		(Seetha *et al.*, 2017)
Tanzania	AFB_1	Maize	38.3 ± 65.4		(Anitha et al., 2019a)
Tanzania	Total AFs	Maize flour	6.0 †	0.33–69.47	(Magoha *et al.*, 2016)
Kenya	Total AFs	Households maize a. 2005 b. 2006 c. 2007 d. Total	 a. 12.9 b. 26.0 c. 2.0 d. 9.1	 0.11-48,000 0.30-24,400 0-2,500 0-48,000	(Daniel *et al.*, 2011)
Kenya	Total AFs	Maize kernel	53.0, (18-480)		(Kilonzo *et al.*, 2014)
Kenya	Total AFs	Households maize a. Case village		 a. 0-13,000	(Mwihia *et al.*, 2008)

		b. Non-case village		b. 0-9,000	
Kenya	Total AFs	Households groundnuts			(Mutegi *et al.*, 2013)
		a. Busia		a. 0-2687.6	
		b. Homa bay districts		b. 0-1838.3	
Kenya	Total AFs	Groundnuts		0-2377.1	(Ndung'u *et al.*, 2013)
Kenya	AFB_1	Maize flour and kernels	57.9 (51.0, 64.7)*		(Nabwire *et al.*, 2019)
	Total AFs		59.6 (52.8, 66.5)*		
Kenya	Total AFs	Maize kernel		18-480	(Kilonzo *et al.*, 2014)
		Muthokoi		12-123	
		Maize meal		6-30	
Zambia	AFB_1	Groundnut kernel		0-11,100	(Njoroge *et al.*, 2017)
		Groundnut powder		1-3000	
Zambia	AFB_1	Peanut butter		0-1070	(Njoroge *et al.*, 2016)
Zambia	Total AFs	Maize	16		(Kachapulula *et al.*,
		Groundnuts	39		2017)
Zambia	Total AFs	Maize			(Mukanga *et al.*,
		Luangwa	5.4	0.2-10	2010)
		Chongwe	2.5	0.8-7.8	
		Namwala	0.02	0.01-4	

Western Africa					
Nigeria	AFB_1	Rice	37.2 ± 14.0	4.1-309.0	(Makun *et al.*, 2011)
Nigeria	Total AFs	Cashew nut	0.01-0.28		(Adetunji *et al.*, 2019)
Nigeria		Maize			(Liverpool-Tasie *et*
	AFB_1	At harvest	1.4		*al.*, 2019)
		Stored for 4 m	27.9		
	Total AFs	At harvest	4.2	2.7-26.5	
		Stored for 4 m	42.7	2.7-1460	
Nigeria	AFB_{1+2}	Red chilies	0.54	0.003-4.96	(Singh & Cotty, 2019)
	Total AFs		0.77		
Nigeria	AFB_1	Ginger		0.11-8.76	(Lippolis *et al.*, 2017)
	AFB_2			0.13-1.01	
	Total AFs			0.11-9.52	
Nigeria	Total AFs	Family cereal	3.3 ± 2.9	0.4-11.1	(Ojuri *et al.*, 2018)
		Peanut butter	9.0 ± 3.3	6.5-13.6	
		Ogi	5.5 ± 13.0	0.4-46.8	
		Tom bran	104 ± 166	0.5-590	
Nigeria	AFB_1	Groundnuts	117.8 ± 194.4	0.9-710	(Oyedele *et al.*, 2017)
	AFB_2		30.8 ± 32.2	0.4-129	

	AFG_1		174.3 ± 366.6	0.4-1202	
	AFG_2		68.3 ± 44.4	18.3-123	
	Total AFs		216.1 ± 496.7	0.4-2076	
Senegal	Total AFs	Groundnuts	4.43 ± 2.13	0.55-15.33	(Diedhiou *et al.*, 2012)
Gambia	AFB_1	Market groundnuts	0.49 (0.27-0.52) †		(Xu *et al.*, 2017)
Ghana	Total AFs	Maize			(Agbetiameh *et al.*, 2018)
		a. Humid Forest		a. 0-135	
		b. Derived Savanna		b. 0-341	
		c. Southern Guinea Savanna		c. 0-190	
		Groundnuts			
		d. Humid Forest		d. 0-387	
		e. Derived Savanna		e. 0-3,868	
		f. Southern Guinea Savanna		f. 0-168	
Togo	AFB_1	Maize	39	4.5-256	(Hanvi *et al.*, 2019)
		Sorghum	9.9	6-16	
Central Africa					

Cameroon	AFB_1	Households			(Ediage *et al.*, 2014)
		a. Maize		a. 6 – 645	
		b. Groundnuts		b. 6 – 125	
		c. Cassava		c. 6 – 194	
DR Congo	AFB_1	Market groundnuts	a. 23.37	1.5 – 390	(Kamika & Takoy,
		a. Dry season	b. 205.7	12 – 937	2011)
		b. Rainy season			
DR Congo	Total AFs	Groundnuts		2.19–1258	(Kamika *et al.*, 2014)
DR Congo	AFB_1	Maize	10.33	1.5 – 51.23	(Kamika *et al.*, 2016)
	Total AFs		20.64	3.1 – 103.89	
DR Congo	Total AFs	Cassava	13.14 ± 1.52		(Udomkun *et al.*,
		Maize	11.92 ± 1.26		2018)
		Sorghum	12.52 ± 1.44		
		Beans	11.85 ± 1.30		
		Soybean	8.74 ± 1.15		
		Groundnut	6.65 ± 1.67		

Notes: GM: geometric mean, 95CI%: 95% confidence interval, IQR: inter quartile range, AFB_1: aflatoxin B_1, Total AFs: mixture of aflatoxins

1.2.3.1 Geographical variation

As in the studies shown in Table 1, AFs concentrations vary in different regions and countries. Rodrigues *et al.* (2011) collected grain and feed samples from animal farms or animal feed production factories from seven African countries and six Middle Eastern countries in 2009. Total AFs levels in samples ranged from 0 to 556 μg/kg. The highest AFs level was detected in a sample from Kenya (556 μg/kg) while the highest mean level of AFs was found in Nigeria (115 μg/kg). It can be seen that samples from northern African country Algeria have no AFs detected and samples from Egypt have low level of AFs (mean value 1 μg/kg). Feed samples from Middle Eastern countries showed significantly lower levels of AFs, ranging from 0 to 18 μg/kg. Such findings are likely due to the lower humidity in the Northern African countries. This study provides a good overview of the variation of AFs contamination in different geographical regions as they collected samples during the same period. Even though in the same country, different latitude and terrain might also contribute to a specific climate condition in different agricultural zones. Kaaya and Kyamuhangire (2006) determined the AFs concentration in maize kernels collected from three agro-ecological zones in Uganda. The highest and lowest AFs were found in moist mid-altitude zone (20.54 μg/kg) and dry highland zone (12.35 μg/kg), respectively. Their results also showed that the variation of climate in different regions could affect fungal growth and aflatoxin contamination in crops. On the whole, AFs contamination is more severe in Sub-Saharan African countries than in North African countries, due to the different climate conditions.

1.2.3.2 Seasonal change and yearly variation

In sub-Saharan Africa, there are two main distinct seasons: the rainy season (Nov – May) and the dry season (June – October), which also referred to harvest and post-

harvest season, respectively. While, in some areas, considering both temperature and rainfalls, it can also divided into three seasons namely, wet season (Nov – Apr), cool dry season (May – Aug) and hot dry season (Aug – Nov). The period for each season varies slightly in different regions, with regional factors affecting climate conditions. The variation in aflatoxin contamination levels in different seasons have been investigated in many studies. Kamika and Takoy (2011) collected groundnuts samples from Congo during dry (May – June) and rainy (October – December) seasons and the AFB_1 concentrations were measured. The mean level of AFB_1 was approximately 10-fold higher in samples collected during the rainy season than those in the dry season (205.7 µg/kg vs 23.37 µg/kg). This result indicates that warmer and moister conditions during the rainy season promotes fungal growth and/or toxin production.

It is worth noting that the aflatoxin contamination is more critical in East and West African countries comparing to other regions in Africa. This could be due to the longer rainy season in those countries which is from April to June and from October to December. The long period of rain might result in higher humidity in those areas, therefore, promoting fungal growth and resulting in high frequency and concentration of aflatoxin contamination in countries such as Kenya, Tanzania, Uganda, Gambia, Benin and other East and West African countries.

Whilst warm and humid conditions promote aflatoxin production in stored food, it has been shown that hot and dry conditions, leading to drought, promote growth of the fungi on crops in the field due to the drought inducing stress on the plants (Wilson & Payne, 1994). A long period of drought occurred in Kenya in 2004 and 2005. This drought led to an outbreak of high levels of aflatoxin contamination in homegrown maize and consequently resulted in the most severe aflatoxicosis crisis in the local residents. Mwihia *et al.* (2008) and Daniel *et al.* (2011) measured AFs concentration

in maize samples collected from Kenya in 2005. Dramatically high levels of AFs were reported in both studies with the highest level of 48,000 and 13,000 µg/kg in maize samples, respectively. Daniel *et al.* (2011) conducted a cross-sectional study which measured the maize samples collected from 2005, 2006 and 2007. The concentration of AFs decreased year by year, range from 0.11 to 48,000 µg/kg in 2005, 0.3 to 24,400 µg/kg in 2006 and drop to <LOD – 2,500 µg/kg in 2007. After the drought in 2004 and 2005, the AFs contamination dropped to its usual level in 2007. Season and climate in the region play a crucial role in aflatoxin contamination as it directly influences the fungal growth and aflatoxin production.

1.2.3.3 Other factors influencing aflatoxin contamination

Food type

Most of the studies conducted in Africa determined AFs concentration in maize and groundnuts, as those are the most susceptible crops for *Aspergillus* species of fungi and the most common staple foods. Based on the data summarized in Table 1, AFs levels ranged from 0 to 11,900 µg/kg in groundnuts and from 0 to 48,000 µg/kg in maize, respectively. A study conducted in Malawi collected 1189 and 519 groundnuts and maize samples and detected the AFB_1 concentration by ELISA method (Waliyar *et al.*, 2010). Higher levels of AFB_1 were determined in groundnuts (0-3871 µg/kg) than in maize samples (0-1335 µg/kg). In contrast, food samples collected from Cameroon showed the highest level of AFB_1 in maize (645 µg/kg) followed by cassava (194 µg/kg) and groundnuts (125 µg/kg) (Njumbe Ediage *et al.*, 2014). Additionally, other crops such as rice, barley and wheat were determined to have much lower aflatoxin levels (Ayalew *et al.*, 2006; Makun *et al.*, 2011).

Storage conditions

Aflatoxin contamination could happen in any stage from crops growing in the field, to harvest, as well as during storage. An upward trend of aflatoxin concentration with storage time has been detected in maize samples, and the storage environment can also influence fungal growth. Kaaya and Kyamuhangire (2006) collected maize samples from mid-altitude agricultural zones with either moist climate or dry climate and highland zone with dry climate. Samples were collected at two time points: stored within two to six months and more than six months. The total AFs concentration increased from 20.5 µg/kg (stored within two to six months) to 30.2 µg/kg (stored more than six months) in samples collected from mid-altitude (moist) zone. While samples from the other two places had no big increase of AFs level, from 18.0 µg/kg to 22.5 µg/kg in mild-altitude (dry) zone and from 12.4 µg/kg to 12.8 µg/kg in highland zone. This study provides further evidence that long-term storage under moist storage conditions could aggravate AFs contamination.

Apart from the environment of storage, the crops storage location, storage form and container could also affect the fungi contamination level. There are two studies summarized in Table 1 that investigated the influence of storage place and form on aflatoxin contamination of crops (Monyo *et al.*, 2012; Waliyar *et al.*, 2010). The highest AFB_1 concentration was detected in samples from the farmhouse (0-2197 µg/kg) and the lowest AFB_1 level was found in samples from the supermarket (0-367 µg/kg). When comparing different storage forms, the highest AFB_1 level was found in unshelled groundnuts (0-3871 µg/kg) followed by shelled groundnuts (0-2273 µg/kg), and the lowest AFB_1 concentration was determined in peanut based ready-to-use food products (2.8 to 57.8 µg/kg) (Waliyar *et al.*, 2010). Furthermore, storage forms also can affect fungal growth during storage stage. An earlier study in Nigeria investigated different storage methods used by local farmers (Udoh *et al.*, 2000). It was found that

most of the farmers stored their maize in bags or in clay rhumbu, and only 13% of farmers used improved cribs (improved system with rat guards, wire mesh, and corrugated iron sheet roof, recommended by FAO) for storage (FAO, 1979). High prevalence (71%) of AFs were determined in maize samples stored in bags. To prevent the AFs contamination during storage stage, proper drying procedure and improved storage tools would be the most cost-efficient methods for local farmers in African area. Another storage intervention study conducted by Turner *et al.* (2005) also implemented a serious of postharvest measures including hand sorting, sun drying, drying on mats, storage in natural-fibre bags, and apply of wooden pallets and insecticides. Significant lower aflatoxin contamination in groundnuts (median 17 vs 55 μg/kg, $p<0.0001$) and AF-alb level (GM 8.0 vs 18.7 pg/mg, $p<0.0001$) in local residents were determined in intervention villages compared to control villages after 5 months storage after harvest.

1.3 Aflatoxin exposure in humans

It has been estimated that approximately 4.5 billion people are exposed to aflatoxins around the world (Williams *et al.*, 2004; Revankar, 2003). Populations in Africa might be exposed to higher levels of AFs than residents in Asian and Western countries. An estimated daily consumption of AFs in inhabitants in Europe and the USA are less than 3 ng/kg bw/day, about 0.3 – 53 ng/kg bw/day in Asians and range from 3.5 to 180 ng/kg bw/day in Africans (JECFA, 2019). Whilst the measurement of AFs in food or feed can give an indication of overall contamination levels and be used in estimations of exposure, in order to measure the exposure in people, it is preferable to measure the biomarkers of aflatoxins.

1.3.1 Aflatoxin metabolism

In animals and humans, aflatoxin B_1 is mainly metabolized in the liver by a superfamily of enzymes, the cytochrome P450s (CYP450s) (Essigmann *et al.*, 1982). There are several key cytochrome enzymes that participate in AFB_1 metabolism in humans, namely: CYP1A2, CYP3A4, CYP3A5 and CYP3A7 (Gallagher *et al.*, 1996; Wang *et al.*, 1998; Kitada *et al.*, 1989). After a series of hydroxylation, O-dealkylation and epoxidation reactions, AFB_1 will be transformed to AFM_1, AFQ_1, AFP_1 and AFB_1-exo-8, 9-epoxide (Raney *et al.*, 1992). After conjugation, AFQ_1 and AFP_1 will be eliminated in urine. AFM_1, which has weak carcinogenic activity, can be excreted through both urine and milk (Neal *et al.*, 1998). AFB_1-exo-8,9-epoxide is the key reactive intermediate in relation to the carcinogenicity of AFB_1. It binds with bases in DNA, particularly guanine, to form aflatoxin-DNA adducts such as aflatoxin-N_7-guanine (Essigmann *et al.*, 1977). This adduct is mutagenic, giving rise to G to T transversions which is crucial in the initiation stage of carcinogenesis (Hsu *et al.*, 1991; Bressac *et al.*, 1991). Aflatoxin metabolites also react with albumin in blood, and the

aflatoxin-lysine (AF-lysine) adduct has been identified as the major type (Wild *et al.*, 1986; Sabbioni *et al.*, 1990). Both DNA and albumin adduct, and AFM_1, have been used as biomarkers of exposure.

In terms of the detoxification of AFB_1, AFP_1 and AFQ_1 can bind to glucuronic acid and eliminated through urine and faeces, while another metabolite AFB_1-8,9-epoxide-glutathione (GSH) conjugate was identified as being involved in detoxification of AFB_1. The AFB_1-epoxide-GSH conjugate is catalyzed by glutathione-S-transferases which can reduce the amount of AF-epoxide available to bind to DNA, and subsequently, reduce the genotoxicity of AFB_1 (Lotlikar *et al.*, 1980). Early studies also clarified the variation of the extent of GSH conjugation in different species, in which humans showed lower conjugation rates than mouse and rat (EFSA, 2007). It is noteworthy that the GSH encoding gene *GSTM1* was associated with protection against occurrence of HCC.(Wild *et al.* (2000)reported the *GSTM1*-null genotype carriers had an increased risk of HCC in a high AFs exposure region.

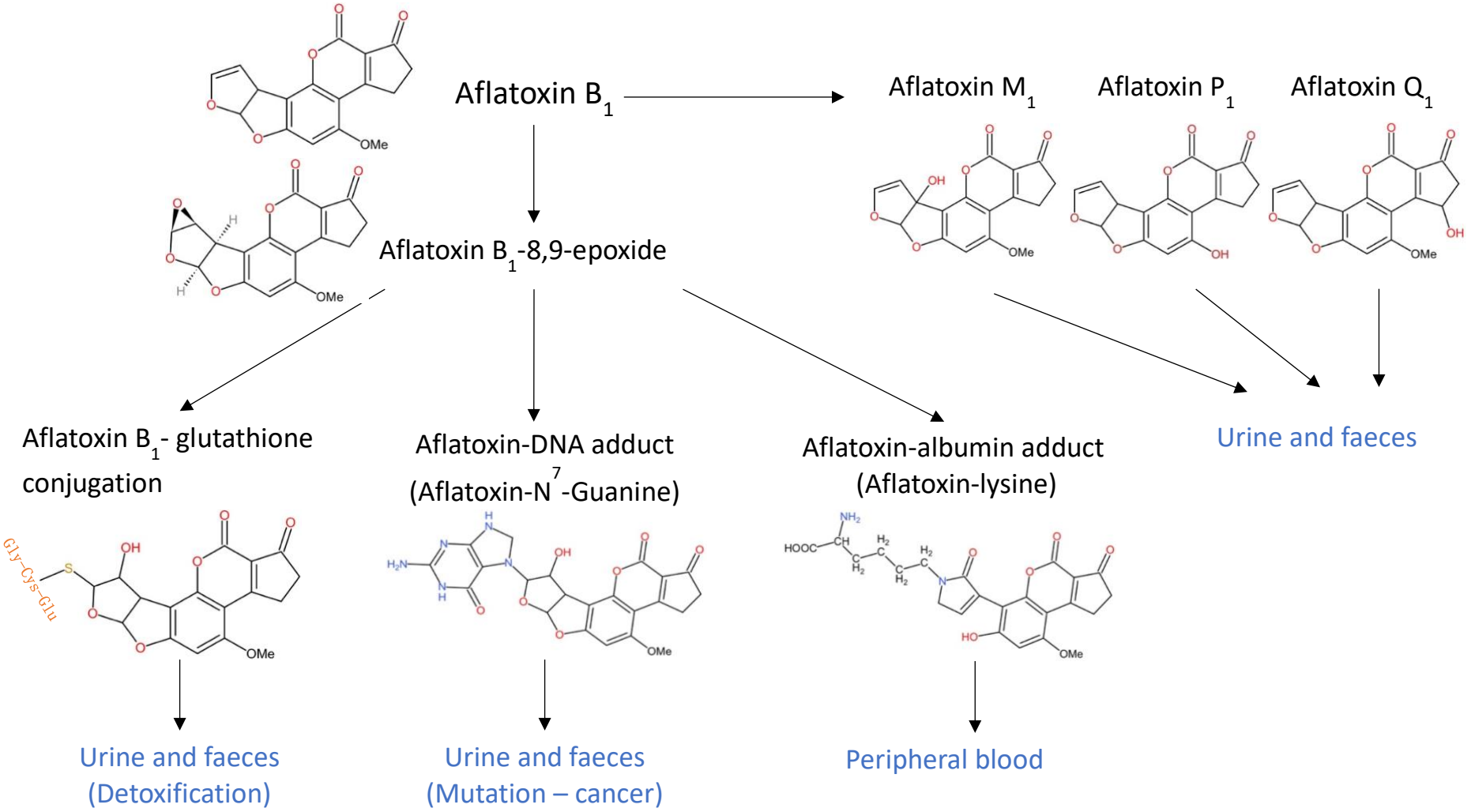


Figure 2 Metabolism of aflatoxin B1.

1.3.2 Biomarkers of aflatoxin exposure

Metabolites of AFB_1, such as AFM_1, AF-DNA adduct and AF-alb adduct have been widely used as biomarkers to examine the AFB_1 exposure in humans and animals, which can provide a more comprehensive view of aflatoxin ingestion *in vivo*. AFM_1 and AF-DNA-adduct are used to evaluate an acute and short-term exposure. A statistical correlation between AFB_1 intake and AFM_1 and AFB_1-N_7-Guanine adduct in urine have been determined. Studies measured the concentration of AFB_1 in food samples and aflatoxin metabolites AFM_1 and AFB_1-N_7-Guanine adduct in urine of the participants (Zhu *et al.*, 1987; Groopman *et al.*, 1992). Comparing to the intake of AFB_1, a correlation coefficient of 0.55 ($P<0.000001$) in AFM_1 and 0.82 ($P<0.0000001$) in AFB_1-N_7-Guanine were reported, respectively, and the estimated excretion ratio was approximately 1.23-2.18% of AFB_1 intake as AFM_1 and 0.2% as AFB_1-N_7-Gua adduct (Zhu *et al.*, 1987; Groopman *et al.*, 1992). Nevertheless, AF-alb have longer half-life, which can reflect the previous two to three months exposure (Wild *et al.*, 1996). The conversion ratio from ingested AFB_1 to AF-alb has been estimated to be approximately 1.4 – 2.3% (Gan *et al.*, 1988).

Early studies used high-performance liquid chromatography (HPLC) separation combined with fluorescence to measure the level of AF-DNA adducts in human urine samples (Autrup *et al.*, 1983). At the same time, the enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA) method were developed by Groopman *et al.* (1984) who developed specific monoclonal antibodies for detection of AFB_1 and its metabolites, such as AFM_1 and AF-DNA adduct.

However, the biomarker of albumin adduct is preferred to be used in human studies due to its long half-life and high correlation with the internal dose (Wild *et al.*, 1990). Chapot and Wild (1991) developed an ELISA method measuring aflatoxin-albumin

level in serum with a LOD at 3 pg/mg, which has been applied in many human studies in Africa (Xu *et al.*, 2018; Gong *et al.*, 2016).

The variation of different methods were evaluated by testing 102 human serum samples collected from an aflatoxicosis outbreak in Kenya (McCoy *et al.*, 2008). The AF-alb levels were measured using three different methods, carried out in three different laboratories: ELISA, HPLC with fluorescence detection (HPLC-f) and HPLC with isotope dilution mass spectrometry (IDMS) (McCoy *et al.*, 2008). IDMS is a detection method based on the stable isotope of target elements. A known amount of isotope is added to a sample as internal standard, and the ratio between the isotopes is measured by MS (Ciccimaro and Blair, 2010). For aflatoxin-albumin measurement, aflatoxin-D_4-lysine was commonly used as a standard isotope (McCoy *et al.*, 2008). All of these three methods showed good precision with the percentage of coefficient of variation (CV %) all lower than 11%. The IDMS performed with highest sensitivity and lowest LOD (0.25 pg/mg), follow by ELISA (3 pg/mg) and HPLC-f (9 pg/mg). The mean value of samples measured by ELISA was 7.5 fold higher than samples measured by HPLC-f and 5 fold higher than samples measured by IDMS, but high correlations were determined between these methods which verify their suitability for aflatoxin exposure measurement in humans. A similar correlation (r=0.88) also found in another study which used ELISA and IDMS measuring the AF-alb level among 20 samples, in which the level of samples measured by ELISA was approximately 2.6 fold higher than those measured by IDMS (Scholl *et al.*, 2006).

It is clear that HPLC-f and IDMS have higher specificity for the aflatoxin-lysine adduct, as they rely on chromatographic separation and comparison to known standards. The ELISA method used involves a polyclonal antibody, which is therefore more likely to cross react with other aflatoxin moieties, possibly explaining the higher

responses. But the acceptable agreement between those methods in previous studies haveconfirmed that they are all valid for determination of AFs biomarkers in human serum samples.

1.3.3 Aflatoxin exposure in humans

As previously discussed, aflatoxin contamination is particularly high in sub-Saharan Africa (SSA) and because staple foods are often contaminated, high levels of aflatoxin biomarkers have been determined in populations in SSA. Aflatoxin exposure levels in humans also varies with age, geographical area and season. Table 2 summarizes the reports of aflatoxin exposure in African populations during the recent decade.

Table 2 Aflatoxin-albumin biomarker levels in human studies in Africa during the recent 10 years (2010—2020).

Country	Sample age & number (Positive %)	Mean ± SD; *GM (95% CI); †Median (pg/mg)	Range	Reference
Aflatoxin-albumin adduct levels measured by ELISA				
Uganda	>18 years / 100 (100%)	11.5 (10.2-13.0)*		(Asiki *et al.*, 2014)
	<3 years / 96 (100%)	9.7 (8.2-11.5) *		
Gambia	18–45 years	Dry 52.8*		(Castelino *et al.*, 2014)
	99 (100%)	Rainy 29.6*		
Tanzania	6-14 months / 146			(Shirima *et al.*, 2013, 2015;
	Recruitment (67%)	4.7*	3.9-5.6	Routledge *et al.*, 2014; Chen
	6 months later (84%)	12.9*	9.9-16.7	*et al.*, 2017)
	12 months later (99%)	23.5*	19.9-27.7	
Guinea	10-46 months	Harvest 12.7*		(Watson *et al.*, 2016)
	Harvest 305 (88.2%)	Post-harvest 16.3*		
	Post-harvest 288 (93.4%)			
Egypt	Pregnant women 18-40 years	4.9 (4.1–5.8)	3.0–35.1	(Piekkola *et al.*, 2012)
	98 (35%)			
Aflatoxin B_1-lysine level measured by HPLC-fluorescence				
Kenya	Women	7.82 (6.04-8.90)*		(Leroy *et al.*, 2015)

	884 (100%)			
Uganda	Pregnant women 23.9 ± 4.3 years 220 (100%)	5.89 (5.25-6.60)*	0.71-95.6	(Lauer *et al.*, 2018)
Ghana	30-39 years 307	14.95 ± 15.86	0.2–109.9	(Jolly *et al.*, 2015)
Uganda	15–49 years 713 (90%)	1.58†	0.40–168	(Kang *et al.*, 2015)
Aflatoxin B_1-lysine level measured by LC-MS/MS				
Nigeria	6-48 months 58 (81%)	2.4*	0.2-59.2	(McMillan *et al.*, 2018)
Kenya	15-64 years 595 (78%)	1.78†	0-211	(Yard *et al.*, 2013)

Notes: GM: geometric mean, 95CI%: 95% confidence interval. ELISA: enzyme-linked immunosorbent assay; HPLC-fluorescence: high-performance liquid chromatography-fluorescence; LC-MS/MS: Liquid chromatography-mass spectrometry

1.3.3.1 Aflatoxin exposure in different geographical areas

In keeping with known aflatoxin food contamination patterns, lower frequency and lower levels of aflatoxin biomarkers have been found in people from European or American countries than those from African or Asian countries. AF-alb levels in forty-four subjects from France and thirty from Poland were all lower than LOD (Wild *et al.*, 1990). One study conducted in the United Kingdom somewhat surprisingly reported mean AF-alb levels of 29.3 pg AF-alb per mg in males and 26.9 pg/mg in females, which were higher than the average level found in other European countries (Turner *et al.*, 1998). In south America, 50 blood samples collected from local residents in Brazil had a mean AF-alb level of 14.9 pg/mg with 19 (38%) of them had nondetectable level (Scussel *et al.*, 2006).

In comparison, levels of AF-alb in Asian countries have been shown to be high in some studies. A study conducted in Malaysia recruited 71 participants with all of them having detectable AF-alb in blood samples, with a geometric mean level of 6.13 pg/mg (Mohd Redzwan *et al.*, 2014); 100% positive AF-alb detection was also found in 63 two-year-old children in Nepal (Groopman *et al.*, 2014). High school students in Taiwan also showed AF-alb above LOD in 95% of participants with mean level of 56.5 pg/mg albumin (Wu *et al.*, 2009).

Exposure in many populations from sub-Saharan Africa tends to be high (Xu *et al.*, 2018). Studies in Gambia, Ghana, Uganda and Senegal all reported 100% of samples as being positive for aflatoxin exposure in local participants (Turner *et al.*, 2000; Turner *et al.*, 2007; Jolly *et al.*, 2006; Asiki *et al.*, 2014; Castelino *et al.*, 2014; Jiang *et al.*, 2005; Watson *et al.*, 2015). Levels of exposure can vary by countries and between regions within country. In a study conducted in Senegal comparing the aflatoxin exposure in residents recruited from three villages (Watson *et al.*, 2015), the

highest AF-alb level was determined in participants from South Senegal (GM 80.0 pg/mg) and was lower in people from Western and North-West Senegal (33.3 pg/mg vs 15.6 pg/mg). These variations indicated that aflatoxin exposure condition is significantly influenced by geographic factors.

1.3.3.2 Aflatoxin exposure in different seasons and years

Seasonal differences in aflatoxin contamination of crops and/or exposure levels in people are common. Kamika and Takoy (2011) reported ten-fold higher levels of aflatoxin in peanuts collected in the rainy season than in the dry season. However, in Gambia, AF-alb levels in pregnant women were higher in serum collected in the dry season (GM 52.8 pg/mg) than in the rainy season (GM 29.6 pg/mg) (Castelino *et al.*, 2014). In Africa, crops are harvested during the dry season, and people have sufficient foods during this time therefore, the consumption of maize and groundnuts will be increased. In addition, as AF-alb level can represent exposure integrated over the previous two months, the high AF-alb level determined in the dry season could also due to the accumulation of highly contaminated food consumption before harvest, when the food had been stored for a long period. However, other two studies conducted in Guinea and Senegal did not found the same trend which reported similar AF-alb level in participants' blood collected in harvest or post-harvest season (Watson *et al.*, 2015; Watson *et al.*, 2016).

In addition to variation with season, aflatoxin contamination also varies in different years. A study conducted in Qidong, China, a region with high risk of aflatoxin exposure and liver cancer, recruited participants from the year 1989 to 2012 and detected the AF-alb levels every 4 to 6 years (Chen *et al.*, 2013b). In 1989, a median of 19.3 pg/mg AF-alb was reported with all participants showing positive for aflatoxin exposure. The levels then decreased with time, with the median level dropping to 3.6

pg/mg in 1995, 2.3 and 1.4 pg/mg in 1999 and 2003, respectively, and after 2009, there was no detectable AF-alb found in participants. This significant reduction of aflatoxin exposure could be due to the change of dietary habit with the introduction of rice as a staple food to replace maize in this region since the later 20th century. In Africa, people still eat groundnuts and maize as daily staples, hence the aflatoxin exposure level in local residents are significantly influenced by the contamination of aflatoxin in foods in that year. In 2004, a severe aflatoxin contamination outbreak occurred in Kenya, which resulted in a dramatically high level of aflatoxins in maize, and subsequently, significantly high levels of AF-alb were determined in local residents. Gong *et al.* (2012) recruited 218 school children in Kenya in 2002 and followed for 2 years, the GM (95%CI) level of AF-alb increased approximately five-fold from 114.5 (99.7, 131.4) pg/mg in 2002 to 539.7 (463.3, 628.7) pg/mg in 2004, reflecting this increase in aflatoxin exposure.

1.3.3.3 Aflatoxin exposure in different age groups

Aflatoxin exposure in humans can happen in every stage of life. As the metabolites of aflatoxin can be transferred through the placenta, the fetus could be exposed to aflatoxin during gestation, and also during the early infancy through breastfeeding. In Gambia, Turner *et al.* (2007) detected the AF-alb level in maternal, cord blood and infant blood with the mean level of 40.4, 10.1 and 8.7 pg/mg, respectively. In addition, in high risk regions of Africa, there is an increase in exposure during early childhood, as children begin to eat family foods. In Tanzania, the mean AF-alb increased in children recruited between 6 to 14 months of age during the subsequent 6 and 12 months (Routledge *et al.*, 2014; Shirima *et al.*, 2015). The mean AF-alb level (and prevalence of samples >LOD) increased with age, from 4.7 pg/mg at recruitment, to 12.9 pg/mg after 6 months and 23.5 pg/mg after 12 months. Although breast milk does

contain AFM_1 as a source of aflatoxin exposure, Gong *et al.* (2002) showed that breast fed children had lower AF-alb levels than weaned children and children aged two to three years old (21.1 vs 37.7 vs 42.9 pg/mg). However, as children get older and start to eat the same food as adults, they soon get similar exposure. Gong *et al.* (2003) investigated the AF-alb level in children aged from 9 months to 5 years old in Benin and Togo. An increasing trend of AF-alb level was reported with the GM of 9.5 pg/mg in children less than 1 year old and increased to 21.1 pg/mg in children at 1-year-old, and stabilized at around 40 pg/mg after 2 years old.

1.4 The effect of aflatoxin on health

Aflatoxin B_1 has been classified as a group one carcinogen and its metabolite aflatoxin M_1 also determined as a class 2B carcinogen (IARC, 1993, 2002). Acute aflatoxicosis in animals includes symptoms such as anorexia, weight loss, liver failure and death. An early study reported deaths and collapse in swine after a few hours ingested high AFs contaminated diet, and those with subacute aflatoxicosis also dead within three weeks (Ketter *et al.*, 1982). Histologic injury caused by acute aflatoxin exposure has also been found in other animals such as periportal fatty infiltrations and hemorrhages in chickens (Newbern & Butler, 1969), and liver fibrosis and bile dult proliferation in cattle (Haschek & Voss, 2013). The median lethal dose (LD50) of AFB_1 has been examined in different animal species, ranging from 0.3 mg/kg in rabbit to 17.9 mg/kg in female rats (Edds *et al.*, 1973; WHO, 1979). Rats and mice are the most tolerable species to AFB_1 (LD50 5.5 - 17.9 mg/kg), follow by chickens (6.3 mg/kg) and much lower in pigs (0.6 mg/kg for 6 - 7 kg pig) and duckling (0.34 mg/kg). However, chronic and long-term exposure is more common in poultries and livestock in daily cultivation. Early animal studies reported chronic AFs ingestion induced immune dysfunction, reduction of feed consumption and nutrients uptake (Eaton and Groopman, 1994). Chickens fed with low contaminated aflatoxin feed for 56 days, suffered reduced growth rate and weight in the experimental group compared to control group (Okiki *et al.*, 2010). Suppressed antibody titres, reduced egg production and weight and elevated mortality was also reported in layer hens fed with AFs contaminated feed for 22 weeks (Azzam & Gabal, 1998).

1.4.1 Acute aflatoxicosis

Since the recognition of acute and chronic toxicity of aflatoxin in animals, aflatoxin influences on human health has received much attention.

Acute exposure to high levels of aflatoxin causes severe illness and is often fatal, with such outbreaks occurring sporadically in countries with frequent high exposures (Okoth, 2016). Although presumably having occurred previously, the earliest recorded aflatoxicosis outbreak in humans was in India in 1974, with 397 patients reported and causing 106 deaths (Krishnamachari *et al.*, 1975). Most of affected patients showed severe jaundice and ascites symptoms, and bile duct proliferation was also found from one liver specimen obtained at necropsy. After determination of the AFs in maize, it was estimated that those affected residents had consumed approximately 2 to 6 mg AFs every day over more than one month (Krishnamachari *et al.*, 1975). Another 20 hepatitis patients with 12 deaths were reported in Kenya in 1981, where a contamination level of up to 12,000 µg/kg AFB_1 was determined in maize samples collected from Mutinda (Ngindu *et al.*, 1982). More than 20 years later, a severe outbreak of aflatoxicosis was reported in Kenya in 2004, which caused 125 deaths and more than 300 patients had nausea, fever or liver damage symptoms (CDC, 2004). This is documented as one of the largest aflatoxin contamination outbreak and occurred in the year high AF-alb levels were measured in Kenyan populations (Gong *et al.*, 2012, see Section 1.3.3.2 above). The most recent severe aflatoxin contamination event was in Tanzania, where more than 14 deaths occurred (Buguzi, 2016). Subsequently, dramatically high levels of aflatoxin biomarkers were determined in patients' blood and urine samples (Kamala *et al.*, 2018). While high contamination of food and/or high levels of biomarkers of aflatoxin exposure are often found when such outbreaks occur, it is not known precisely what levels may be fatal but the LD50 of AFB_1 in humans has been estimated to be 0.54 – 1.62 mg/kg bodyweight (Wild & Gong, 2009).

Currently, there is no specific therapy for acute aflatoxicosis. Supportive care involving antibiotics and antifungal treatment, transfusion of plasma, and therapeutic medications for liver disease and other aflatoxin poisoning manifestations were used for aflatoxicosis therapy (Mwanda *et al.*, 2005).

1.4.2 Aflatoxin effects on liver disease

Liver cancer is one of the most prevalent cancer around the world. During 2018, there were more than eight hundred thousand new liver cancer cases diagnosed and nearly the same number of deaths due to liver cancer (Bray *et al.*, 2018). It is noteworthy that 73.3% of the liver cancer cases were reported in Asia and around 8% cases in Africa and Europe, respectively (Bray *et al.*, 2018). Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. There are approximately 600,000 new HCC cases every year, in which 4.6 to 28.2% could be related to AFs exposure (Liu *et al.*, 2012). As a group one carcinogen, AFB_1 has been determined to be associated with liver damage and cancer in humans and animals through both acute and chronic exposure (IARC, 2002).

The chronic exposure of AFs has been classified as a great risk of HCC, as well as hepatitis B virus (HBV) and the hepatitis C virus (HCV) (Bosetti *et al.*, 2014). Early studies also elucidated that populations living in the high HBV infection and high AFs exposure region have dramatically greater risk of liver cancer than those exposed to AFs alone (Liu *et al.*, 2010; Groopman *et al.*, 2008; Qian *et al.*, 1994). Turner *et al.* (2000) investigated the association between AFs exposure and HBV infection status in children from Gambia. A significant difference of AF-alb level in children in different stage of HBV infection were reported. Uninfected children had the lowest AF-alb level of 31.6 pg/mg, which was higher in chronic carriers and highest in acutely infected children (44.9 and 96.9 pg/mg, respectively). A systematic review and meta-

analysis study which summarized seventeen studies from mainland China, Taiwan (China) and sub-Saharan Africa with more than 3000 controls and 1680 HCC cases (Liu *et al.*, 2012) examined the Odds Ratio (OR) and population attributable risk (PAR) of AF- and/or HBV positive- related HCC. The OR of HCC was significantly higher in aflatoxin and HBV combined (73) than aflatoxin only (11.3) or HBV only (6.4). In addition, the PAR of AF-related HCC was higher in HBV positive populations than HBV negative populations (21% vs 8.8%).

The potential molecular mechanism of HCC has been investigated before. Hsu *et al.* (1991) determined a mutation at the third base position of codon 249 in the tumour-suppressor gene p53 in HCC patients in Qidong, China. A G to T transversion in several HCC positive DNA samples was found, which is consistent with the type of mutation induced by aflatoxin (Bressac *et al.*, 1991; Slagle *et al.*, 1991).

It is clear that chronic aflatoxin exposure and HBV infection have a synergistic effect on HCC. A recent study in China summarized the AFs exposure data from seven cohort studies from 1982 to 2012 (Chen *et al.*, 2013). During the three decades, China had a great agricultural reform with diet change from eating maize as staple to rice, which has lower susceptibility to AFs contamination. The mean level of AF-alb in populations was reduced to less than the LOD of 0.5 pg/mg in 2012 from 19.3 pg/mg in 1989. Along with the mass HBV immunization program carried out in China, the PAR of primary liver cancer was reduced approximately 65% over the thirty years. This study provided great evidence that reduced risk of HCC can be achieved by decreasing exposure of AFs and enhancing the immunization against HBV. This gives a great example for further interventions.

1.4.3 Aflatoxin effects on child growth

Child growth impairment is a global health burden especially in poor nations such as African countries. The Global Burden of Diseases, Injuries, and Risk Factors Study 2016 reported that among those under five years old children in SSA, approximately 36.6% of them were stunted, 19.5% were underweight and 8.6% were wasted (GBD, 2017). A great amount of studies in animals and humans have proven the association between AFs exposure and growth impairment. Reduction in feed intake and reduced weight gain in animals fed with aflatoxin contaminated feed were reported (Khlangwiset *et al.*, 2011).

An early study showed that growth retardation in new-born rats after the mothers were fed with AFs during late pregnancy (Butler and Wigglesworth, 1965). Another rat study also found a similar decrease in birth weight, as well as increased mortality at birth in AFs dosed group compared to control group (Kihara, 2000). In humans, aflatoxin exposure *in utero* also impacts child growth. Female babies from Kenyan mothers who had detectable AF-alb levels had a significant reduction in birth weight (255 g) compared to those from mothers with AF-alb below the limit of detection (Vries *et al.*, 1989). There were two stillbirths reported in this study for which both of the maternal and cord blood had detectable AF-alb levels (Vries *et al.*, 1989). Turner *et al.* (2007) examined the AF-alb level in maternal, cord and infant blood in 138 mothers and infants from The Gambia. It was found that mothers with higher AF-alb level were correlated to lower weight and height gain in neonates. It was calculated that an increase of AF-alb level in mothers from 10 to 110 pg/mg could lead to a 0.8 kg reduction in weight and 2cm decrease in height during the first year of life in infants (Turner *et al.*, 2007).

Gong *et al.* (2016) state that the first one thousand days of life, from conception to two years old is a crucial period for growth and immune system establishment that may be impacted by aflatoxin exposure. Breast milk feeding is another route for exposure to aflatoxin during early infancy. Milk can contain AFM_1, a metabolite of AFB_1 that is eliminated through milk and urine within 24 hours. AFM_1 has also been determined to be a weak carcinogen and to have potential effect on health. A Tanzanian study recruited 143 lactating mothers and their babies examining the potential effect of AFM_1 in breastmilk on children's growth (Magoha *et al.*, 2014). All of the breast milk samples showed detectable AFM_1 ranging from 0.01 to 0.55 ng/ml. The average AFM_1 level in children was around 11 ng/kg bw per day at all time points (1, 3 and 5 months). Significant negative correlations between AFM_1 level and HAZ and WAZ ($p<0.05$) were reported, which indicated that chronic AFM_1 exposure through breastfeeding could also contribute to growth faltering in children.

A recent meta-analysis study reviewed 196 studies that measured the AFM_1 in human breastmilk from 1983 to 2017 (Fakhri *et al.*, 2019). It was found that the highest prevalence of AFM_1 (100%) was in breast milk collected from two African countries (The Gambia and Tanzania) and one Middle East country (Jordan). Among all of the studies, Africa had the highest prevalence area with an average of 52% of samples positive for AFM_1. Interestingly, the prevalence of AFM_1 in breastmilk was significantly increased with the higher average rain and poverty of the location ($p<0.05$). Overall, the prevalence of AFM_1 in breastmilk was reduced by time, which could be due to the improvement of economy, hygiene of environment and the awareness of food safety.

Although the previous studies illustrated some potential harm on animal and human growth from AFM_1 in breast milk, breast milk contains nutritional content and

immunological substances which are crucial for child growth. WHO recommends exclusive breastfeeding for at least 6 months. Studies also determined that children with exclusive breastfeeding had much lower AF-alb level in the body than partially or fully weaned children. Gong *et al.* (2003 & 2004) found an increase in AF-alb level with age during the first three years of life, approximately two-fold higher of AF-alb level measured in fully weaned children than in exclusive or partially breastfed children (37.7 vs 21.1 pg/mg) in Benin and Togo. In Africa, maize and peanuts are the major ingredients of supplementary foods for young children, and those are the most susceptible foods for aflatoxin contamination. Almost all child studies conducted in African countries reported a high prevalence of AF-alb occurrence in children (Gong *et al.*, 2003; Gong *et al.*, 2004; Turner *et al.*, 2000; Turner *et al.*, 2003; Turner *et al.*, 2005; Shirima *et al.*, 2015; Watson *et al.*, 2018).

The potential mechanism of aflatoxin induced child growth impairment might be due to the interruption of insulin-like growth factors (IGFs) pathway. This was investigated in 199 schoolchildren from Kenya and determined the AF-alb level and IGFs protein expression by ELISA (Castelino *et al.*, 2015). A significant inverse correlation was determined between AF-alb level and IGF_1 and $IGFBP_3$ level ($p<0.05$), in addition, the IGF_1 and $IGFBP_3$ also showed significant association with height and weight of children ($p<0.01$). This putative pathway was further examined in HHL-16 cells which treated with AFB_1 and determined the same inverse association between AFB_1 doses and IGFs gene and protein expression (Castelino *et al.*, 2015). Aflatoxin exposure related malnutrition have also been investigated (Khlangwiset *et al.*, 2011). Several studies determined the contribution of aflatoxin on kwashiorkor and marasmus in children which could subsequently influence the nutrient absorption and status in their body (Khlangwiset *et al.*, 2011; Mupunga *et al.*, 2017; Watson *et al.*, 2017).

Alongside malnutrition, aflatoxin induced immune dysfunction could be another potential mechanism of child growth impairment which may inhibit the intestinal protein synthesis and disturb the absorption of nutrients (Smith *et al.*, 2012).

1.4.4 Aflatoxin effects on immune function

The immunomodulatory effects of aflatoxin have been well established in cell and animal models (IARC, 1993, 2002). However, only a few studies explored the aflatoxin effect on immune function in humans (Gong *et al.*, 2016). An animal study indicated the low dose of AFB_1 induced change of antigen-presenting capacity of dendritic cells in pigs could be the potential pathway to cell-mediated immunity impairment (Mehrzad *et al.*, 2014). Further, aflatoxin compromises the integrity of intestinal epithelium thus affecting the innate immune system of intestine which plays an important role in pathogen defense. Some of the reported effects involve damaged murine intestinal mucosal linings, genotoxicity in isolated rat jejunal epithelial cells and decreased trans-epithelial electrical resistance in Caco-2 cell line (Gratz *et al.*, 2007; Yunus *et al.*, 2011). Consequently, the altered intestinal integrity disturbs nutrient absorption which along with a dysregulated immune response is suggested to contribute to the severely impaired growth associated with aflatoxin exposure (Wild & Gong, 2009).

Aflatoxin has been shown to hamper the functional activities of all the components of innate immunity which include phagocytic leukocytes, dendritic cells, NK cells and circulating plasma proteins (Bbosa *et al.*, 2013; Rushing & Selim, 2019). Macrophages are important sentinel cells which play a vital role in both innate and adaptive immune systems. Macrophages can recognize and engulf dead cells, cell debris and pathogens through phagocytosis (Gordon, 2007). The effect of aflatoxin on macrophages has been investigated since the 1970s. A dose-dependent damage in

reticuloendothelial system in phagocytic cells was determined in chicks fed with aflatoxin (Michael *et al.*, 1973). Richard and Thurston (1975) concluded that aflatoxin affects serum factors required for macrophage phagocytic activity. This was based on their observation of highly reduced number of alveolar macrophages ingesting *Aspergillus fumigatus* spores when serum from rabbits fed a daily dose of 0.09 mg AFB_1/day was added to the *in vitro* macrophage culture. Later studies also found aflatoxin exposure could modify cytokines expression in macrophages. Decreased expression of IL-1, IL-6 and TNF-α, reduction of oxygen metabolite (i.e. NO), and also suppression of toll-like receptor 2 (TLR2) and CD14 were demonstrated in an aflatoxin fed rat model (Moon *et al.*, 1999, 2000; Bianco *et al.*, 2012; Bruneau *et al.*, 2012). These results indicated a potential suppression of the killing ability of macrophages' which could subsequently damage the resistance of immune system.

An inhibitory effect of AFB_1 has also been observed in dendritic cells (DC). A low dose of AFB_1 not only impaired the phagocytic capacity of porcine monocyte-derived dendritic cells but also diminished the capacity to induce T-cell proliferation thereby implicating the toxin's impact on antigen-presenting functions of DCs (Mehrzad *et al.*, 2014). In contrast, a mixture of naturally occurring levels of AFB_1, AFB_2, AFG_1 and AFG_2 increased the phagocytic potential of swine monocyte derived DCs. Further, the aflatoxin mixture increased the expression of DCs activation markers CD25 and CD80/86 resulting in an observed increase in T-cell proliferation inducing capacity (Mehrzad *et al.*, 2015). Those two studies indicated that aflatoxin exposure can cause dysfunction of DCs which might vary with the treated dose and type of aflatoxins. Further, exposure of a mixture of aflatoxin B_1, B_2, G_1 and G_2 in human peripheral blood mononuclear cells (PBMCs) resulted in several fold up-regulation of TLR signalling pathway molecules including MyD88, TLR2, TLR4 and CD14 at the

mRNA level (Mohammadi *et al.*, 2014). This up regulation corresponded with toxicity to the PBMCs after prolonged exposure to the AFs mixture. The enhanced expression of the key immune surveillance molecules thus indicated proinflammatory status of the cells induced by AFs.

The natural killer (NK) cell as a type of cytotoxic cell is responsible for eliminating virally infected cells and tumours, and in addition it can provide immunoregulatory cytokines to macrophages and other cytotoxic cells (Vivier *et al.*, 2008). An early study that investigated the immunotoxic effects of AFB_1 on male mice reported a dose-related reduction of cell count and DNA synthesis in splenic lymphocytes and suppression of NK cells function in the treated group (Reddy & Sharma, 1989). Jiang *et al.* (2005) recruited 64 people in Ghana and collected blood samples for measuring AF-alb levels and immune function parameters. They reported a lower percentage of CD56 bright NK cells, which is a subset of NK cells, expressing a higher affinity form of IL-2R in the high AF-alb level group. However, the percentage of NK cells in peripheral blood was not significantly different to those with low and high AF-alb level (Jiang *et al.*, 2005; Jiang *et al.*, 2008). Therefore, the influence of aflatoxin on NK cells function varies in different exposure conditions and species.

Studies in animals and humans have also illustrated that aflatoxin induces alterations in cell mediated immune response. A study in Ghanaians showed inhibitory effects of AFB_1 on the percentages of activated T and B cells (Jiang *et al.*, 2005). The group reported decreased percentages of not only $CD3^+CD69^+$ and $CD19^+CD69^+$ lymphocytes in participants with high AFB_1 levels but also of $CD8^+$ T cells containing perforin or both perforin and granzyme A. These findings are suggestive of an impaired immune response with AFB_1 exposure attributed to lack of lymphocyte activation and proliferation. In a significant finding on HIV-infected Ghanaians,

aflatoxin exposure was shown to cause further immune perturbations and hence increase HIV disease severity (Jiang *et al.*, 2008). T regulatory cells, which are pivotal in controlling immunopathology during chronic viral infections, were found to be significantly decreased in HIV positive participants with high AF-alb adduct levels thus facilitating HIV-associated hyper activation and severity (Jiang *et al.*, 2008). The similar effects of aflatoxin exposure on immune response were also seen in animal studies. A study on chicks showed dietary AFB_1 to affect T-cell subset populations along with excessive thymic apoptosis (Peng *et al.*, 2016). The latter effect was proposed to be mediated by AFB_1-induced mitochondrial and death receptor pathways. Another study on chicks showed decreased percentages of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ populations induced by 0.3 mg/kg dietary AFB_1 compared to untreated controls (Chen *et al.*, 2013b).

There is varied evidence to support a depressed immunity with aflatoxin exposure culminating in decreased antibody production, reduced vaccine efficacy and consequent increased risk to infections. A study conducted in Gambia reported that in children with detectable AF-alb adducts, secretory IgA levels in saliva were found to be reduced, thus compromising the mucosal barrier for pathogen defence and hence increasing susceptibility to infections (Turner *et al.*, 2003). A similar marked reduction in the IgA and IgG titre in AFB_1 dosed mice was reported by Choi *et al.* (2010). Jiang *et al.* (2015) also determined the aflatoxin induced reduction in IgA^+ cell counts and salivary IgA (sIgA), IgA, IgM and IgG secretion in broiler's ileum. Another human study in Gambia found that children who were positive for hepatitis B surface antigens as well as with *Plasmodium falciparum* parasitemia had higher AF-alb levels than in uninfected children (Allen *et al.*, 1992).

To date, the available data tell us that aflatoxin modulates the immune response at the level of innate cell functions, antibody production, lymphocyte activation and proliferation and regulation of cytokine/chemokine expression. At the same time, the available reports are inconclusive and inconsistent owing to cross-sectional design, limited sample size and the very small number of human studies. It is thus imperative that large-scale immunological investigations are carried out keeping in consideration the sample size, aflatoxin dose, exposure timings, longitudinal study design and *in vivo* mimicry of chronic aflatoxin exposure. In turn, this will facilitate an understanding of the molecular mechanisms of aflatoxin impact on immune response and hence will provide leads for therapeutic interventions for populations at risk of aflatoxin-related diseases.

1.5 Project Aims

The main goal of this project was to further examine the potential for aflatoxin to impair immune responses, in particular through application of the aflatoxin biomarker AF-alb to existing human studies.

The main aims of this project were:

1. To investigate the effect of aflatoxin exposure on immune development in children through examining the association between aflatoxin exposure levels and thymus growth and antibody response to vaccination in Gambian children.
2. To assess the impact of aflatoxin on gastrointestinal health and immune response by examining the association between aflatoxin exposure and environmental enteropathy in children from Malawi.
3. To explore the potential molecular mechanism of aflatoxin impact on immune function through measuring gene and protein expression changes in the IL-6/STAT3 pathway in three cellular models (PBLs, Jurkat T cells and HHL-16 cells) *in vitro*.
4. To evaluate the effect of acute aflatoxin exposure in liver and kidney function by measuring the AF-alb level and biochemical parameters in patients and healthy participants in the Tanzanian aflatoxicosis incident in 2016.

Chapter 2: Methods

2.1 Materials and equipment

Aflatoxin B_1 (AFB_1; purity ≥98.0%), aflatoxin B_2 (AFB_2; purity ≥98.0%), absolute ethanol, dimethyl sulfoxide (DMSO, 99.99%), general foetal bovine serum, 3-[4,5-dimethylthiazol-2-yl]-2,5-dipenyltetrazolium bromide solution (MTT), sodium dodecyl sulfate (SDS), 1 M hydrochloric acid (HCL), hydrogen peroxide solution (H_2O_2, 30 % (w/w) in H_2O, contains stabilizer), Dulbecco's phosphate buffered saline (PBS), penicillin-streptomycin, Trypan Blue, 3,3',5',5'-Tetramethylbenzidine, ammonium sulphate, acetic acid, human serum albumin, citric acid trisodium salt, Tween20 were purchased from Sigma-Aldrich (Dorset, UK). Goat anti-rabbit IgG peroxidase-labelled antibody, Minimum Essential Medium (MEM) 500ml, TaqMan Reverse Transcription Reagents, SYBR GREEN PCR Master Mix were purchased from Thermo Fisher Scientific (UK). RNeasy Mini Kit, Human IL-6/STAT3 pathway PCR array kit were purchased from QIAGEN (UK). C_{18} Sep-pak cartridge was purchased from Waters (UK). Pronase was purchased from Roche (UK). RPMI-1640 Medium and Fetal bovine serum for Jurkat T cells (ATCC 30-2020) were purchased from ATCC (UK). Cell Staining Buffer, Fixation Buffer, Intracellular Staining Perm Wash Buffer, Brefeldin A, PE anti-human CD69, APC-Cy7 anti-human CD19, PerCP/Cy5.5 anti-human IL-2, Pacific Blue™ anti-human IL-6, PerCP/Cy5.5 anti-human IL-8, PE/Cy7 anti-human IL-10, APC anti-human IFN-γ, PE ant-human STAT3, Alexa Fluor 488 anti-STAT3 Phospho (Tyr705), were purchased from BioLegend (San Diego, CA). RNase/DNase/pyrogen free pipette tips were purchased from StarLab, UK. 96 Well ELISA Microplates (Cat. 655061) for ELISA was purchased from Greiner Bio-One Ltd (UK). Marvel Dried Skimmed Milk Powder was purchased from Tesco.

iEMS microplate reader and SPD1010 Speed Vac (Thermo Scientific, Langenselbold, Germany), ELISA plate shaker (Thermo Scientific, Vantaa, Finland), LightCycler® 480 Instrument (Roche, UK), CytoFLEX S (Beckman Coulter, UK), Eppendorf 5415C microcentrifuge and Eppendorf centrifuge 5810R (Palo Alto, Califonia, USA), Olympus inverted microscope, Water bath, Freezers (-20°C and -80°C), refrigerator, Cryostat and cell culture incubator, NanoDrop ND-1000 Spectrophotometer (ThermoScientific).

2.2 Aflatoxin-albumin adduct measurement ELISA

2.2.1 Aflatoxin-albumin adduct extraction

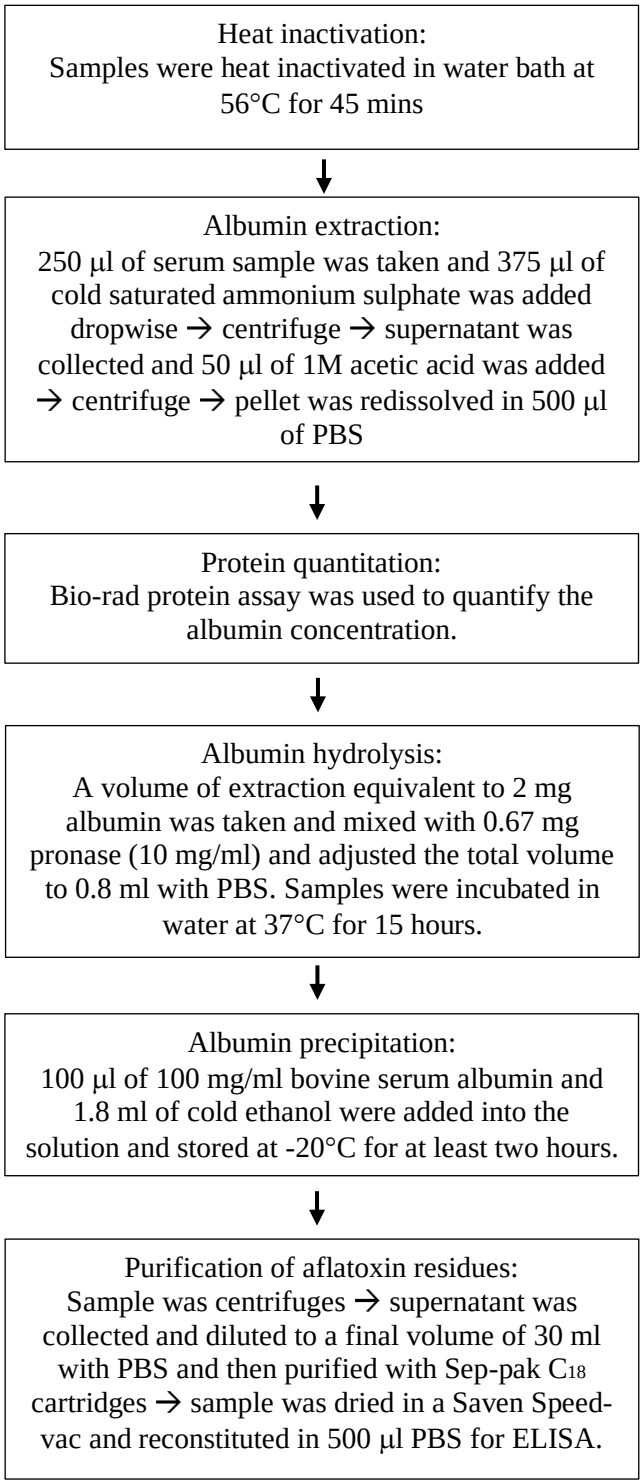


Figure 3 Aflatoxin-albumin adduct extraction procedure.

Heat deactivation

Serum samples collected from participants were heat inactivated in a preheated water bath at 56°C for 45 minutes. Samples were then stored at -20°C for further analysis.

Albumin extraction

An aliquot of 250 µl of inactivated serum sample was added into a 1.5 ml microtube, and then 375 µl of cold saturated ammonium sulphate was added dropwise into the serum. The mixture was vortexed and centrifuged at 9,000 g, 4°C for 15 mins. The supernatant was collected into another microtube and 50 µl of 1M acetic acid was added to adjust the solution to pH 5, to precipitate the albumin. The mixture was vortexed and centrifuged at 9,000 g, 4°C for 15 mins. The supernatant was discarded, and the pellet was redissolved in 500 µl of PBS.

Protein quantitation

Bio-rad protein assay was used to quantify the albumin concentration. Samples were first diluted 1:50 in distilled water (20 µl of samples + 980 µl of distilled water), and then further diluted in distilled water to make a final 1:500 dilution. Human serum albumin standard solution was prepared as the follow concentrations: 0, 2, 5, 10, 15, 20, 25, and 30 µg/ml. 40 µl of filtered Bio-rad reagents was added into each 96 well microplate and mixed with 160 µl of standard / samples. The plate was read immediately at 620 nm in a plate reader. The protein concentrations were then calculated in Excel software.

Albumin hydrolysis

A volume of extraction equivalent to 2 mg albumin was taken into a 15 ml Falcon tube. This was mixed with 0.67 mg pronase (10 mg/ml) and the total volume was adjusted to 0.8 ml with PBS. Samples were incubated in water at 37°C for 15 hours. Then, 100

μl of 100 mg/ml bovine serum albumin and 1.8 ml of cold ethanol were added into the solution, and samples were stored at -20°C for at least two hours.

Before purification with Sep-pak C_{18} cartridges, samples were centrifuged at 1000g for 15 minutes and the supernatant was diluted to a final volume of 30 ml with PBS, in order to dilute the final ethanol concentration to about 5% (v/v). Sep-pak C_{18} cartridge was used as follows: the cartridge was washed with 5 ml 80% (v/v) methanol and 10 ml distilled water; and then the sample (totally 30 ml) was loaded onto the column. After that, the cartridge was washed with 5 ml distilled water and 5 ml 5% (v/v) methanol, and lastly, aflatoxin residues were eluted using 5 ml of 80% (v/v) methanol. Samples were collected and dried in a Saven Speed-vac overnight at room temperature. Thereafter, the residue was reconstituted in 500 μl PBS for ELISA analysis.

2.2.2 Aflatoxin-albumin adduct competitive ELISA

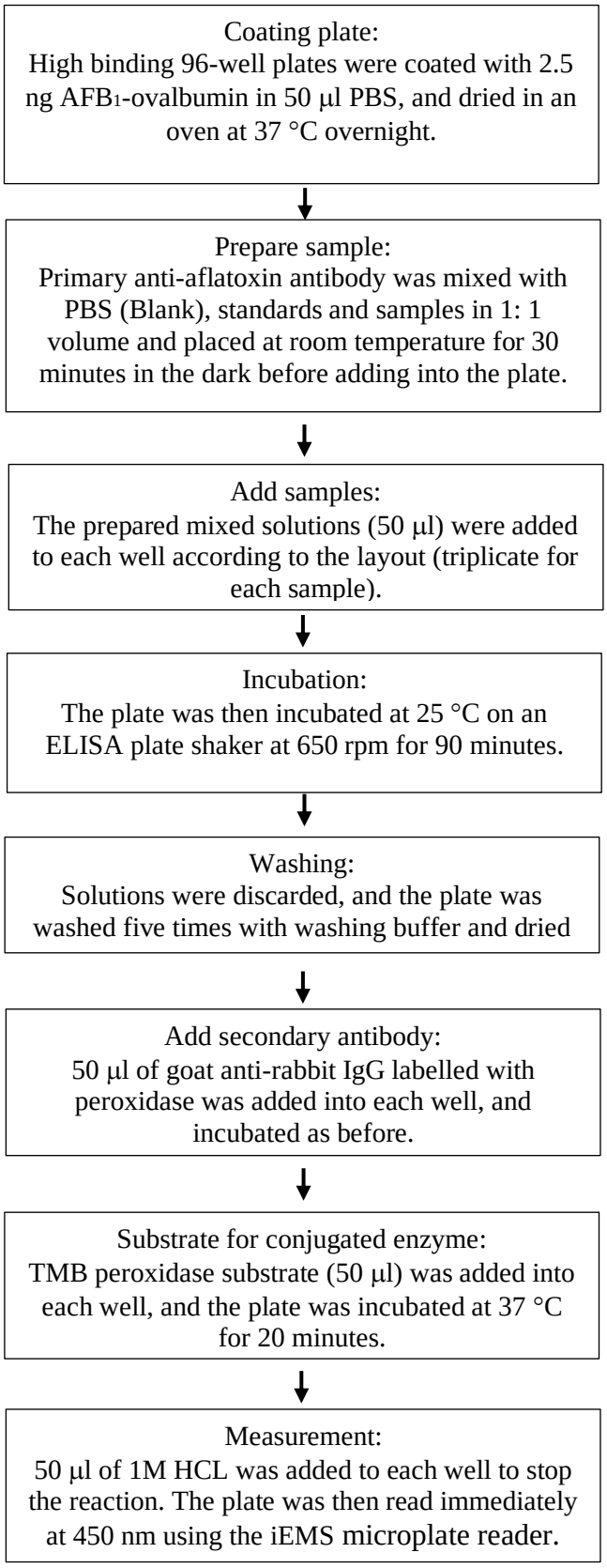


Figure 4 Outline of aflatoxin-albumin ELISA procedure.

AFB_1-ovalbumin was synthesised by reacting either AFB_1-8,9-dichloride or -8,9-dibromide with ovalbumin in our laboratory by Dr Gaoyun Chen. Primary anti-aflatoxin antibody is from a stock generated by immunisation of New Zealand White rabbits with conjugate which was prepared by reacting AFB_1-Cl_2 with bovine serum albumin by Brigitte Chapot and Chris Wild (Chapot and Wild, 1991). AFB_1-lysine standards were synthesised in our laboratory by Dr Gaoyun Chen following the method of Sabbioni *et al.* (1987).

Coating plate

High binding 96-well plates were coated with 2.5 ng AFB_1-ovalbumin in 50 μl PBS, the plate was dried in an oven at 37 °C overnight. Coated plates were stored at room temperature for up to two months in dry and light-protected conditions.

ELISA measurement

The plate was washed five times with washing buffer (PBS + 0.05% Tween 20) before use, and then blocked with 200 μl of 5% (w/v) milk solution in PBS at room temperature for 1 hour in the dark.

Primary anti-aflatoxin antibody was diluted in PBS, and mixed with PBS (Blank), standards and samples in 1: 1 volume. The mixture was placed at room temperature for 30 minutes in the dark before add into the plate.

After one hour of incubation, the milk solution was discarded and the plate was washed five times with washing buffer. The plate was then dried with tissues. The prepared mixed solutions (50 μl) were added to each well according to the plate layout (triplicate for each sample). The plate was then covered by plate protection film and incubated at 25 °C on an ELISA plate shaker at 650 rpm for 90 minutes.

After incubation, solutions were discarded and the plate was washed five times with washing buffer. The plate was then dried with tissues. The secondary antibody (50 μl

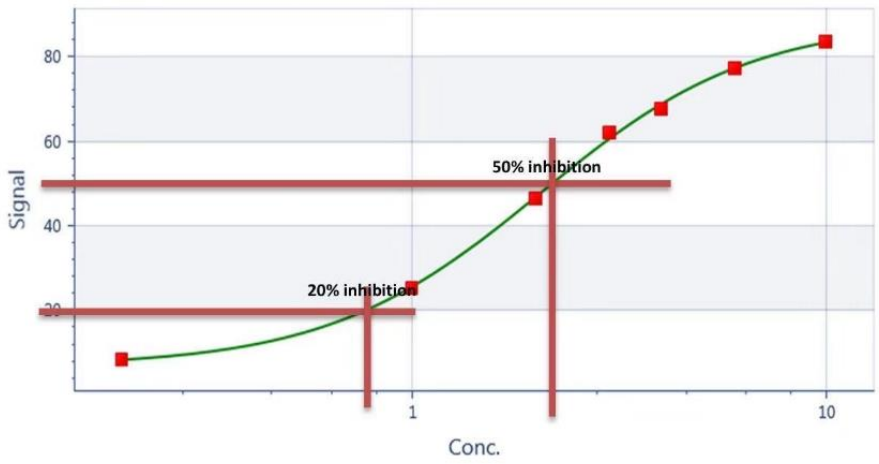
of goat anti-rabbit IgG labelled with peroxidase) was added into each well, and incubated at 25 °C on an ELISA plate shaker at 650 rpm for 90 minutes.

After incubation with the secondary antibody, solutions were discarded and the plate was washed five times with washing buffer. The plate was then dried with tissues. TMB peroxidase substrate (50 µl) was added into each well, and the plate was incubated at 37 °C for 20 minutes. Finally, 50 µl of 1M HCL was added to each well to stop the reaction. The plate was then read immediately at 450 nm using the iEMS microplate reader.

2.2.3 Data analysis

The percentage of inhibition (% inhibition) was calculated based on the optical density (OD) value using the following formula and a standard curve was generated to calculate the corresponding concentrations of samples.

$$\% \text{ inhibition} = \frac{Control\ mean\ OD - sample\ OD}{Control\ mean\ OD}$$



The calculated samples concentration was then transformed to the final concentration in pg/mg using the formula below:

$$\text{Mean in pg/mg} = \text{mean in fmol/25 µl}$$

The 4.57 factor is derived from the molecular weight of aflatoxin-albumin (457 g/mol), the amount of albumin used (2 mg) and the volume it was reconstituted in prior to ELISA. This formula is only valid for the standard 2 mg method.

The detection limit of the method is 3 pg AF-alb adduct/ mg albumin (Gong *et al.*, 2003). If the % inhibition of the sample is above 60%, the sample was diluted accordingly. If the % inhibition of the sample is below 20%, the sample was recorded as <LOD. For subsequent analytical purposes samples <LOD were assigned a value of 1.5 pg/mg in the database.

For each batch of samples, four quality controls were used along with the detection and results were checked after each day's test. These controls were prepared by spike blank human serum with a corresponding concentration of AFB_1-lysine. The controls should be within the following range of concentrations so that the result of samples can be accepted:

Control A: 2.5 – 3 fmol/25 µl
Control B: 1.2 – 2.0 fmol/25 µl
Control C: 0.8 – 1.2 fmol/25 µl
Control D: < 0.7 fmol/25 µl (negative)

All sample tests were repeated at least twice on two different days. Both control and sample results were judged to be acceptable when values had a percent coefficient of variation (%CV) below 10% within each day, and below 25% between days. If not, more repeats were conducted until between-day %CV < 25%.

2.3 Cell maintenance

All the cell culture procedures were conducted inside a class II biological laminar airflow cabinet. Strict aseptic technique was followed in all procedures.

The HHL-16 cell line is a non-tumorigenic human liver cell line that was kindly provided by Dr. Arvind H. Patel (MRC Virology Unit, Glasgow). Cells were routinely cultured in tissue culture flasks in MEM medium containing 10% foetal bovine serum and 1% penicillin-streptomycin. Cells were incubated at 37 °C and 5% CO_2. Passage 10 to 20 were used for all cell experiments.

Jurkat, Clone E6-1 T cells from ATCC was kindly provided by Dr Gina Scott, University of Leeds. Cells were routinely cultured in tissue culture flasks in RPMI-1640 Medium supplemented with 5% foetal bovine serum (ATCC 30-2020) and 1% penicillin-streptomycin. Cells were incubated at 37 °C and 5% CO_2.

2.4 Cytotoxicity assay

The cytotoxicity of AFs was measured by 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. The mechanism of MTT assay is that the succinate dehydrogenase produced by mitochondria in viable cells can catalyse MTT to an insoluble MTT-formazan. After incubation with solubilising solution, the formazan will be solubilised and can the resultant colour measured spectrophotometrically.

HHL-16 cells were seeded in a 96-well plate at a density of 2 x 10^4 in each well and incubated at 37 °C and 5% CO_2 overnight. Cells were treated with AFB_1, AFB_2 or DMSO in serum free MEM medium for 24 or 48 hours.

Jurkat T cells were seeded in a 96-well plate at a density of 1 x 10^5 in each well and treated with AFB_1, AFB_2 or DMSO and incubated at 37 °C and 5% CO_2 for 24 and 48 hours.

After the treatment for specific concentration and times, 10 μl of MTT solution was added into each well, and plates were incubated at 37 °C and 5% CO_2 for 4 hours. Then, 100 μl of solubilising solution was added into each well and plates were incubated overnight before reading the plate at 540 nm and 690 nm using the iEMS plate reader.

The percentage of viability was calculated using the formula below:

$$\%\ \text{of cell viability} = \frac{(A540-A690)sample}{(A540-A690)control} \times 100$$

2.5 Quantitative Real-time PCR assay

2.5.1 RNA extraction

Cells were treated with various concentrations and time periods. Cells were harvested and washed once with PBS to remove medium residues. RNA extraction followed the RNeasy Mini Kit instructions.

350 μl Buffer RLT (kit provided) was added and the Falcon tubes briefly vortexed to lyse the cells completely. The same volume of 70% ethanol (350 μl) was added into the lysed cells, and mixed gently by pipetting. The 700 μl of mixture was then transferred into the RNeasy Mini spin column and centrifuged at 4000 g for 1 minute and 6000 g for 15 seconds. The solution was then discarded and 700 μl Buffer RW1 (kit provided) was added to the column and centrifuged as before. The washed out Buffer RW1 was discarded. The column was then washed with 500 μl Buffer RPE (kit provided) twice. An RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 minute to dry the membrane.

The RNeasy spin column was placed in a new 1.5 ml microtube (kit provided). To elute the RNA sample from the column, 30 to 50 μl of RNase-free water was added and centrifuged at 4000 g for 1 minute and 8000 g for 15 seconds.

The concentration and purification of RNA was measured by NanoDrop ND-1000 Spectrophotometer (ThermoScientific).

2.5.2 Reverse transcription

Taqman Reverse Transcription Reagents (Life Technologies, UK) were used to reverse transcribe the total RNA to cDNA. The RNA sample was diluted to 100 ng/μl. The mix reaction solution was prepared as below:

Table 3 Reverse transcription reagents preparation

Component	Volume	Final Concentrations
DNase-RNase Free H_2O	1.6 µl	-
10x RT Buffer	2 µl	1x
25 mM $MgCl_2$	1.4 µl	1.75 mM
10 mM dNTP mix	1 µl	0.5 mM
100 mM DTT	1 µl	5mM
RNase Inhibitor (20 U/µl)	1 µl	1 U/µl
MultiScribe RT (50 U/µl)	1 µl	2.5 U/µl
50 µM Random Hexamers	1 µl	2.5 µM
Template RNA (100 µg/µl)	10 µl	50 µg/µL

Following the kit instruction, the reaction solution was incubated in a PCR machine as below:

Table 4 Reverse transcription thermal cycle setup

Temperature	Time
25 ^{o}C	10 minutes
37 ^{o}C	30 minutes
95 ^{o}C	5 minutes
4 ^{o}C	Indefinitely

After the incubation, the total RNA was reverse transcribed to cDNA. Samples were stored at -20 ^{o}C for further analysis.

2.5.3 Quantitative Real-time PCR (qRT-PCR)

SYBR Green Master Mix (Thermo Fisher, UK) was used in the qRT-PCR for fluorescence quantification. 8 µL (40 ng) of cDNA solution was mixed with 10 µl 2x SYBR Green PCR Master Mix, 1 µl (500 nM) forward primer and 1 µl (500 nM) reverse primer to compose a 20 µl volume of reaction solution. Samples were added in 96-well plates in duplicate. LightCycler 480 (Roche) instrument was used for the qPCR reaction. Settings are given below in Table 5. Relative gene expression was calculated by the comparative 2-^^Ct method (Livak & Schmittgen, 2001).

Table 5 Quantitative real-time PCR thermal cycle setup

Program	Analysis Mode	Cycle	Segment	Temperature	Time	Acquisition Mode
Denaturation	None	1		95 °C	10 min	None
Amplification	Quantification	40	Denaturation	95 °C	15 s	None
			Annealing	60 °C	1 min	Single
Melting Curves	Melting Curves	1	Denaturation	95 °C	5 s	None
			Annealing	60 °C	1 min	None
			Melting	95 °C		Continuous
Cooling	None	1		40 °C	30 s	None

2.6 Human IL-6/STAT3 pathway PCR array

2.6.1 RNA extraction

RNA extraction used the same method as described in Section 2.5.1.

2.6.2 Reverse transcription

The RT_2 First Strand Kit (QIAGEN) was used for RNA reverse transcription. An amount of 1 μg RNA sample was mixed with 2 μl of Buffer GE (kit provided) and variable RNase free water to make a final volume of 10 μl genomic DNA mix solution. The genomic DNA mix was incubated at 42 ^{o}C for 5 minutes and placed immediately on ice for more than 1 minute.

The reverse transcriptase (RT) mix solution (10 μl) was mixed with 4 μl of 5X Buffer BC3, 1 μl of Control P2, 2 μl of RE3 RT mix and 3 μl of RNase free water (all of the reagents are provided by the kit). RT mix was added into each tube containing genomic DNA mix (10μl). The mixture was mixed gently by pipetting up and down and centrifuged briefly.

Samples were then incubated at 42 ^{o}C for 15 minutes and 95^{o}C for 5 minutes. After the incubation, 91 μl of RNase free water was added to each sample. Samples are ready to do PCR or store at -20 ^{o}C for further analysis.

2.6.3 Quantitative Real-time PCR

The Human IL-6/STAT3 signalling pathway PCR array Kit (QIAGEN, Cat. 330231) was used for this experiment. The PCR components was mixed with 1350 μl of 2X RT_2 SYBR Green Mastermix (QIAGEN, Cat. 330502), 1248 μl of RNase free water and 102 μl of cDNA synthesis reaction. Added 25 μl of the PCR components mix to each well of the RT_2 Profiler PCR Array. Roche LightCycler 480 was used for qRT-PCR running and setup as below:

Table 6 Quantitative real-time PCR thermal cycle setup for PCR array

Program	Analysis Mode	Cycle	Segment	Temperature	Time	Acquisition Mode
Heat Activation	None	1		95 °C	10 min	None
PCR Cycling	Quantification	45	Denaturation	95 °C	15 s	None
			Annealing	60 °C	1 min	Single
Melt Curve	Melting Curves	1	Annealing	60 °C	15 s	None
			Melting	95 °C		Continuous

2.6.4 Data analysis

The cycle threshold (C_T) values were uploaded to the QIAGEN Web-based data analysis portal. The average C_T value of the three times testing was used to calculate the fold change. A volcano plot and heat map were generated.

2.7 Flow cytometry

Intracellular flow cytometry was used to measure intracellular protein and cytokine expression in cells. All target antibody and solutions were purchased from BioLegend Ltd.

Cells were cultured and treated with selected treatment and time in 6 wells plate. Brefeldin A was added four hours before harvest to inhibit protein secretion. Cells after corresponding treatment were collected and washed once with Cell Staining (CS) Buffer (BioLegend Ltd.) to remove the residue of medium. 0.5 to 1 ml of Fixation buffer (BioLegend Ltd.) were added to each well with gentle pipetting to resuspend cells. Cells were incubated at room temperature in the dark for 20 minutes, then centrifuged to remove the fixation buffer. Cells were then washed twice with CS buffer. Intracellular Staining Permeabilization Wash (ISPW) Buffer (10x) (BioLegend Ltd.) was diluted with distilled water to 1x. Cells after fixation were then washed twice with 2 ml of diluted 1x ISPW buffer and centrifuged at 350g for 5 to 10 minutes. The ISPW buffer was then discarded and cells were resuspended in 100 µl of fresh ISPW buffer. Corresponding antibodies were added to cells and incubated at room temperature in the dark for 30 minutes. After binding with antibodies, cells were washed twice with CS buffer. Finally, cells were resuspended in 0.5 ml of CS buffer and analysed with CytoFLEX S flow cytometer (Beckman Coulter, UK).

2.8 Blood biochemistry parameters measurement

The liver and kidney function parameters were evaluated in blood samples from Tanzania. The level of urea, creatinine, ALT (alanine aminotransferase), AST (aspartate aminotransferase), total bilirubin, direct bilirubin and total protein were measured by the Blood Sciences Centre in Leeds General Infirmary.

2.8.1 The surface antigen of hepatitis B virus measurement

The surface antigen of hepatitis B virus (HBs) was measured and confirmed by Monolisa HBs Ag ULTRA kit and Monolisa HBs Ag ULTRA Confirmatory kit (Bio-RAD).

A monoclonal anti-HBs antibody pre-coated 96-well plate was provided. 100 μl of negative control (quadruplicate, kit provided), positive control (kit provided) and unknown samples were added in corresponding wells, and 50 μl of conjugate solution (kit provided) was added into each well. The plate was then incubated in the dark at 37 °C for 90 minutes. After incubation, solutions were removed and the plate was washed four times with washing buffer. Next, 100 μl of development solution (kit provided) were added into each well, and incubated in the dark for 30 minutes at room temperature. Another 100 μl of stopping solution (kit provided) were added into each well. After waiting for at least 4 minutes, the plate was read at the optical density of 450 and 620 nm by iEMS plate reader.

Interpretation of the results

The OD of negative control should be ≤ 0.080, and the OD of positive control should be ≥1.000. The cut-off value is equal to the mean OD of negative and control plus 0.050. Ratio of OD of sample / Cut-off value was calculated. When the ratio ≥ 1, the sample was considered to be initially positive. The results between 0.9 – 1 were

interpreted with caution. These samples were then determined again by Monolisa HBs Ag ULTRA Confirmatory kit.

Positive sample confirmation

Negative, positive and tested samples were added in quadruplicate. 20 µl of the negative control diluent solution (kit provided) were added in one well of each sample. 50 µl of neutralization reagent (kit provided) were added in another well of each sample. Plates were then incubated in the dark at 37 °C for 90 minutes. After incubation, solutions were removed and the plate was washed four times with washing buffer. Next, 100 µl of development solution was added into each well, and incubated in the dark for 30 minutes at room temperature. Another 100 µl of stopping solution was added into each well. After waiting for at least 4 minutes, plates were read at the optical density of 450 and 620 nm by iEMS plate reader.

Interpretation of the results

The Inhibition percentage was calculated as below:

$$Inhibition\ percentage\ = \frac{(\text{Mean OD samples with NCD}) - (Mean\ OD\ samples\ with\ NR)}{(\text{Mean OD samples with NCD})}\ X\ 100$$

Notes: NCD: negative control diluent; NR: neutralization reagent
Samples with inhibition percentage higher than 50% can be confirmed as positive sample.

Chapter 3: Aflatoxin effect on child immune function in Gambia

3.1 Introduction

Malnutrition and high morbidity in children are still common in African countries. The slow development of the economy and the widespread use of subsistence agriculture results in the lack of sufficient staple foods in many African populations. Aflatoxin, as the majority contaminant in crops, pose a great health risk to populations living in sub-Saharan Africa especially in regions where groundnuts and/or maize are staple foods.

Earlier studies conducted in Gambia that measured either food contamination and/or aflatoxin biomarkers, showed that local residents are at a high risk of aflatoxin exposure through food consumption (Hudson *et al.*, 1992; Wild *et al.*, 1993; Turner *et al.*, 2007; Castelino *et al.*, 2014; Watson *et al.*, 2018). Hudson *et al.* (1992) determined a mean level of 9.7 μg/kg AFs from maize samples and 162 μg/kg AFs from groundnut sauces and estimated that local resident might intake 3.5 μg of AFs every day through food consumption. Our recent intervention study in Gambia also determined a dramatically high level of AFB_1 in mouldy groundnuts with a median (IQR) of 141.38 (8.22–813.86) μg/kg (Xu *et al.*, 2017).

Exposure to aflatoxin can begin in very early life; *in utero* through trans-placental exposure, during early infancy through breast feeding, and then increasing as they are weaned onto family food such as maize porridge or peanut sauces (Gong *et al.*, 2003). Turner *et al.* (2007) reported a geometric mean level of AF-alb 40.4 pg/mg in maternal blood and 10.1 and 8.7 pg/mg in cord and infant blood, respectively.

Aflatoxin is a human liver carcinogen (IARC, 2002) and has been associated with child growth impairment (Gong *et al.*, 2002, 2004; Turner *et al.*, 2007; Watson *et al.*, 2018). An inverse relationship between AF-alb levels in pregnant women and growth

of their infants was reported (Turner *et al.*, 2007) as well as between AF-alb and growth in infants below 2 years old (Gong *et al.*, 2004; Watson *et al.*, 2018).

While data from animal studies suggested that aflatoxin modulates the immune response at the level of innate cell functions, antibody production, lymphocyte activation and proliferation and regulation of cytokine/chemokine expression, there have been few studies in humans. A reduction of salivary IgA expression (Turner *et al.*, 2007) and lower percentage of lymphocytes in children (Jiang *et al.*, 2005; Jiang *et al.*, 2008) with high aflatoxin exposure have been reported.

As the development of an active immune system is critical to good health, anything that impacts on immune function can increase susceptibility of children to infectious disease, with potentially profound short- and long-term outcomes (MacGillivray & Kollmann, 2014). To investigate the immune function of children, thymus index is often used as an indicator as thymus is a crucial organ for T lymphocyte maturation and development and plays a vital role in immune function. The thymus is a bi-lobed and pyramid-shaped gland composed of cortex and medulla. It starts to develop from the 6th gestational week, and has completed the differentiation by 14 to 16 weeks of gestation (Nishino *et al.*, 2006). It will grow rapidly during the first year of postnatal life, while after puberty, it begins to involute, during which the parenchyma will be replaced by adipose tissue (Rezzani *et al.*, 2014). The cortex is mainly made up of lymphocytes, while the medulla is composed primarily of epithelial cells which build the framework of the thymus (Nishino *et al.*, 2006).

The thymus is a main component of the immune system and plays a dominant role in cellular immunity, generating circulating T cells during the early years of life. It has also been found that inadequate development of the thymus is strongly associated with mortality and morbidity in young children. Previous studies from Guinea Bissau and

Bangladesh demonstrated the strong correlation between smaller thymus size and higher mortality in children in the first year (Aaby *et al.*, 2002; Garly *et al.*, 2008; Moore *et al.*, 2014).

In addition, the status of immune function could directly influence the response to vaccines. Therefore, antibody (Ab) response to vaccination in children can be used as another indicator of immune function. Combined DTP (diphtheria, tetanus and pertussis) vaccines were the first targets involved in the Expanded Programme on Immunization that was established in 1977 by the WHO. By 2010, 85% of children around the world had received at least three doses of DTP within the first year of life. To build the immunity to disease, Ab response to vaccination could also be influenced by different factors include age, gender, maternal Ab level, nutritional status and sometimes seasons of administration. Maternal Ab which transport from mother to infant through either placenta or breastmilk showed dipolar effect in previous studies (Siegrist, 2003; Silfverdal *et al.*, 2002). Other factors such as nutritional status and seasonal variations were investigated by Moore *et al.* (2001, 2003 & 2006), in which they illustrated the association between Ab response and nutrition level and seasonal impact.

The current study conducted in the Gambia is aimed to investigate whether high level of aflatoxin exposure could be a reason that impact the immune function in children through impair thymus growth and alter Ab response to vaccination.

3.2 Methods

3.2.1 Study subjects

The current sub-study (ENID-Growth sub-study) was embedded within the Early Nutrition and Immune Development (ENID) trial (ISRCTN49285450). Full details of the ENID trial protocol have been published (Moore *et al.*, 2012) and details of the association between aflatoxin and child growth has been described elsewhere (Watson *et al.*, 2018).

Ethical approval for the ENID trial, and the ENID-Growth add-on was obtained from the joint Gambian Government/Medical Research Council (MRC) Unit The Gambia ethics committee (SCC1126v2, L2013.40). Written informed consent was obtained from all participants.

In brief, a total of 875 pregnant women at less than 20 weeks of gestation were recruited into the ENID trial from early 2010 to the end of 2012 from the West Kiang region of The Gambia (Figure 5). Children born between May 2011 and December 2012 were enrolled into the current sub-study.

Figure 5 Study location – the West Kiang region of the Gambia

The primary objective of the ENID trial was to investigate whether early pre-natal and infant nutritional intervention could improve child immune development. At enrolment, pregnant women were randomly allocated to four different trial arms: Iron-folate (FeFol), multiple micronutrients (MMN), protein-energy (PE) combined with FeFol and PE combined with MMN. Supplementation continued until delivery. From 6 to 12 months of age, infants were further randomised into two further supplementation arms: lipid-based nutritional supplementation (LNS) or LNS+MMN. Compliance of the supplementation was also recorded, through weekly interview.

Anthropometric measurements were conducted at birth (week 1), week 8, 24 and 52. Serum samples were collected at week 12, 24 and 52. The infants' morbidity and feeding practices were recorded by field assistants who visited the infants weekly at home. Infants who had experienced any diarrhoea, rapid breathing, vomiting, cough or fever or other symptoms during the last week were recorded. At the same time, food practices were also recorded, especially when the infants start to access weaning foods.

3.2.2 Aflatoxin exposure measurement

AF-alb levels in serum taken at 24 and 52 weeks of age were measured using an inhibition based indirect ELISA method (Chapot and Wild, 1991) (see Chapter 2, Section 2.2).

3.2.3 Thymus size measurement

Thymus size of infants was assessed sonographically at week 1, 8, 24 and 52 of age using a validated method by the same observer (PN) to ensure the accuracy and consistency of the results. The transverse diameter of the thymus and the sagittal area of its largest lobe were detected and multiplied to give a volume-related thymic index (TI) (Hasselbalch *et al.*, 1996). Ultrasound examinations were performed by one

observer (PN) to ensure consistency of measurement at the MRC Keneba clinic in The Gambia.

3.2.4 Antibody response to vaccination measurement

All infants were immunized vaccines in accordance with the Gambia EPI programme (Moore *et al.*, 2012). Three injections of combined DTP (diphtheria, tetanus, pertussis) were gave to infants at week 8, 12 and 16. Venous blood samples of infants were collected at 12 and 24 weeks and were used to determine the antibody (Ab) response to vaccination. A multiple immunoassay based on Luminex xMAP technology was used to detect serum-specific IgG antibody responses against diphtheria toxoid, pertussis toxin and tetanus toxin (Colombet *et al.*, 2005; Gageldonk *et al.*, 2008). All antibody assays were performed at the MRC Unit in The Gambia by MRC staff.

Full details of the primary (thymic index) and secondary outcomes (antobody response to vaccination) of the ENID trial can be found in Moore *et al.* (2018) and Okala *et al.* (2019).

3.2.5 Statistical analysis

Statistical analysis was performed using STATA version 15 (StataCorp LP). Distribution AF-alb adduct level, TI and Ab response to vaccination data were skewed and were therefore log transformed prior to further analysis. AF-alb values <LOD were allotted values of 1.5 pg/mg albumin for statistical analysis. Ab fold change means the Ab level at 24 weeks divided by Ab level at 12 weeks. Low and high aflatoxin exposure groups was divided at both 24 weeks and 52 weeks based on the median of AF-alb level at each time point. Student's *t*-test was used to compare the association of parameters in different seasons or aflatoxin exposure level groups.

For other covariates, the season of sampling was categorised as dry season from June to October, and as rainy season between November and May based on the date of TI

measurement and blood sample collection for Ab and AF-alb analysis. The age (months) of introduction of non-breast milk foods to infants were recorded. The supplementation group infants were assigned to LNS+MMN or LNS only. The infant morbidity was coded as a pooled score of the number of morbidity episodes (diarrhoea, vomiting, cough, rapid breathing and fever).

Relationships between AF-alb level and TI or Ab titre were explored using ordinary least squares regression for individual time points, or random effects model for the pooling data of all time points. Data are presented in different models for TI and Ab titres. The correlation between AF-alb level and TI were analysed as model 1: unadjusted; model 2: adjusted for infant size (length), sex and season at TI measurement; model 3: adjusted for infant size (length), season at TI measurement, sex and maternal supplement groups (for age <= 24 weeks) and infants supplement groups (for age at 52 weeks). The correlation between AF-alb level and Ab response were analysed as model 1: unadjusted; model 2: adjusted for weight for height z-score (WHZ) and season of sample collection; model 3: adjusted for WHZ and season of sample collection, sex, maternal supplement group, Hb levels and morbidity.

3.3 Results

3.3.1 Characteristics of the participants

A total of 875 pregnant women were enrolled in the ENID trial followed by 800 live-born babies. 374 out of 800 infants were included in the current sub-study. Details of characteristics of the participants have been published (Moore *et al.*, 2019, Watson *et al.*, 2018) and are summarised in Table 7. Most of the participants (87%) belong to the Mandinka tribal group. About 85% of mothers had less than one year of formal education. Twenty-five out of 374 (6.7%) children were born with a low birth weight (<2500g). The mean (SD) duration of exclusive breast milk feeding was 5.2 (1.3) months with 34% of children had more than 6 months of exclusive breastfeeding.

Infants were provided with one of the two different supplementations from 6 to 12 months of age. 192 out of 374 (51.3%) infants were treated with LNS+MMN, while another 182 (48.8%) were treated with LNS only. Among these two groups, children were nearly equally from four different maternal supplement treatment groups.

AF-alb levels were measured at 24, 52 and 72 weeks of age, while only 24- and 52-weeks results were used in the current study for further analysis. Approximately half of serum samples (170/352) collected at 24 weeks have an AF-alb level below the LOD; in contrast, only 2% of samples collected at 52 weeks have AF-alb level lower than the LOD. The geometric mean (GM) level of AF-alb was 3.52 and 25.39 pg/mg at 24 and 52 weeks of age, respectively.

Table 7 Characteristics of the participants in the ENID-Growth sub-study

Variable	n	Mean ± SD / n (%)
Gender, n (%)	374	
Male		192 (51.3)
Female		182 (48.7)
Ethnicity, n (%)	348	
Fula		30 (8.6)
Jola		11 (3.2)
Mandinka		304 (87.4)
Other		3 (0.9)
Mother's education, n (%)	352	
<1 year formal education		235 (66.8)
>1 year formal education		117 (33.2)
Birth weight (kg)	335	3.0 ± 0.4
Birth length (cm)	335	49.7 ± 2.0
Age of introduction of non-breast milk foods (months)		
<6 months	248	4.6 ± 1.3
>6 months	125	6.2 ± 0.1
Morbidity		
sum of first 12 weeks of life	360	8.3 ± 11.0
sum of first 24 weeks of life	361	18.7 ± 18.3
Infant supplementation groups (6 months)	374	
LNS+MMN		192 (51.3)
LNS only		182 (48.7)
AF-alb GM level (pg/mg)		
Week 24	352	3.52 (3.15, 3.94)
Week 52	331	25.39 (22.37, 28.82)

3.3.2 Thymic index and antibody response to vaccination

Table 8 compares the mean TI value and GM of Ab titres in the current sub-study and the ENID trial. The TI increased significantly from birth to 8 weeks of age and reached maximum size at 24 weeks, and then showed a decrease by 52 weeks. Infants included in our sub-study showed similar TI when compared with the overall 800 infants in the ENID trial.

TI is significantly associated with infant's weight, and infant birthweight was also significantly associated with TI at both 1 and 8 weeks of age ($p<0.001$) (Appendix 3.1). Neither maternal nor infant supplementation had a significant effect on AF-alb level, TI or Ab titre at any time point (Appendix 3.2). It is of note that a significantly larger TI at 52 weeks was determined in children who received LNS+MMN supplement than those took LNS only supplement in the ENID trial (Moore *et al.*, 2019).

Antibody response to vaccination was increased significantly at 24 weeks of age, after the three doses of vaccine, as expected. Very weak Ab response to vaccination were determined at 12 weeks as this is the response to the first dose. More than a fivefold increase of anti-diphtheria and anti-tetanus Ab levels were determined after the third does of vaccination. For pertussis vaccine, the Ab response increased approximately 20-fold at week 24 compare with at week 12. Except for the Ab response to diphtheria at 24 weeks, both the GM of Ab titre and the percentage of samples with protective antibody titres were higher in the sub-group studied here than in the full cohort of ENID infants. However, morbidity was higher among the ENID trial subjects than in our sub-samples at both 12 and 24 weeks.

Table 8 The comparison of thymic index and antibody response to vaccination in the main ENID trial and the ENID-Growth sub-study.

	ENID-Growth sub-study		ENID trial	
	n	GM (95%CI) / Mean ± SD / (%)[a]	n	GM (95%CI) / Mean ± SD / (%)[a]
Thymic index (cm^3)				
Week 1	371	9.21 ± 3.13	765	9.18 ± 3.08
Week 8	369	13.97 ± 4.01	752	13.9 ± 4.09
Week 24	372	14.77 ± 4.25	747	14.7 ± 4.20
Week 52	362	13.59 ± 3.4	707	13.2 ± 3.71
Ab response to vaccination				
Pertussis (EU/ml)				
Week 12	355	7.61 (6.77, 8.57) / 69.3%	711	5.52 (5.04, 6.03) / 50.1%
Week 24	322	132.37 (111.44, 157.22) / 96.0%	663	89.81 (77.71, 103.79) / 88.2%
Diphtheria (IU/ml)				
Week 12	355	0.26 (0.22, 0.32) / 72.4 %	711	0.12 (0.11, 0.14) / 55.5%
Week 24	322	1.18 (1.04, 1.34) / 94.1%	663	1.40 (1.30, 1.51) / 96.8%
Tetanus (IU/ml)				
Week 12	355	0.83 (0.75, 0.91) / 98.9%	711	0.64 (0.59, 0.68) / 97.3%
Week 24	322	4.34 (3.81, 4.94) / 99.7%	663	3.71 (3.40, 4.05) / 99.6%
Morbidity				
Sum of 12 weeks	360	8.3 ± 10.9	729	9.6 ± 12.1
Sum of 24 weeks	361	18.7 ± 18.3	730	22.8 ± 23.5

Notes: International standards protective antibody titres (WHO): diphtheria and tetanus > >0.1 IU/ml (Efstratiou and Maple, 1994; Colombet *et al.*, 2005); pertussis >5.0 EU/ml (Gageldonk *et al.*, 2008). The (%)[a] presented the percentage of samples had protective antibody titres.

3.3.3 Seasonal variation of parameters

Seasonal variations were observed in both the exposure and outcome measures (Table 9, Fig. 6 & 7). Around two-fold higher levels of AF-alb were observed in samples collected during the dry season than in the rainy season at both 24 and 52 weeks of age ($p<0.0001$). TI measured during the dry season were slightly but steadily higher than those measured in the rainy season at all time points, but this difference was only statistically significant in infants when measured at 8 weeks of age ($p=0.0136$). Ab titres were slightly higher in samples collected during the rainy season at 12 weeks of age, but an inverse trend was determined at 24 weeks of age.

Table 9 Seasonal variation of aflatoxin-albumin level, thymic index and antibody response to vaccinations.

	Rainy season (June – October)		Dry season (November – May)		
	n	GM (95%CI) / Mean ± SD	*n*	GM (95%CI) / Mean ± SD	*p value*
AF-alb level (pg/mg)					
Week 24	125	2.49 (2.13, 2.91)	227	4.27 (3.68, 4.94)	<0.0001
Week 52	155	19.34 (15.01, 20.03)	176	35.52 (29.39. 42.94)	<0.0001
Thymus index (cm^3)					
Week 1	142	8.93 ± 2.95	229	9.39 ± 3.23	0.2693
Week 8	138	13.34 ± 3.81	230	14.37 ± 4.09	0.0136
Week 24	100	14.26 ± 4.34	271	14.96 ± 4.21	0.1192
Week 52	135	13.47 ± 3.14	226	13.63 ± 3.53	0.9242
Ab response to vaccination					
Pertussis (EU/ml)					
Week 12	145	8.26 (6.98, 9.78)	192	7.67 (6.47, 9.10)	0.5506
Week 24	98	81.71 (57.53, 116.04)	214	171.65 (142.10, 207.35)	0.0001
Diphtheria (IU/ml)					
Week 12	145	0.44 (0.31, 0.62)	192	0.20 (0.16, 0.24)	0.0001
Week 24	98	0.75 (0.56, 1.00)	214	1.45 (1.29, 1.64)	<0.0001
Tetanus (IU/ml)					
Week 12	145	0.96 (0.93, 1.11)	192	0.74 (0.65, 0.85)	0.0101
Week 24	98	3.94 (2.85, 5.43)	214	4.50 (3.98, 5.08)	0.352

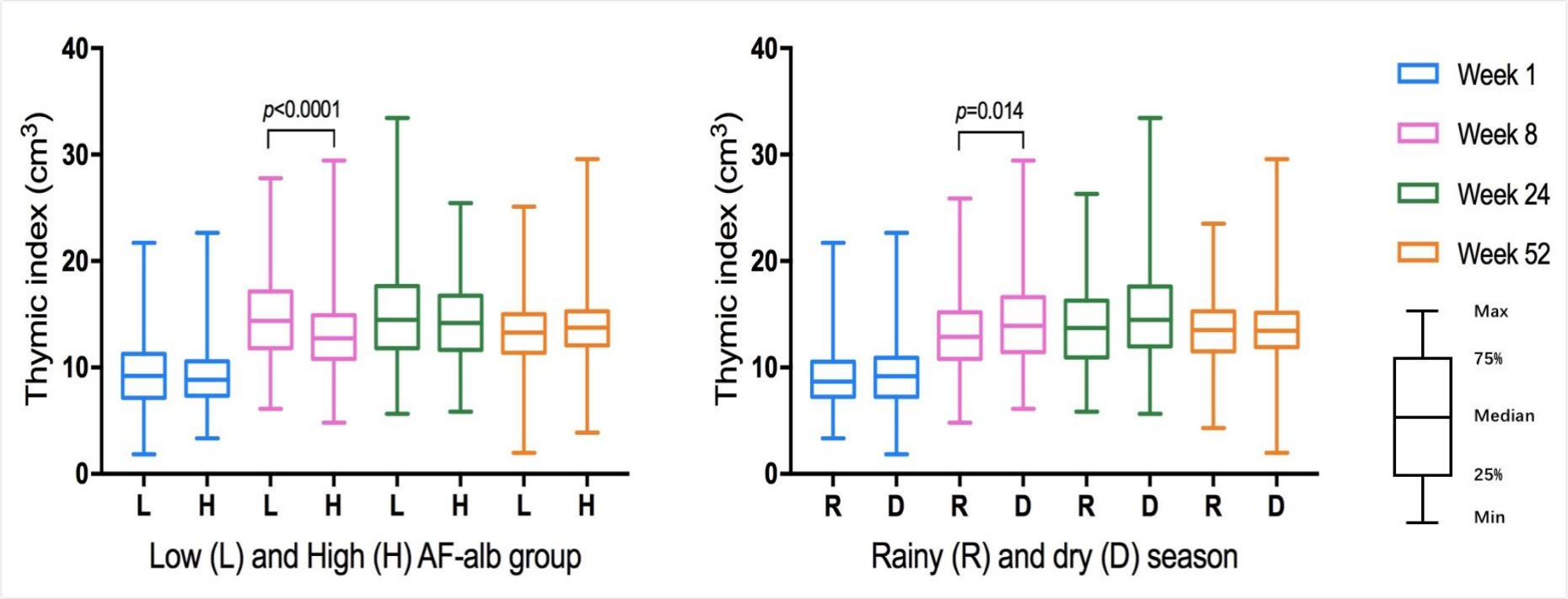


Figure 6 Thymic index in children in low or high Aflatoxin-albumin group or in different seasons.

The low and high AF-alb group was divided by the median level at each time point (1.5 pg/mg at wk24, 23.01 pg/mg at week 52). TI at week 8, 12 and 24 were group by AF-alb at week 24, TI at 52 weeks was group by AF-alb level at week 52. The season of TI was defined as the time of measurement conducted. *P* value presented student's *t*-test result between samples.

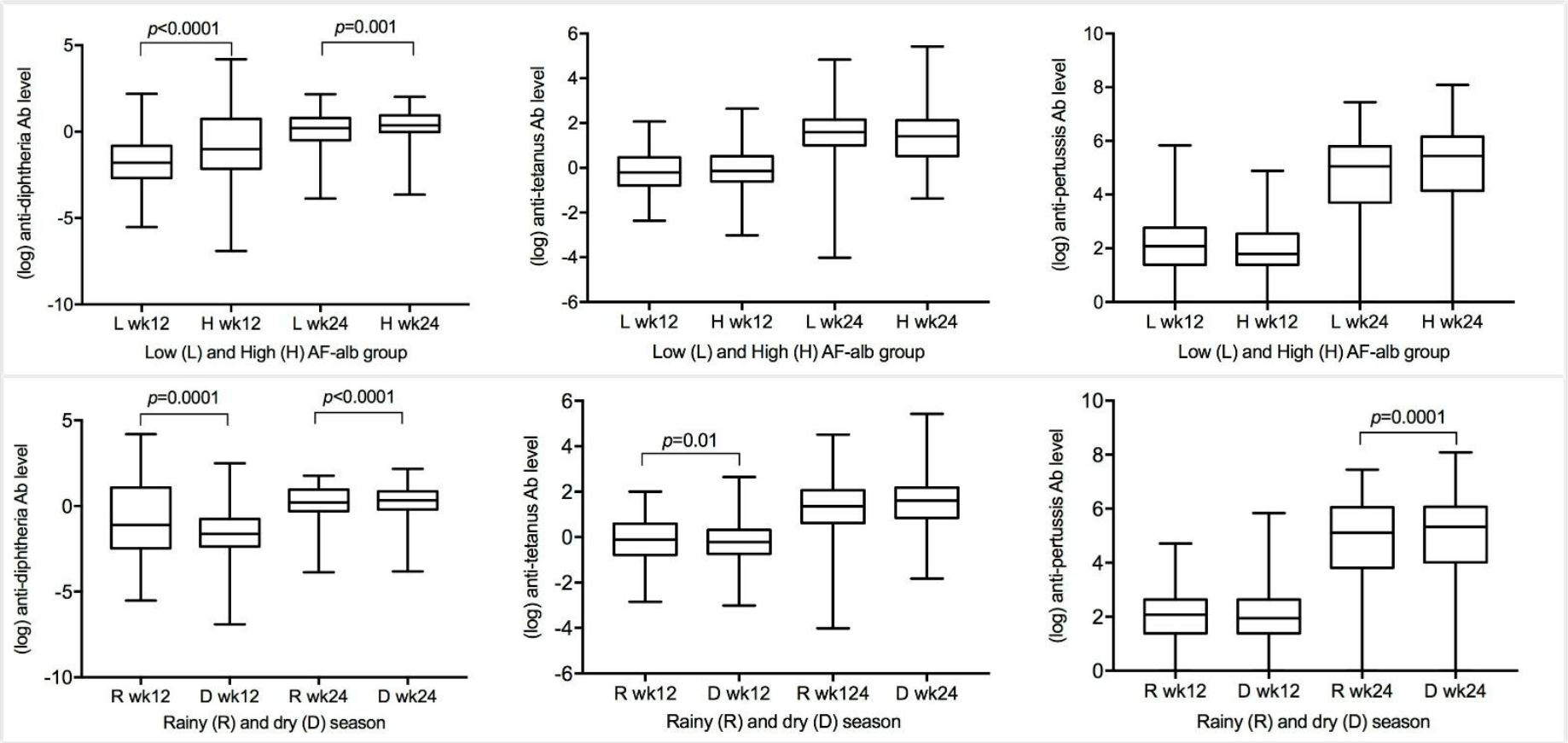


Figure 7 Antibody level to vaccination in low or high aflatoxin-albumin group and in different seasons.

Antibodies against diphtheria, pertussis and tetanus were measured in serum samples taken at 12 and 24 weeks. The low and high AF-alb group was divided by the median level at week 24 (1.5 pg/mg). The season of Ab level was defined as the date of blood sample collection. *P* value presented student's *t*-test result between samples.

3.3.4 Aflatoxin effect on thymic index and antibody response to vaccination

Table 10 shows the level of AF-alb and the TI value and Ab titres in the low and high AF-alb groups. The mean TI was lower in the high AF-alb group than in the low AF-alb group during the first 24 weeks, but the difference was only statistically significant at 8 weeks, and not present at 52 weeks. The effect of aflatoxin exposure level on Ab response varied for different vaccines. There were no significant differences for Ab response to pertussis and tetanus vaccines between the low and high AF-alb groups. However, a significantly higher Ab titre against diphtheria vaccine was determined in infants in the high AF-alb group compared to those in the low AF-alb group at both time points ($p<0.0001$ & $p=0.0011$ for Ab titre at 12 and 24 weeks, respectively).

Table 10 Geometric mean of aflatoxin-albumin level and Ab response to vaccination and mean thymic index in low and high aflatoxin-albumin level groups (unadjusted)

	n	Low AF-alb group	*n*	High AF-alb group	*p* value
AF-alb level (pg/mg)					
Week 24	182	1.5 (1.5, 1.5)	170	8.8 (7.7, 10.0)	<0.0001
Week 52	166	10.19 (9.27, 11.21)	165	63.59 (55.97, 72.23)	<0.0001
Thymus index (cm^3)					
Week 1	180	9.47 ± 3.45	170	9.05 ± 2.79	0.5774
Week 8	180	14.89 ± 4.0	161	13.08 ± 3.84	<0.0001
Week 24	181	14.92 ± 4.44	170	14.49 ± 3.87	0.4691
Week 52*	166	13.24 ± 3.42	164	13.79 ± 3.50	0.1335
Ab response to vaccination					
Pertussis (EU/ml)					
Week 12	176	8.58 (7.17, 10.28)	164	6.83 (5.80, 8.04)	0.0659
Week 24	158	114.22 (90.02, 144.94)	151	150.80 (115.62, 196.68)	0.1243
Diphtheria (IU/ml)					
Week 12	176	0.16 (0.13, 0.20)	164	0.46 (0.33, 0.65)	<0.0001
Week 24	158	0.96 (0.79, 1.17)	151	1.46 (1.26, 1.69)	0.0011
Tetanus (IU/ml)					
Week 12	176	0.79 (0.69, 0.90)	164	0.90 (0.78, 1.04)	0.1818
Week 24	158	4.69 (3.86, 5.69)	151	3.89 (3.24, 4.67)	0.1666

Notes: The low and high AF-alb group was divided by the median level at each time point. *Only TI at 52 weeks was grouped by AF-alb level at 52 weeks.

To compare the effect of other covariates, table 11 and 12 present the association between AF-alb level and TI and Ab titres with or without adjusting for the other variables. There were no significant associations between AF-alb level and TI at 1, 24 and 52 weeks individually. However, a significant negative correlation was determined between AF-alb and TI at 8 weeks in all unadjusted and adjusted models. In addition, the random effect model, which measured the AF-alb correlation with all time points TI pooling data also showed significantly negative correlations at 24 weeks in both unadjusted and adjusted models, and at 52 weeks in adjusted models.

There were no significant association between AF-alb level and Ab response to pertussis and tetanus, while significant positive correlations were determined between aflatoxin exposure level and Ab response to diphtheria at both 12 and 24 weeks of age. When the Ab fold change between 12 and 24 weeks was used, a significant negative correlation was found between AF-alb level and Ab fold change to diphtheria in unadjusted model, but the significant correlation diminished after adjusted with other variates.

Table 11 Relationship between aflatoxin-albumin level and thymic index

	Model 1		Model 2		Model 3	
	Coef. (SE)	*p*	Coef. (SE)	*p*	Coef. (SE)	*p*
Week 1	-0.0083 (0.018)	0.637	-0.0012 (0.018)	0.95	-0.0012 (0.018)	0.948
Week 8	-0.0606 (0.014)	<0.001	-0.056 (0.014)	<0.001	-0.056 (0.014)	<0.001
Week 24	-0.0098 (0.014)	0.49	-0.015 (0.015)	0.3	-0.015 (0.015)	0.301
Week 52	-0.0115 (0.014)	0.399	-0.014 (0.014)	0.296	-0.014 (0.014)	0.301
all time points to infant age <24 weeks	-0.027 (0.011)	0.014	-0.023 (0.010)	0.022	-0.023 (0.010)	0.021
all time points to infant age <52 weeks	0.0076 (0.007)	0.28	-0.053 (0.007)	<0.001	-0.052 (0.007)	<0.001

Notes: TI at 1, 8 and 24 weeks were analysed with AF-alb level at 24 weeks. TI at 52 weeks was analysed with AF-alb level at 52 weeks.
Model 1 unadjusted
Model 2 adjusted for infant size (length), sex and season at TI measurement
Model 3 adjusted for infant size (length), season at TI measurement, sex and maternal supplement groups (for age <= 24 weeks) & infants supplement groups (for age at 52 weeks)

Table 12 Relationship between aflatoxin-albumin level and antibody response to vaccination

	Model 1		Model 2		Model 3	
	Coef. (SE)	*p*	Coef. (SE)	*p*	Coef. (SE)	*p*
Pertussis						
Week 12	-0.093 (0.058)	0.11	-0.093 (0.060)	0.123	-0.088 (0.070)	0.207
Week 24	0.143 (0.083)	0.085	0.133 (0.086)	0.124	0.099 (0.096)	0.301
Fold change	0.190 (0.109)	0.083	0.197 (0.113)	0.084	0.127 (0.127)	0.319
Diphtheria						
Week 12	0.421 (0.093)	<0.001	0.418 (0.094)	<0.001	0.495 (0.109)	<0.001
Week 24	0.186 (0.058)	0.002	0.190 (0.061)	0.002	0.204 (0.064)	0.002
Fold change	-0.239 (0.097)	0.014	-0.124 (0.097)	0.204	-0.149 (0.106)	0.162
Tetanus						
Week 12	0.065 (0.048)	0.175	0.065 (0.049)	0.182	0.096 (0.056)	0.089
Week 24	-0.034 (0.062)	0.578	-0.064 (0.064)	0.322	-0.071 (0.070)	0.316
Fold change	-0.111 (0.081)	0.172	-0.096 (0.084)	0.252	-0.127 (0.092)	0.168

Notes: Antibody response to vaccination were analysed with AF-alb level at 24 weeks only.
Model 1 unadjusted
Model 2 adjusted for WHZ and season of sample collection
Model 3 adjusted for WHZ and season of sample collection, sex, maternal supplement group, Hb levels and morbidity

3.4 Discussion

There have been a number of studies that have demonstrated the association between high levels of aflatoxin exposure and child health problems in Africa. This is the first study to investigate the impact of aflatoxin exposure on immune function of infants less than 12 months of age by using the thymic index and antibody response to vaccination as indicators of immune function.

The ENID trial was conducted in The Gambia, where subsistence agriculture is the norm. Under the subsistence farming, residents are more likely to face food shortages and lack of food diversity. High levels of aflatoxin exposure have been determined in both children and adults in Gambia in previous studies (Turner *et al.*, 2000; Turner *et al.*, 2007; Castelino *et al.*, 2014). The observed high prevalence of aflatoxin exposure in the present study is consistent with previous findings in the same region of the Gambia (Turner *et al.*, 2000, 2007). An increasing prevalence and upward trend of AF-alb level were determined in our study. Infants at 52 weeks showed more than seven-fold higher of AF-alb level than at 24 weeks of age. The percentage of samples with detectable levels also increased from 51% at 24 weeks to 98% at 52 weeks. Aflatoxin exposure could happen in the foetus through trans-placental exposure, and the exposure level will increase during the early year when infants are gradually introduced to supplementary foods such as maize and peanuts porridge.

The Gambia has pronounced dry and rainy seasons, and previous studies have shown a seasonal impact on both growth and aflatoxin exposure (Castelino *et al.*, 2014). In the present study, serum samples collected during the dry season had significantly higher levels of AF-alb than those samples collected during the rainy season. The annual dry season in The Gambia is a time of relative food availability, as food supplies from the previous harvest are available. Aflatoxin contamination in foods and

crops tends to elevate after a period of storage (Pitt, 2000). Populations are more likely to consume old grains towards the end of the dry (harvest) season, which foods have been stored for a period and more likely to be contaminated. Our findings are also consistent with previous studies in Benin and Guinea (Gong *et al.*, 2004; Turner *et al.*, 2005). Watson *et al.* (2015) in a study conducted in Senegal also demonstrated that populations from one of the villages, Nioro du Rip, had significantly higher AF-alb level in the harvest season than in the post-harvest season, which was consistent with the recorded high consumption (four or more days a week) of contaminated groundnuts during harvest.

In the present study, TI showed a growing trend from the first week (9.21 cm^3) to 24 weeks (14.77 cm^3), but fell to 13.59 cm^3 at 52 weeks. In addition, a seasonal trend in TI was also found in our study, as larger TI was determined in infants measured in the dry season than in the rainy season, reaching statistical significance at 8 weeks ($p=0.0136$). The same growth trend and seasonal variation of TI were reported before in Gambia by Collinson *et al.* (2003) who also reported the consistently smaller thymus size in the rainy season, and significant difference at 8 weeks of age ($p=0.001$). In both the main ENID trial and our sub-study, significant correlations were identified between TI and child weight and height. A recent published retrospective cohort study investigated the growth faltering in Gambian children during 36 years (1976-2012) (Nabwera *et al.*, 2017). After analysis of the growth data in more than 3500 children from birth to two years old, a seasonal growth failure was identified in this area with growth retardation found in the rainy season. Less infection such as diarrhoea in the dry season might contribute to the better growing of infants during this time (Rowland, 1986).

The antibody titre against vaccines were elevated dramatically at 24 weeks after three doses. The immune response mechanism is varied for different vaccines. Plotkin (2001) reviewed and categorised the mechanism of antibody response to different vaccines. DTP as protein antigens can induce IgG antibody by B cells with the help of $CD4_+$ T cells (Rothman *et al.,* 1998). The possible reason for induced seasonal variation in Ab response to vaccination could be the seasonality changes in immune status and infectious diseases. Collinson *et al.* (2003) and Dopico *et al.* (2015) determined higher numbers of white blood cells, lymphocytes and monocytes during the rainy season in Gambian populations. Studies conducted in Gambia also determined striking seasonal patterns in disease infectious such as malaria which has the highest infection rate during the rainy season (Brewster & Greenwood, 1993; Kasasa *et al.*, 2013).

Some early studies also indicated that the Ab status in the mother could also influence the Ab response in neonates. Newborns have immature immune systems (Simon *et al.*, 2015). Infants will get immune protection such as antibodies from their mother through the placenta and breastmilk. A recent Meta study analysed a total of 7830 infants and concluded that high levels of maternal antibodies can inhibit antibody response to vaccine in infants (Voysey *et al.*, 2017). According to that analysis, 2-fold higher maternal antibodies could induce 11% lower Ab response to pertussis, 13% to tetanus and 24% to diphtheria. Another study conducted in the UK also confirmed that children with high level of anti-tetanus antibody at birth showed significantly lower response post-immunization for tetanus ($p=0.009$) (Jones *et al.,* 2014). In our sub-study, we did not measure the maternal antibodies or the antibody level in infants before vaccination. This could be a potential factor that affects the Ab response to vaccination in early life.

The ENID trial was designed to explore the effects of combined maternal and infant nutritional supplementation on immune parameters in the infants, such as thymus size (measured as thymic index) and Ab response to vaccination (Moore *et al.*, 2019; Okala *et al.*, 2019b). In this sub-study, we examined whether aflatoxin exposure had any impact on these immune outcomes. A negative correlation between infant aflatoxin exposure level and TI was observed. A smaller mean TI was observed in infants at 6 months of age in the high aflatoxin exposure group, compared to the low exposure group, but a statistically significant difference ($p<0.001$) was only determined at 8 weeks, which is the time point that the thymus grows fastest. However, this effect was diminished by 52 weeks. Previously, aflatoxin induced damage on the thymus has only been investigated in animal models. A recent study reported aflatoxin caused thymic histopathological lesions and pathological impairments in chickens which had been fed with aflatoxin contaminated feeds (34.3–134 μg AFB_1/kg corn feed) for 21 and 42 days (Peng *et al.*, 2017). The reduction in thymus size and number of apoptotic lymphocytes occurred in a dose-dependent manner. It is interesting that they also found an alleviation of histological lesions of the thymus at 42 days than at 21 days, which they considered could be due to increasing tolerance of the chickens to aflatoxin with growth (Peng *et al.*, 2017).

The sonographic method used in the current study to assess TI only represents an anatomical feature, but not function. However, early studies in animals and infants have demonstrated a correlation between thymus size and lymphocyte proportion and function (Jeppesen *et al.*, 2004; Peng *et al.*, 2017). Therefore, smaller TI in infants could predict a lower immunity in the future.

One limitation of the current study was that the AF-alb levels were not measured at the birth of infants or in the maternal blood. Therefore, aflatoxin exposure level at 24

weeks were used as a predictor of previous exposure condition. Turner *et al.* (2007) reported a close association of AF-alb level in maternal and infants' cord blood, but the AF-alb in the first few months of infancy is difficult to detect due to the low level. In addition, Turner *et al.* (2007) also reported a significant negative correlation between maternal aflatoxin biomarker level and child weight and height gain at the first year of life. In our study, we found the TI was significantly correlated with the weight of infants at the first year. Considering the aflatoxin associated growth retardation was determined in infants (Watson *et al.*, 2018), it is reasonable to suggest that aflatoxin could be a potential factor that influenced child growth and thymus development during the early stage.

The ENID trial also used antibody response to vaccination as another indicator of immune function in children. In our study, we found a significant positive association between aflatoxin biomarker level and Ab titre to diphtheria. There was no previous study that investigated the influence of aflatoxin exposure on Ab response to DTP vaccines. Another study conducted in Gambian infants determined a similar weak but significant positive correlation between AF-alb level and one serotype pneumococcal Ab (Turner *et al.*, 2003). Adjuvants are widely used in vaccines design to enhance immune response to vaccines (Coffman *et al.*, 2010). An animal study that tried to develop a conjugated vaccination to induce anti-aflatoxin B_1 antibody in dairy cows used combined AFB_1 with recombinant diphtheria toxin molecules and injected into heifers which boost the generation of anti-AFB_1 Abs (Giovati *et al.*, 2014). The mechanism of how these two toxins interact with each other is still unknown, but a potential adjuvants-like activity might be suggested between aflatoxin and diphtheria vaccine. The effect of aflatoxin on Ab response could vary depending on the features of the vaccines, as early animal studies conducted in chickens and rabbits reported

inverse influence of aflatoxin exposure level on Ab response to different vaccines (Dimitri & Gabal, 1996; Azzam & Gabal, 1997).

A few previous studies have examined the effect of high levels of aflatoxin exposure on markers of immunity in human. Turner *et al.* (2007) reported a decrease in expression of salivary IgA in children from rural Gambia with detectable AF-alb level compared with those with undetectable levels. A lower percentage of T and B cells and lower albumin levels have been observed in participants with high concentrations of AF-alb in Ghana (Jiang *et al.*, 2005, 2008). However, studies on aflatoxin induced effects on the immune system are inconsistent. Li *et al.* (2014) illustrated that there is no big effect of aflatoxin B_1 on humoral immunity function. While other studies found increased expression of IgM, IgG and IgA in swine after dosed high aflatoxin contaminated feeds (Panangala *et al.*, 1986; Meissonnier *et al.*, 2008). The mechanism of the immunoglobulin rise is unclear, but it is reasonable to suggest that low levels of aflatoxin exposure could stimulate the immune response and will not induce a significant damage in humoral immunity.

In conclusion, an increasing prevalence of aflatoxin exposure was determined in children after 6 months when most of them start to consume complementary foods. A strong adverse effect of aflatoxin on thymus growth at the fastest growth stage was found in our study, which suggests the potential detrimental effect on immune development from aflatoxin in early infancy. For further examination of the aflatoxin's impact on antibody response, other potential factors, such as maternal antibody status, health condition of infants and/or feeding stage should all considered.

Chapter 4: Impact of aflatoxin on biomarkers of environmental enteropathy in Malawian children: a pilot study

4.1 Introduction

Childhood mortality is high in low-income countries, largely due to poor sanitary conditions and high burden of infectious disease. Nearly 22% of children under 5 years old (149 million) globally were stunted in 2018 (WHO, 2019). Children living in Southern and Eastern Africa showed the highest stunting prevalence (> 30%), and despite a large number of interventions, prevalence of stunting has remained high during recent years. Wasting, stunting and underweight are defined by the WHO as weight for height (WHZ), height for age (HAZ) or weight for age (WAZ) Z-score less than -2, respectively (WHO, 2006). It has been estimated that approximately half of child deaths under 5 years old are attributable to malnutrition (UNICEF, 2016). Recent studies have indicated that environmental enteropathy (EE) could be a crucial contributor to impaired growth in children (Lin *et al.*, 2013; Weisz *et al.*, 2012; Kosek *et al.*, 2013).

EE is defined as a subclinical condition with symptoms mainly including intestinal inflammation likely caused by frequent faecal-oral contamination, although exact contributors are ill defined (Korpe & Petri, 2012). Children who live in low-resource communities are the most susceptible. EE leads to intestinal leakiness and increased permeability, gastrointestinal inflammation, bacterial translocation and nutrient malabsorption (Sullivan *et al.*, 1991; Ramakrishna *et al.*, 2006), and thus contributes to malnutrition, anaemia, growth stunting and high mortality in children (Watanabe & Petri, 2016; Harper *et al.*, 2018).

In sub-Saharan Africa, prevalence of aflatoxin contamination in crops poses a chronic, and sometimes acute, risk to human health. Studies have demonstrated impaired child growth associated with exposure to aflatoxin *in utero* (Turner *et al.*, 2007) or in infancy (Gong *et al.*, 2004; Watson *et al.*, 2018). One possible mechanism by which

aflatoxin exposure could impact child growth is through impaired immune function and EE (Gong *et al.*, 2003).

Stool biomarkers, such as α-1 antitrypsin (AAT), myeloperoxidase (MPO) and calprotectin (CP), have been widely used as indicators of EE in human studies. AAT is a protease inhibitor which can protect tissues and cells from inflammatory enzymes; an increase in AAT suggests abnormal intestinal permeability. AAT was also used as a biomarker of protein-losing enteropathy in early studies (Hill *et al.*, 1981; Karbach *et al.*, 1983). MPO is an enzyme mainly secreted by neutrophils during acute inflammation, and which participates in the antibacterial response (Wagner *et al.*, 2008). The high level of MPO in the stool indicated the potential occurrence of lymphocytic infiltration, which is considered to be related to EE (Keusch *et al.*, 2014). CP is another antimicrobial protein that is mainly released by macrophages and neutrophils. The presence of CP in stool suggests the occurrence of intestinal inflammation (Tibble *et al.*, 2000, 2002). Although the dual sugar absorption test, also called the lactulose: mannitol (L:M) test were used as a indicator of EE, the serum endotoxin core antibody (EndoCab) test has been used as an alternative to the L:M test recently, as the test is low cost and easy to perform. EndoCab is an indicator of exposure to lipopolysaccharide (LPS), which can reflect intestinal inflammation and integrity, and has been used in a previous study as an indicator of EE (Benzoni *et al.*, 2015).

A previous study conducted in Malawian children demonstrated the association between the host transcriptome in faeces and severity of EE (Yu *et al.*, 2016), and the authors recommended that immune related gene expression can be explored as potential biomarkers of EE. Therefore, we also examined immune related genes expression in infants in comparison with the aflatoxin exposure.

The pilot study described here aimed to characterise aflatoxin exposure in Malawian infants and to determine the potential association between aflatoxin exposure condition and EE and immune responses in children.

4.2 Methods

4.2.1 Study site and participants

The study sites were three villages in the Mangochi district, a lakeside district located in the South-Eastern region of Malawi (Figure 8). Mangochi is one of the poorest districts in Malawi with high birth rates and low literacy levels (National Statistical Office (NSO) & ICT, 2017). The main sources of sustenance and income are farming maize and fishing. Maize is the staple food in Malawi which accounts for more than 80% of the cultivated land. In Malawi, three annual weather seasons have been defined, namely hot and dry season (August—November), wet and warm season (November—April), and cool and dry season (May—August), with the maize harvest occurring from April to June (USAID, 2013).

Figure 8 Study location – the Mangochi district of Malawi.

A total of 98 children aged 5 to 7 months were enrolled in a study to explore associations between biomarkers of EE and antibacterial responses in peripheral blood. The children were identified by door-to-door visits conducted by study field workers and the village health surveillance assistant. Children who were severely malnourished, defined as having either a mid-upper arm circumference (MUAC) <115mm or bilateral pedal oedema, were excluded from the study and referred to the nearest health centre for evaluation and nutritional rehabilitation. Children who were HIV positive were also excluded to avoid confounding by HIV-related enteropathies.

This was an opportunistic study that utilized samples collected under the framework of the Childhood mortality reduction after oral azithromycin in Mangochi Malawi trial approved by the London School of Hygiene and Tropical Medicine (reference no. 6500) and Malawi College of Medicine Research Ethics Committee (reference no. P.02/14/1521). Subsequent permission to export the samples from Malawi for aflatoxin testing was approved by the Malawi College of Medicine Research Ethics Committee. Written informed consent was collected from a parent or guardian of each participant prior to enrolment.

4.2.2 Anthropometry measurements

Anthropometry measures were recorded by study nurses. Height, weight, MUAC and head circumference (HC) were measured in triplicate. Length was measured using a Seca 417 measuring board (Seca GMBH & Co. Germany); weight was measured by a Seca 336 weighing scale; MUAC and HC were measured using non-stretch tapes (Chasmors Limited, UK).

4.2.3 Blood and stool Sample collection

Up to 3 ml of venous blood was collected by a study nurse, who then aliquoted the sample into PAXGene preservative (Qiagen, UK) and serum separation tubes (BD

Diagnostics, UK). Serum was isolated following 10 min centrifugation in a Hettich EBA270 bench-top centrifuge (Sartorius, UK) at 3,000 rpm. All samples were stored at -80°C until further processing.

Stool sample collection containers were given to mothers the day before sampling was to take place along with instructions on how to collect the sample. Mothers were asked to collect the sample in the morning of the scheduled visit and to bring it to the clinic directly. Stool samples were kept on ice before being transferred to -80°C storage until further processing.

4.2.4 Aflatoxin biomarker analysis

Serum samples were sent, on dry ice, to the University of Leeds for analysis of aflatoxin-albumin adduct levels, determined using a competitive ELISA method (Chapot & Wild, 1991) (see chapter2. Section 2.2).

4.2.5 RNA isolation and gene expression

RNA isolation and gene expression analysis was performed by David Chaima at London School of Hygiene and Tropical Medicine in the UK. Total RNA was extracted from whole blood using the PAXgene PreAnalytix blood RNA kit (Qiagen) following the modified procedures for small sample volumes extraction (Carrol *et al.*, 2007). Taqman array custom microfluidic cards (Thermo Fisher-Life technologies, UK) were used to measure host cell gene expression in extracted samples. Forty-seven target genes were selected based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and canonical pathways in EE.

4.2.6 Biomarkers of environmental enteropathy

All EE biomarker measurements were performed at the College of Medicine in Blantyre in Malawi by Ken Maleta and Khumbo Kalua. Commercially available ELISA kits were used to measure three biomarkers of EE in stool: myeloperoxidase

(Immundiagnostik, Germany), calprotectin (Hycult) and α-1 antitrypsin (GenWay, USA). IgG and IgM against endotoxin core in serum samples were measured using the ELISA kit from Hycult Biotech (Netherlands).

4.2.7 Statistical Analysis

Statistical analyses were performed using STATA version 15 (StataCorp LP). The data for AF-alb, AAT, MPO, CP, EndoCab IgG and IgM showed skewed distribution and were therefore log transformed for further analysis. AF-alb values <LOD were allotted values of 1.5 pg/mg albumin for statistical analysis. The anthropometric indices (Z-scores) were calculated based on the World Health Organization's 2006 Child Growth Standards using Anthro v 3.1 (WHO, Geneva). The geographical and seasonal variation of parameters were analysed using ANOVA. The relationship between EE biomarkers were performed using unadjusted Pearson's correlation. The correlation between AF-alb level and EE biomarkers or growth parameters were performed using simple linear regression model (Model I: unadjusted) or multiple regression model (Model II: adjusts for age, gender, feeding (categorised as exclusive feeding or mix feeding at six months), village and season). The gene expression was calculated by $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The mean value of each gene was used as control, and the target gene expression level was normalised using GAPDH as housekeeping gene. Results were compared with other parameters using Spearman's correlation with or without adjustment by Bonferroni correction.

4.3 Results

4.3.1 Characteristics of the participants

A total of 98 children aged 5 to 7 months were originally enrolled in a study to explore associations between biomarkers of EE and antibacterial responses in peripheral blood. Samples from 78 infants had sufficient serum volume remaining for inclusion in this pilot study of effects of aflatoxin exposure.

Table 13 presents the demographic description of infants in different villages and overall. The average age of the children was 24.6 ± 3.1 weeks with more males (63%) than females. The mean weight and height was 7.2 kg and 64.8 cm, respectively. Stunting (HAZ<-2) was identified in 10 children, underweight (WAZ<-2) was reported in 7 children, and none of them had wasting (WHZ<-2). Five children showed both stunting and underweight. Approximately half of the children were from village 3 (54%), 26% from village 2 and 21% from village 1. Only 26% of children at six months maintained exclusive breastfeeding.

Table 13 Demographic description of the study participants in Malawi

Variable (Mean ± SD/ GM (95%CI))	Village 1	Village 2	Village 3	Overall
No. of participants	16	20	42	78
Age (weeks)	23.8 ± 3.2	25.2 ± 4.3	24.7 ± 2.4	24.6 ± 3.1
Gender, male n (%)	11 (69%)	12 (60%)	26 (62%)	49 (63%)
Season at examination				
Hot season (Aug—Nov) n (%)	4 (25%)	0	14 (33%)	18 (23%)
Wet season (Nov—Apr) n (%)	12 (75%)	19 (95%)	5 (12%)	36 (46%)
Cool season (May—Aug) n (%)	0	1 (5%)	23(55%)	24 (31%)
Weight (kg)	7.2 ± 0.8	7.3 ± 1.0	7.1 ± 0.8	7.2 ± 0.9
Height (cm)	64.9 ± 3.7	65.6 ± 3.5	64.3 ± 1.8	64.8 ± 2.8
Mid-upper arm circumference (cm)	14.5 ± 0.7	14.1 ± 1.2	13.6 ± 1.0	13.9 ± 1.0
Head circumference (cm)	42.9 ± 1.3	43.2 ± 1.4	43.0 ± 1.8	43.0 ± 1.6
Weight for age Z-score (WAZ)	-0.36 ± 0.93	-0.40 ± 1.29	-0.61 ± 1.0	0.72 ± 1.27
Height for age Z-score (HAZ)	-0.56 ± 1.69	-0.37 ± 1.60	-0.94 ± 0.82	0.51 ± 1.06
Weight for height Z-score (WHZ)	0.14 ± 1.49	-0.14 ± 1.19	0.02 ± 1.09	0.01 ± 1.19
Exclusive breastmilk feeding n (%)	5 (31%)	3 (16%)	12 (29%)	20 (26%)
AF-alb (pg/mg)	3.78 (2.38, 6.0)	9.93 (5.63, 17.52)	29.56 (19.10, 45.75)	14.66 (10.46, 20.55)
IgG (Gmu/ml)	39.05 ± 23.81	53.32 ± 34.94	66.8 ± 3.56	57.66 ± 39.30
IgM (Mmu/ml)	31.92 ± 14.19	48.60 ± 32.88	46.36 ± 33.37	43.97 ± 30.62
α-1 antitrypsin (AAT, mg/g)	0.21 (0.14, 0.31)	0.18 (0.13, 0.26)	0.12 (0.07, 0.18)	0.15 (0.11, 0.19)
Calprotectin (CP, μg/g)	350.48 (212.73, 577,42)	655.94 (356.87, 1205.65)	617.56 (442.51, 861.85)	554.18 (431.44, 711.82)
Myeloperoxidase (MPO, ng/ml)	7880.36 (3708.19, 16746.71)	16059.85 (10359.34, 24897.22)	11163.40 (7645.40, 16300.19)	11319.57 (8612.39, 14877.71)

Notes: n: number of sample, %: percentage of samples; SD: standard deviation; GM: geometric mean; 95%CI: 95% of confidence interval. Feeding record was missing for one child, stool biomarkers measurement for nine. Western standard for EE biomarkers: MPO>2,000 ng/ml, AAT>0.27 mg/g, CP>200 μg/g.

4.3.2 Blood and stool biomarkers of environmental enteropathy

There are no official standards for the indicators of EE in African or low-income countries. Comparing our results with the only published Western standards (CP > 200 µg/g, MPO > 2000 ng/ml and AAT >270 µg/g), there are 78%, 93% and 28% of samples had levels of CP, MPO and AAT higher than the Western standards, respectively. Seventeen children (25%) had all of the three biomarkers higher than the standards.

The Pearson's correlation analysis (unadjusted) demonstrated a significant positive correlation between the level of IgG and IgM (coef.=0.4088, p=0.0002), and the level of CP also showed significant correlation with AAT (coef.=0.3050 , p= 0.0108) and MPO (coef.=0.3908, p=0.0009), but there are no association between AAT and MPO, or between the blood and stool biomarkers.

4.3.3 Geographical variation

The AF-alb GM in infants from village 3 was significantly higher than in the other two villages, with or without adjustment for feeding and season (unadjusted p<0.0001, adjusted p=0.0013). MUAC and the level of IgG also showed similar geographical variation. Smaller MUAC and higher IgG was reported in infants in village 3 (unadjusted p=0.0116 & p=0.0253).

4.3.4 Seasonal variation

Figure 9 presents the results divided into the different seasons. AF-alb showed a significant seasonal variation in these populations, with samples collected during the cool season having significantly higher levels of AF-alb than those samples collected during the hot and wet seasons (Fig. 7a) with or without adjusting for feeding (p<0.0001). For the faecal biomarkers, only the concentration of AAT (Fig. 7d) showed seasonal variation. Children examined during the hot season showed

significantly lower levels of AAT than children examined at wet seasons with or without adjusting for feeding (unadjusted p=0.0042, adjusted p=0.0009).

4.3.5 Relationship between aflatoxin exposure and EE biomarkers

There was no difference in AF-alb levels between the sexes. Table 14 presents the correlation between aflatoxin exposure level and other parameters. AF-alb levels showed significant negative correlations with WAZ and MUAC in the unadjusted model (p=0.047 & p=0.004), but no significant correlation was found after adjusting for other covariates. No significant association was found between aflatoxin exposure level and other anthropometric parameters. For the biomarkers of intestinal inflammation and permeability in blood and stool, a negative correlation was determined between AF-alb level and AAT in those two models ($p<0.05$).

4.3.6 Relationship between aflatoxin exposure and immune related gene expression

The association between AF-alb level and inflammatory response related gene expression is presented in appendix 4.1. Significant correlations were determined between AF-alb and expression levels in several target genes, including: *AMICA1, AQP9, BIN2, CD53, CR1, CSF2RB, CSF3R, CST7, CXCR2, FCGR2A, FCGR3B, FFAR2* and *IL1RN* ($P<0.05$). While after adjustment by Bonferroni correction, the significant correlation was eliminated (Table 15).

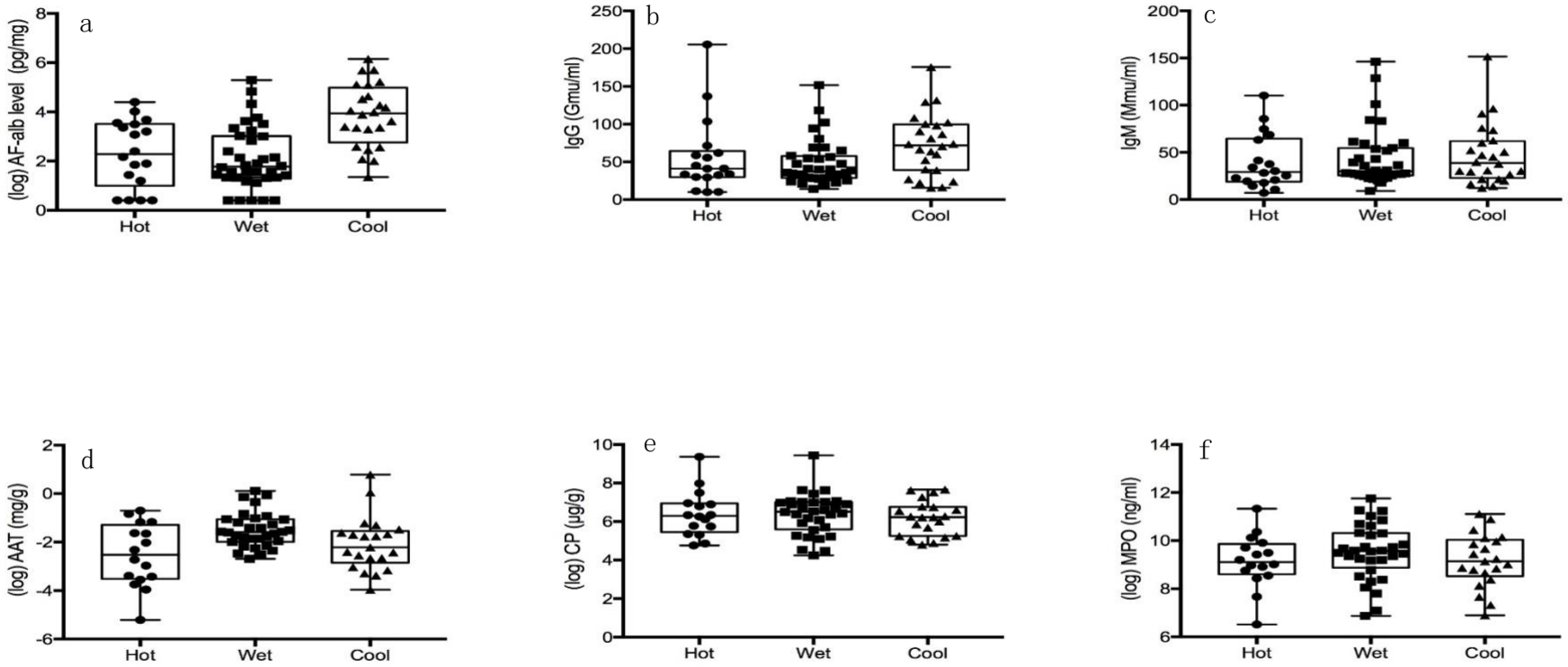


Figure 9 Seasonal variation of parameters in Malawian infants

Box and whisker plot showing the levels of (log) AF-alb for infants grouped by season of sampling.
a) (Log) AF-alb level (pg/mg), b) level of IgG (Gmu/ml), c) level of IgM (Mmu/ml), d) (log) AAT (mg/g), e) (log) CP (µg/g), f) (log) MPO (ng/ml). AF-alb level: aflatoxin-albumin level; AAT: α-1 antitrypsin; CP: calprotectin; MPO: myeloperoxidase. AF-alb level, AAT, CP and MPO were log transformed. Hot season (August—November), Wet season (November—April) and Cool season (May—August).

Table 14 Relationship between aflatoxin-albumin level and anthropometric parameters and EE biomarkers

	Model I		Model II	
	Coefficient (95%CI)	*p* value	Coefficient (95%CI)	*p* value
WAZ	-0.16 (-0.32, -0.002)	0.047	-0.17 (-0.37, 0.03)	0.092
MUAC	-2.25 (-3.77, -0.74)	0.004	-1.49 (-3.43, 0.45)	0.130
IgG	0.10 (-0.003, 0.20)	0.057	0.02 (-0.11, 0.14)	0.774
IgM	0.09 (-0.01, 0.18)	0.069	0.08 (-0.04, 0.20)	0.206
AAT	-0.23 (-0.40, -0.06)	0.010	-0.25 (-0.48, -0.02)	0.033
CP	0.03 (-0.15, 0.20)	0.764	-0.07 (-0.31, 0.18)	0.590
MPO	-0.06 (-0.25, 0.13)	0.552	-0.14 (-0.40, 0.12)	0.284

Notes:
WAZ: weight for age Z-score; MUAC: mid-upper arm circumference; AAT: α-1 antitrypsin; CP: calprotectin; MPO: myeloperoxidase.
Model I: unadjusted
Model II: adjust for age, gender, feeding, village and season of sample collection.

Table 15 Correlations between aflatoxin-albumin level and gene expression fold change

Gene symbol	Description	Coefficient	*P* value [1]	*P* value [2]
AMICA1	Adhesion molecule, interacts with CXADR antigen 1	0.226	0.048	1
AQP9	Aquaporin-9	0.381	0.001	0.661
BIN2	Bridging Integrator 2	0.284	0.012	1
CD53	Leukocyte surface antigen CD53	0.314	0.005	1
CR1	Complement component (3b/4b) receptor 1 (Knops blood group)	0.312	0.005	1
CSF2RB	Colony-stimulating factor 2 receptor, 1 (Knops blood g	0.312	0.005	1
CSF3R	Colony-stimulating factor 3 Receptor (granulocyte)	0.417	0.0002	0.169
CST7	Cystatin-F (leukocystatin)	0.417	0.0002	1
CXCR2	Chemokine (C-X-C motif) Receptor 2	0.373	0.0008	0.858
FCGR2A	Fc fragment of IgG, low-affinity IIa, receptor (CD32)	0.349	0.002	1
FCGR3B	Fc fragment of IgG, low-affinity IIIb, receptor (CD16b)	0.312	0.006	1
FFAR2	Free fatty acid receptor 2	0.307	0.007	1
IL1RN	Interleukin-1 receptor antagonist	0.232	0.042	1

1 Spearman's correlation
2 Adjusted for Bonferroni correction

4.4 Discussion

Malawi is a big peanut producing and exporting country which ranked in the top 15 in Africa for this commodity (Simtowe *et al.*, 2010). Around 90% of its total yield are produced by smallholder farmers who culture peanuts for both family diet and selling as a cash crop. Previous studies conducted in Malawi determined high levels of aflatoxins in maize and groundnut samples, which was up to 3871 µg/kg in peanuts and 1335 µg/kg in maize, much higher than the maximum limit for aflatoxins in foods in Malawi (3 µg/mg) and the EU (4 µg/mg) (Monyo *et al.*, 2012; Mwalwayo & Thole, 2016; Waliyar *et al.*, 2010).

The current study is the first one that examined the aflatoxin exposure condition in infants less than 6 months. Another study conducted in Malawi recently determined the AFB_1 in food samples and AF-alb level in 230 local residents aged older than 14 years old (Seetha *et al.*, 2018). Sixty seven percent of human serum samples showed AF-alb positive with a mean level of 20.5 ± 23.4 pg/mg. It is interesting that all participants in this study ate maize based porridge every day (Seetha *et al.*, 2018). The high frequency of maize consumption in Seetha's study provided a possible explanation for the higher AF-alb level determined in infants in the current study than the same age infants in Gambia (Chapter 3) or other African countries. More than four-fold higher of AF-alb level was determined in infants in the current study than the level in the same age infants in the Gambia study (Chapter 3) (14.7 vs 3.5 pg/mg). Lower AF-alb level also reported in previous studies, Shirima *et al.* (2015) determined the GM (95%CI) level of AF-alb 4.7 (3.9, 5.6) pg/mg among 166 children aged between 6 and 14 months in Tanzania, and McMilan *et al.* (2015) reported a GM level of AF-alb 2.4 pg/mg in children aged between 6 to 48 months in Nigeria.

A similar increase in aflatoxin exposure with age in this study was as reported in the Gambia study (Chapter 3). For 65 of the infants in the current study, samples were also available from 9 months of age to examine the AF-alb level. The GM (95%CI) level of AF-alb is 52.4 (39.9, 68.8) pg/mg at 9 months, which is more than three-fold higher than the level detected at 6 months. In addition, only a few infants (12%) had AF-alb level less than LOD at 6 months in Malawi study compared to more than half of infants had undetectable AF-alb in the Gambia study (Chapter 3). The high frequency of maize or maize-based foods consumption might contribute to the higher aflatoxin exposure level in Malawi.

It is known that breastfed infants can be exposed to aflatoxin from AFM_1 in breast milk, but that much higher exposure occurs once family food is given during weaning (Magoha *et al.*, 2014). Here, a significantly lower AF-alb level was detected in infants with exclusive breastfeeding than those with mixed feeding (GM 8.26 vs 18.20 pg/mg, $p=0.0425$), but the statistically significant difference was diminished after adjusting for age ($p>0.05$). Previous research in Benin and Togo demonstrated that AF-alb was low during breastfeeding but increased significantly after six months when weaning foods were introduced (Gong et al, 2002). Another study conducted in Benin also found that AF-alb levels increased with age during the first 2 to 3 years of life, from a GM of 9.5 pg/mg at < 1 year old, to 42.9 and 44.7 pg/mg at 2 and 3 years old, respectively (Gong *et al.*, 2003).

Aflatoxin associated growth retardation was determined in the Gambia study and published before (Watson *et al.*, 2018). A negative association between AF-alb level and anthropometric parameters also found in the current study. MUAC has been used by the WHO as an indicator of malnutrition (WHO & UNICEF, 2009). For children aged between 6 and 60 months, a MUAC less than 110 mm indicates severe wasting

and Sever Acute Malnutrition; between 110 mm and 125mm Moderate Acute Malnutrition is indicated. In this study, children who had a MUAC less than 115 mm were excluded, nevertheless a significant negative association was still seen between AF-alb level and MUAC in children ($p<0.05$). Similarly, a significant negative correlation was identified between AF-alb levels and WAZ in our study ($p<0.05$). As described in the introduction section, many previous studies have indicated that high levels of aflatoxin exposure could retard child growth. Gong *et al.* (2004) and Turner *et al.* (2007) illustrated that children with high aflatoxin exposure levels had less height and weight gain, especially during the first year of life. Association of high AF-alb with low insulin-like growth factor (IGF) in older children was previously reported as a possible mechanism for stunting *(Castelino et al.*, 2015), but this was not corroborated in a subsequent cohort study (Watson *et al.*, 2018).

Here, we also saw seasonal and geographical variation of aflatoxin exposure levels in children. Both the contamination of aflatoxin in crops and aflatoxin exposure in humans have been reported to be associated with season in previous studies (Castelino *et al.*, 2014; Watson *et al.*, 2015; Kamika & Takoy, 2011). It is known that aflatoxin contamination in foods can happen in every stage from pre-harvest to storage, and improper storage conditions and long storage time could dramatically increase the contamination of AFs. Drought stress has been identified as a major factor that contributes to elevated aflatoxins contamination in the field (Guo *et al.*, 2008), as most of the aflatoxicosis outbreaks happened during severe droughts. Therefore, two possible reasons could explain the significantly higher level of AF-alb determined in participants during the cool season. The first could be the increased food consumption during the cool season (also called harvest season) leading to elevated intake of AFs, and the second could be that the AF-alb levels, which represent exposure integrated

over the previous 2 months, could reflect the accumulation of AF-alb from consumption of more highly contaminated food during the wet (post-harvest) season, food which had been stored for a longer period.

The study described here assessed EE by measuring intestinal permeability and inflammatory biomarkers. Currently, there are no official standards for the indicators of EE in African or low-income countries. Comparing our results with the Western threshold values indicating EE, most of the infants had CP (78%) and MPO (93%) level higher than the Western standards. Similar high levels of intestinal inflammatory biomarkers was also seen in the earlier MAL-ED (malnutrition and enteric diseases) study, which recruited 537 children from eight low-income countries and examined the level of stool biomarkers during the first 15 months of infancy (Kosek *et al.*, 2013). Previous animal studies reported the impact of aflatoxins on the gastrointestinal tract, such as an increase in carriage of gram-negative bacteria and aerobic bacteria (Galarza-Seeber *et al.*, 2016), reduced weight and length of intestine (Hossein & Gurbuz, 2016), increased lymphocyte and leucocyte infiltration in intestinal mucosa (Akinrinmade *et al.*, 2016), and increased apoptosis in the jejunum (Peng *et al.*, 2014). In addition, some *in vitro* studies which treated Caco-2 cells, a colon cell line with AFB_1 also demonstrated the inhibition of cell growth, inactivity of lactate dehydrogenase, damage to cell membranes and genetic injury caused by AFB_1 (Zhang *et al.*, 2015). Although, aflatoxin exposure could, therefore be a potential contributor to intestinal injury and EE, leading to malabsorption and child growth retardation (Campbell *et al.*, 2003), we did not see a significant association between the biomarkers and AF-alb in this study.

Earlier studies had demonstrated that increased intestinal permeability could contribute to child growth retardation. A Gambia study conducted by Campbell *et al.*

(2003) determined the negative association between children's weight and height gain and increased intestinal permeability, which was measured by both dual sugar absorption (lactulose : mannitol test) and EndoCab test. A weak but significant negative correlation also found between the level of IgG and WAZ in our study ($p < 0.05$, data not shown) which indicates the potential effect of intestinal injury on child growth. As it has been proved before that children with EE could lead to malabsorption and other intestinal dysfunction. However, some other studies found contradictory results with children in different ages (from birth to 12 or 18 or 48 months or between 2 and 5 years) all showed no association between the growth and EndoCab (Mondal *et al.*, 2012; Lin *et al.*, 2013; Prendergast *et al.*, 2014; Benzoni *et al.*, 2015). Hence, Benzoni *et al.*, (2015) suggested that EndoCab might not a sensitive biomarker for investigating child growth or EE in children older than 2 years old.

However, significant correlations between AF-alb level and the expression of several immune response related genes were identified in the current study. High aflatoxin exposure is mainly associated with the elevation of granulocyte colony–stimulating factor (G-CSF) signalling genes *CSF2RB* and *CSF3R* and genes in the phagocytosis pathway *FCGR2A* and *FCGR3B*, and cytokine receptors such as *CXCR2*. These target genes participate in the bacterial and/or viral response. The increased expression of G-CSF genes could contribute to the defence of bacterial and virus infection, and villous tissue repair in the intestine (Yu *et al.*, 2016). The IgG receptor genes (*FCGR2A* and *FCGR3B*) can present on several types of immune cells, and play an important role in antibody production by B cells and phagocytosis of immune complexes in macrophages (Hibbs *et al.*, 1988). The surface antigen CD53 can express on all peripheral leukocytes and its expression is highly associated with lymphocyte activation (Amiot, 1990). A previous study conducted in Malawi with 259 children

aged between 12 and 61 months also found an association between EE and expression of immune related genes, such as G-CSF pathway target genes and phagocytosis related genes (Yu *et al.*, 2016). Therefore, our findings suggest that aflatoxin exposure could be another factor that contributes to the modification of immune response and EE in children.

There are a number of limitations to our study. This was only a pilot study conducted in a limited number of villages with small sample size. Here we did not identify a significant association between aflatoxin exposure level and blood and stool EE biomarkers. This was an opportunistic study that made use of samples already collected. That there was only one examination time point also limited the analysis, and ruled out considerations of long-term effects. For further exploration, long-term follow-up research and additional data such as socio-economic status, food consumption, food preparation and storage, health condition (morbidity and clinical symptoms) will be helpful for analysis.

In conclusion, this is the first study that examined the effect of aflatoxin exposure on child growth, EE and immune gene expression in infants (less than 6 months) in Malawi. Children in the current study had higher levels of AF-alb than those reported from other countries in Africa. It is a concerning observation that the exposure is associated with the activation of immune response related genes. There is no significant association between aflatoxin exposure level and biomarkers of EE determined in this study, which might due to the small sample size and narrow age range. The finding that aflatoxin exposure was inversely associated with MUAC and WAZ growth indicator, coincide with many other research findings, suggesting a possible pathway link and potential detrimental impact from aflatoxin exposure, EE

to child malnutrition. However, the conclusive evidence requires a purposely-designed human study.

Chapter 5: Immunomodulation effect of aflatoxin *in vitro* through the IL-6/STAT3 pathway

5.1 Introduction

The immunotoxicity of aflatoxin has been explored in many animal and *in vitro* studies (IARC, 2002; *Wild et al.*, 2015). Aflatoxin has been shown to effect both humoral and cellular immune function such as suppressed phagocytic activity, damage to intestinal integrity, depressed antigen presenting and impairment of lymphocyte function (Bbosa *et al.*, 2013). In rats fed with aflatoxin, the reduction in the percentage of NK cells and $CD8^+$ T cells, decrease in the expression of cytokines such as IL-1, IL-6, TNF-α and suppression of TLR2 signalling pathway have been reported (Bruneau *et al.*, 2012; Moon & Pyo, 2000; Qian *et al.*, 2014). Choi *et al.* (2010) also found the reduction in levels of IgA and IgG in AFB_1 treated mice. In human, a similar reduction in salivary IgA was associated with detectable AF-alb compared to those with non-detectable AF-alb (Turner *et al.*, 2003). In the Gambia study reported in Chapter 3 and Malawi study reported in Chapter 4, we also observed an association between aflatoxin exposure and retardation of thymus growth, alteration of antibody response to vaccination in children and modulation of immune response related genes, which could also suggest the potential effect of aflatoxin on both innate and adaptive immune function. However, such human population studies are limited as most of them are cross-sectional including with sample size and require repeatability in different populations (Wild *et al.*, 2015). Also, the molecular mechanism of aflatoxin induced immune modulation is still unclarified and needs further investigation.

Signal transducer and activator of transcription (STAT) proteins are crucial regulators of anti-tumour immunity or pro-oncogenic inflammation. Among the STAT family, STAT1 and STAT2 function as anti-tumour proteins which activate the T_H1-type cell and subsequently increase the production of IL-2, TNF-β and IFNγ to promote anti-tumour immunity (Lim & Cao, 2006). However, STAT3 is a key protein that

contributes to cancer inflammation and tumour growth and survival. Numerous studies have found persistent activation of STAT3 in tissues in which a tumour developed (Corvinus *et al.*, 2005). Interlukin-6 (IL-6), a key activator of STAT3, has also been shown to be elevated in cancer patients (Tanaka *et al.*, 2014). Liu *et al.* (2011) found that elevated IL-6 expression led to continuous activation of STAT3, consequently aggravating the progress of hepatocellular carcinoma. In an earlier study on the impact of aflatoxin exposure *in utero* on gene expression in Gambian children, whole genome gene expression assay showed an association between high AF-alb level in pregnant women and higher expression of four immune related genes (*STAT3*, *NFκB*, *IL-6* and *CCL20*) in white blood cells of the children at six months old (Castelino, 2013).

In this study, we applied three types of cells, namely, human hepatocyte line 16 (HHL-16), Jurkat T cell line and peripheral blood lymphocytes (PBLs) from healthy human donors. HHL-16 is a novel human hepatocyte line which was established from healthy human liver, unlike the cancerous cell lines such as HepG2 or Huh-7. HHLs retain the phenotype of primary hepatocytes, and can synthesize cytokines, albumins and Cytochrome P450 enzymes (Clayton *et al.*, 2005). The Jurkat T cell line was first derived from the peripheral blood of a 14 year old boy who suffered from leukemia (Schneider *et al.*, 1977). It has been widely used as a classic model to explore T cell signalling. PBLs, which were collected from healthy volunteers, could possibly give a more realistic immune response to aflatoxin exposure.

This study aimed to examine whether AFs impact on human immune function through modification of the IL-6/STAT3 pathway. In addition, expression changes in mRNA level of the four immune related genes identified as showing changes *in vivo* (Castelino, 2013) was also measured.

5.2 Methods

5.2.1 Cell maintanence

The HHL-16 and Jurkat T cells were cultured as described in Chapter 2, Section 2.3. PBLs were extracted from three healthy volunteers (conducted by Dr Anusree Mahanta). Cells were then cultured in T25 flask overnight in RPMI 1640 (+Glu) medium supplemented with 10% ATCC FBS and 1% Penicillin-Streptomycin.

5.2.2 Isolation of peripheral blood lymphocytes

Blood samples were collected by a phlebotomist and mixed with anticoagulant (0.5M EDTA, pH 8.0 in 1M PBS) at a ratio of 1:10. Samples were then diluted with RPMI media at a ratio of 1:1, and layered slowly on top of Histopaque at a ratio of 1:1. The mixture was then centrifuged at 400g for 30mins at 20°C. The upper yellow plasma was removed, and the opaque interface layer was collected carefully into a tube. The collected PBLs were then washed once with PBS and twice with RPMI media. PBLs were then cultured in a T25 flask for further experiments.

5.2.4 Cytotoxicity test

HHL-16 cells were seeded in 96 well plates in the density of 2 x 10^4 cells per well in a volume of 200 µl media. Jurkat T cells were seeded in 96 well plates in the density of 1x10^5 cells per well in a volume of 100 µl media. Cells were treated with AFB_1 and AFB_2 with the concentrations of 0.5 µg/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml and 50 µg/ml for 24 and 48 hours. Untreated cells were used as the baseline for DMSO controls, and the DMSO controls were used as baseline for the corresponding AFB_1 or AFB_2 treatment. The AFB_1 and AFB_2 used for treatment were purchased from Sigma with purification ≥ 98.0% HPLC, and the standard powder was dissolved in sterile-filtered DMSO. The original stock concentration of AFB_1 and AFB_2 is 20

mg/ml and 5 mg/ml, respectively. Cytotoxicity was measured by MTT assay as described in Chapter 2, Section 2.4.

5.2.5 Human IL-6/STAT3 pathway PCR Array

HHL-16 cells and PBLs were cultured in a T75 flask for culture and RNA extraction. Both cell types were treated with medium (control), 10 µg/ml AFB_1 or 10 µg/ml AFB_2 for 24 hours. The RNA of each sample was harvested using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions (see Chapter 2, Section 2.5.1). RNA reverse transcription was performed using RT_2 First Strand kit (QIAGEN), according to the manufacturer's instructions (see Chapter 2, Section 2.6.2). The gene expression change in the IL-6/STAT3 pathway was determined by RT^2 Profiler™ PCR Array Human IL6/STAT3 Signalling Pathway Kit (QIAGEN) which contained 83 target genes on this pathway. The quantitative real-time PCR was performed in LightCycle 480 PCR machine (see Chapter 2, Section 2.6.3).

5.2.6 Gene expression level assessment in HHL-16 and Jurkat T cells by qRT-PCR

HHL-16 cells were cultured in a T75 flask and treated with 5, 20 and 30 µg/ml AFB_1. Cells were treated for 24 and 48 hours and RNA samples for each treatment were harvested for PCR assay. Levels of mRNA of four immune function related genes (*IL-6*, *STAT3*, *NFKB* and *CCL20*) were measured. Primers are shown in Table 15.

Jurkat T cells were cultured in 6 well plates and treated with 5 µg/ml and 10 µg/ml AFB_1, and 10 µg/ml AFB_2 for 6, 12 and 24 hours. The expression changes in the main target genes in IL-6/STAT3 pathway were measured by qRT-PCR. Primers are shown in Table 16.

Table 16 Primers for genes analysis by qRT-PCR

Gene	Primer	Sequence (5'—3')
GAPDH	Forward	AAGCCTGCCGGTGACTAAC
NM_001256799.2	Reverse	GCATCACCCGGAGGAGAAAT
IL-6	Forward	AGGACATGACAACTCATCTC
NM_000600.3	Reverse	GGTGCCCATGCTACATTTGCC
STAT3	Forward	CTCTGCCGGAGAAACAGG
NM_003150.3	Reverse	CTGTCACTGTAGAGCTGATGGAG
NF- κB	Forward	CTGGCAGCTCTTCTCAAAGC
NM_001165412.1	Reverse	TCCAGGTCATAGAGAGGCTCA
CCL20	Forward	GCTGCTTTGATGTCAGTGCT
NM_004591.2	Reverse	GCAGTCAAAGTTGCTTGCTG
JAK2	Forward	GATGGATGCCCAGATGAGAT
NM_001322194.1	Reverse	TTGATCCACTCGAAGAGCTAGA
IL-8	Forward	TGGACCCCAAGGAAAACTGG
NM_000584.3	Reverse	TTTGCTTGAAGTTTCACTGGC
IL-10	Forward	GGCTTGGGGCTTCCTAACTG
NM_000572.2	Reverse	GGGAATCCCTCCGAGACACT
Bcl2	Forward	CCTATCTGGGCCACAAGTGAA
NM_000633.2	Reverse	ACAGCCTGCAGCTTTGTTTC
PIM1	Forward	AAGGACCGGATTTCCGACTG
NM_002648.3	Reverse	AAGAGATCTTGCACCGGCTC
SOCS3	Forward	GGACGGAGACTTCGATTCGG
NM_003955.4	Reverse	AACTTGCTGTGGGTGACCAT
MYC	Forward	AGAGTTTCATCTGCGACCCG
NM_002467.5	Reverse	GAAGCCGCTCCACATACAGT

5.2.7 Protein expression level assessment by flow cytometer

The protein expression change were measured by flow cytometry assay. HHL-16 cells were treated with 5, 10 and 20 µg/ml AFB_1, and 10 µg/ml AFB_2 for 24 and 48 hours. PBLs were treated with 10 µg/ml AFB_1 and AFB_2. Jurkat T cells were treated with 5 and 10 µg/ml AFB_1 and 10 µg/ml AFB_2. Brefeldin A was added four hours before harvest to inhibit intracellular protein transport. PBLs were first combined with the surface antibody anti-CD69 and CD19 to distinguish activated lymphocytes and B cells, respectively, and then combined with anti-IL-2, IL-4, IL-6 and IFN antibodies. Jurkat T cells were combined with anti-STAT3, STAT3-Phospho (P-STAT3), IL-6 and IL-10 antibodies. HHL-16 cells were combined with anti-STAT3, STAT3-Phospho (P-STAT3), IL-6, IL-8 and IL-10 antibodies. Cells after corresponding treatment were collected and measured protein expression as described in Chapter 2, Section 2.7.

5.2.8 Statistical analysis

The data were presented as mean with standard error. Comparisons between two groups were analysed using Student's *t*-test. $P < 0.05$ was considered as statistically significant as indicated in the figures. GAPDH was used as housekeeping gene, and relative gene expression was calculated by the comparative 2-^^Ct method (Livak and Schmittgen, 2001).

5.3 Results

5.3.1 Cytotoxicity of aflatoxin B_1 and B_2 in HHL-16 cells and Jurkat T cells

Viability of HHL-16 cells following 24 and 48 hours AFB_1 and AFB_2 treatment was determined (Figure 10). Different DMSO treatment were examined and used as control for the calculation of its corresponding concentrations of AFs. Cells treated with 0.1% - 0.6% DMSO showed more than 80% viability at both 24 and 48 hours, but the reduction was determined with 1% DMSO at 24 hours. Dose-dependent decrease of viability was detected in both AFB_1 and AFB_2 treated cells. Less than 80% of viability remained at 10 µg/ml or above for AFB_1 and AFB_2 at both 24 and 48 hours. Figure 11 presented the percentage of cell viability of Jurkat T cells after 24 and 48 hours AFs treatment. Decrease of viability were found at 10 µg/ml of AFB_1 and above doses at 24 and 48 hours. Less than 80% of viability was determined in cells treated with 20 and 30 µg/ml of AFB_1, and lower than 50% at 50 µg/ml of AFB_1 at 48 hours. Compared with HHL-16 cells, AFB_2 has much weaker cytotoxicity in Jurkat T cells. More than 80% of viability was found in cells treated with all doses of AFB_2.

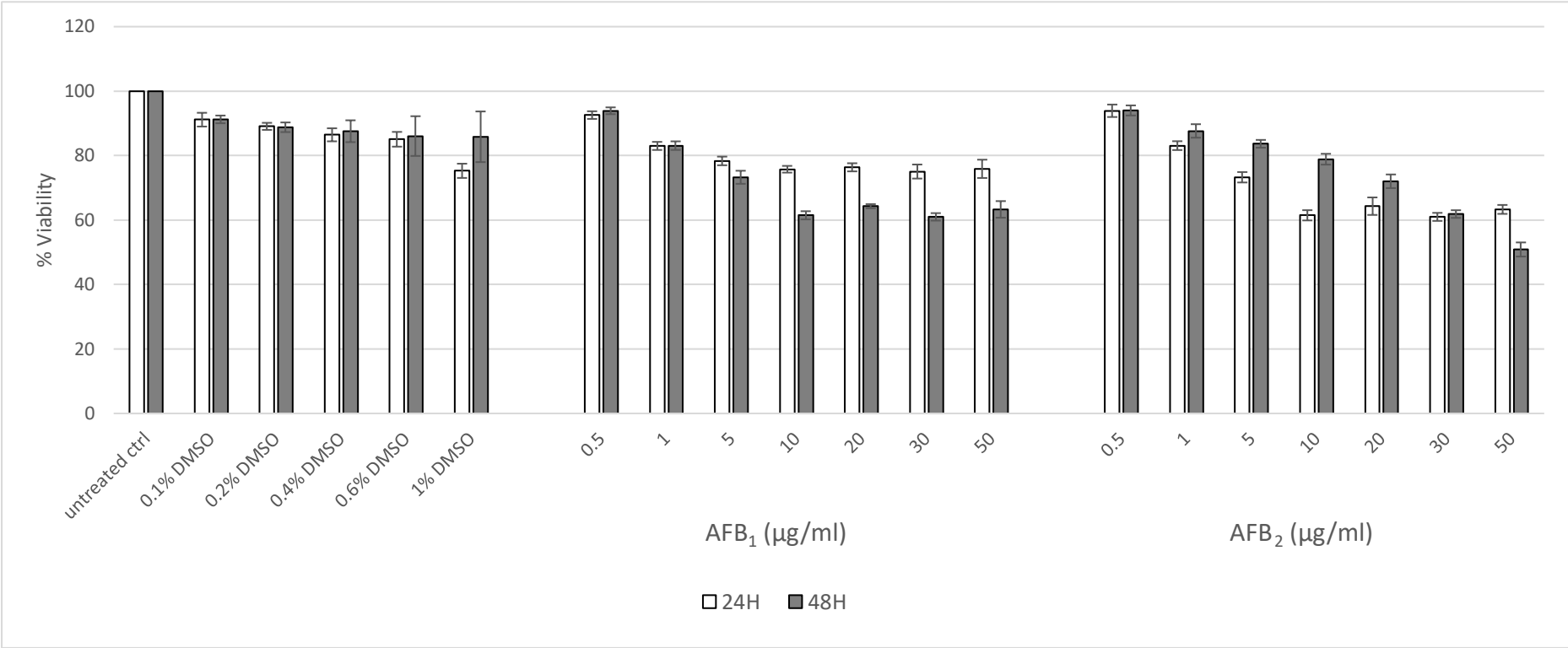


Figure 10 Cytotoxicity test at 24 and 48 hours of treatment with seven AFB_1 and AFB_2 doses for HHL-16 cells.

Test substances were assayed in quadruplicate, in at least three independent experiments. Error bars represent standard error of mean from at least three separate experiments. The original stock concentration of AFB_1 and AFB_2 is 20 mg/ml and 5 mg/ml, respectively. The 50 μg/ml AFB_2 treated cell was normalized with 1% DMSO, 30 μg/ml AFB_2 treated cell was normalized with 0.6% DMSO, 20 μg/ml AFB_2 treated cell was normalized with 0.4% DMSO, 10 μg/ml AFB_2 and 50 μg/ml AFB_1 treated cell were normalized with 0.2% DMSO, 10 μg/ml AFB_2, 20 and 30 μg/ml AFB_1 treated cell were normalized with 0.1% DMSO, other lower treatment concentration were normalized with untreated medium control.

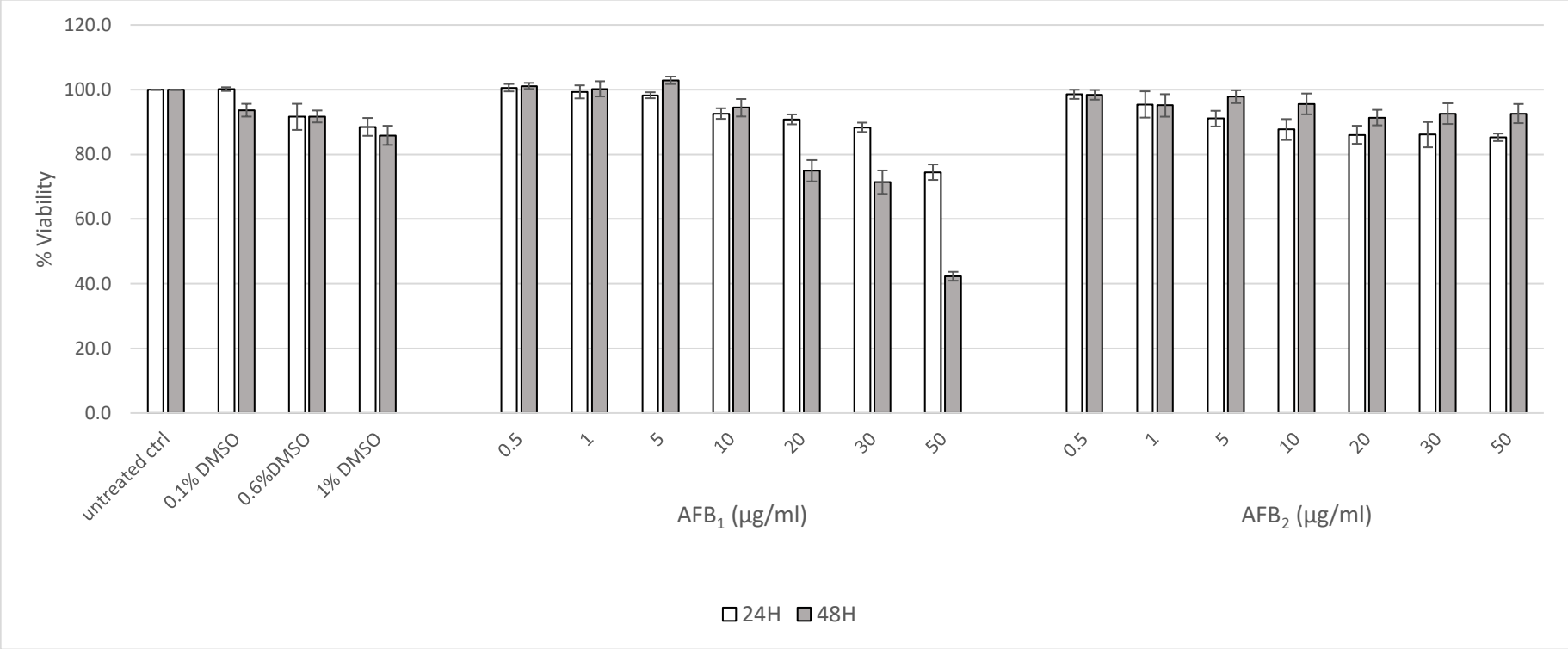


Figure 11 Cytotoxicity test at 24 and 48 hours of treatment with seven AFB_1 and AFB_2 doses for Jurkat T cells.

Test substances were assayed in quadruplicate, in at least three independent experiments. Error bars represent standard error of mean from at least three separate experiments. The original stock concentration of AFB_1 and AFB_2 is 20 mg/ml and 5 mg/ml, respectively. The 50 μg/ml AFB_2 treated cell was normalized with 1% DMSO, 30 μg/ml AFB_2 treated cell was normalized with 0.6% DMSO, 20, 10 and 5 μg/ml AFB_2 treated cell was normalized with 0.1% DMSO, 50, 30 and 20 μg/ml AFB_1 treated cell were normalized with 0.1% DMSO, other lower treatment concentration were normalized with untreated medium control.

5.3.2 Gene expression change in the IL-6/STAT3 pathway in PBLs and HHL-16 cells

The gene expression changes in the IL-6/STAT3 pathway in PBLs and HHL-16 cells were measured by Human IL-6/STAT3 pathway PCR array kit. Figure 12 shows the heat map of the fold changes of several selected genes in both cells, the full data were presented in Appendix 5.1-4. By contrast, higher gene expression elevation was determined in PBLs than in HHL-16 cells.

For PBLs, even though both AFB_1 and AFB_2 treatment induced elevation in RNA levels, the rise was less in cells treated with AFB_2 compared to those treated with AFB_1. An approximately 14-fold increase in *STAT3* with AFB_1 treatment in comparison to about six fold increase in *STAT3* with AFB_2 treatment. IL-6, the crucial activator of STAT3, and the pathway transducer *JAK3* also showed big elevation. In addition, a more than 20-fold increase expression was determined in *NFκB*, which is another important transcription factor in the regulation of the immune response and has been found to interact with STAT3. Downstream targets regulated by both of them showed significant increase such as *IL-1*, *IL-2*, *IL-4* and *IL-10* etc. Other target protein-encoding genes of STAT3 pathway also showed dramatic elevation including, *BCL2* and *PIM1*.

However, in HHL-16 cells, most of the target genes showed no significant change after treatment. For cells treated with AFB_1, an approximately three fold increase in *IL-6* mRNA level was detected. The expression of *STAT3* and *NFκB* was elevated around 1.5-fold. Cytokines encoding genes such as *IL-8* and *IL-12* showed a more than two fold increase. In addition, there were no notable gene expression changes in HHL-16 cells treated with AFB_2.

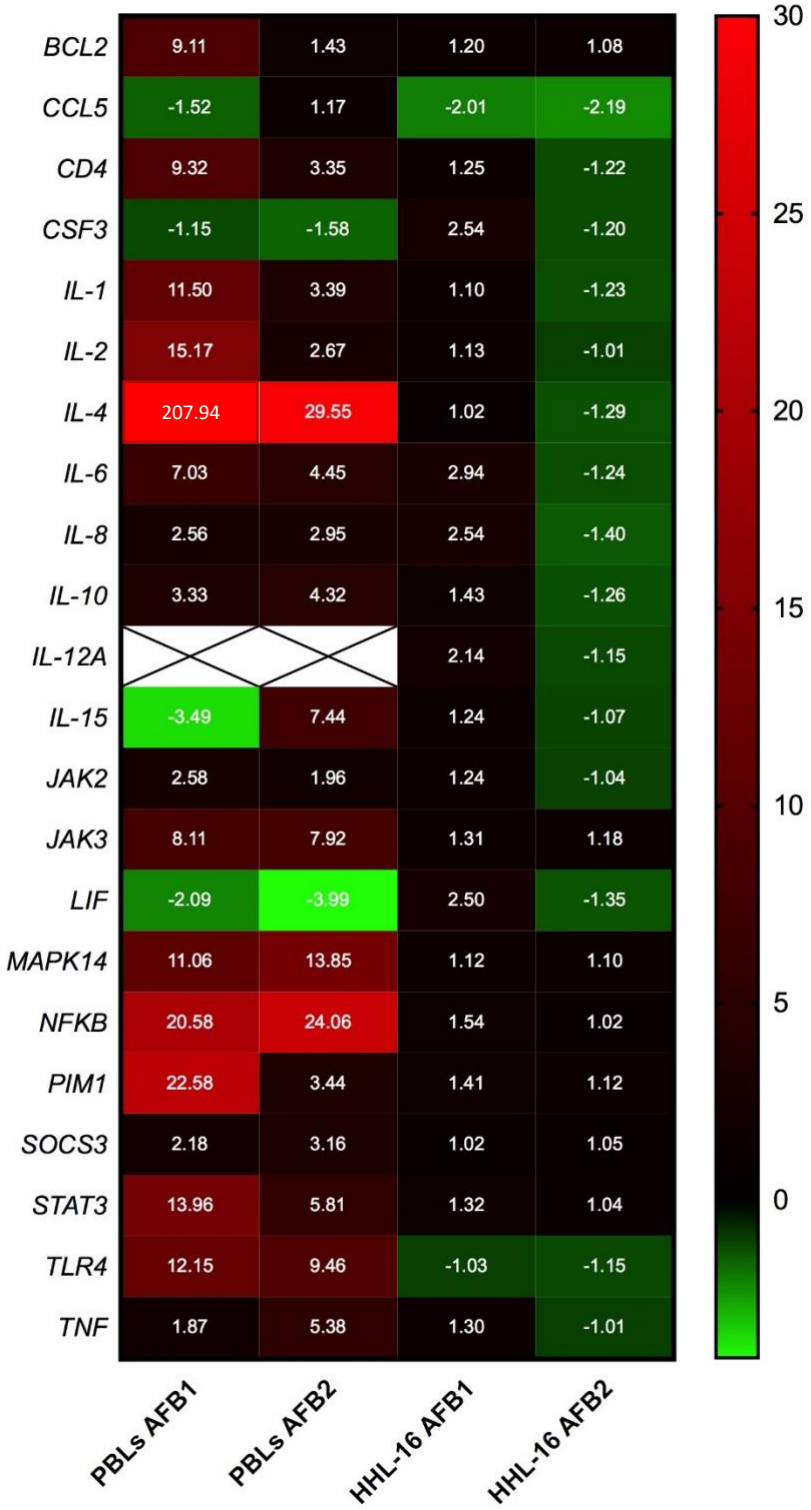


Figure 12 Heat map of gene expression fold change in IL-6/STAT3 pathway in PBLs and HHL-16 cells.

5.3.3 Gene expression change in IL-6/STAT3 pathway in Jurkat T cells

According to the gene expression results in PBLs, several target genes in the IL-6/STAT3 pathway were selected and used to measure the gene expression change in Jurkat T cells with treatment of AFB_1 and AFB_2. Figure 13 presents the first pilot test of gene expression in Jurkat T cells at 24 hours treatment with different concentration of AFB_1. Cells treated with 0.5, 10 and 20 µg/ml AFB_1 showed suppression in gene expression at 24 hours, however, cells treated with 5 µg/ml AFB_1 showed an increase in RNA level. There was no dose-dependent trend at 24 hours treatment.

In previous studies, immune response of Jurkat T cells appeared after short time exposure to a stimulator (Gertsch *et al.*, 2002; Aupanun *et al.*, 2016). Therefore, we redesigned a time-series treatment for 6, 12 and 24 hours (Figure 14-16). Small and transient changes in gene expression in Jurkat T cells were observed for target genes in the IL-6/STAT3 pathway for both compounds. A time dependent increase of gene expression was observed in several genes during the first 12 hours. Significant increases in *STAT3*, *JAK2*, *IL-6*, *IL-10* and *SOCS3* appeared at 12 hours AFB_1 treatment. Expression of *IL-6*, *STAT3* and *IL-10* was elevated in cells treated with AFB_2 at 6 hours. Whilst the gene expression levels returned to normal by 24 hours for both compounds treatment which might due to the increased expression of *SOCS3*, an inhibitor of STAT3, stimulated the negative feedback control of the STAT3 expression.

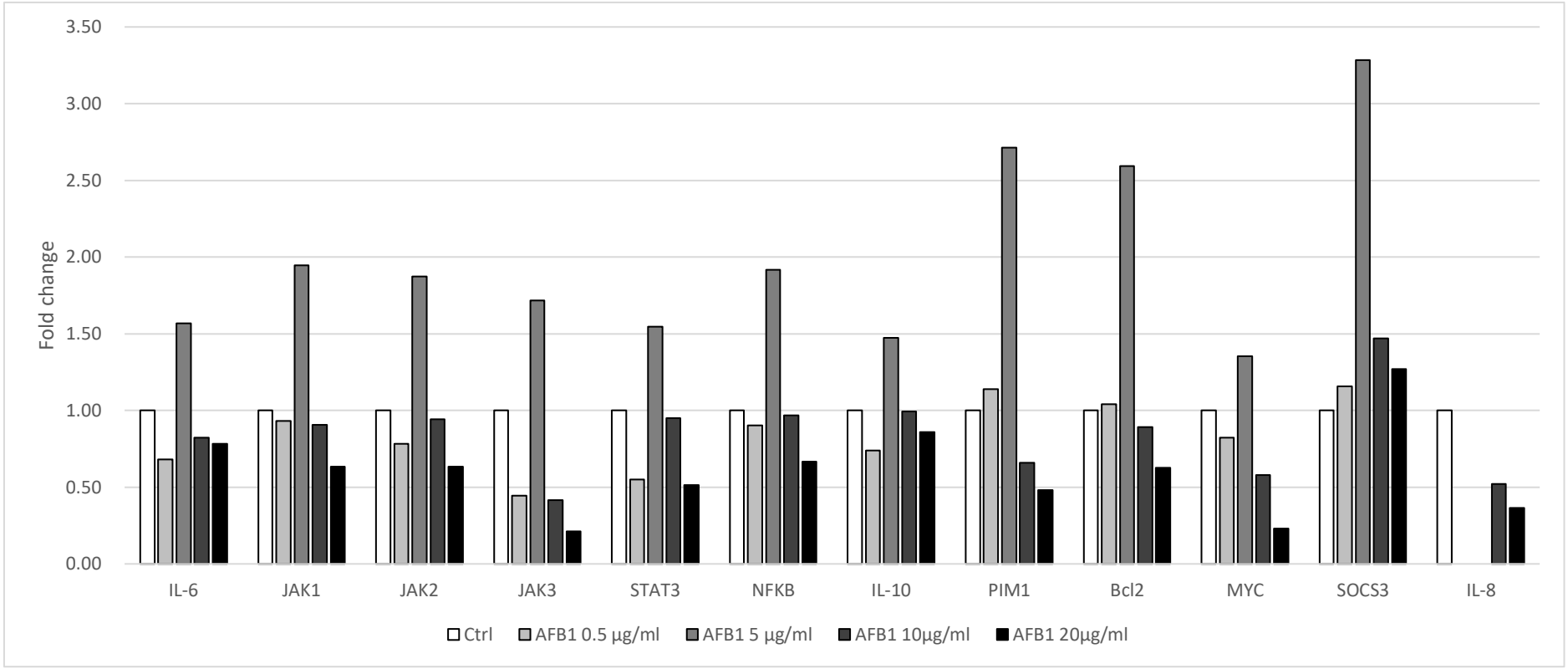


Figure 13 Gene expression fold change in Jurkat T cells treated with different doses of AFB_1 for 24 hours.

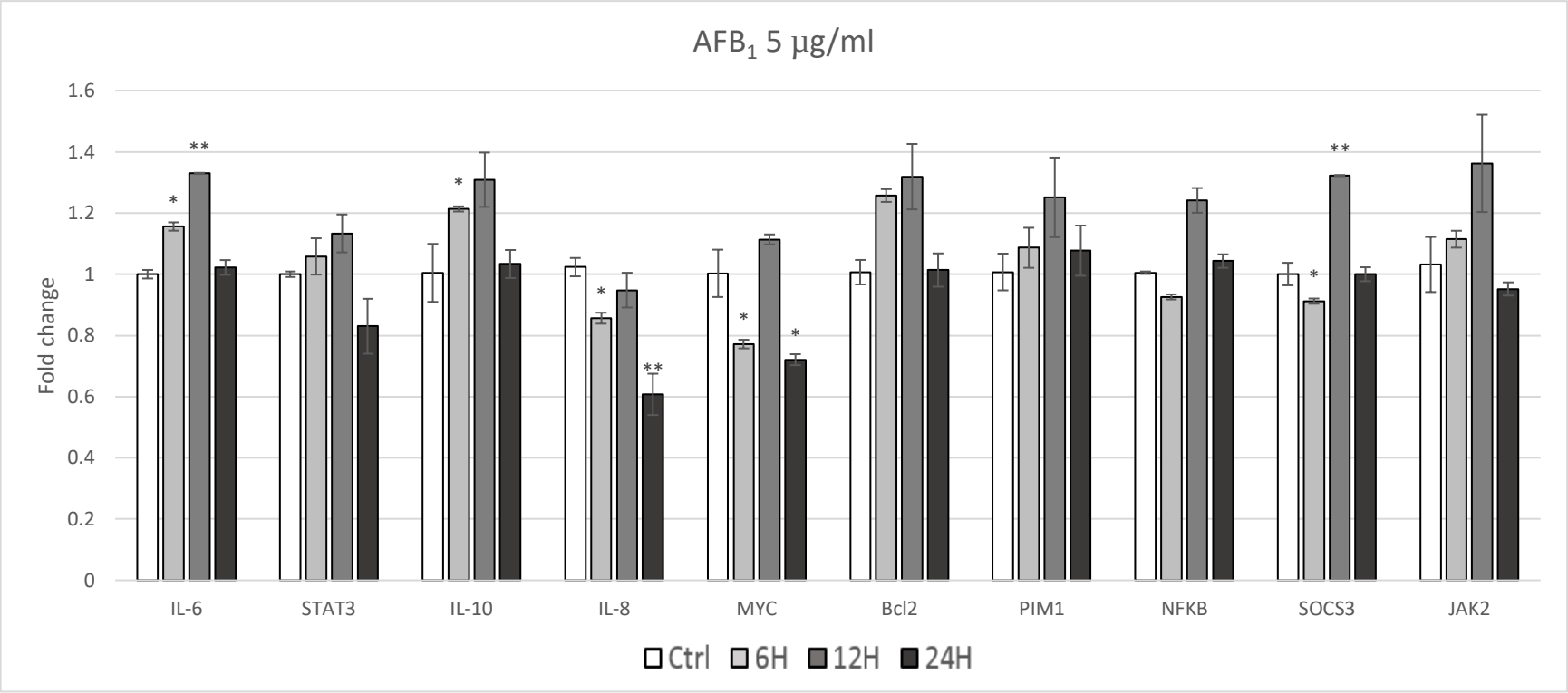


Figure 14 Gene expression fold change in Jurkat T cells treated with 5 µg/ml AFB_1 at 6, 12 and 24 hours.

Test substances were assayed in duplicate in at least three independent experiments. Error bars represent standard error of mean from at least three separate experiments. *signifies $p \leq 0.5$ and ** signifies $p \leq 0.005$ based on student's *t*-test compared to the control sample.

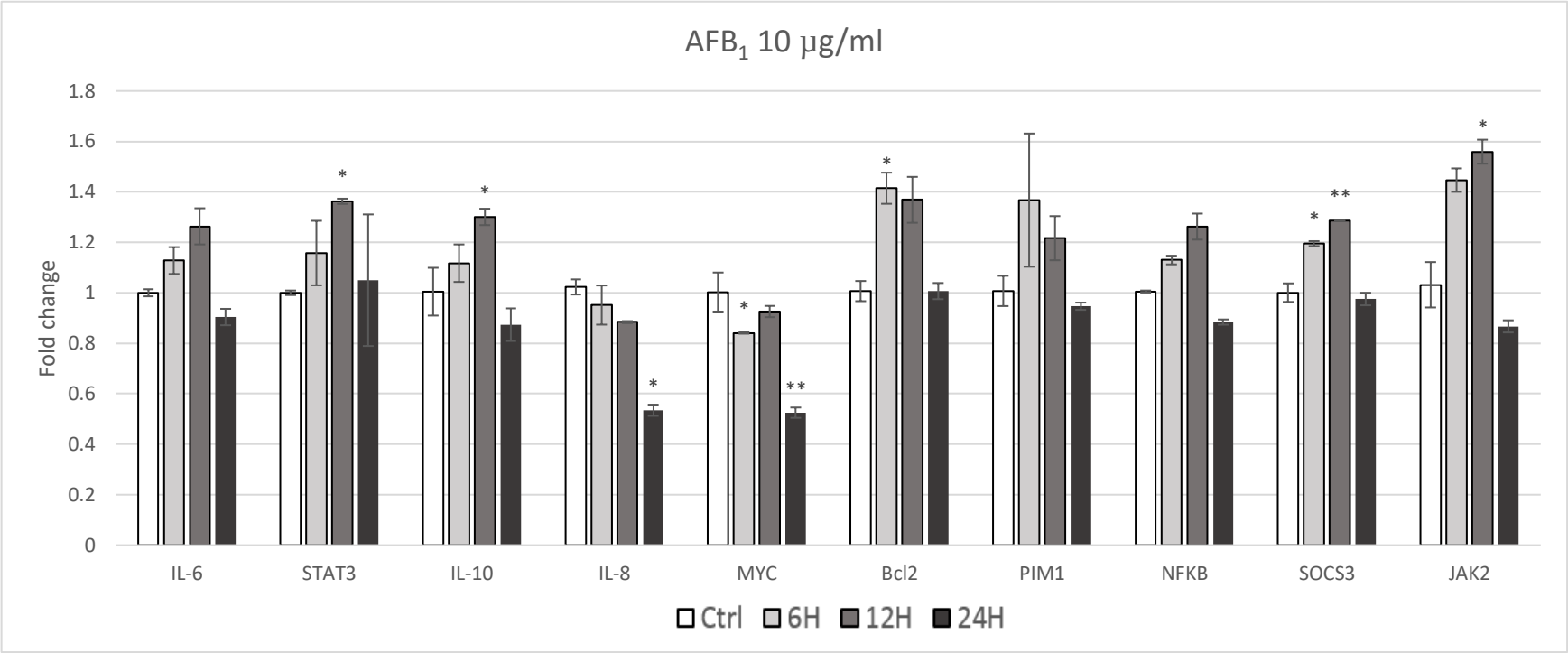


Figure 15 Gene expression fold change in Jurkat T cells treated with 10 µg/ml AFB_1 at 6, 12 and 24 hours.

Test substances were assayed in duplicate in at least three independent experiments. Error bars represent standard error of mean from at least three separate experiments. *signifies $p \leqslant 0.5$ and ** signifies $p \leqslant 0.005$ based on student's *t*-test compared to the control sample.

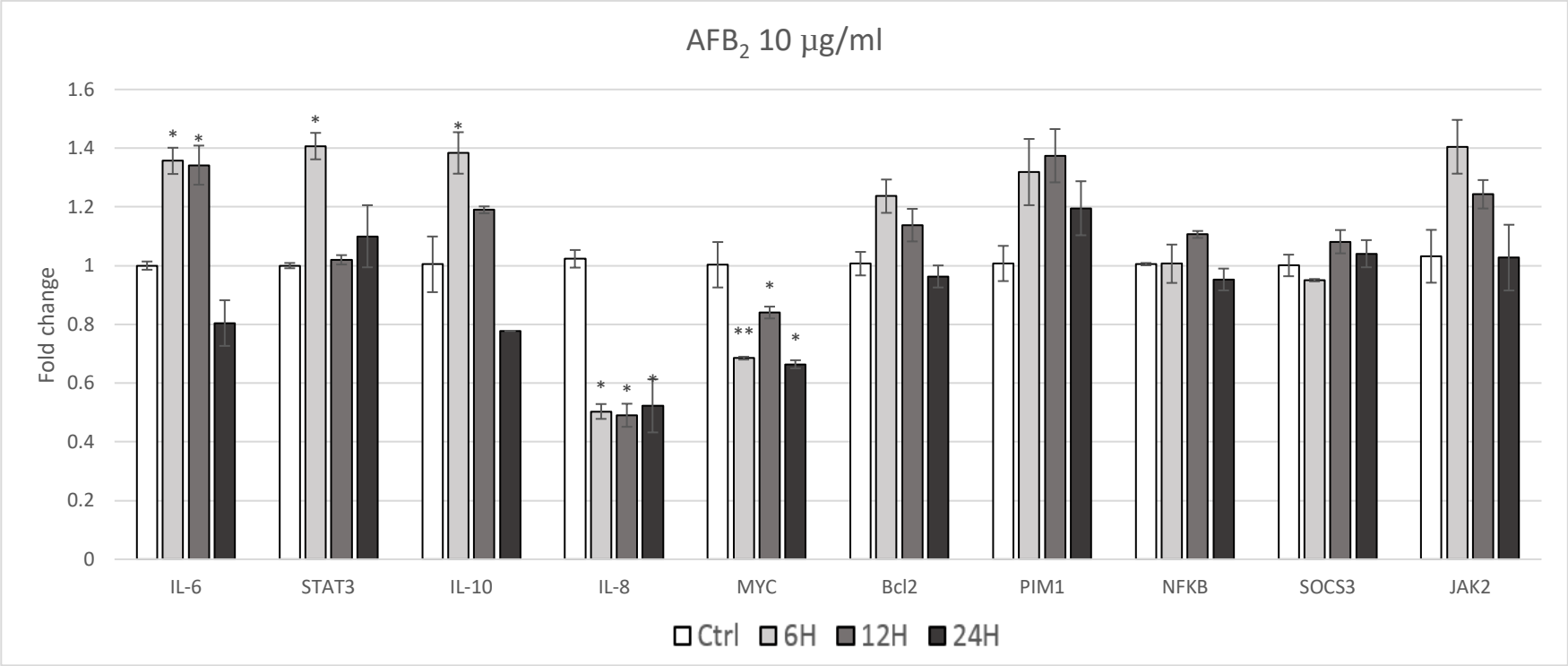


Figure 16 Gene expression fold change in Jurkat T cells treated with 10 µg/ml AFB_2 at 6, 12 and 24 hours.

Test substances were assayed in duplicate in at least three independent experiments. Error bars represent standard error of mean from at least three separate experiments. *signifies $p \leq 0.5$ and ** signifies $p \leq 0.005$ based on student's *t*-test compared to the control sample.

5.3.4 Selected immune gene expression change in HHL-16 cells

There were very small gene expression changes in HHL-16 cells measured by the IL-6/STAT3 pathway PCR array kit. Four immune related genes (*IL-6*, *STAT3*, *NFκB* and *CCL20*) were then selected and measured separately in HHL-16 cells treated with different doses of AFB_1 (5, 20 and 30 µg/ml) for 24 and 48 hours (Figure 17). All of the four immune related target genes showed significant increase at 24 hours at all doses. Approximately four-fold increases were determined in *IL-6* and *CCL20*. About a two-fold increase was detected in *STAT3* and *NFκB*. While after 48 hours, the increases in gene expression were less than at 24 hours. *IL-6*, *NFκB* and *CCL20* showed approximately 1.5 fold increases, while *STAT3* was suppressed at 48 h at 20 and 30 µg/ml AFB_1 treatment.

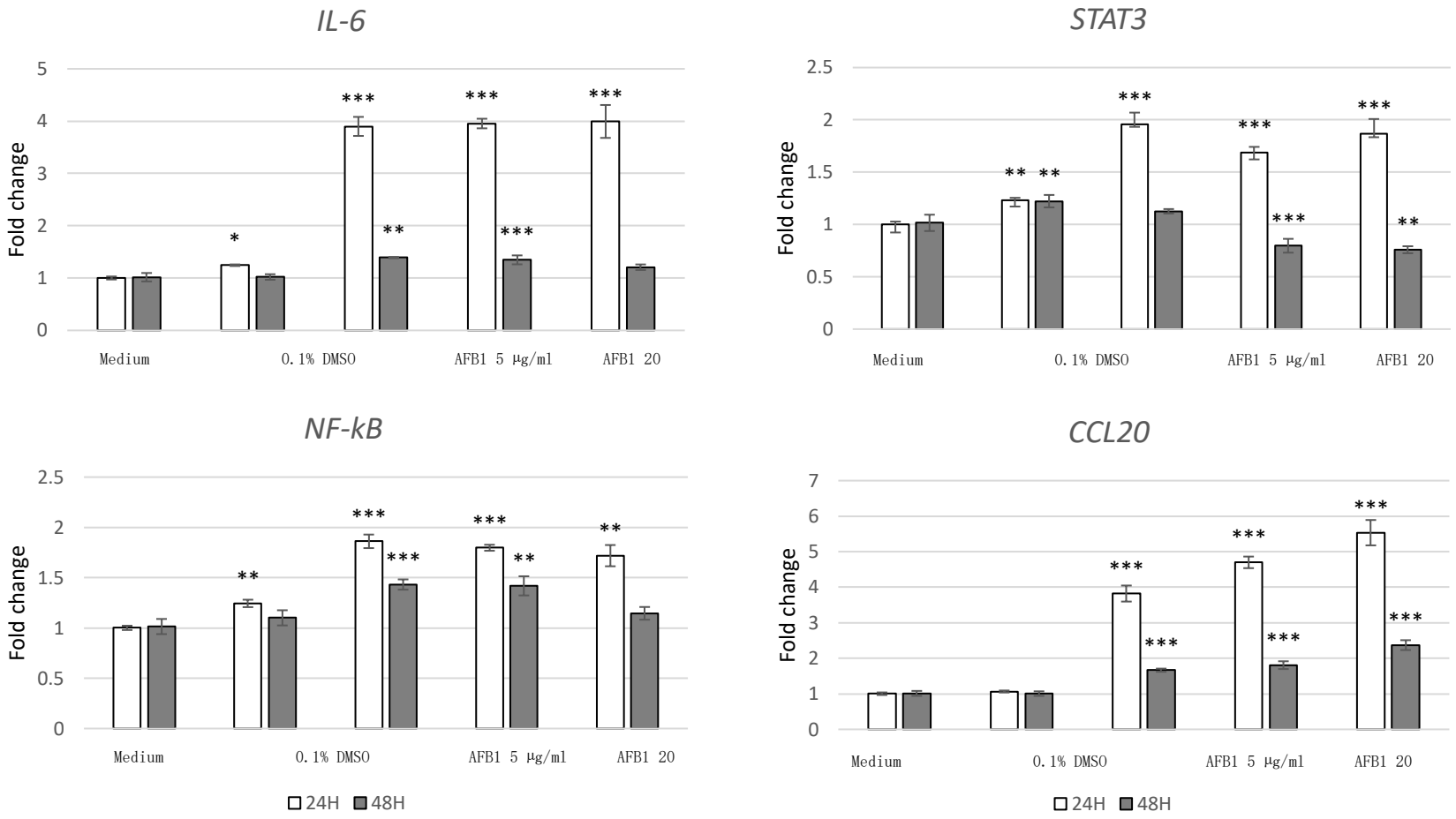


Figure 17 Gene expression fold change in four immune related genes in HHL-16 cells treated with 5, 20 and 30 µg/ml of AFB_1 for 24 and 48 hours.

Test substances were assayed in duplicate in at least three independent experiments. Error bars represent standard error of mean from at least three separate experiments. *signifies $p \leq 0.5$, ** signifies $p \leq 0.005$ and *** signifies $p \leq 0.001$ based on student's *t*-test compared to the control sample.

5.3.5 Protein expression change in three cellular models

The expression levels of several target proteins and cytokines in the IL-6/STAT3 pathway in these three types of cells with 10 μg/ml AFB_1 and 10 μg/ml AFB_2 treatment were measured by flow cytometry assay.

Figure 18 - 21 showed protein expression of four cytokines (IL-2, IL-4, IL-6 and IFNγ) in PBLs. Compared to control samples, both AFB_1 and AFB_2 treatment resulted in an increase in cytokines expression. Higher cytokines expression was determined in cells treated with AFB_1 than AFB_2, in addition, activated lymphocytes appeared to express higher cytokines than B cells.

Figure 22 shows the protein expression changes in HHL-16 cells treated with AFB_1 and AFB_2. The protein expression results are consistent with gene expression results from Figure 12. There is slight increase in STAT3 and phosphorylated-STAT3 (P-STAT3) protein. Dose-dependent increase of protein level were determined in IL-6 and IL-8 after treatment with AFB_1. Cells treated with AFB_2 did not show any significant changes in cytokine expression at the protein level.

For Jurkat T cells, the protein expression of STAT3, P-STAT3 and IL-10 had no significant change during 24 hours treatment for either AFB_1 or AFB_2. Cytokine IL-6 showed a dose-dependent and time-dependent increase in expression, and AFB_2 treatment induced higher protein expression than AFB_1. However, due to the large standard error between experiments, the increases are not statistically significant (Figure 23).

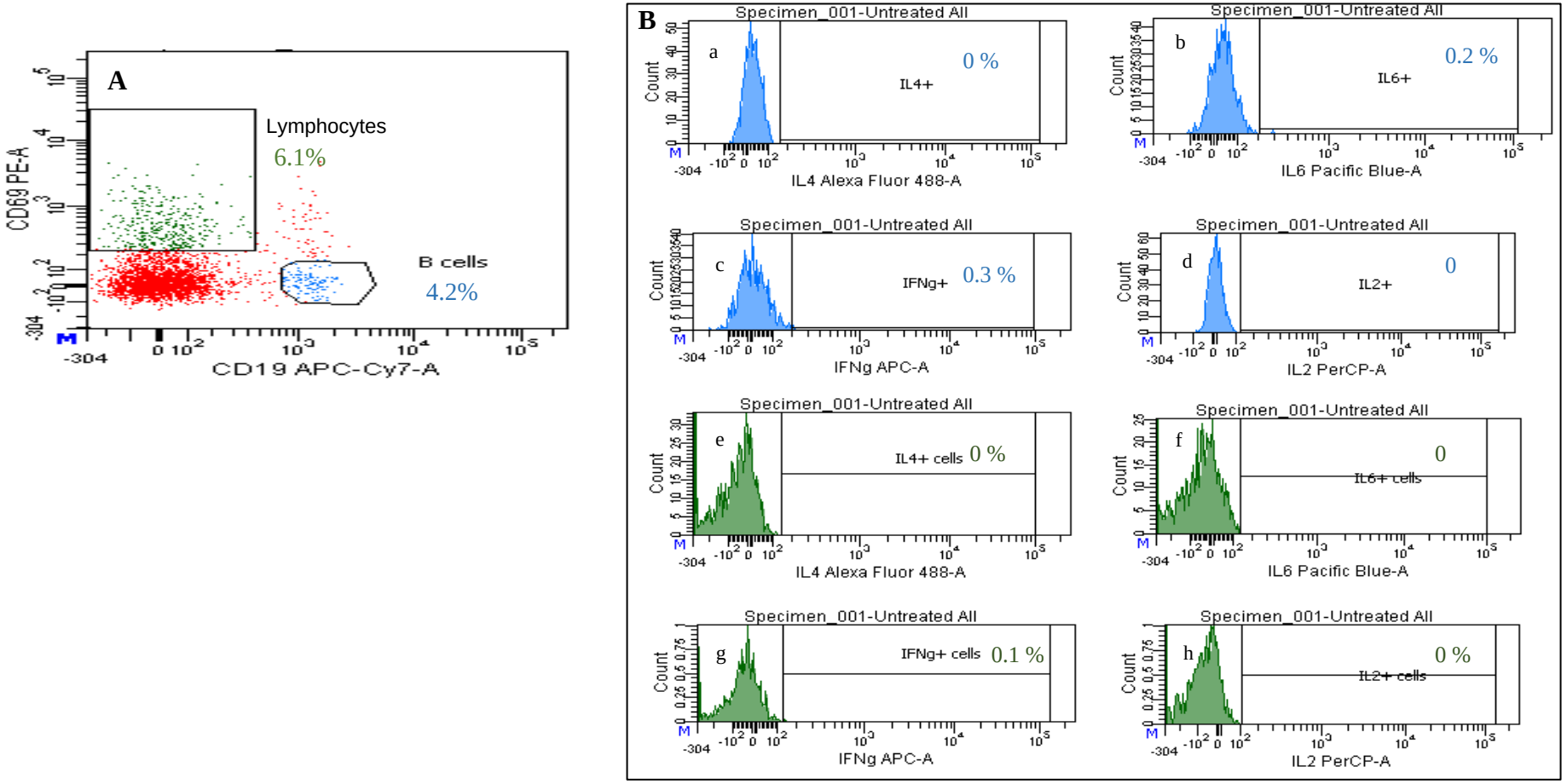


Figure 18 Flow cytometry plots of untreated PBLs.

A) Gating of activated B cells (Blue) and lymphocytes (Green) from untreated PBLs; B) Activated B cells (Blue) and lymphocytes (Green) specific cytokine production from untreated PBLs. B a&e) Interlukin-4; B b&f) Innterlukin-6; B c&g) IFNγ; B d&h) Interlukin-2.

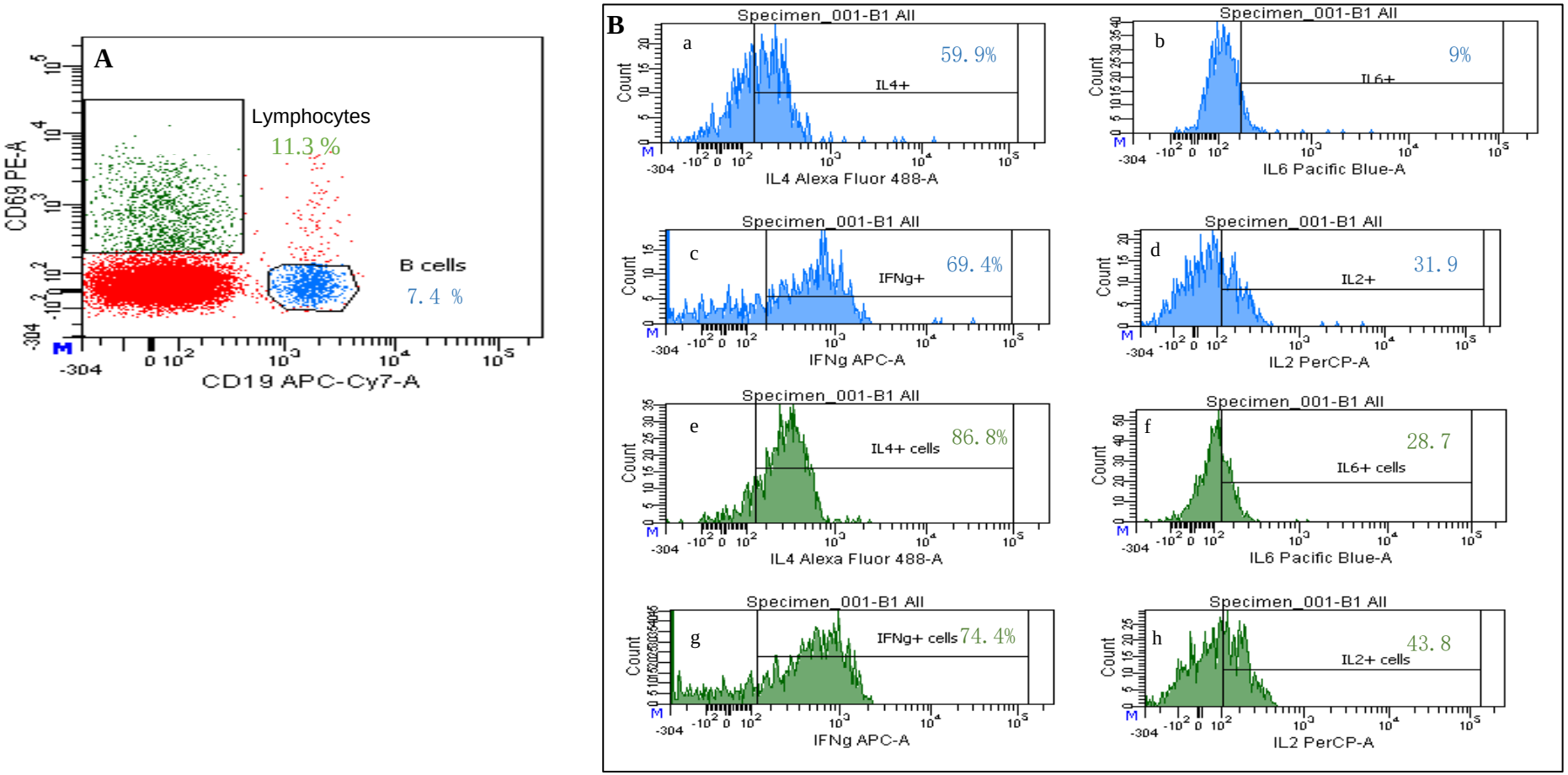


Figure 19 Flow cytometry plots of PBLs treated with 10 µl AFB_1.

A) Gating of activated B cells (Blue) and lymphocytes (Green) from 10 µl AFB_1 treated PBLs; B) Activated B cells (Blue) and lymphocytes (Green) specific cytokine production from 10 µl AFB_1 treated PBLs; B a&e) Interlukin-4; B b&f) Innterlukin-6; B c&g) IFNγ; B d&h) Interlukin-2.

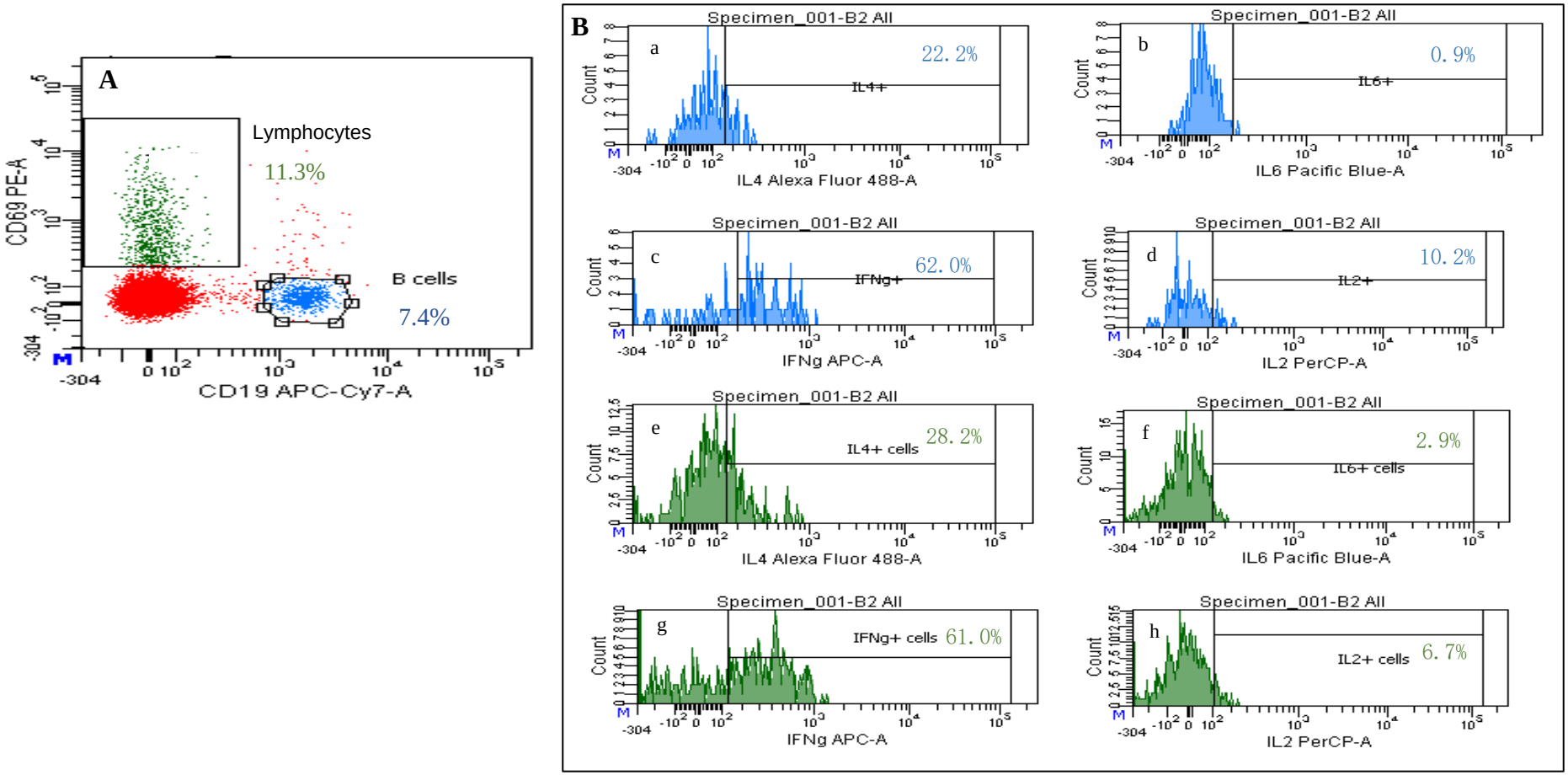


Figure 20 Flow cytometry plots of PBLs treated with 10 µl AFB_2.

A) Gating of activated B cells (Blue) and lymphocytes (Green) from 10 µl AFB_2 treated PBLs; B) Activated B cells (Blue) and lymphocytes (Green) specific cytokine production from 10 µl AFB_2 treated PBLs; B a&e) Interlukin-4; B b&f) Innterlukin-6; B c&g) IFNγ; B d&h) Interlukin-2.

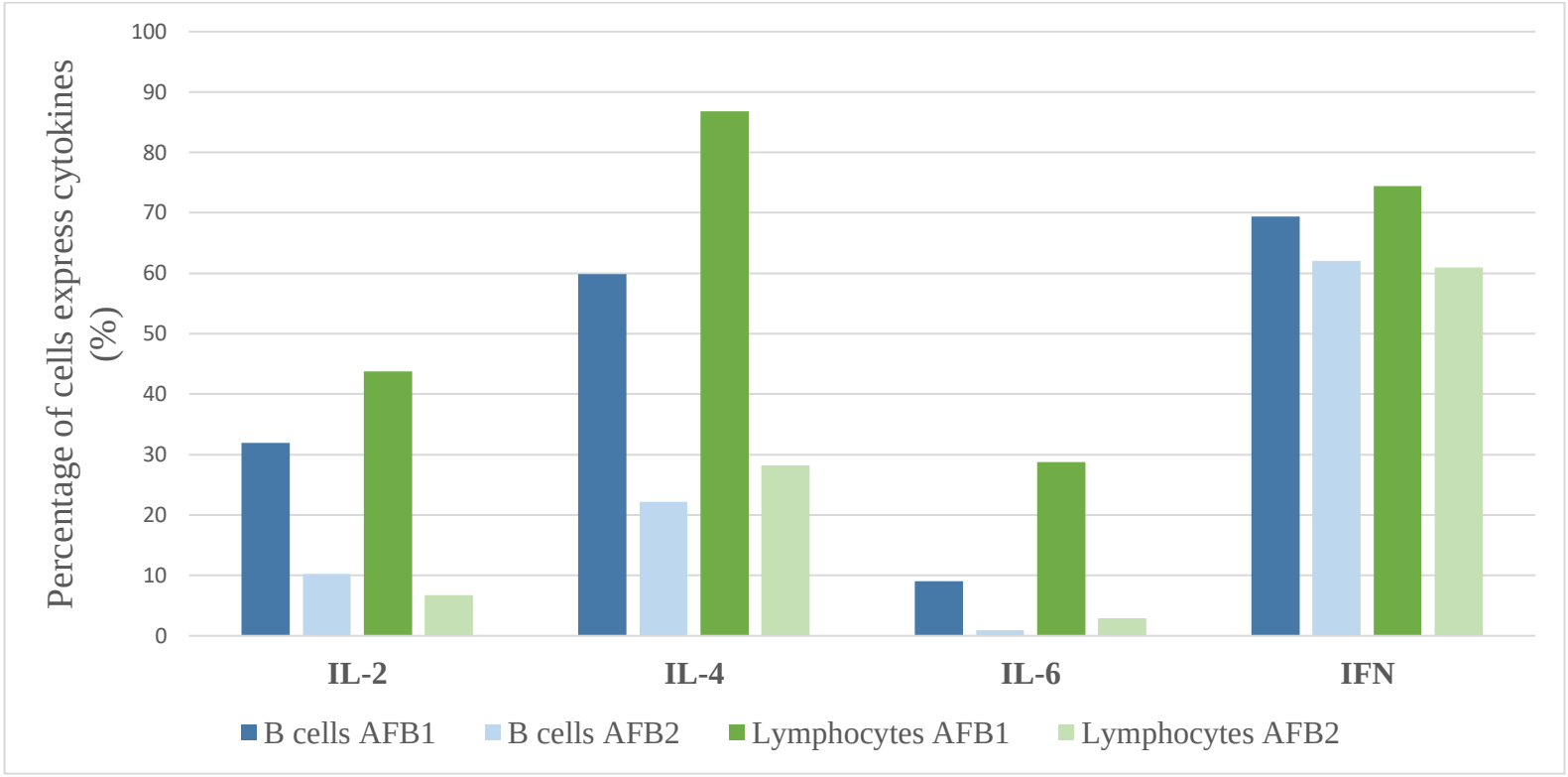


Figure 21 Percentage of B cells and activated lym1phocytes expressing four cytokines after treatment with 10 μg/ml AFB_1 and 10 μg/ml AFB_2 treatment.

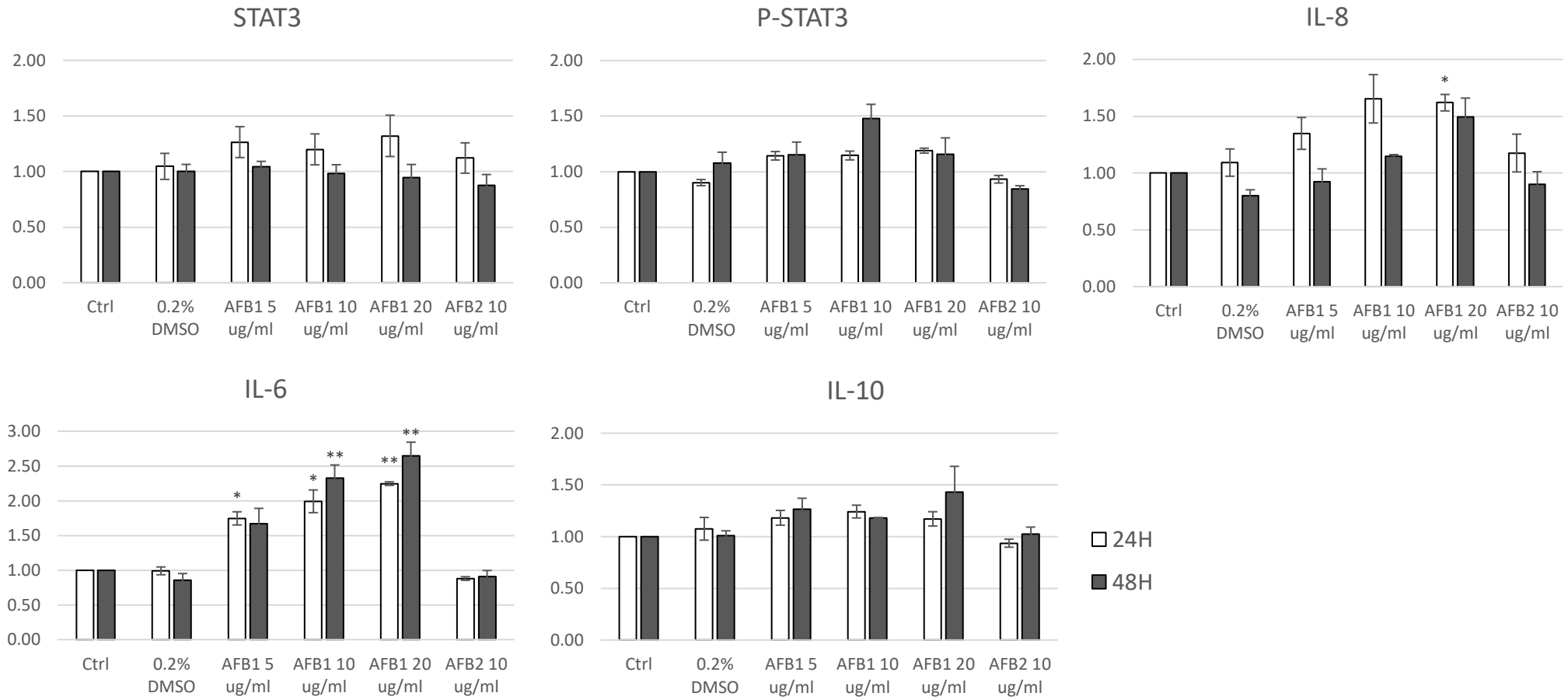


Figure 22 Protein expression fold change in HHL-16 cells with different doses of AFB_1 and AFB_2 treatment

Test substances were assayed in at least three independent experiments. Error bars represent standard error of mean from at least three separate experiments. *signifies $p<0.05$ and ** signifies $p<0.01$ based on student's t-test compared to the control sample.

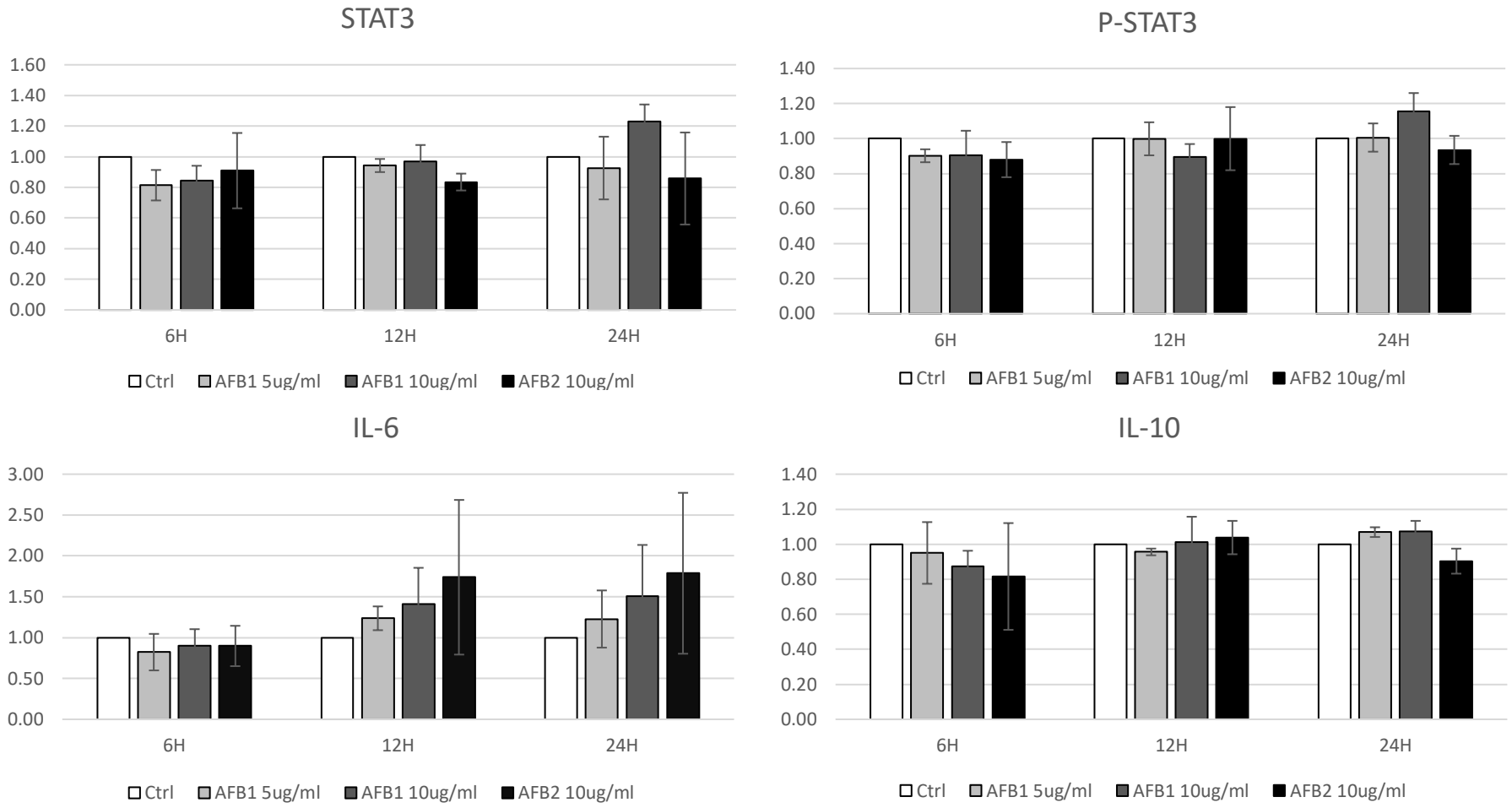


Figure 23 Protein expression fold change in Jurkat T cells with different doses of AFB_1 and AFB_2 treatment

Test substances were assayed in at least three independent experiments. Error bars represent standard error of mean from at least three separate experiments.

5.4 Discussion

As a number of studies in animals and cells *in vitro* have shown that AFB_1 can modulate immune response, we investigated the ability of aflatoxins to alter gene and protein expression of key immune regulatory genes in cultured hepatocytes and lymphocytes. We included AFB_2 as a presumed less toxic aflatoxin, which due to the inability to form epoxide does not react with DNA or proteins to form adducts.

Comparing the cytotoxicity of AFs between HHL-16 and Jurkat T cells, liver cells appeared more sensitive and susceptible to AFs than the T cells. Even the less toxic AFB_2 showed significant reduction of viability in HHL-16 cells in high doses. Jurkat T cells are more tolerant to AFB_2 as even at 50 µg/ml of treatment, they still presented more than 80% viability. The similar weak cytotoxicity of AFB_2 was previously determined in J774A.1 murine macrophages, for which the IC_{30} was higher than 200 µM for AFB_2 treatment compared with 50 µM for AFB_1 (Bianco *et al.*, 2012). Bianco *et al.* identified the synergistic effect of the combination of AFB_1 and AFB_2 with their own metabolite AFM_1 and AFM_2 on nitric oxide (NO) inhibition, which subsequently might reduce cell life span, alter immune response and increase carcinogenesis. An early study compared the cytotoxicity of AFB_1 in primary reticuloendothelial non-parenchymal cells (NPC) and hepatocytes isolated from male rats (Jennings *et al.*, 1994). A dose and time-dependent increase of cytotoxicity was determined in hepatocytes, but not in NPC. In addition, significantly stronger (90-fold higher) macromolecular-binding ability of AFB_1 was determined in hepatocytes versus NPC. Hepatocytes metabolized around 50 - 70% of the treated AFB_1 compared to 28% by NPC, and more AF-DNA adduct was generated by hepatocytes than NPC (Jennings *et al.*, 1994). This study provided a good overview of the cell type-specific metabolism of AFs and suggested the close correlation between cytochrome P450 enzymes and

cytotoxicity of AFB_1. This was also confirmed in another study which examined the cytotoxicity of AFB_1 in normal and cytochrome P450-deficient mutant mouse hepatoma cells (Hepe1). The mutant cells showed significantly higher resistant to AFB_1 than normal Hepe1 cells, therefore they clarified that cytochrome P450 enzymes have a dominant effect on cytotoxicity of AFs (Kärenlampi, 1987).

The response in gene expression changes in the IL-6/STAT3 pathway following aflatoxin treatment was greater in the immune cells compared to the HHL-16 cells, which may reflect the importance of this pathway in controlling immune response. The primary response to aflatoxin is generally held to be in the liver, the site of most metabolism and the target of aflatoxin induced carcinogenesis. It is not surprising therefore that the highest toxicity was seen in the liver cells, where metabolism to the reactive aflatoxin 8,9-epoxide that binds to DNA and proteins would be expected. What was unexpected was that AFB_2 was equally toxic to the HHL-16 cells, even though this compound cannot be metabolised to the epoxide owing to the lack of the carbo-carbon double bond at the 8,9 position of the furan ring. It would be of interest to determine which metabolites were formed when the liver cells were treated with AFB_2, although this was beyond the scope of the current study. Presumably there is a difference in metabolism capability of AFB_2 for the HHL-16 cells and Jurkat T cells, as AFB_2 was not toxic in the latter. AFB_1 was toxic to the Jurkat T cells, suggesting they can also metabolise the aflatoxin to reactive intermediates.

The aim of this study was to investigate whether changes in gene expression seen in some genes in white blood cells of children from Gambia as part of a previous thesis (Castelino, 2013) would be seen in liver cells and lymphocytes exposed *in vitro*. In addition to looking at four genes of interest from that study; IL-6/STAT3 PCR, a

pathway array was also used to examine changes in related genes that may be important in the immune response.

STAT3 plays a crucial role in pro-inflammatory immunity and regulates expression of many cytokines. Continuous high levels of STAT3 have been associated with tumours and tumour promotion (Corvinus *et al.*, 2005). Carey *et al.* (2008) found an elevation of IL-6 in inflammatory bowel disease (IBD) patients, and consistent increase in STAT3 activation in peripheral blood granulocytes. The subsequent influence on the immune system such as control of leukocyte recruitment, alteration of the leukocyte antigen expression and modulation of angiogenesis were also identified in patients associated with the upregulated IL-6/STAT3 (Carey *et al.*, 2008). It has been suggested that the IL-6/STAT3 pathway could be a potential target to reduce mucosal inflammation in disease therapy.

In addition, the adverse effect of AFs on intestinal health has been reported in many *in vitro* and animal studies (Robert *et al.*, 2017; Grenier & Applegate, 2013). Zhang *et al.* (2015) reported an AFB_1 induced DNA damage and reduction in cell viability in human Caco-2 cells. Ledoux *et al.* (1999) and Peng *et al.* (2014) found a decrease in nutrition absorption, less weight and length of the intestine, increased apoptosis in jejunal cells and elevation of Bax and caspase-3 gene expression in broilers fed with AFs contaminated feed. Considering the adverse impact of AFs on the gastrointestinal tract and the stimulation of the IL-6/STAT3 pathway by AFs exposure, we could speculate that AFs exposure might contribute or even aggravate intestinal disease or cancer via activate IL-6/STAT3 pathway.

There are only a few studies that have investigated the effect of mycotoxins on STAT3. Cano *et al.* (2013) reported a significant increase of mRNA expression of T cells regulation pathway (*STAT3*, *IL-6*, *IL-1β* and *IL-17A*) in IPEC-1 (non-transformed

intestinal porcine epithelial cells) after treated with deoxynivalenol (DON). A recent study conducted by Liu *et al.* (2015) also found an upregulation of *p-STAT3*, *NFκB* and cytokines in mice after chronic oral gavage of AFG_1. It is well documented that pattern recognition receptors play an important role in immune response to microbial infection (Suresh and Mosser, 2013). Studies demonstrated the stimulation of toll-like receptors via doses of AFs and C-type lectin receptors due to the exposure of *Aspregillus* species of fungi (Malvandi *et al.*, 2013; Goyal *et al.*, 2018). Our current study is the first one that investigated the effect of both AFB_1 and AFB_2 on IL-6/STAT3 pathway in hepatocyte and lymphocytes. The elevated gene and protein expression in short exposure time in Jurkat T cells suggested that IL-6/STAT3 pathway could be the first immune response that stimulated by AFs exposure.

The expression of other downstream cytokines in both liver and lymphocytes could also interact with each other *in vivo* and maybe activate other part of the immune response. IL-8 is a major chemotactic cytokine which is primarily produced by macrophage and endothelial cells. The increased IL-8 level in HHL-16 cells might induce chemotaxis of lymphocytes. CCL20 (Chemokine (C-C motif) ligand 20) has been found to play an important role in inflammatory response and leukocyte migration. Significant overexpression of CCL20 and its receptor CCR6 were determined in colorectal cancer and it is highly associated with the organ-selective liver metastasis of colorectal cancer (Frick *et al.*, 2016). Here we showed an approximately 3- to 6-fold increase in CCL20 gene expression following AFB_1 treatment, suggesting a new potential target through which aflatoxin could stimulate the immune response.

In PBLs, both *IL-2* and *IL-2 receptor* were increased at mRNA level, and IL-2 at protein level. IL-2 is a vital T lymphocyte growth factor which has been found can

stimulate and amplify the T cells response *in vivo* (Nelson, 2004). IL-4, another mediator that can stimulate the activation of B cells and the differentiation of Th2 cells, was determined to increase more than 200-fold in PBLs with AFB_1 treatment. It is also an activator of STAT6, which is another member of the STAT family and is involved in anti-apoptosis and cell differentiation (Brown & Hural, 1997, 2017; Kis *et al.*, 2011). For those downstream target genes, *BCL2* is well known as a pro-apoptosis agent and *PIM1* plays a crucial role in cell proliferation and survival, and also participates in cytokine regulation (Marie Hardwick & Soane, 2013; Bachmann & Möröy, 2005). According to the gene expression change, not only is the IL-6/STAT3 pathway activated by AFs exposure, but also the NFκB pathway was stimulated at the same time which is another crucial pathway in modulation of immune response and tumour-surveillance. The elevated expression of *TLR4* gene confirmed that the NFκB pathway was also stimulated by AFs in PBLs.

It is surprising that the less toxic AFB_2, which received little attention before, also induced the activation of STAT3 pathway, even though the level of increase for most genes were lower than with AFB_1 treatment. However, the mRNA level of *IL-15* was significantly increased by AFB_2 treatment, but was suppressed after treatment with AFB_1. IL-15 regulates the activation and proliferation of T and NK cells. It is also known as an anti-apoptotic factor that can inhibit the apoptosis induced by BCL2 (Malamut *et al.*, 2010). Further investigation of the effect of AFB_2 on immune function will be needed.

Low expression level of phosphorylated STAT3 protein were determined in both Jurkat T and HHL-16 cells after 24 and/or 48 hours aflatoxin treatment. This could due to the missing of peak timing of STAT3 phosphorylation which was reported within in the first 30 to 120 minutes (Wang *et al.*, 2013). In addition, some recent

studies identified that even unphosphorylated STAT3 (uSTAT3) can work as a transcriptional regulator in cells (Yang *et al.*, 2005, 2007; Yang & Stark, 2008; Timofeeva *et al.*, 2012). Yang *et al.* (2007) found that several target genes such as *IL-6, IL-8, CCL5* and *MET*, etc., can be stimulated by uSTAT3. In addition, the interaction between uSTAT3 and unphosphorylated NFKB (uNFκB) was also determined, in which the complex of uSTAT3 and uNFκB can activate kB-dependent genes (Yang *et al.*, 2007). These findings could well explain the low expression level of phosphorylated STAT3 protein detected in our cell models.

In conclusion, our findings suggested that IL-6/STAT3 pathway can be stimulated by AFs exposure within a short time. The elevated downstream target proteins and cytokines could subsequently activate other immune response genes, such as *NFκB.* In addition, we could also speculate that AFs exposure might interfere the immune response in gastrointestinal disease through modulation of the IL-6/STAT3 pathway. Further investigation will be needed especially in populations who are chronically exposed to AFs.

Chapter 6: Aflatoxicosis outbreak in Tanzania

6.1 Introduction

Tanzania is a major producer of peanuts and maize in the East African area, which produces approximately 2% of world maize (Suleiman & Rosentrater, 2015). As their staple foods, maize and peanuts provide about half of the daily energy intake. The reported daily maize consumption in Tanzania is 128 g/person/day (Ranum *et al.*, 2014). More than half of Tanzanian households rely on subsistence agriculture. Crops cultured such as maize and peanuts are highly susceptible to *Aspergillus* species of fungi infestation and therefore, in a high risk of potential aflatoxins contamination.

A recent study conducted in Tanzania collected 101 maize porridge samples from three villages, from which the range of AFB_1 were found to be between 0.2 and 34.5 µg/kg (Geary *et al.*, 2016). AFB_1 in samples increased after six months storage when compared to the samples collected at initial harvest time. In another study, maize samples from 300 households in different agriculture zones in Tanzania were collected, and levels of eleven mycotoxins were assessed (Kamala *et al.*, 2015). More than half of samples were found to be contaminated with AFB_1, DON and fumonisin B_1 (FB_1), with AFB_1 ranging between 3 and 1081 µg/kg. In addition, 45% of samples were contaminated with more than one mycotoxin.

Populations in Sub-Saharan Africa are at a high risk of aflatoxin exposure as maize and groundnuts constitute the staple food. There have been many aflatoxicosis outbreaks in African countries in the past. The most severe one was in Kenya in 2004, which resulted in more than 125 deaths (Probst *et al.*, 2007). The latest aflatoxicosis outbreak in Tanzania reported 68 cases and 20 deaths (Kamala *et al.*, 2018). Almost all of the reported patients appeared jaundice, with abdominal pain and vomiting, and around half of them also had diarrhoea and ascites. Food samples were collected from the affected areas, from which aflatoxins in case households were found at levels up

to 51,100 µg/kg compared to 285 µg/kg in control families (Kamala *et al.*, 2018). Here, the levels of aflatoxin albumin biomarker in patients and controls were determined. In addition, the liver and kidney functions were also examined to further verify the effects of acute aflatoxin exposure.

6.2 Methods

6.2.1 Study population

An epidemiological survey to investigate the aflatoxicosis outbreak in Tanzania was carried out in July 2016 and reported by Kamala *et al.* (2018). Residents living in Dodoma and Manyara regions were surveyed to record presentation of jaundice, abdominal pain, diarrhoea, vomiting or general body weakness since April of 2016 (Figure 24).

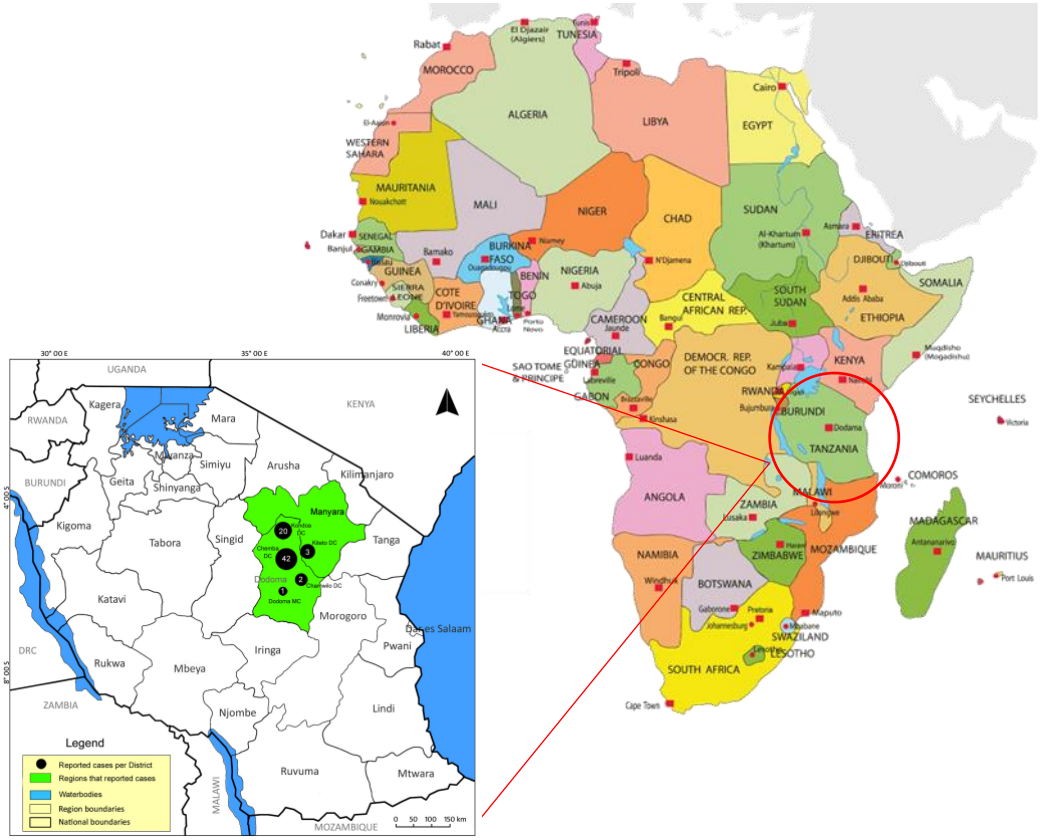


Figure 24 Study location – Dodoma and Manyara regions of Tanzania.

The demographic details and clinical symptoms were recorded in patients. Blood samples of patients and healthy participants from the same districts were collected for aflatoxin exposure and other biochemistry measurements.

6.2.2 Aflatoxin exposure measurement

Aflatoxin-albumin adduct levels were determined using a competitive ELISA method at the University of Leeds. Details of the method showed in Chapter 2, Section 2.2.

6.2.3 Biochemistry analysis

Hepatitis B surface antigen (HBsAg) was measured by Monolisa HBs Ag ULTRA and Confirmatory Assay kit to identify positive HBV subjects. Details of the method showed in Chapter 2, section 2.8.1.

Indicators of liver and kidney functions were measured in the Blood Science Department in Leeds General Infirmary. Parameters including alanine amino-transferase (ALT), aspartate amino –transferase (AST), creatinine, urea, total bilirubin (TB) and directed (conjugated) bilirubin (DB) and total protein were assessed.

6.2.4 Statistical analysis

Statistical analyses were performed using STATA version 15. Aflatoxin-albumin level was log transformed. If the concentration of biochemistry parameters were less than the LOD a value of half of the LOD was used in calculations, i.e. samples have less than 9 U/L ALT recorded as 4.5 U/L for statistical analysis. Student's *t*-test was employed to compare the biochemistry changes between patients and healthy participants. Scatterplot matrix was performed in all parameters to identify the outliers. Simple linear regression was employed to compare the correlation between AF-alb level and biochemistry parameters.

6.2.5 Ethical review

Ethical approval for the current study was obtained from the Muhimbili University of Health and Allied Sciences, Tanzania (2016-07-05/AEC/Vol.XI/67) and the University of Leeds MaPS and Engineering joint Faculty Research Ethics Committee.

6.3 Results

6.3.1 Characteristics of subjects

A total of 47 samples including 24 patients and 23 healthy participants were enrolled in this study. The characteristic data of the 24 patients are shown in Table 17. There is no detailed information of those healthy participants. The age of patients ranged from 2 to 70 years old, with 21% patients less than 5 years old, 29% between 5 to 15 years old and 50% older than 25 years old. All the patients came from two adjacent districts, Chemoba (n=19) and Kondoa (n=5). There were more males (67%) than females (33%) among the patients. All the patients were hospitalised between May and July 2016. More than 80% of them appeared with vomiting, abdominal pain and jaundice during this period. Nine (38%) of them also had diarrhoea and four (17%) showed ascites.

Table 17 Characteristics of patients from the aflatoxicosis in Tanzania

Variables	*n*	*n* (%) / Mean ± SD
Participants	47	
Patients		24 (51%)
Non-patients		23 (49%)
age of patients (ys)	24	23.1 ± 18.8
Children <5 ys	5	3.1 ± 1.2
Student <15 ys	7	9.9 ± 2.4
Subsistence farmer >25 ys	12	39.1 ± 12.7
Sex		
Male		16 (67%)
Female		8 (33%)
District	24	
Chemoba		19 (79%)
Kondoa		5 (21%)
Clinical symptoms	24	
Diarrheal		9 (38%)
Vomiting		21 (88%)
Abdominal pain		20 (83%)
Jaundice		20 (83%)
Ascites		4 (17%)
Fever		0 (0%)

6.3.2 Aflatoxin exposure

The AF-alb level ranged from 10.0 to 7152.1 pg/mg in controls and ranged from 35.9 to 32,791.0 pg/mg in patients (Table 18). Figure 25 shows the distribution of AF-alb levels in participants. Significantly higher levels of AF-alb were determined in patients than in healthy participants with the geometric mean (GM) level of 3402.9 pg/mg and 285.9 pg/mg, respectively.

Table 18 Aflatoxin-albumin level in patients and healthy participants

AF-alb level (pg/mg)	GM (95%CI)	Min	Max
All samples	1012.7 (501.9, 2043.5)	10.0	32791.0
Healthy participants	285.9 (137.1, 1710.3)	10.0	7152.1
Patients	3402.9 (7211.6, 15536.5)	35.9	32791.0

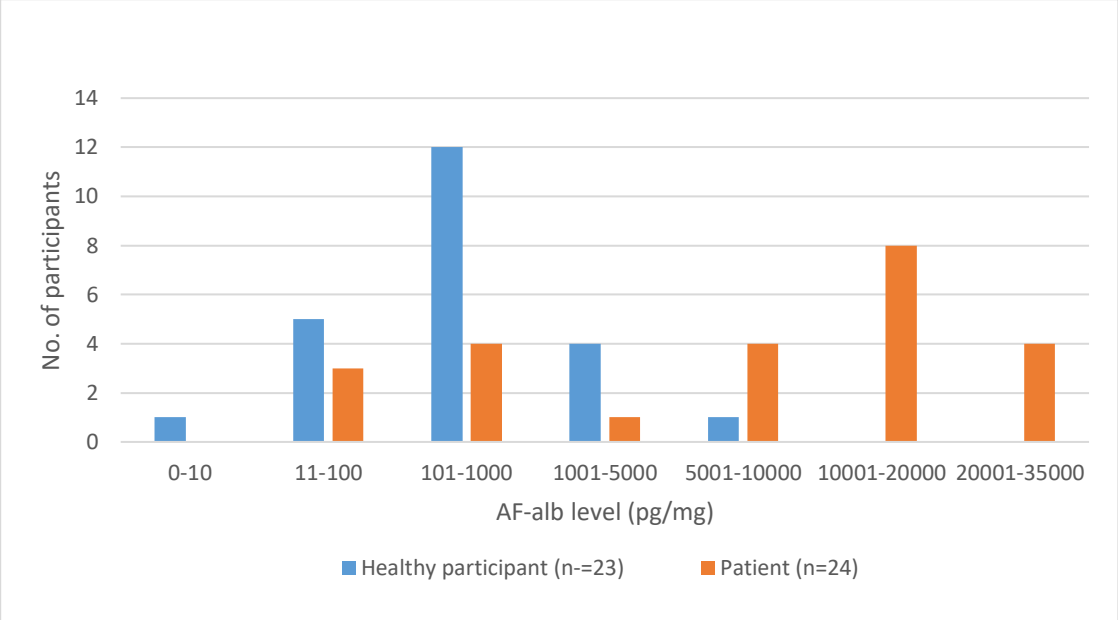


Figure 25 Distribution of aflatoxin-albumin level among patients and healthy participants.

6.3.3 Blood biochemistry indicators

HBsAg of all 47 samples were examined and three of them showed as positive. There was insufficient serum volume available for one of the patient samples to be tested for biochemical endpoints. The reference range of the parameters were shown in appendix 6.1. It is notable that except for two controls with creatinine slightly higher than the reference level (4.7 and 5.2 μmol/L), the rest of all healthy participants had a normal level across all indicators, while the concentration of ALT, AST, TB and DB were significantly elevated in patients ($p<0.0005$; Table 19 & Figure 26). There were no big differences in TP, urea and creatinine between patients and healthy participants.

Table 19 Biochemistry characteristics of patients and healthy participants

Mean ± SD	Healthy participant	No.>LOD (N=23)	Patient	No.>LOD (N=23)	Min of all samples	Max of all samples	*p* value
ALT (U/L)	9	1	46.2 ± 68.2	13	<9	255.5	0.0002
AST (U/L)	21.9 ± 6.6	23	75.4 ± 98.6	23	13.2	440.1	0.0002
Creatinine (µmol/L)	47.1 ± 19.9	23	55 ± 48.5	22	<9	259	0.8112
Total bilirubin (µmol/L)	5.4 ± 2.4	22	49.7 ± 63.2	21	<2	213.9	0.0001
Direct bilirubin (µmol/L)	3.7 ± 1.5	4	32.1 ± 49.4	20	<2	158.6	<0.0001
Total protein (g/L)	69.5 ± 7.4	23	69.8 ± 5.4	23	42.5	81	0.8511
Urea (mmol/L)	3.9 ± 1.0	23	4.1 ± 4.3	23	2	18.7	0.2147

Notes: The limited of detection (LOD) was 9.0 U/L for ALT, 9.0 µmol/L for creatinine, and 2.0 µmol/L for total bilirubin and direct bilirubin. Samples lower than LOD will calculated as half of LOD value for statistical analysis. P value represent effects based on student's *t*-test compared between patients and healthy participants.

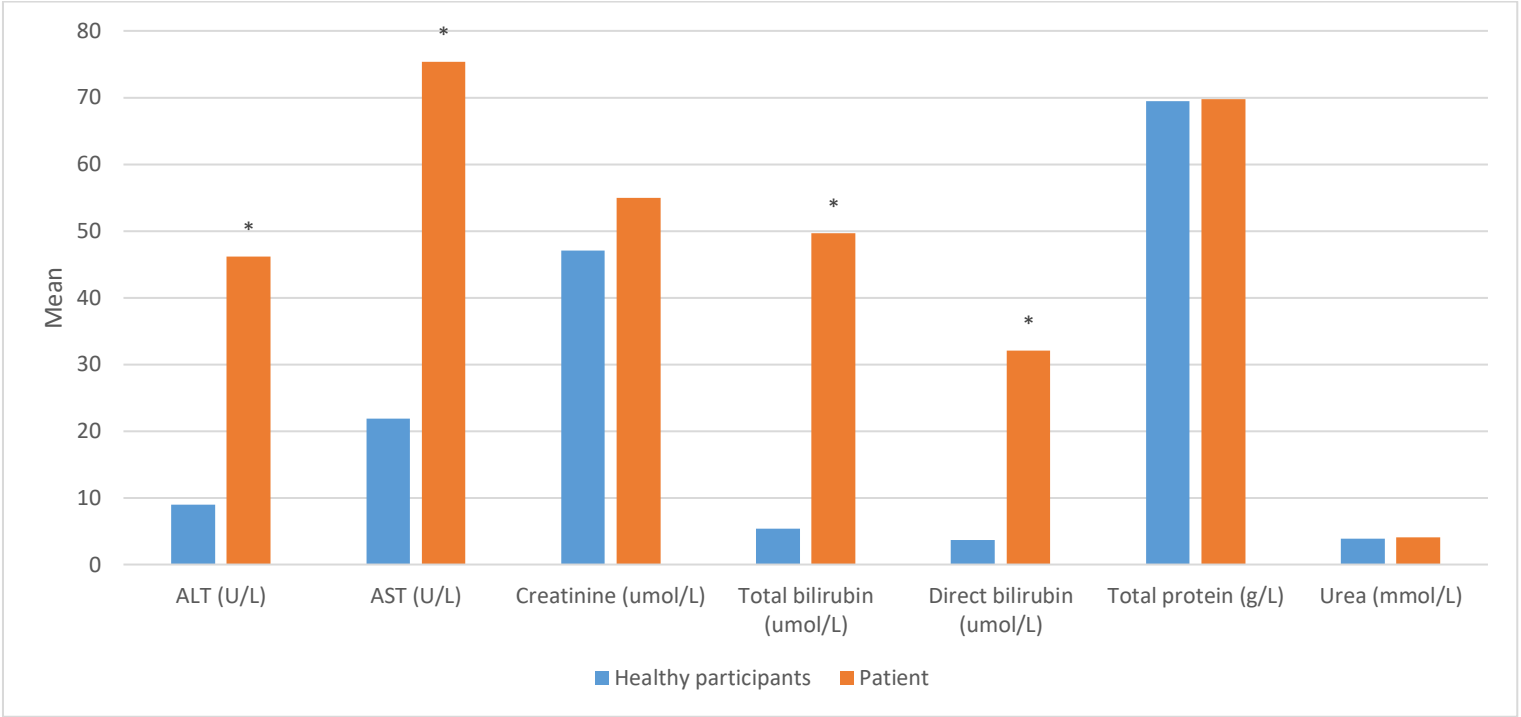


Figure 26 Mean level of biochemistry parameters in patients and healthy participants

* signifies $p \leq 0.0002$ based on student's *t*-test compared between patients and healthy participants.

6.3.4 Relationship between aflatoxin exposure and blood biochemistry indicators

According to the scatterplot matrix (appendix 6.2), sample T12, T22 and T42 were considered as outliers. Those samples have high level in most of all parameters, but low in AF-alb level, which were considered as special cases and excluded from further analysis.

After excluding the three outliers, significant positive correlations were found between AF-alb level and ALT, AST, TB and DB (Table 20 & Figure 27 A-D). Patient's occupation had no effect on either AF-alb level or any of the serum biochemical indicators.

Table 20 Correlation between aflatoxin-albumin level and biochemistry parameters

Biochemistry parameters	Coefficient (*r*)	*p* value
ALT (U/L)	0.376	0.0129
AST (U/L)	0.544	0.0002
Creatinine (μmol/L)	0.075	0.6338
Direct bilirubin (μmol/L)	0.405	0.0071
Total bilirubin (μmol/L)	0.435	0.0036
Total protein (g/L)	-0.033	0.8333
Urea (mmol/L)	-0.060	0.7042

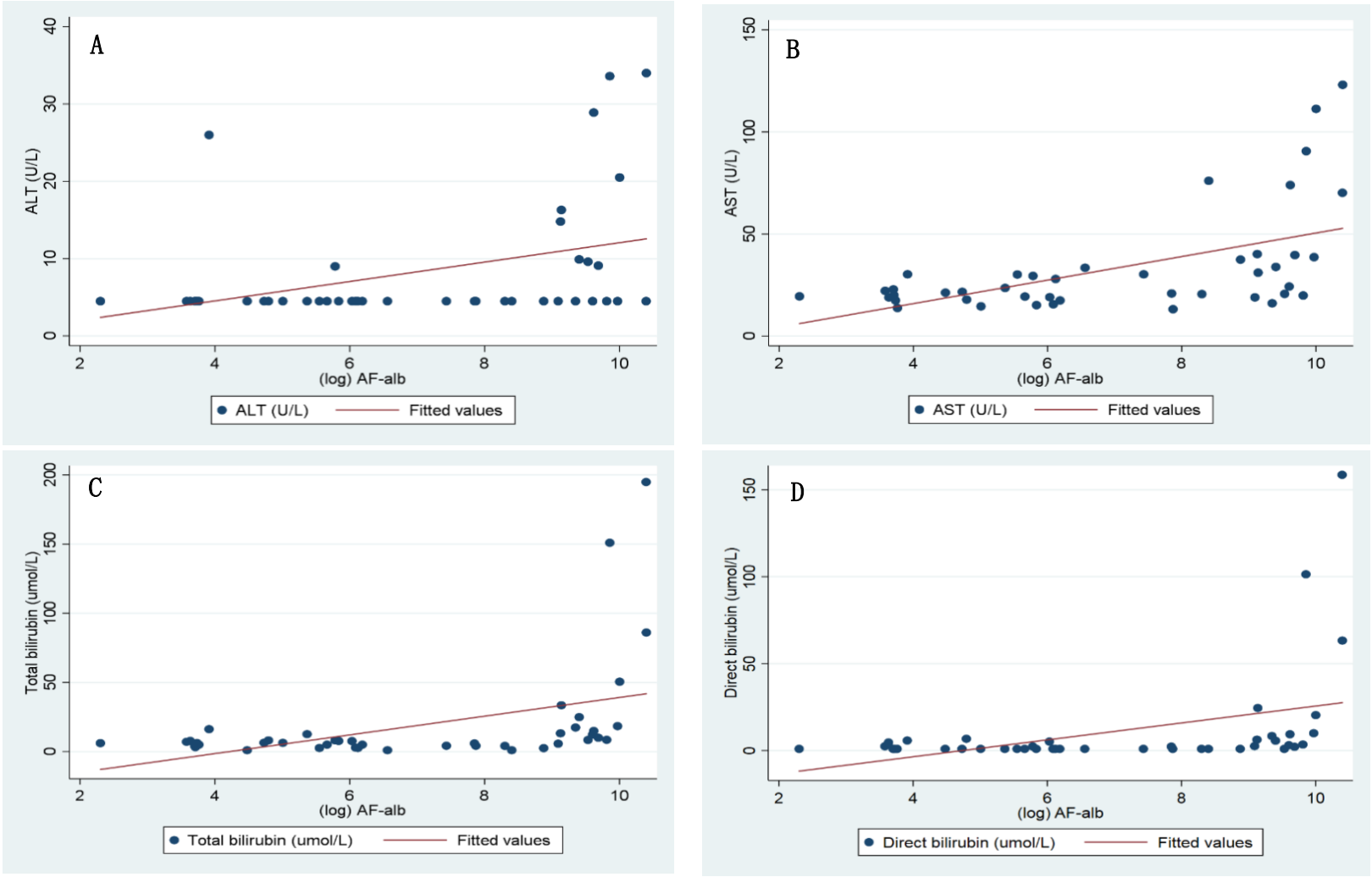


Figure 27 A-D presented the correlation between AF-alb level and biochemistry parameters.

6.4 Discussion

Populations living in rural areas of Africa and South-east Asia are at a great risk of aflatoxin exposure. This study results from the most recent aflatoxicosis outbreak in humans, which was focused on two adjacent districts in Tanzania. Symptoms such as those seen during the outbreak can also be due to other causes, such as infectious disease, so it was important for the authorities to determine that aflatoxicosis was responsible. Food samples from the affected area were collected and examined for AFs. Higher levels of AFs were determined in food samples collected from affected families than non-affected ones supporting the initial hypothesis that aflatoxin was responsible for the acute illness. Our subsequent analysis of serum for AF-alb provided further confirmation. The highest AFs level in foods (51,131 μg/kg) corresponded with the highest AF-alb level 32,791 pg/mg in a patient from that household (Kamala *et al.*, 2018). This reflects a common pattern that eating home-grown contaminated foods such as maize and peanuts is the majority cause of aflatoxicosis due to lack of safety checks. The most severe recorded aflatoxicosis incident in Kenya in 2004 also resulted from the consumption of AFs contaminated maize, following which dramatically high levels of AFs in maize and AF-alb level in affected residents were determined (Azziz-Baumgartner *et al.*, 2005). The GM level of AF-alb level among 29 patients in the 2004 Kenya outbreak was 1200 pg/mg which was lower than the GM level of AF-alb found in our study (3402.9 pg/mg). In the Kenya case, the highest AF-alb level was approximately 19,000 pg/mg which was identified as the highest AFs exposure level in humans measured up to that time. However, in the 2016 Tanzania outbreak, the highest AF-alb level in patients was higher than 35,000 pg/mg which was approximately two fold higher than those determined in Kenya in 2004. In addition, we found that the acute aflatoxicosis cases

tended to happen in family clusters, where they share contaminated foods. The same manifestation was also reported in an earlier outbreak in Malaysia (Munn Sann Lye *et al.*, 1995).

In the current study, more than half of the controls from the same district were determined to have over 100 pg/mg AF-alb and the highest exposure level among controls was up to 7152.1 pg/mg. The similar high AFs exposure in controls were also reported in Kenya outbreak. It is still unclear why residents with high exposure level did not show clinical symptoms, although it may be that this suggests some people are more resistant to the exposure than others. The concentration of AFs exposure required to cause acute poisoning has not been determined in humans. Azziz-Baumgartner *et al.* (2005) concluded that people with over 250 pg/mg AF-alb exposure level were in a high risk group for developing aflatoxicosis.

Among the 47 serum samples we collected from both cases and controls, we examined the HBsAg of all samples, and determined the liver and kidney function biochemical indicators in 46 samples (serum volume insufficient for one case sample). Three samples were found to be HBV positive and all came from the patients group. In the Kenya study, 44% of patients were HBV positive compared to 7% of positive in controls (Azziz-Baumgartner *et al.*, 2005). Therefore, they illustrated that having positive HBV was a risk factor for acute hepatic damage with an odds ratio (OR) of 9.8 (95%CI 1.5 – 63.1). Only three positive subjects were identified in our study which is not enough for statistically significant effects to be observed. HBV positivity has previously been associated with increased AF-alb levels. In two child studies conducted in Gambia AF-alb levels were higher in HBV infected subjects than non-infected ones. Allen *et al.* (1992) reported higher AF-alb level in HBV positive children than controls (mean log 4.41 vs 4.04 pg/mg, $p<0.1$). In addition, Turner *et al.*

(2000) detected the highest GM level of AF-alb in children with acute HBV infection (96.9 pg/mg) followed by chronic carriers (44.9 pg/mg) and lowest in uninfected children (31.6 pg/mg). It is known that there is a synergistic effect of AFs exposure and HBV infection in developing hepatocellular carcinoma (Kew, 2003, 2013). Several cohort studies conducted in China found multiplicative effect in the occurrence of HCC when exposed to both factors comparing to either AFB_1 or HBV positive alone (Qian *et al.*, 1994; Ross *et al.*, 1992; Wang *et al.*, 1996). One of the potential mechanism proposed was that chronic hepatitis induced by HBV infection could modulate or elevate the expression of cytochrome P450, the specific enzyme for AFs metabolism in body, and then, increase the generation of metabolite AFB_1-epoxide which subsequently can react to form aflatoxin albumin or DNA adduct. The mutagenic AFB_1-DNA adduct is involved in the initiation of cancer. This hypothesis might also explain the higher level of AF-alb adduct determined in HBV positive subjects than negative ones. However, in this cohort, very high levels of AF-alb were observed in HBV negative individuals.

This is the first case-control study in an aflatoxicosis outbreak case which explored the correlation of acute AFs exposure in human and liver and kidney function changes. Significant positive correlation between AF-alb level and ALT, AST, TB and DB ($p<0.05$) were found in this study. Liver ALT and AST were used as early indicators of acute hepatic injury and hepatocyte necrosis. In this study, all controls have normal levels of the blood biochemical indicators. Two patients showed dramatically high levels of ALT (111.3 and 255.5 U/L), and approximately 43% of patients had AST over the reference level (40 U/L) and the highest one was up to 440.1 U/L. There are no previous studies examined the liver biochemistry parameters among aflatoxicosis patients. However, acute aflatoxin exposure induced elevation of ALT and AST and

histological changes in liver were illustrated in some animal studies before. A recent rat study mimicked both single acute high doses (50 - 1000 µg AFB_1/kg bw) and repeated chronic low doses (5 - 75 µg AFB_1/kg bw) of AFB_1 treatment (Qian *et al.*, 2013). The liver biochemistry parameters ALT, AST and ALP (alkaline phosphatase) and histological changes in liver and bile were examined (Qian *et al.*, 2013). The highest single dosed rats (1000 µg/kg) dead within 7 days. For the single acute dose group, AST and ALT level elevated immediately at day 1 and reached the highest level at day 3, in which, the highest dosed rats showed more than hundreds fold increase than controls. They also identified that the dramatically elevated enzymes levels were due to the substantial liver necrosis at day 3. Hence, patients in our study with elevated ALT and AST level could also have resulted from the liver necrosis induced by acute AFs exposure. Further histological examination in humans will be needed.

TB and DB are another two indicators that can indirectly reflect the liver function. High levels of bilirubin reveal either damage of red blood cells or the dysfunction of liver that is unable to clear bilirubin from blood. There were 9 and 13 patients with high levels of TB and DB, respectively, which also corresponded with the high ratio of jaundice in patients (79%). This result further predicted the liver injury caused by the acute aflatoxicosis. Two early studies dosed rat with a single high level of AFB_1 (7 mg/kg bw by gastric irritation or 2 mg/kg bw intraperitoneally) also reported significantly elevation in bilirubin level (Clifford and Rees, 1967; Rastogi *et al.*, 2001).

The two kidney function indicators creatinine and urea tended to be normal in both patients and controls. Only one patient showed high level of creatinine (259.0 umol/L) and urea (16 mmol/l) which suggested potential damage in kidney. Several animal studies determined elevation of creatinine, urea, ALT and AST levels in rats after chronic AFB_1 treatment along with histological injury in liver and kidney tissues

(Rotimi *et al.*, 2018; El-Nekeety *et al.*, 2011; Eraslan *et al.*, 2017). A recent *in vivo* and *in vitro* combined study indicated the potential mechanism of AFs induced kidney damage might through stimulate the activation of oxidative stress, upregulate proapoptotic genes and downregulate apoptosis inhibitors, which therefore, resulted in cells apoptosis (Li *et al.*, 2018).

In conclusion, this is the latest aflatoxicosis outbreak reported in Africa. Victims were mainly from two adjacent districts in Tanzania. Even though the scale of the current aflatoxicosis was smaller than the Kenya outbreak in 2004, higher levels of AFB_1 in maize samples and AF-alb level in patients were determined this time. This is the first study investigated the biochemistry changes in patients who suffered with acute aflatoxin poisoning. Significant positive correlation were determined between AF-alb level and the liver function parameters (ALT, AST, TB and DB). The elevation of the biochemistry parameters indicated the lesion of liver caused by acute AFs exposure. Less damage to kidney was found in current study as the kidney function indicators creatinine and urea had no significant change in patients. Early animal studies demonstrated the similar elevation of liver and kidney function indicators along with histological changes such as hepatocyte necrosis and bile duct proliferation induced by high dose of AFB_1 treatment. Further histological examination will be needed in human study.

Chapter 7: Overall discussion and conclusion

This thesis examined the aflatoxin exposure condition by measuring a biomarker in the blood of exposed people in Tanzania, Gambia and Malawi, and associated the exposure condition with acute liver damage, child growth and immune development. Afterwards, the potential mechanism of AF's effect on immune function *in vitro* was explored in different cell models.

The Gambia and Malawi studies suggested the high prevalence of aflatoxin exposure and highlighted that people can expose to aflatoxin from a very early age. The aflatoxin exposure level in infants in Malawi study (GM at 6m 14.7 & at 9m 52.4 pg/mg) were much higher than those in the Gambia study (GM at 6m 3.5 and at 12m 25.4 pg/mg), and also in previous studies conducted in other African countries (Shirima *et al.*, 2013; Watson *et al.*, 2016; Gong *et al.*, 2003). As we all know, consuming contaminated food is a major way that human exposes to aflatoxin. During the first two years of infancy, an upward trend of aflatoxin exposure level was identified in our current and previous studies as weaning foods were introduced to them which are mainly made up of maize and peanuts. The early Tanzania study recruited children aged between 6 and 14 months and followed up for 1 year, reported the increase of AF-alb level with age from 4.7 pg/mg at recruitment and went to 12.9 and 23.5 pg/mg at 6 and 12 months later (Routledge *et al.*, 2014; Shirima *et al.*, 2013). The growth of fungi and AFs production can be influenced by the climate and environment of the region. Both of our Gambia and Malawi studies determined seasonal variation of aflatoxin biomarker level *in vivo*. Higher levels of AF-alb were determined in serum samples collected during the dry season than in the rainy season. A similar trend also reported in previous studies conducted in sub-Saharan Africa. In addition, the geographic variation of aflatoxin exposure was also found in children from Malawi, in which children from village 3 had significantly higher AF-alb levels

than those from village 2 and 1. These three villages are adjacent to each other and have similar climate condition. The difference in aflatoxin exposure level could due to other potential factors such as local sanitary conditions, socio-economic status or food storage and consumption. The global climate change issue could also lead to increased aflatoxin contamination in wider geographical spread in the future.

It has been reported in several previous studies that higher aflatoxin exposure at early life time is associated with child growth impairment. Lower birth weight and growth stunting were found in early studies in which children expose to aflatoxin *in utero* via the trans-placental pathway (Turner *et al.*, 2007; Shuaib *et al.*, 2010). In the Gambia and Malawi study, we also found significant negative correlation between aflatoxin exposure and child growth indicators. Inverse relationships between AF-alb level and WAZ, WLZ and LAZ z-score were determined in children before 18 months of age in Gambia (Watson *et al.*, 2018). Children from Malawi also showed a negative association between AF-alb level and WAZ and mid-upper arm circumference, an indicator that has been used by WHO for categorising malnutrition. The effect of AFs on child growth impairment has been identified in many previous studies including the effect on IGFs expression, or on the DNA methylation level of growth and immune related genes (Hernandez-Vargas *et al.*, 2015; Castelino *et al.*, 2015). Gong *et al.* (2016) emphasized that the first one thousand days from conception to two years old is a crucial period for the growth effect of aflatoxin exposure. Hence, breastfeeding and weaning could directly influence the health outcomes and needs to pay more attention to.

The impact of AFs on immune function has been investigated before, mainly in *in vitro* and animal studies. Both cell-mediated and humoral immune response induced by AFs exposure were all reported, but less consistent in different studies. Our Gambia

study examined the thymus size and antibody response to vaccination as indicators of immune function and also provided a novel perspective on research. Thymus is the primary organ for T lymphocyte development and maturation, which plays a crucial role in adaptive immunity. The significant slower growth and smaller size of thymus in children with high aflatoxin exposure at the first 8 weeks of infancy suggested the potential detrimental effect of aflatoxin on immune development. There is no previous study that investigated the effect of aflatoxin on TI in human, but a dose-dependent manner of aflatoxin associated reduction in thymus size and increase in lymphocyte apoptosis was reported in a chicken study (Peng *et al.*, 2017). A study by Jiang *et al.* (2005) in Ghanaians determined inhibitory effects of AFB_1 on the percentages of activated T and B cells, and suppression on the expression of perforin and granzyme A, protein/enzyme that can lyse infected cells to inhibit pathogen spread, in $CD8^+$ T cells. Taken together these studies are suggestive of an impaired immune function with aflatoxin exposure attributed to retardation of thymus development and lymphocyte activation and proliferation.

On the other hand, aflatoxin exposure could also impact the antibody response to vaccination. B cells as the antibody production lymphocytes also play an important role in the adaptive immune system. The pathway of antibody response to different vaccination was varied and complex. Here we determined a significant association between AF-alb level and anti-diphtheria antibody titre, which predicted that aflatoxin exposure might have a specific effect on particular type of antibody response. An early study by Turner *et al.* (2003) examined the impact of AF-alb level on antibody response to rabies and pneumococcal in more than 400 children aged between 6 and 9 years old. Only a weak association was found between AF-alb and antibody response to pneumococcal serotype 23 ($p<0.05$). The mechanism of antibody response to

different vaccines is various. DTP as protein antigens can induce IgG antibody by B cells with the help of $CD4^+$ T cell (Rothman *et al.*, 1998). The status of the immune system could directly influence the Ab response to vaccination, other factors, such as the infant's health status could also modify the immune response. An early study reported significant lower level of IgG and IgM Ab to tetanus in stunted children than in nonstunted children (Brussow *et al.*, 1995). Higher Ab level to diphtheria were determined in children who received vitamin A supplements than in those received placebo (Rahman *et al.*, 1999). The mechanism of aflatoxin effect on Ab response to different vaccines needs further investigation.

It is known that liver is the primary organ for metabolism and accumulation of AF. While a lot of animals and *in vitro* studies also determined an adverse effect of aflatoxin on gastrointestinal health, such as an increased transfer of bacteria (Galarza-Seeber *et al.*, 2016), reduced weight and length of intestine (Hossein & Gurbuz, 2016), increased lymphocyte and leucocyte infiltration in intestinal mucosa (Akinrinmade *et al.*, 2016), and increased apoptosis in the jejunum (Peng *et al.*, 2014b). In addition, some *in vitro* studies which treated Caco-2 cells, a human colon cell line with AFB_1 also demonstrated the inhibition of cell growth, damage of cell membrane and genetic injury caused by AFB_1 (Zhang *et al.*, 2015). So far there is no human study that investigated the AFs effect on gastrointestinal health. But the clinical symptoms abdominal pain and vomiting in acute aflatoxicosis patients suggested the potential effect of AFs on gastrointestinal tract.

Our Malawi study, in chapter 4, is the first study that associated the aflatoxin exposure condition with environmental enteropathy and the expression of antibacterial response related genes. EE is a type of subclinical disease without obvious symptoms, but happened frequently in children in low-income areas. In the Malawi study, the blood

and stool biomarkers were used to examine the intestinal health which provided a non-invasive sampling method and the available commercial measurement kits also simplify the sample test. The much higher level of stool markers than the western standards suggested a great threaten of EE in infants, and might increase the exposure chance to gram-negative bacteria and subsequently, induce immune response. In addition, the results also showed high aflatoxin exposure associated elevation of several immune-related genes, which are involved in response to bacteria and/or viruses, modulate antibody production by B cells, or lymphocyte activation. These findings suggested several potential pathways that might influenced by aflatoxin on immune system.

To sum up the findings in the Gambian and Malawi study, aflatoxin exposure could impact both cell-mediated and humoral immune response, and also influence gastrointestinal health and modulate the expression of immune response related genes. An early study conducted in Gambian children reported the upregulation of DNA expression in IL-6, STAT3, and NFKB in children with aflatoxin exposure in early infancy (Castelino, 2013). This provided a potential route that could be modulated by aflatoxin and subsequently regulate the immune response. Here we further examined the AFs induced gene and protein expression change in IL-6/STAT3 pathway in three different cell models. The quick and transient increase in gene expression in Jurkat T cells suggested that the IL-6/STAT3 pathway might be the first immune response to AFs exposure. The higher elevation of the target genes in this pathway in PBLs than in Jurkat T and liver cell lines suggested the interaction of different lymphocytes. In addition, the upregulated downstream target genes such as *PIM1*, *BCL2*, and cytokines *IL-4*, *IL-2* could then activate its corresponding immune response.

Unlike the chronic exposure impact, acute and high levels of aflatoxin exposure could result in acute clinical symptoms, severe liver damage or even death. The levels of biochemistry parameters examined in both patients and controls from the Tanzania aflatoxicosis outbreak proved the severe liver and bile injury in patients induced by the dramatically high aflatoxin exposure. The current data along with the recorded symptoms such as jaundice, abdominal pain, vomiting etc. in patients further highlighted the severe liver and bile damage induced by acute exposure. Measures to reduce and control aflatoxin contamination is urgently required in Africa to avoid the serious consequences induced by acute aflatoxicosis.

Limitations and future works

In our Gambia study, the AFs effect on TI was determined significantly during the first 8 weeks of life. Here we only measured the AF-alb level from 24 weeks, which can only use as a predictor of previous exposure condition. For further study, the examination of AF-alb level during early infancy, for example, during pregnancy or after gestation, can provide a more comprehensive view of the early exposure condition. In addition, early studies have been used the amount of T cells and its development as an indicator of thymus health. For further analysis, accounting the number of different lymphocytes and their development and function can give a better understanding of the immune modulation of AFs.

The Malawi study as a pilot study was conducted in a limited number of villages with a small number of participants, which reduces study power and may not be fully representative of the population. As ours was an opportunistic study that made use of samples already collected, some potential confounding factors such as socio-economic status, food storage and consumption, infants feeding stage, house sanitary condition,

morbidity and clinical symptoms etc. were not recorded. For further investigation, more information will be helpful.

The biochemistry analysis of both controls and patients from the Tanzanian aflatoxicosis outbreak provided evidence of the liver, bile and kidney damage after acute exposure. However, there is no histologic examination in patients in the current study. For further research, if conditions permit, histologic examination of the gastrointestinal tract, liver and kidney will provide visible evidence of AFs induced damage in human *in vivo*.

Conclusions

The aflatoxin-albumin data determined in Gambian and Malawian children highlighted that the aflatoxin exposure happens in early life and the exposure level is directly associated with the stage of weaning. Aflatoxin related growth impairment was also found in Malawian infants in which higher AF-alb level was linked to smaller MUAC and lower WAZ. Both the Gambia and Malawi studies explored the aflatoxin impact on immune function, in which the Gambia study is the first one that identified the aflatoxin associated thymus growth retardation in humans. In addition, the aflatoxin related change in antibody response to vaccination and immune response related genes expression found in our studies also provides a novel direction for further exploration. The *in vitro* analysis further confirmed the mechanism involving IL-6/STAT3 pathway plays a crucial role in aflatoxin-related immune function on the molecular level. Furthermore, the dramatically high AF-alb level and liver damage in patients from the acute aflatoxicosis incident in Tanzania (in which there were a number of fatalities) points to the urgent need for controlling and reducing aflatoxin contamination problem in Africa. More investment, educational and agricultural

intervention and policy change could help to improve aflatoxin contamination problem and further reducing cancer burden and improving child health.

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Appendices

Appendix 3.1 Correlation between thymus index and infant's weight analysed by simple linear regression model.

	Birth weight		Weight at the week	
Thymus index (cm^3)	Coef.	*p* value	Coef.	*p* value
Week 1	0.175	<0.001	0.253	<0.001
Week 8	0.168	<0.001	0.179	<0.001
Week 24	0.069	0.088	0.097	<0.001
Week 52	0.068	0.084	0.067	<0.001

Appendix 3.2 Parameters in four different maternal supplements groups.

GM (95%CI) / Mean ± SD	FeFol	MMN	PE+FeFol	PE+MMN	*p* value
n	95	96	89	93	
AF-alb level (pg/mg)					
week 24	4.0 (3.0, 5.3)	3.0 (2.5, 3.6)	3.6 (2.9, 4.6)	3.6 (2.9, 4.3)	0.3489
Week 52	27.0 (21.7, 33.8)	24.7 (19.5, 31.2)	22.4 (17.5, 28.6)	27.8 (20.1, 38.5)	0.639
Thymus index (cm3)					
Week 1	9.4 ± 3.3	9.5 ± 3.0	9.3 ± 3.4	8.6 ± 2.8	0.1415
Week 8	13.7 ± 3.8	14.4 ± 4.4	14.1 ± 4.3	13.7 ± 3.6	0.7407
Week 24	14.9 ± 4.0	14.6 ± 4.1	15.0 ± 4.4	14.7 ± 4.6	0.9192
Week 52	13.9 ± 3.2	13.6 ± 3.6	13.3 ± 3.7	13.5 ± 3.1	0.6567
Pertussis (EU/ml)					
Week 12	7.7 (6.0, 10.0)	7.8 (6.2, 10.0)	6.6 (5.3, 8.3)	8.3 (6.5, 10.5)	0.6215
Week 24	159.2 (116.4, 217.8)	99.8 (68.2, 146.1)	154.6 (109.0, 219.1)	124.0 (87.8, 175.3)	0.2059
Diphtheria (IU/ml)					
Week 12	0.21 (0.14, 0.31)	0.29 (0.20, 0.42)	0.28 (0.19, 0.40)	0.29 (0.19, 0.45)	0.5841
Week 24	1.20 (0.95, 1.52)	1.08 (0.82, 1.43)	1.33 (1.02, 1.71)	1.13 (0.89, 1.44)	0.7001
Tetanus (IU/ml)					
Week 12	0.77 (0.64, 0.93)	0.91 (0.75, 1.10)	0.84 (0.69, 1.02)	0.80 (0.65, 0.98)	0.6621
Week 24	4.8 (3.8, 6.1)	3.7 (2.7, 5.0)	4.6 (3.6, 5.9)	4.3 (3.3, 5.6)	0.5291

Notes: p value was calculated by *ANOVA*. FeFol: Iron-folate, MMN: multiple micronutrients, PE: protein-energy.

Appendix 4.1 Correlation between gene expression fold change and aflatoxin-albumin level analysed with Spearman's correlation with or without adjusted by Bonferroni correction.

No	RefSeq ID	Gene	Name	Coefficient	Spearman's correlation *p*	Adjusted by Bonferroni correction *p*
1	NM_001098526	AMICA1	Adhesion molecule, interacts with CXADR antigen 1	0.2264	0.0477	1
2	NM_020980	AQP9	Aquaporin-9	0.3806	0.0006	0.6607
3	NM_001190266	ATG16L1	Autophagy related 16 like 1	0.0761	0.5109	1
4	NM_001114735	BCL2A1	Bcl-2-related protein A1 (B-cell lymphoma 2)	0.1249	0.2791	1
5	NM_016293	BIN2	Bridging Integrator 2	0.2836	0.0124	1
6	NM_001725	BPI	Bactericidal Permeability Increasing Protein	0.0045	0.9689	1
7	NM_000734	CD247	T-cell surface glycoprotein CD3 zeta chain	0.126	0.2747	1
8	NM_000560	CD53	Leukocyte surface antigen CD53	0.3139	0.0054	1
9	NM_000573	CR1	Complement component (3b/4b) receptor 1 (Knops blood group)	0.3195	0.0046	1
10	NM_000567	CRP	C-reactive protein	(undetectable)		
11	NM_000395	CSF2RB	Colony-stimulating factor 2 receptor, 1 (Knops blood g	0.3195	0.0046	1
12	NM_000760	CSF3R	Colony-stimulating factor 3 Receptor (granulocyte)	0.4167	0.0002	0.1693
13	NM_003650	CST7	Cystatin-F (leukocystatin)	0.4167	0.0002	0.1693
14	NM_001168298	CXCR2	Chemokine (C-X-C motif) Receptor 2	0.3732	0.0008	0.8577
15	NM_001136219	FCGR2A	Fc fragment of IgG, low-affinity IIa, receptor (CD32)	0.3494	0.0018	1
16	NM_000570	FCGR3B	Fc fragment of IgG, low-affinity IIIb, receptor (CD16b)	0.3116	0.0058	1
17	NM_005306	FFAR2	Free fatty acid receptor 2	0.3072	0.0066	1
18	NM_001193306	FPR1	Formyl peptide receptor 1	0.1565	0.1742	1
19	NM_002033	FUT4	Fucosyltransferase 4	0.214	0.0616	1
20	NM_021175	HAMP	Hepcidin antimicrobial peptide	0.2291	0.0512	1

21	NM_000619	IFNG	Interferon gamma	0.0379	0.7433	1
22	NM_002187	IL12B	Subunit beta of interleukin 12	0.0192	0.8894	1
23	NM_002190	IL17A	interleukin-17A	0.0059	0.9626	1
24	NM_173843	IL1RN	interleukin-1 receptor antagonist	0.2323	0.042	1
25	NM_144701	IL23R	Interleukin-23 receptor	0.0186	0.8724	1
26	NM_000600	IL6	Interleukin 6	0.0118	0.9188	1
27	NM_006762	LAPTM5	Lysosomal protein transmembrane 5	0.0534	0.6445	1
28	NM_002298	LCP1	Lymphocyte cytosolic protein 1 (L-plastin)	0.1544	0.1801	1
29	NM_001111097	LYN	V-yes-1 Yamaguchi sarcoma viral-related oncogene homolog	0.1619	0.1594	1
30	NM_000239	LYZ	Lysozyme	0.0364	0.7535	1
31	NM_000250	MPO	Myeloperoxidase	0.0986	0.3937	1
32	NM_021950	MS4A1	Membrane spanning 4-domains A1	0.2054	0.0731	1
33	NM_001018016	MUC1	Mucin 1	0.1976	0.085	1
34	NM_000433	NCF2	Neutrophil cytosol factor 2	0.1472	0.2014	1
35	NM_144687	NLRP12	NLR Family Pyrin Domain Containing 12	0.2093	0.0678	1
36	NM_001276700	NLRP6	NLR Family Pyrin Domain Containing 6	0.0443	0.702	1
37	NM_022162	NOD2	Nucleotide-binding oligomerization domain-containing 2	0.2025	0.0773	1
38	NM_000607	ORM1	Orosomucoid 1	0.0326	0.7781	1
39	NM_152309	PIK3AP1	Phosphoinositide-3-kinase adaptor protein 1	0.2181	0.0567	1
40	NM_005621	S100A12	S100 calcium-binding protein A12	0.111	0.3367	1
41	NM_002964	S100A8	S100 calcium-binding protein A8	0.052	0.6532	1
42	NM_022136	SAMSN1	SAM domain, SH3 domain and nuclear localization signals 1	0.0104	0.9292	1
43	NM_003955	SOCS3	Suppressor of cytokine signalling 3	0.1971	0.0857	1
44	NM_213662	STAT3	signal transducer and activator of transcription 3	0.1725	0.1336	1

45	NM_138554	TLR4	Toll-like receptor 4	0.1547	0.179	1
46	NM_006068	TLR6	Toll-like receptor 6	0.1098	0.3416	1
47	NM_002046	GAPDH	Glyceraldehyde 3-phosphate dehydrogenase			
48	NM_000194	HPRT1	Hypoxanthine Phosphoribosyltransferase 1			

Appendix 5.1 Primers of RT² Profiler™ PCR Array Human IL6/STAT3 Signalling Pathway

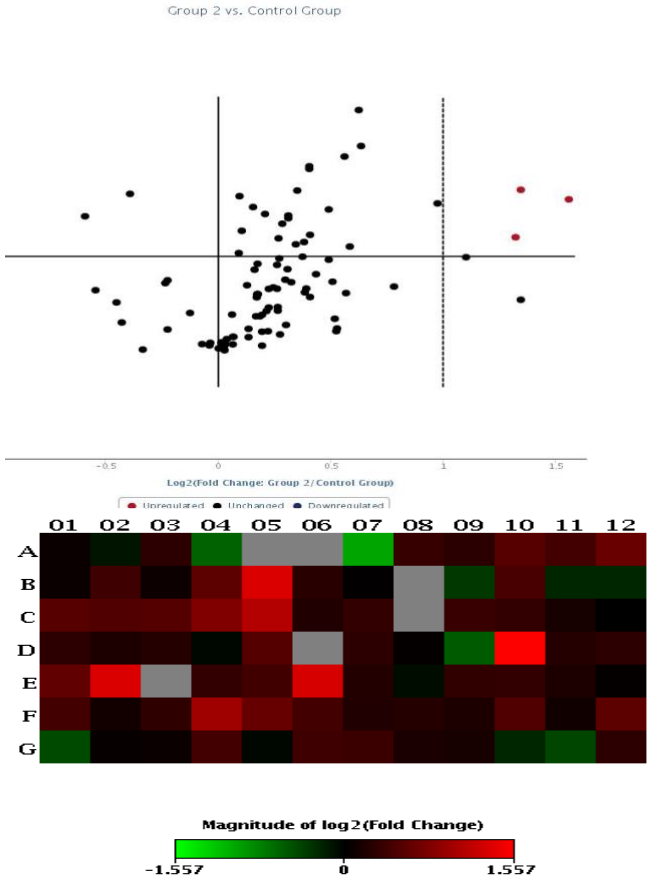
Position	*Refseq*	*Symbol*	*Description*
A01	NM_005163	AKT1	V-akt murine thymoma viral oncogene homolog 1
A02	NM_004324	BAX	BCL2-associated X protein
A03	NM_000633	BCL2	B-cell CLL/lymphoma 2
A04	NM_002982	CCL2	Chemokine (C-C motif) ligand 2
A05	NM_002983	CCL3	Chemokine (C-C motif) ligand 3
A06	NM_002984	CCL4	Chemokine (C-C motif) ligand 4
A07	NM_002985	CCL5	Chemokine (C-C motif) ligand 5
A08	NM_000616	CD4	CD4 molecule
A09	NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5
A10	NM_000074	CD40LG	CD40 ligand
A11	NM_005191	CD80	CD80 molecule
A12	NM_001789	CDC25A	Cell division cycle 25 homolog A (S. pombe)
B01	NM_000389	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
B02	NM_005195	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta
B03	NM_000757	CSF1	Colony stimulating factor 1 (macrophage)
B04	NM_000758	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)
B05	NM_000759	CSF3	Colony stimulating factor 3 (granulocyte)
B06	NM_000760	CSF3R	Colony stimulating factor 3 receptor (granulocyte)
B07	NM_001565	CXCL10	Chemokine (C-X-C motif) ligand 10
B08	NM_000609	CXCL12	Chemokine (C-X-C motif) ligand 12
B09	NM_003467	CXCR4	Chemokine (C-X-C motif) receptor 4
B10	NM_005228	EGFR	Epidermal growth factor receptor
B11	NM_000043	FAS	Fas (TNF receptor superfamily, member 6)
B12	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)
C01	NM_000601	HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)
C02	NM_001556	IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta

C03	NM_000572	IL10	Interleukin 10
C04	NM_000641	IL11	Interleukin 11
C05	NM_000882	IL12A	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)
C06	NM_002188	IL13	Interleukin 13
C07	NM_000585	IL15	Interleukin 15
C08	NM_002190	IL17A	Interleukin 17A
C09	NM_001562	IL18	Interleukin 18 (interferon-gamma-inducing factor)
C10	NM_003855	IL18R1	Interleukin 18 receptor 1
C11	NM_000575	IL1A	Interleukin 1, alpha
C12	NM_000576	IL1B	Interleukin 1, beta
D01	NM_000877	IL1R1	Interleukin 1 receptor, type I
D02	NM_000586	IL2	Interleukin 2
D03	NM_021803	IL21	Interleukin 21
D04	NM_020525	IL22	Interleukin 22
D05	NM_016584	IL23A	Interleukin 23, alpha subunit p19
D06	NM_000417	IL2RA	Interleukin 2 receptor, alpha
D07	NM_000588	IL3	Interleukin 3 (colony-stimulating factor, multiple)
D08	NM_000589	IL4	Interleukin 4
D09	NM_000879	IL5	Interleukin 5 (colony-stimulating factor, eosinophil)
D10	NM_000600	IL6	Interleukin 6 (interferon, beta 2)
D11	NM_000565	IL6R	Interleukin 6 receptor
D12	NM_002184	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)
E01	NM_000880	IL7	Interleukin 7
E02	NM_000584	CXCL8	Interleukin 8
E03	NM_000590	IL9	Interleukin 9
E04	NM_004972	JAK2	Janus kinase 2
E05	NM_000215	JAK3	Janus kinase 3

E06	NM_002309	LIF	Leukemia inhibitory factor (cholinergic differentiation factor)
E07	NM_002310	LIFR	Leukemia inhibitory factor receptor alpha
E08	NM_000595	LTA	Lymphotoxin alpha (TNF superfamily, member 1)
E09	NM_002755	MAP2K1	Mitogen-activated protein kinase kinase 1
E10	NM_002745	MAPK1	Mitogen-activated protein kinase 1
E11	NM_001315	MAPK14	Mitogen-activated protein kinase 14
E12	NM_002746	MAPK3	Mitogen-activated protein kinase 3
F01	NM_002750	MAPK8	Mitogen-activated protein kinase 8
F02	NM_000245	MET	Met proto-oncogene (hepatocyte growth factor receptor)
F03	NM_004958	MTOR	Mechanistic target of rapamycin (serine/threonine kinase)
F04	NM_002467	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
F05	NM_003998	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
F06	NM_020529	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
F07	NM_020530	OSM	Oncostatin M
F08	NM_003999	OSMR	Oncostatin M receptor
F09	NM_006099	PIAS3	Protein inhibitor of activated STAT, 3
F10	NM_002648	PIM1	Pim-1 oncogene
F11	NM_006908	RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
F12	NM_021975	RELA	V-rel reticuloendotheliosis viral oncogene homolog A (avian)
G01	NM_003745	SOCS1	Suppressor of cytokine signaling 1
G02	NM_003955	SOCS3	Suppressor of cytokine signaling 3
G03	NM_005417	SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
G04	NM_003150	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)
G05	NM_138554	TLR4	Toll-like receptor 4
G06	NM_000594	TNF	Tumor necrosis factor
G07	NM_003842	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b
G08	NM_001065	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
G09	NM_001066	TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B

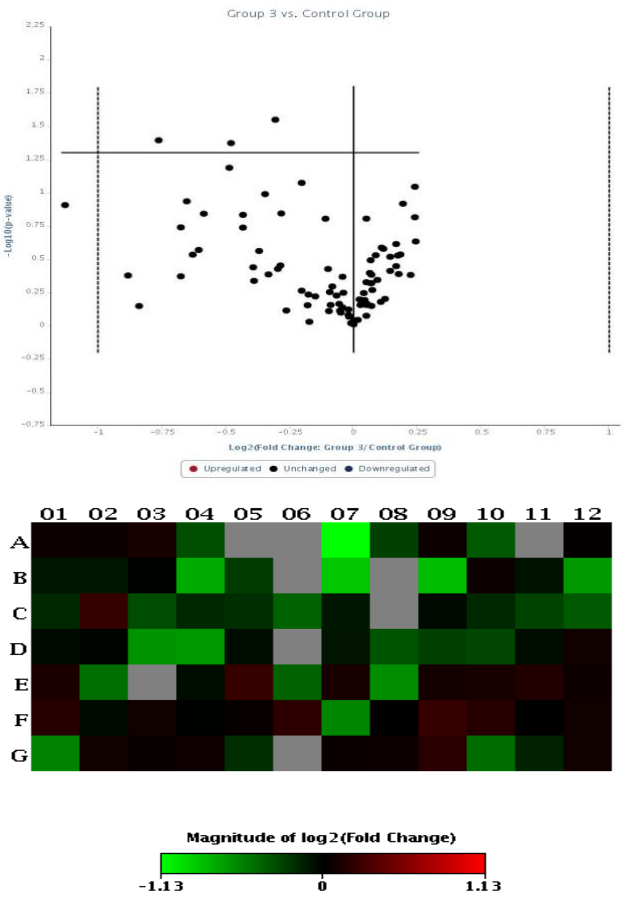
G10	NM_003810	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
G11	NM_003701	TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11
G12	NM_003331	TYK2	Tyrosine kinase 2
H01	NM_001101	ACTB	Actin, beta
H02	NM_004048	B2M	Beta-2-microglobulin
H03	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H04	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1
H05	NM_001002	RPLP0	Ribosomal protein, large, P0
H06	SA_00105	HGDC	Human Genomic DNA Contamination
H07	SA_00104	RTC	Reverse Transcription Control
H08	SA_00104	RTC	Reverse Transcription Control
H09	SA_00104	RTC	Reverse Transcription Control
H10	SA_00103	PPC	Positive PCR Control
H11	SA_00103	PPC	Positive PCR Control
H12	SA_00103	PPC	Positive PCR Control

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AKT1 1.04	BAX -1.09	BCL2 1.20 B	CCL2 -1.51	CCL3 1.13 C	CCL4 1.13 C	CCL5 -2.01 A	CD4 1.25 B	CD40 1.20	CD40LG 1.44 B	CD80 1.33 B	CDC25A 1.55
B	CDKN1A 1.05	CEBPD 1.30	CSF1 1.05	CSF2 1.48	CSF3 2.54	CSF3R 1.18 B	CXCL10 1.01 B	CXCL12 1.13 C	CXCR4 -1.26 B	EGFR 1.35	FAS -1.17	FASLG -1.17 B
C	HGF 1.44	IKBKB 1.41	IL10 1.43 B	IL11 1.72	IL12A 2.14	IL13 1.14	IL15 1.24	IL17A 1.13 C	IL18 1.27	IL18R1 1.23	IL1A 1.10	IL1B 1.00
D	IL1R1 1.20	IL2 1.13 B	IL21 1.17 B	IL22 -1.03 B	IL23A 1.42	IL2RA 1.13 C	IL3 1.21 B	IL4 1.02 B	IL5 -1.46 B	IL6 2.94	IL6R 1.16	IL6ST 1.21
E	IL7 1.50	CXCL8 2.54	IL9 1.13 C	JAK2 1.24	JAK3 1.31	LIF 2.50	LIFR 1.15	LTA -1.05 B	MAP2K1 1.23	MAPK1 1.24	MAPK14 1.12	MAPK3 1.02
F	MAPK8 1.32	MET 1.07	MTOR 1.22	MYC 1.96	NFKB1 1.54	NFKBIA 1.33	OSM 1.14 B	OSMR 1.17	PIAS3 1.13	PIM1 1.41	RAC1 1.07	RELA 1.47
G	SOCS1 -1.37	SOCS3 1.02	SRC 1.05	STAT3 1.32	TLR4 -1.03	TNF 1.30 B	TNFRSF10 B 1.28	TNFRSF1A 1.11	TNFRSF1B 1.10	TNFSF10 -1.18	TNFSF11 -1.35 B	TYK2 1.20



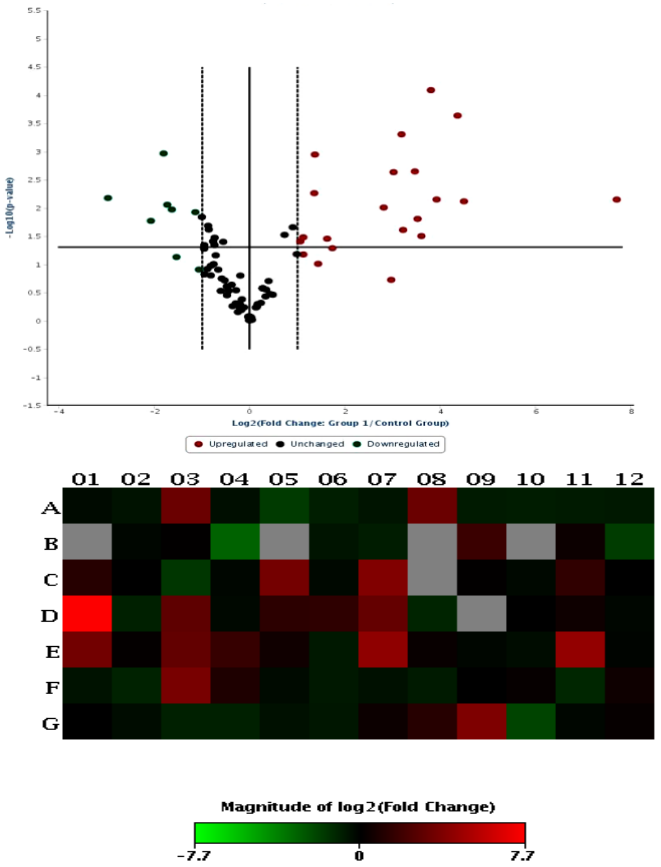
Appendix 5.2a Gene expression fold change in IL-6/STAT3 pathway in HHL-16 at 10 µg/ml AFB_1 treatment at 24 hours.

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AKT1 1.04	BAX 1.03	BCL2 1.08 B	CCL2 -1.27	CCL3 -1.01 C	CCL4 -1.01 C	CCL5 -2.19 A	CD4 -1.22 B	CD40 1.04	CD40LG -1.31 B	CD80 -1.01 C	CDC25A 1.01
B	CDKN1A -1.07	CEBPD -1.07	CSF1 -1.01	CSF2 -1.70	CSF3 -1.20	CSF3R -1.01 C	CXCL10 -1.84 B	CXCL12 -1.01 C	CXCR4 -1.79 B	EGFR 1.04	FAS -1.06	FASLG -1.60 B
C	HGF -1.13	IKBKB 1.18	IL10 -1.26 B	IL11 -1.13	IL12A -1.15	IL13 -1.35 A	IL15 -1.07	IL17A -1.01 C	IL18 -1.03	IL18R1 -1.13	IL1A -1.23	IL1B -1.31
D	IL1R1 -1.03	IL2 -1.01 B	IL21 -1.57 B	IL22 -1.60 B	IL23A -1.04	IL2RA -1.01 C	IL3 -1.06 B	IL4 -1.29 B	IL5 -1.22 B	IL6 -1.24	IL6R -1.04	IL6ST 1.05
E	IL7 1.09	CXCL8 -1.40	IL9 -1.01 C	JAK2 -1.04	JAK3 1.18	LIF -1.35	LIFR 1.08	LTA -1.55 B	MAP2K1 1.06	MAPK1 1.07	MAPK14 1.10	MAPK3 1.04
F	MAPK8 1.12	MET -1.03	MTOR 1.05	MYC -1.00	NFKB1 1.02	NFKBIA 1.14	OSM -1.52 B	OSMR 1.00	PIAS3 1.18	PIM1 1.12	RAC1 1.00	RELA 1.05
G	SOCS1 -1.50	SOCS3 1.05	SRC 1.02	STAT3 1.04	TLR4 -1.15	TNF -1.01 C	TNFRSF10 B 1.03	TNFRSF1A 1.04	TNFRSF1B 1.13	TNFSF10 -1.39	TNFSF11 -1.11 B	TYK2 1.05



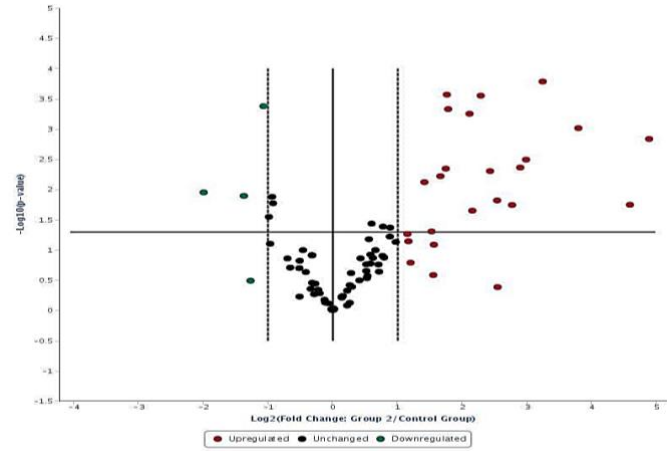
Appendix 5.2b Gene expression fold change in IL-6/STAT3 pathway in HHL-16 at 10 µg/ml AFB_2 treatment at 24 hours.

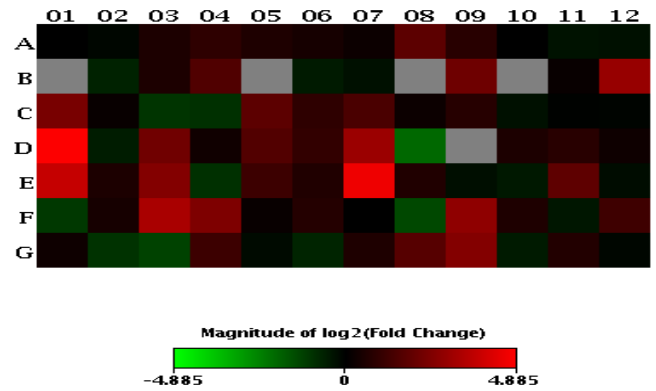
Layout	1	2	3	4	5	6	7	8	9	10	11	12
A	AKT1 -1.22	BAX -1.44	BCL2 9.11	CCL2 -1.35	CCL3 -3.31 A	CCL4 -1.85	CCL5 -1.52	CD4 9.32	CD40 -1.70	CD40LG -1.83	CDKN1A -1.76	CSF1 -1.64
B	CSF2 1.21	CSF3 -1.15	CSF3R 1.04	CXCL10 -7.84	EGFR 1.21 C	FAS -1.50	FASLG -1.81	HGF 1.21	IL10 3.33	IL11 1.21	IL13 1.27	IL15 -3.49
C	IL17A 2.18	IL18 1.02	IL18R1 -3.10	IL1A -1.13 B	IL1B 11.50	IL1R1 -1.18	IL2 15.17	IL21 1.21	IL22 1.02	IL23A -1.16	IL2RA 2.71 A	IL3 -1.01
D	IL4 207.94	IL5 -1.94	IL6 7.03	IL6R -1.16	CXCL8 2.56	JAK2 2.58	JAK3 8.11	LIF -2.09	LIFR 1.21	LTA -1.02	MAP2K1 1.32	MAPK1 -1.14
E	MAPK14 11.06	MAPK3 1.10	MAPK8 7.84	MET 3.09 A	MTOR 1.40	MYC -1.67	NFKB1 20.58	NFKBIA 1.18	OSM -1.11	PIAS3 -1.30	PIM1 22.58	RELA -1.08
F	SOCS1 -1.47	SRC -2.00	TLR4 12.15	TNF 1.87	TNFRSF10B -1.22	TNFRSF1B -1.69	TNFSF10 -1.39	TYK2 -1.75	BCL3 -1.00	CASP4 1.11	CEBPD -2.20	GLRX 1.34
G	IFITM2 -1.01	IL6ST -1.28	JUNB -1.92	NRP1 -1.93	PHF21A -1.39	PROS1 -1.58	PVRL2 1.26	SOCS3 2.18	STAT3 13.96	TGM2 -4.20	TNFRSF1A -1.12	TUBB2A 1.13



Appendix 5.2c Gene expression fold change in IL-6/STAT3 pathway in PBLs at 10 µg/ml AFB_1 treatment at 24 hours.

Layout	1	2	3	4	5	6	7	8	9	10	11	12
A	AKT1 1.01	BAX -1.09	BCL2 1.43	CCL2 1.85	CCL3 1.50	CCL4 1.33	CCL5 1.17	CD4 3.35	CD40 1.70	CD40LG 1.01	CDKN1A -1.27	CSF1 -1.25
B	CSF2 -1.25	CSF3 -1.58	CSF3R 1.44	CXCL10 2.88	EGFR -1.25	FAS -1.43	FASLG -1.22	HGF -1.25	IL10 4.32	IL11 -1.25	IL13 1.11	IL15 7.44
C	IL17A 4.87	IL18 1.10	IL18R1 -1.98	IL1A -1.90	IL1B 3.39	IL1R1 1.84	IL2 2.67	IL21 1.17	IL22 1.64	IL23A -1.23	IL2RA -1.02	IL3 -1.05
D	IL4 29.55	IL5 -1.43	IL6 4.45	IL6R 1.23	CXCL8 2.95	JAK2 1.96	JAK3 7.92	LIF -3.99	LIFR -1.25	LTA 1.45	MAP2K1 1.71	MAPK1 1.20
E	MAPK14 13.85	MAPK3 1.43	MAPK8 5.84	MET -1.92	MTOR 2.22	MYC 1.51	NFKB1 24.06	NFKBIA 1.54	OSM -1.21	PIAS3 -1.38	PIM1 3.44	RELA -1.17
F	SOCS1 -2.10	SRC 1.35	TLR4 9.46	TNF 5.38	TNFRSF10B 1.10	TNFRSF1B 1.63	TNFSF10 1.01	TYK2 -2.59	BCL3 6.81	CASP4 1.50	CEBPD -1.34	GLRX 2.30
G	IFITM2 1.22	IL6ST -1.96	JUNB -2.41	NRP1 2.25	PHF21A -1.15	PROS1 -1.63	PVRL2 1.47	SOCS3 3.16	STAT3 5.81	TGM2 -1.43	TNFRSF1A 1.58	TUBB2A -1.09





Appendix 5.2d Gene expression fold change in IL-6/STAT3 pathway in PBLs at 10 µg/ml AFB_2 treatment at 24 hours.

Appendix 6.1 The reference range of biochemistry parameters

Analyte	Units	Age	Reference range
Alanine amino-transferase (ALT)	U/L	<26wk	<55
		27wk - 4yr	<60
		4wk – 12yr	<50
		12 – 14yr	F <45; M <70
		14 - 16yr	F <45; M <60
		16 – 19yr	F <45; M <55
		>19yr	<40
Asparatate amino-transferase (AST)	U/L		<40
Creatinine	umol/L	7 – 9 yr	30 – 48
		>15 yr M	64 – 104
		>15 yr F	49 – 90
Urea	mmol/L	2 – 17yr	2.5 – 6.5
		>17yr	2.5 – 7.8
Total bilirubin	Umol/L		2--21
Conjugated (direct) bilirubin	Umol/L		<4
Total protein	g/L		60 – 80

Appendix 6.2 Scatterplot matrix of parameters