PREVALENCE OF CHRONIC AFLATOXIN EXPOSURE AND THE RESULTANT CLINICAL-IMMUNOLOGICAL EFFECTS IN CHILDREN IN MAKUENI COUNTY, KENYA

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A thesis submitted in partial fulfillment of the requirements for the degree of PhD in Medical Immunology

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

This work is dedicated to all the children that I have served professionally over the years as well as their parents who accorded me the privilege of allowing me to care for their precious children. The children continue to give me a reason to love my work.

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In paraphrasing an African saying, it took more than my village to carry out this study beginning the earlier exploratory days in 2011. Since many people have helped me along the way, there is a risk of inadvertently failing to acknowledge them all. I ask for forgiveness from those that fall in that category.

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LIST OF ABBREVIATIONS

ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AST	Aspartate Aminotransferase
AUC	Area Under Curve
BAZ	Basal Mass Index for Age
BMI	Body Mass Index
CCD	Charged Coupled Device
CMI	Cell-Mediated Immunity
DDS	Dietary Diversity Score
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPT	Diphtheria, Pertussis , and Tetanus
EA	Enumeration Area
EDTA	Ethylenediaminetetraacetic Acid
EPI	Expanded Program on Immunization
ERC	Ethical Review Committee
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GVAP	Global Vaccine Action Plan 2011
HB	Hepatitis B
HDI	Human Development Index
HIV	Human Immuno-Deficiency Virus
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research in Cancer
IFNy	Interferon Gamma
IQC	Internal Quality Control
KDHS	Kenya Demograpic and Housing Survey
KNH	Kenyatta National Hospital
LED	Light-Emitting Diode
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume MFI
Median Fluorescence Intensity MUAC	
Mid-Upper Arm Circumference OPV	
Oral Polio Vaccine	
PBMC	Peripheral Blood Mononuclear Cell
PC	Principal Component
PCA	Principal Component Analysis

PMT	Photomultiplier Tube
PSU	Primary Sampling Unit
QC	Quality Control
RBC	Red Blood Cell
RDW	Red Blood Cell Distribution Width
ROC Curve	Receiver Operating Characteristic Curve
SA-PE	Streptavidin-Phycoerythrin
SDGs	Sustainable Development Goals
TMB	Tetramethylbenzidine
TNFa	Tumor Necrosis Factor Alpha
UN	United Nations
UNICEF	The United Nations Children's Fund
UON	University of Nairobi
USFDA	United States Food and Drug Administration
WAZ	Weight-for-Age
WBCs	White Blood Cells
WHO	World Health Organization
UN	United Nations
UNICEF	The United Nations Children's Fund
UON	University of Nairobi

ABSTRACT

Background:

Globally, approximately three million children die each year from vaccine preventable infectious diseases mainly in developing countries. Despite the success of the expanded immunization program, not all infants and children around the world develop the same protective immune response to the same vaccine. An ideal vaccine must be able to induce a response over the basal immune response that may be largely driven by environmental and other population specific and socio-economic factors. Mycotoxins such as aflatoxins and fumonisins are immune suppressants and are confirmed to interfere with both cell-mediated and acquired immunity. The mechanism of aflatoxin toxicity of aflatoxin is linked to the binding of the bio-activated AFB_1-8, 9-epoxide to cellular macromolecules.

Methods: We studied Hepatitis B surface antibodies [anti-HBs] levels to explore the immune modulation effects of dietary exposure to aflatoxins in children aged between one and fourteen years in Kenya. Hepatitis B vaccine was introduced for routine administration for Kenyan infants in November 2001. To assess the effects of Aflatoxin on immunogenicity of childhood vaccines Aflatoxin B_1-lysine in blood serum samples were determined using High Performance Liquid Chromatography with Fluorescence detection while anti-HBs were measured using Bio-ELISA anti-HBs kit.

Main Objective: To determine prevalence and clinical-immunologic effects of chronic exposure to aflatoxin in 410 asymptomatic children aged 1-14 years in Makueni County, Kenya.

Methodology: This was a Cross sectional study. Serum aflatoxin-B1 lysine adduct (AFB1) was the measure of exposure and High Performance Liquid Chromatography with Fluorescent detection was used for this. Hepatitis Bs antibody levels (anti-HBs)- measured using Bio-Elisa anti-HBs kit-was the outcome measure. Wealth index and dietary diversity scores, anthropometry, liver function tests, blood counts, 8 cytokine markers, grain aflatoxin levels and fumonisin levels in urine were recorded.

Results: AFB1 was detected in 100% of the children; geometric mean 20.4, median 19.98 (range of 0.74-901.15) all in pg/mg albumin. Only 98/205 (47.8%) of the study population had protective antibodies for Hepatitis B surface antibodies. High AFB1 was associated with 73% increased odds of low anti-HBs. For every unit rise in AFB1, anti-HBs dropped by .91mIU/ml (OR 0.35; 95% CI 0.15-0.81, p<0.01). Females had 65% reduced odds of having low antibodies (OR 0.35; 95%CI 0.15-0.81 p<0.01). Malnourished children had a 35-fold increased risk of having high AFB1. 7 out of 8 cytokines showed a down-regulated trend that was not statistically significant. Socio-economic status was significantly associated with AFB1 exposure (p< 0.01) and dietary diversity scores (p< 0.003). There was high co-occurrence of fumonisin and AFB1 in the study population.

Conclusions

Aflatoxin was found in the serum of 100% of the studied population of children of Makueni County.

More than half of the studied population (52%) did not have protective anti-HBs antibodies possibly associated with aflatoxin exposure.

Despite the decline in the rate of stunting of children in Makueni County, the malnourished children were more at risk of having high aflatoxin.

This study showed not only a high exposure to aflatoxin in asymptomatic children in the study area but also a direct relationship between poverty, dietary diversity and aflatoxin exposure. Co-occurrence of
fumonisins with aflatoxin was high in this community. Fumonisins independently depressed immune response in this study. There is a compelling need to investigate if the other routinely administered vaccines behave similarly in the children highly exposed to aflatoxin.

CHAPTER 1: INTRODUCTION

1.1 Background

The World Health Organization [WHO] recommended "Expanded Program on Immunization" [EPI], has been one of the most cost-effective public health interventions in history(1). This is exemplified by the eradication of smallpox, significantly lowering the prevalence of poliomyelitis and the dramatic reduction in morbidity and mortality from several other illnesses such as measles, rotavirus infection and tetanus(2). Although a great deal has been achieved in diagnosis and treatment of many medical conditions, emerging and re-emerging infectious diseases remain a major threat to global health, causing severe morbidity and mortality worldwide(2,3). To address these diseases, the World Health Organization put in place the Global Vaccine Action Plan 2011 to 2020 (WHO-GVAP) that was endorsed by 194 countries. This plan aims at strengthening current routine immunization to accelerate control of vaccine preventable diseases by introducing new and improved vaccines and spurring development of the next generation of vaccines and technologies(1).

Unfortunately, approximately three million children die each year of infectious diseases that are easily preventable with currently available vaccines globally(4). Failure of vaccines to prevent infections and/or diseases may be due to suboptimal vaccine coverage, breakdown in cold chain of vaccine storage and delivery. In Kenya, diminished vaccine effectiveness is suspected to be partly due to the fact that the Kenyan population differs from the populations studied in the original vaccine clinical trials(4). Furthermore, many infants do not receive recommended vaccines either on time or the required number of doses to provide optimal protection. Moreover, not all infants around the world develop the same protective immune response to the same vaccine. To tackle these issues requires identification of age and population-optimized vaccine schedules and

formulations that can only be developed through research aimed at understanding the reasons why some children do not mount an efficacious immune response following vaccination.

An ideal vaccine must be able to induce a response over the basal immune response that may be largely driven by environmental and other population specific and socio-economic factors. The importance of environmental factors modulating immunity is most readily recognized in early life, a period of rapidly changing environments(5). Understanding the environmental engines that drive development and evolution of the immune system naturally and in response to childhood vaccines is not only necessary to address specific pediatric diseases but also to identify the strategies to change trajectories toward long-term, life-long protection from disease(6). Malnutrition, Soil-Transmitted Helminth infections, early microbial exposure and exposure to mycotoxins through the diet are few environmental factors that must be taken into consideration as they are likely to have an impact on the overall vaccine induced immunological response(7–11).

Epidemiological studies have shown that exposure to mycotoxins such as aflatoxins, fumonisins, deoxynivalenol, zearalenone and ochratoxin have significant negative health impacts on pediatric population (12–15). Mycotoxins are naturally occurring fungal metabolites produced by filamentous fungi and commonly contaminate food supplies worldwide. Among the over 300 identified mycotoxins, aflatoxins are considered the most toxic as they are confirmed immune suppressants, and Group I carcinogens besides interfering with growth and development in children(13,16). Moreover, aflatoxins are teratogenic, mutagenic and hepatotoxic(16,17). The mechanism of aflatoxin toxicity is linked to the binding of the bio-activated AFB1-8, 9-epoxide to cellular macromolecules(18). Since the discovery of aflatoxins in the 1960s, exposure to high levels of aflatoxins in the diet have resulted in over 600 deaths in the Kenyan population (19). The effects of acute exposure to high levels of aflatoxins came to the fore in Kenya following major outbreaks

in 1981 and 2004(20,21). A wide range of cereals, legumes, tubers and nuts are contaminated by a ubiquitous Aspergillus fungi –which produce aflatoxin- in the field or in storage(22–24).

Deaths from consumption of aflatoxin contaminated grains has also been reported in India (25). The United States Food and Drug Administration [USFDA] started monitoring aflatoxin levels in food supplies in 1969, concluding that they are unavoidable contaminant and recommended guidelines of 20µg/kg for all foods and animal feeds(26).

In addition to the known genotoxic and carcinogenic effects of mycotoxins, there is emerging evidence of direct effects of mycotoxins to the immune system. Immune responses occur by macromolecular synthesis and cellular proliferation and this gives us an indication of how mycotoxins can cause immune-toxicity(27). A major effect of aflatoxin exposure is the suppression of cell-mediated immunity (CMI) (27–29). Modulation by mycotoxins also increases the susceptibility to bacterial and parasitic infections that adversely affects acquired immunity, as evidenced following experimental challenge with infectious agents after vaccination(27). Biologically reactive mycotoxins tend to inhibit protein synthesis or cell multiplication(13). Ultimately, in order to bridge the gap of knowledge and understanding of vaccine immune responses, it will be important to assess the possible role of environmental and nutritional factors that affect the general vaccine immune responses.

The interaction between Hepatitis B [HB] virus infection, and exposure to aflatoxins through the diet are confirmed to synergistically increase the risk of developing chronic liver diseases and hepatocellular carcinoma(30–33). Hepatitis B vaccine was introduced for routine administration for Kenyan infants in November 2001. In this study, we selected anti-HBs antibody test to explore the immune modulation effects of dietary exposure to aflatoxins in children aged between one and

fourteen years. Assessment of immune memory and measurement of Hepatitis B antibody, is key in assessing immune competence(34). Hepatitis B vaccine was selected as the vaccine for exploration of the effects of aflatoxin on immune response due to the known association between hepatitis B infection and chronic aflatoxin exposure in the pathogenesis of chronic liver disease. The use of Hepatitis B vaccine has increased the usefulness of anti-HBs determination as a tool in monitoring seroconversion after immunization.

Generating vaccine-mediated immune protection is a complex challenge. Currently available vaccines have largely been developed empirically; their early protective efficacy is primarily conferred by the induction of antigen-specific antibodies. The quality of such antibody responses, specifically their avidity have been identified as a determining factor of efficacy. However, there is more to antibody-mediated protection than the peak vaccine-induced antibody titers. In addition, long-term protection requires the persistence of vaccine antibodies and/or the generation of immune memory cells capable of rapid and effective reactivation upon subsequent microbial exposure (35,36). The determinants of immune memory induction, as well as the relative contribution of persisting antibodies and of immune memory to protection against specific diseases, are thus essential parameters of long-term vaccine efficacy(34,37). The overall objective of this study was to estimate the prevalence and determine the clinical and immunological correlates of aflatoxin exposure among children aged between 1-14 years in Muuni sub-location, Makueni County in Kenya.

1.2 Problem Statement

High morbidity and mortality in the Kenyan paediatric population remains largely driven by infective illnesses. These are largely vaccine-preventable. Varied and exerted national efforts to address this problem have made some progress but this is inadequate. It cannot be over-emphasized

that there is an urgent need to explore further into other factors- including the non-traditional ones like ingested environmental contaminants- that may be frustrating the control of vaccine-preventable diseases. These effects are potentially controllable if well understood and brought to the attention of policy makers with convincing data that is easy to understand.

Vaccines are a well-recognized cost-effective method of preventing infectious diseases. Factors that potentially affect vaccine efficiency have a direct effect on the morbidity and mortality patterns and this makes this study important.

1.3 Justification

Vaccines are a well-recognized cost-effective method of preventing infectious diseases. Factors that potentially affect vaccine efficiency have a direct effect on the morbidity and mortality patterns. There is currently no designed for purpose studies that have looked at the prevalence of aflatoxin exposure in a wide range of pediatric population and how this affects routine childhood vaccinations. This creates an imperative for this study important.

1.4 Hypothesis

Exposure to aflatoxin has no effect on immunity of children aged 1-14 years.

1.5 Broad Objectives

To assess the prevalence and clinical-immunological effects of exposure to aflatoxin in children aged 1-14 years in Makueni County, Kenya.

Specific Objectives

i. To estimate aflatoxin levels in blood samples of the study population.
ii. To establish the association between aflatoxin blood levels with 8 cytokines in the study population.
iii. To establish the effect of serum aflatoxin levels on hepatitis B surface antibody response.

CHAPTER 2: LITERATURE REVIEW

2.1 The African Child and the Attendant Mortality

The world has made substantial progress in reducing child mortality in the past few decades. Globally, the under-five year mortality rate dropped from 93 deaths per 1,000 live births in 1990 to 41 deaths per 1,000 live births in 2016 (38). Despite this progress, child mortality remains a critical public health concern. In 2016, 5.6 million children died before their fifth birthday, of which 46 per cent died in the first month of life. These children died mostly from preventable and treatable infectious diseases whose knowledge and technologies for life-saving interventions are available.

The burden of under-five deaths remains unevenly distributed. About 80 per cent of under-five deaths occur in Sub-Saharan Africa and Southern Asia. Six countries account for half of the global-under five deaths namely, India, Nigeria, Pakistan, the Democratic Republic of Congo, Ethiopia and China (38). In 2016, India and Nigeria alone accounted for 32 per cent of the global under-five deaths (38).

Pneumonia, diarrhoea and malaria remain among the leading causes of death among children under age 5 – accounting for almost a third of global under-five deaths - about 40 per cent of under-five deaths in sub-Saharan Africa. It is important to note that malnutrition is closely linked to these deaths, as malnourished children are nine times more likely to die from infectious diseases (39). Repeated diarrhea can disrupt the intestinal absorptive and barrier functions of the gut. This is the cause of up to 43% of the stunted growth affecting one fifth of the children worldwide and one third of children in developing countries (40). It is recognized that vitamin A is essential for a healthy functioning immune system and that children under 2 years are less likely to die from measles when given vitamin A supplements(41). Diarrhea and stunting are closely linked, and therefore, efforts into improving water access and quality, sanitation, nutrition, and access to vaccines should be seen

not just as efforts to prevent and treat diarrhea, but also as efforts to combat malnutrition and its developmental consequences(40).

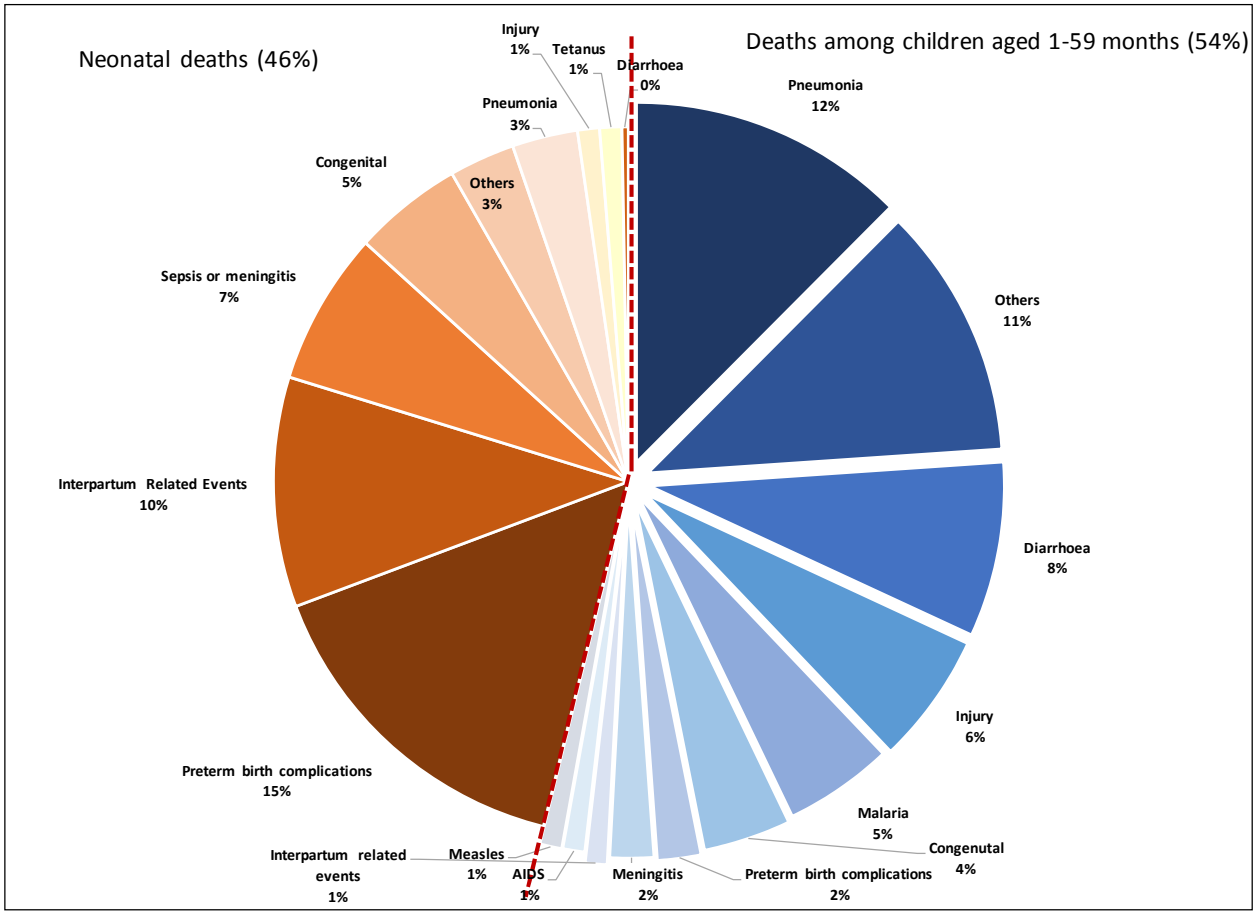


Figure 1: Global Distribution of Deaths among Newborns, by Cause, 2016. Adapted from WHO-MCEE (42)

2.2 Child Mortality in Kenya

The World Health Organization (WHO) estimates that globally 2.5 - 3 million infants born healthy die of acute infections in their first year(43). The Kenya Demographic and Health Survey 2014 estimated that the infant mortality rate in Kenya is 39 deaths per 1,000 live births, and the under-five mortality is 52 deaths per 1,000 births. This means that one in every 26 Kenyan children dies before their first birthday, and about one in 19 children does not celebrate their fifth birthday(44).

A limited number of vaccine-preventable pathogens cause these deaths; the pie chart below shows that the causes of childhood mortality in Kenya are similar to the global landscape. It is notable that pneumonia, diarrhoea and malaria cause about 45 per cent of deaths in young children each year in Kenya.

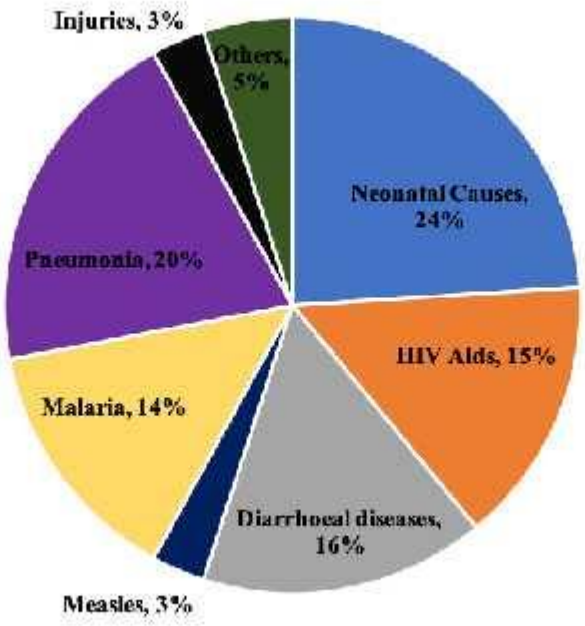


Figure 2: Distribution of Causes of Death among Under-Fives in Kenya, 2000-2003. Adapted from: WHO (43)

Of the above diseases, at least 39% are part of the WHO recommended routine immunization for children, which has been adopted as part of public health practice in many African countries, Kenya included (45). According to the KDHS, Kenya's immunization coverage was slightly over 77 per cent, higher than the continental average of 75 per cent. This has been attributed by concerted efforts by the Ministry of Health in sensitizing the population on the need for immunization besides ensuring ready access of the vaccines. However, there are still areas in the country with lower than average immunization coverage. Six counties, most of which are in Northern Kenya, had

immunization coverage of below 60% in 2014. The figure below shows the distribution between counties for DPT, OPV, and Measles. The target coverage for all counties is 80%, and approximately half of those counties had not met the target as of 2014 (44)

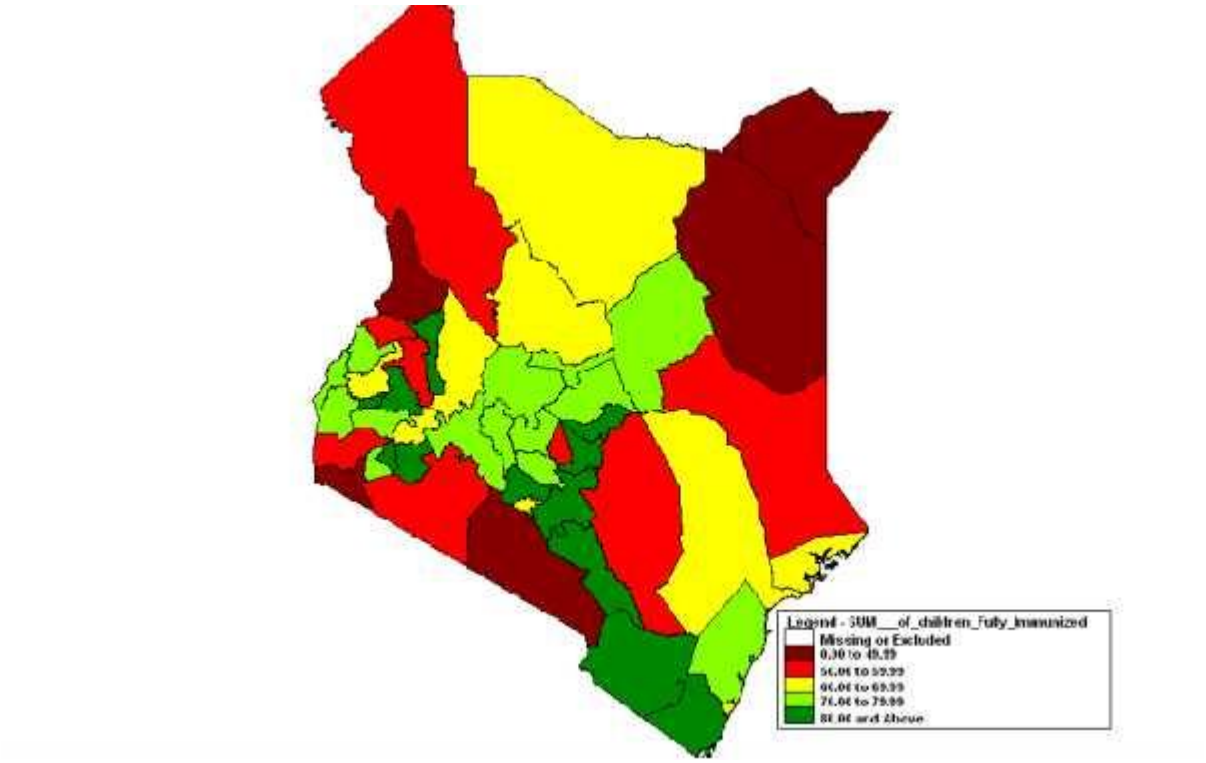


Figure 3: Access to Vaccine for Infants Under Five Years Old. Adapted from the KDHS(44)

Other than access to vaccination, the high mortality rates from vaccine-preventable infective diseases are caused by a less explored area of "vaccine failure". It is therefore imperative that resources are dedicated to this area, especially because reduction of childhood morbidity and mortality is core to achieving Kenya's Vision 2030 as well as the United Nations (UN) Sustainable Development Goals (SDGs) which include "good health and well-being for people"(46).

2.3 Role of Immunization in Reducing Child Mortality

It is well understood that most of the causes of high infant mortality are preventable, and the cost of preventing those diseases is cheaper than the cost of managing the disease once the child or children

are infected. It is also well known that improvement of health services and public health interventions have led to the decline of morbidity and mortality in children. These interventions include improved management of childhood illnesses, immunizations, mass distribution of insecticide treated nets, improved access to peri-partum care, safe drinking water and sanitation, and general decline in poverty level that tends to be associated with reduction in use of biomass fuel for cooking (household air pollution) and under-nutrition.

Except perhaps provision of safe drinking water and sanitation, no other interventions, including antibiotics, have had such a major effect on mortality reduction like vaccinations in spite of the implementation and technical challenges that are closely interlinked (47). Vaccines are indeed one of the greatest achievements of biomedical science and public health(48).

Access to immunization globally is not uniform. A child in a developing country is ten times more likely to die of a vaccine-preventable disease than a child from an industrialized one. In some countries, up to 70% of children do not receive the full set of vaccines; the lowest coverage is found in sub-Saharan Africa. In Africa as a whole, over 40% of children are not immunized against measles, a major cause of infant mortality that kills one child every minute.

Vaccine efficacy indicates direct protection to the individual vaccinated under optimal condition. By the time a vaccine is administered to a patient, it will have gone through steps that prove its efficacy to an acceptable degree. Vaccine effectiveness connotes protection conferred by immunization in a defined population in day-to-day circumstances. This is impacted by other non-vaccine factors such as vaccine coverage, access to health centres, cultural/religious attitudes to vaccinations, cost of the vaccines and cold chain integrity. There are other biological factors that include differential uptake of vaccinations, prior infections and underlying immunocompetence that

affect vaccine efficiency. The resulting immunological response heterogeneity leaves some children unprotected from vaccine preventable conditions and consequent ill health if not death.

Effectiveness is proportional to efficacy but these two are not usually similarly matched for the reasons indicated above. Immunization for primary prevention of illness is value for money. For every $1 spent on immunization, there is a saving of $44 in low and middle income countries(49). Given their importance and cost- effectiveness while considering the considerable costs of the delivery systems to the children, it is not an unreasonable proposition to address factors that may interfere with the intended sero-conversion rates.

2.4 Immunology of the Child from Conception to Teenage

The role of a properly regulated immune system is that of protecting the host from pathogens and other environmental challenges without causing damage to self-tissues. Simply put, the immune system distinguishes danger from stranger. It is made of physical barriers, nonspecific and specific mechanisms as demonstrated in Figure 4 below.

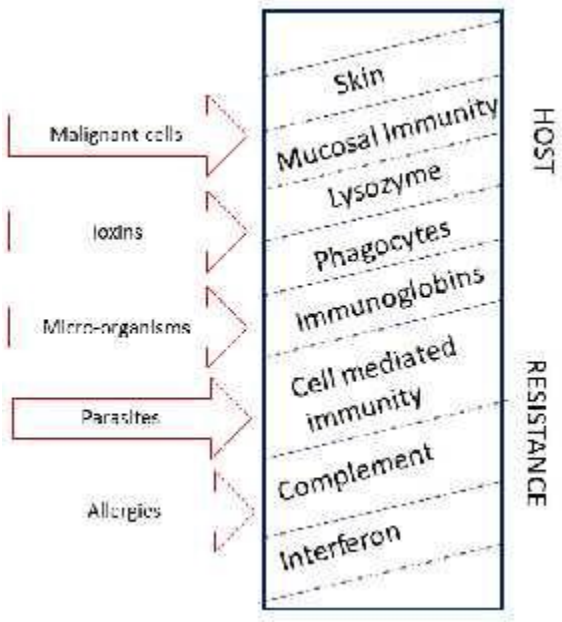


Figure 4: Host Defenses Play a Protective Role in the Body. Adapted from Chandra R(50)

A simple view of host defenses consisting of physical barriers (skin and mucous membranes), nonspecific mechanisms (complement, interferon, lysozyme, and phagocytes), and antigen-specific processes (antibodies of five immunoglobulin isotypes and cell-mediated immunity)(50)

The immune system has 3 main physiologic functions namely:

i. Defence against infections. Deficiencies result in increased susceptibility to infections and poor uptake of vaccinations. This was the focus of the study and is the area of discussion of this thesis.

ii. Recognition of tissue grafts and newly introduced proteins thus impacting on transplantation and gene therapy

iii. Defence against tumors; availing the potential for immunotherapy of cancer.

In addition to fighting viruses, bacteria, fungi and parasites, the immune system plays a role in tissue repair, wound healing, elimination of dead and cancerous cells as well as the formation of healthy gut microbiota(51).

Leucocytes are the main immunologic cells. They originate from the reticulo-endothelial and lymphoid systems in the foetus. In postnatal life, neutrophils, basophils, eosinophils, monocytes, macrophages, natural killer cells, T & B lymphocytes originate from the bone marrow. These cells communicate by either ligand-receptor interactions or via secreted molecules called cytokines. T lymphocytes circulate through the blood, regulate antibody and cellular immunity and defend against many types of infections. The B lymphocyctes also called antibody-forming cells and natural killer cells are thymic independent and remain mainly in the peripheral lymphoid organs.

T helper cells are the primary regulators of T cell and B cell mediated responses whose function is to aid the antibody producing cells. There are two types of T helper cells; TH1 cells which aid in the regulation of cellular immunity while TH2 cells aid B cells to produce antibodies IgA and IgE.

The functions of T helper cells depend on the type of cytokines that are generated. TH1 cells trigger IL-2 and IFN gamma while TH2 cells trigger IL-4 and 10.

Innate (natural, native) immunity exists in healthy individuals to block entry of microbes. It consists of natural killer cells, phagocytes and proteins of the complement system. Adaptive (specific, acquired) immunity -there are two types, humoral and cell-mediated immunity, is stimulated by microbes that invade tissues. This occurs through a variety of lymphocytes that recognize antigens.

There is a difference between antigen specific B and T cell responses in the fetal life compared to the mature children. These may be due to antigen naivety of the immune system, immaturity of both B and T cells as well as the microenvironment in which they differentiate(52).

It has been said that more than 1600 genes are involved in innate and adaptive immune responses(53). Part of the innate immune soluble factors called cytokines/chemokines play a significant role by acting via transmembrane cell surface receptors. They trigger signal transduction pathways that result in new gene transcription and synthesis of new cellular proteins(54). Cytokines are small proteins released by a variety of cells in the body usually in response to an activating stimulus. Measurement of the concentrations of the various cytokines accords us the opportunity of exploring the cytokine expression in the subjects that respond to vaccines compared to those that do not. Future studies that focus on looking at cell subsets, immune genes and cellular metabolites, will perhaps further elucidate our understanding of the interplay between vaccinations and infections(55).

TH1 cells (IL-2, IFN gamma, IL-13) drive the immune response towards cell mediated immunity while TH2 cells (IL-4, 5,10, GM-CSF) promote a humoral or allergic response.

The fetal environment requires that the immune system is tolerant to maternal antigens. After birth, the exposure to many antigens in the environment including intestinal and vaginal commensals require appropriate immune response (51).

For the newborn, both the innate and adaptive immunological responses are suppressed. The body's ability to defend against antigens changes throughout the lifespan. It is known that infants respond well to protein-but not glycoprotein and polysaccharide- antigens, and this change after the second year of life (51). Newborns have low phagocytic activity as well as the complement system. The antigen presenting cells poorly activate T cells that produce small quantities of cytokines. This explains why low-virulence organisms like staphylococcus cause infections in neonates. Newborns get passively acquired IgG antibodies since these cross the placenta. Some IgA antibodies can be passed through breast milk. Passively acquired antibodies accord the newborns protection for up to 18 months. These antibodies are also known to dampen response to active immunization (51).

Antigen presenting cells in newborns have reduced capability to activate T cells besides the poor proliferation and production of tiny amounts of IL-2, 4,5, 12 and Interferon gamma (51). The child's immune system begins to respond more strongly to glycoproteins by 6-9 months. Between 12-24 months, there occurs marked improvement in the body's response to polysaccharides.

During the first 5 years, active development of the immune system occurs(56). There is accumulation of immunological memory that persists throughout life that fades away with old age (57). The gut bacteria influence the immune system significantly. About 20% of all lymphocytes reside in the gut where they are exposed to many immunogens(58). As the child grows, the immune repertoire is shaped by vaccinations and intercurrent infections even when the infections are subclinical (59,60).

At adolescence, testosterone and oestradiol impact on the immune system. As age advances, there is expanding memory T and B cells triggered by vaccinations and infections as well as by exposure to the microbiome, food and inhaled antigens (52). Ultimately, immunosenescence sets in and affects the body's capacity to respond to infections and with this, dampened formation of long-term memory cells in old age.

2.5 Vaccinations and their Role in Building the Infant's Immune System

Determinants of primary vaccine antibody responses in healthy individuals include the type of vaccine (live versus inactivated; protein versus polysaccharide; adjuvants), nature of the antigen (polysaccharide, protein) antigen dose, vaccine schedule, genetic determinants, age at immunizations, viability of the vaccines which may be affected by the cold chain for certain vaccines and other environmental factors. This work looked at aflatoxin as an environmental factor while trying to control, as far as possible, the other factors that may also affect vaccine sero-conversion fully recognizing that many other factors may be at play here.

Vaccine-mediated protection is a complex issue that does not only get affected by level of antigen-specific antibodies but also the generation of immune memory cells that are capable of rapid and effective reactivation upon subsequent microbial exposure. B cells have a predominant role in the efficacy of vaccines while T cells are essential to the induction of high-affinity antibodies and immune memory. Identification of immune correlates, or at minimum, surrogate markers, is important in efforts to optimize immunization strategies for the routinely administered vaccines.

Vaccine-induced immune effectors are essentially B lymphocyte-produced antibodies that bind to either a toxin or a pathogen (61). The other effectors include cytotoxic CD8+ T lymphocytes (CTL). These limit the spread of infectious agents by recognizing and killing infected cells or secreting

specific antiviral cytokines. The generation and maintenance of both B and CD8+ T cell responses is supported by growth factors and signals provided by CD4+ T helper (Th) lymphocytes which are divided into T helper 1 (Th1)-which on activation secrete IL-2, IFN-gamma, and TNF beta and support to cytotoxic T cells and macrophages- and T helper 2 (Th2) subtypes-these prompt the secretion of IL-4, IL-6, IL-10, IL-13 and exert antimicrobial functions (parasites) and support to B lymphocytes. Regulatory T cells (Treg) that are involved in maintaining immune tolerance are controlled by these effectors(62). The function of TH cells is assessed by production of cytokines where the level of production indicates the level of activity. Most antigens and vaccines trigger both B (humoral immunity) and T (cellular immunity) cell responses. CD4+ T cells are required for most antibody responses while antibodies exert significant influences on T cell responses to intracellular pathogens (63).

The spectrum of immune system parameters is wide. Those chosen for this study require having 3 characteristics; i) known correlation to clinically relevant endpoints (biological relevance), ii) ability to distinguish within- and- between subject variation (sensitivity) and iii) practical feasibility given that this cross-sectional study was field-based.

Vaccine-specific antibody production is known to have high suitability using the above three criteria while cytokine patterns produced by activated immune cells have medium suitability. By studying both antibody levels and the cytokine profiles, we hoped to obtain accurate information on the relationship between these and aflatoxin exposure across differing age groups. We are aware that vaccine-specific immunity can vary widely within a population due to prior vaccinations or natural infections. By selecting a sample that resides in a relatively homogenous rural environment, we hoped to mitigate the individual differences attributable to the environment.

Hepatitis B Vaccine and its Significance

The association between Hepatitis B infection and chronic liver disease and hepatocellular carcinoma is well recognized (30–33) .

Anti-HBs antibody test was selected as the vaccine for exploration of the effects of aflatoxin on immune response due to the known association of hepatitis B infection and chronic aflatoxin exposure in the pathogenesis of chronic liver disease (30–33). This vaccine was introduced for routine administration for Kenyan infants in November 2001. In 1992, WHO recommended that all countries should incorporate hepatitis B vaccination into their national immunization services. In 2009, Wacheke Nganga in a cross sectional dissertation studied 13-18 year old children in Nairobi and Machakos reporting a prevalence of HepBs IgG antibody of 9.3% with 3% antigen markers (64).

Antibody levels >/= 10mIU/ml are considered protective. 15-50% of fully vaccinated patients have been noted to have low or undetectable concentrations of anti-HBs 5-15 years post vaccination (38). It is known that immune memory, not just HepBs antibody measurement, is a key factor in assessing immune competence (65).

World Health Organization has not identified mycotoxins as a high priority risk factor since that consideration is based on mycotoxin connections with hepatocellular carcinoma (29,66). Mycotoxins are known to have both nutritional and immunologic consequences. The largest contributor to morbidity and mortality in low-income countries is infective illnesses. Were the immunologic consequences of mycotoxins to be considered, WHO would place mycotoxins at their rightful place on priority health risks. Globally, about 2.5-3 million infants that are born healthy die of acute infections in the first year(38).

A limited number of vaccine-preventable pathogens cause these deaths. Figure 2 below shows the causes of childhood mortality in Kenya is similar to the global picture. It is notable that pneumonia, diarrhoea and malaria, cause about 45 per cent of deaths in young children each year in Kenya. Any factors that negatively affect vaccination status of children are an upstream contributory factor to the morbidity and mortality patterns as long as we fail to address those factors.

2.6 Environmental Factors that Affect Immune Indicators: Nutrition, Mycotoxins, Infections

Determinants of immune modulation include poor sanitation and personal hygiene, overcrowding, contaminated food, and water and inadequate nutrition (50). The association between aflatoxin and malnutrition has been widely described(67). Some infections are known to subsequently confer permanent immunity to that specific infection.

Nutrition is a public health concern in children under five years in many countries particularly in Africa, where infant mortality, remains at around 40% with both over- and under-nutrition co-existing even in the same household (68). This review also indicates the co-existence of both over- and under- nutrition even in the same household (dual-burden household).

There is an intricate connection between social, cultural, political and environmental factors with food security at household level. This affects the more vulnerable members of society especially the children. Whether we are looking at rural or urban poverty, the consequences are very similar. The figure below points to basic causes that impact on underlying causes. In essence, malnutrition is derived from wider factors that are out control at the household level.

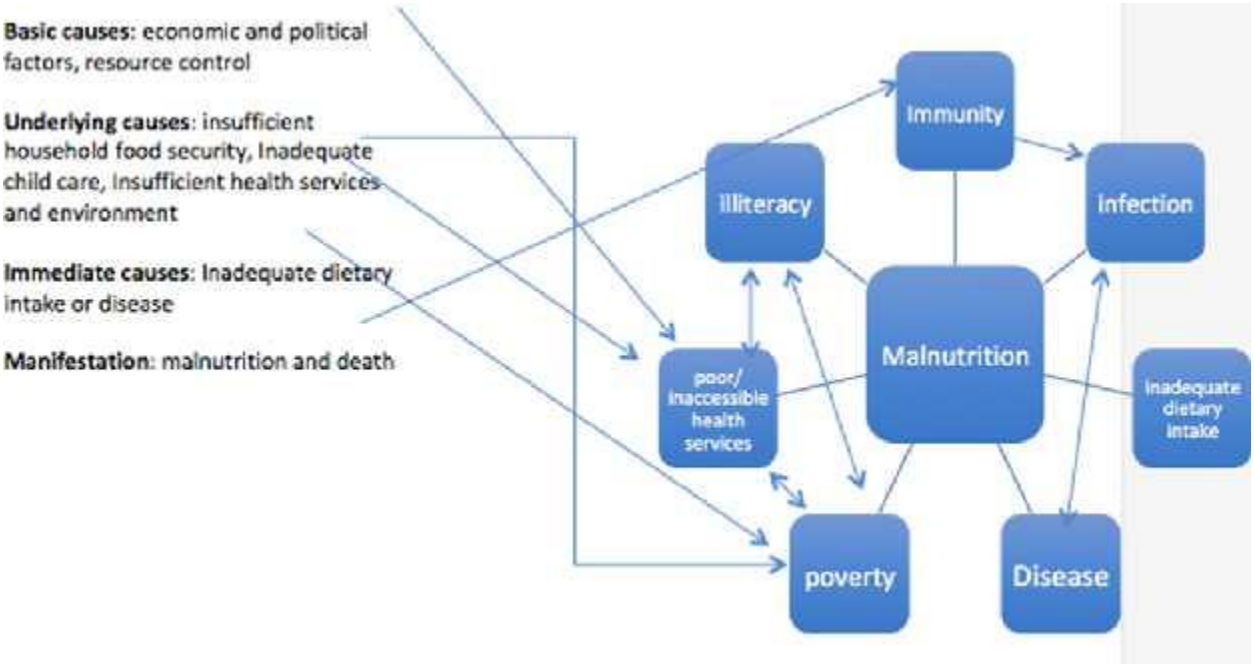


Figure 5: Multifactorial Associations of Malnutrition

The effects of malnutrition on the cognitive development in childhood ultimately affect productivity and the inability to attain full human potential(68). Stunting is the most prevalent form of malnutrition affecting 161 million children in the world, 40% of those in sub-Saharan Africa (69). Malnutrition is associated with half the deaths in children in developing countries (70).

Nutrition is a critical determinant of immune responses and has been mentioned as the most common cause of immunodeficiency globally (50). Protein energy malnutrition is associated with significant impairment of cell mediated immunity, phagocytic function, complement system, secretory IgA antibody concentration, as well as cytokine production (50). Micronutrient deficiency also referred to as hidden hunger is known to influence immune responses. These include iron, copper, selenium, zinc, vitamins A, C, E, B-6 and folic acid. Over-nourished as well as low birth weight infants have immune function impairments (50).

There are over 400 mycotoxins that are secondary metabolites of fungi that exist in nature mainly Aspergillus flavus and Aspergillus parasiticus (71). They contaminate a broad spectrum of foods that make up the staple diet of most people. The most affected by this contamination are populations that lack centralized food systems, which ensure food safety and quality. Toxicity of mycotoxin in exaggerated in children as well as those malnourished (71).

Maize is a widely grown cereal in Kenya and forms the major staple diet in the rural population in particular. Makueni has been documented as having a particularly high aflatoxin levels (72).

Temperatures between 24^{o} and 35^{o} Celsius with moisture content exceeding 7% favour aflatoxin production (72). The now well-recognized phenomenon of extreme weather events globally favors fungal growth and consequently more foodstuff contamination by aflatoxin.

Mycotoxin induced illnesses are not easily recognized by clinicians partially due to their sporadic nature as well as being overshadowed by common illnesses like hepatitis and malarial infections, gastroenteritis. Clinicians are not routinely sensitized about the likelihood of mycotoxicosis as a differential diagnosis. Additionally, diagnostic laboratory tests are not readily available in most hospitals partially caused by lack of demand for the same.

Acute exposure by high amount of mycotoxin especially aflatoxin B1, carries a high mortality rate; the one situation that draws a lot of interest nationally (73). Clinical presentation of acute aflatoxicosis resembles many paediatric acute illnesses. These include, abdominal pains, fever, vomiting, diarrhoea, anorexia and jaundice. Continued exposure progresses to enlargement of the liver and spleen, ascites and coma. In small rural facilities, it is possible to have these symptoms pass as malaria, infective hepatitis or acute poisoning.

Exposure to small doses of aflatoxin over a long period of time is usually insidious and perhaps a significant contributor to impaired growth and protein metabolism. Decreased immunity is the area this work explored in asymptomatic children who reside in a rural area known to have high prevalence of grain contamination by aflatoxin.

2.7 Metabolism and Biotransformation of Aflatoxins.

Aflatoxin is harmful to genetic material (genotoxic) and has no safe limit. It therefore means that keeping the levels as low as reasonably achievable would be a prudent caution. Due to their different metabolic pathways and their body weights, one would expect that the effects on young children would be exaggerated compared to older children and adults.

Aflatoxin is metabolized in 2 phases; the first is an enzymatic oxidation process by cytochrome P450 isoenzymes while the second is a conjugation or non-enzymatic hydrolysis process that produces reactive epoxides as summarized in the figure below (74). Different pathways of metabolism result in varying levels of toxic metabolites the most potent being the one that follows epoxidation that yields AFB1-8, 9-epoxide. This reacts with the N^7 atom of guanine to form pro-mutagenic aflatoxin-N^7 -guanine adducts. Some of the DNA adducts are resistant to DNA repair processes that may culminate in malignancies (75).

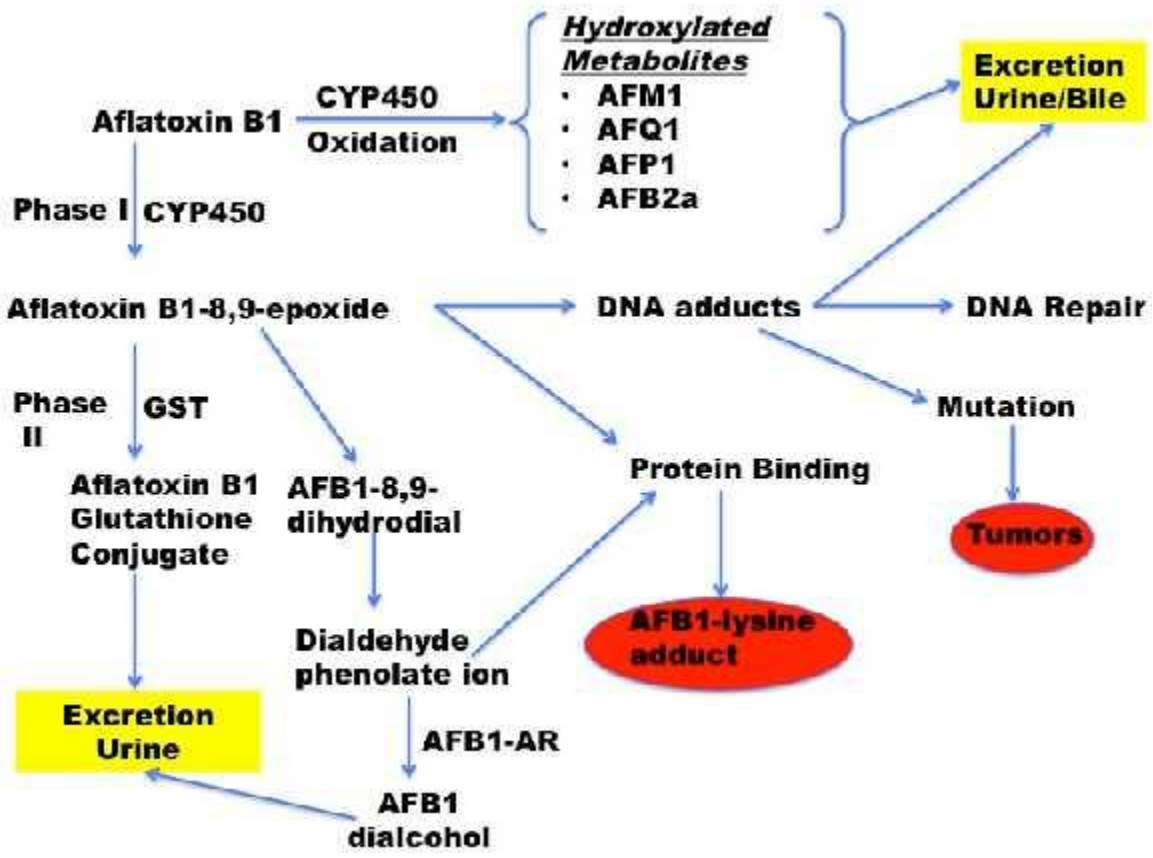


Figure 6: AFB1 Metabolism Process Adapted from: Dohnal V, Wu Q, Kuca K. (76)

Chronic low dose exposure to aflatoxin begins in utero in areas with high prevalence as its fat soluble nature enables aflatoxin to cross the placenta (61,77).

The foetal period has significant vulnerable developmental windows that have the capacity to impact the developing organs that includes the immunological system. The smoldering inflammatory changes triggered by aflatoxin ultimately results in liver cirrhosis. There is a 25 to 30-fold increased risk of hepatocellular carcinoma when exposure to AFB1 is combined with hepatitis B or C viral infections (71).

International Agency for Research in Cancer (IARC) has classified aflatoxin as Group A1 carcinogens (sufficient evidence for human carcinogenicity) while the metabolite of AFB1 that is found in milk has carcinogenic activity 10 times lower than AFB1. It is classified as possibly

carcinogenic under (Group 2B) by IARC and the WHO (17,78). Aflatoxin B1 has been known to up-regulate apoptosis while triggering the expression of death receptors in the endoplasmic reticulum molecules in chicken spleen earning AFB1 the term 'death ligand' (79,80).

In addition to the known genotoxic and carcinogenic effects of mycotoxins, there is emerging evidence of direct effects of mycotoxins to the immune system. Aflatoxin, fumonisins, deoxynivalenol, zearalenone and ochratoxin are the most important in human health, with the first three having significant effects on the paediatric population.

Biologically reactive mycotoxins tend to inhibit protein synthesis or cell multiplication. Immune responses occur by macromolecular synthesis and cellular proliferation and this gives us an indication of how mycotoxins can cause immunotoxicity.

Apart from the independent role malnutrition plays in immunity, mycotoxins in their own right have an immune modulating effect. The bulk of the evidence for this has been derived from laboratory animals and peripheral lymphocytes in vitro (79). These effects may either be a stimulatory or suppressive (80). Aflatoxin has been reported as increasing pro-inflammatory cytokine IL_6 that maintains dendritic cells in immature state (81,82). IL-6 has been reported to decrease antigen specific lymphocyte proliferation without affecting the mitogenic proliferation (83).

Earlier studies have looked at cell mediated and antibody response, natural killer cell activity, macrophage phagocytic function, infectivity and host resistance challenges (84).

Hatori *et al.*, in 1991 looked at a number of factors indicating immune suppression that included expression of regulatory cytokines like IL-2 production by spleenocytes (85). His results showed that much higher doses of aflatoxin were necessary to affect blastogenic response, IL-2 production and primary antibody production. There seems to be inconsistencies in immunotoxicity as well as

the immune response to vaccinations (86,87). In an experiment on piglets, Marin *et al.,* found that low dose aflatoxin decreased the total number of white cells while high dose aflatoxin in animal feed did the opposite (88). In an experiment Turner (89) noted markedly reduced secretory IgA in Gambian children who had elevated serum aflatoxin levels. He also noted no association between cell-mediated immune responses to test antigens and aflatoxin levels (90). Activated T and B cells resulted in proliferation and amplification of immune responses that kill infectious organisms and produce effective antibody response to vaccines. Jiang working with Ghanaian adults showed decreased activated T and B cells in adults with high aflatoxin levels. There was also alteration in different lymphocyte subsets and their cytokine production. Jiang and Turner studies seem to suggest that high levels of aflatoxin B1-albumin adducts and the resultant immune perturbations result in decreased resistance to infections and hepatocellular carcinoma, decreased immune responses to vaccines as well as increased susceptibility to reactivation of chronic infections (29).

Co-occurrence of aflatoxin, fumonisins, trichothecenes and ochratoxin in many food products especially maize has been noted (61,91,92). Although the combined immunotoxic effects of several mycotoxins each via different mechanism is unknown (93), at the very least, one would anticipate an additive if not synergistic effects with multiple mycotoxins (94). Aspergillus, which produces aflatoxin and fusarium, which produces fumonisins, are two of the most common fungi in grains (86). Fumonisins, like aflatoxin, have been reported to alter cytokine profiles and decrease antibody titres to a vaccine in pigs (95). Low dose trichothecenes and Ochratoxin A can up-regulate or down-regulate immune functions via diverse array of cytokines (96). Aflatoxin is lipid soluble and therefore crosses the placenta to affect the foetus at its most vulnerable developmental window. This may result in the blunting of the developing immune system(28,97,98). As aflatoxin is also excreted in breast milk as aflatoxin M1, the newborn continues to get exposed before weaning and

this exposure continues with the introduction of weaning diets that are made from contaminated grains. This continuous and incremental exposure is bound to negatively impact on a rapidly developing immune system.

There is a complex interrelationship between mycotoxins, macro- and micro- nutrient deficiencies, immune suppression, growth impairment and infective conditions.

It may therefore be argued that a substantial investment in vaccine research should be complemented by efforts to address upstream factors that potentially interfere with the ability of vaccines to work effectively. These include the direct immunomodulatory effects of mycotoxins and their indirect effects on nutrition. We remain uncertain about the threshold exposure dose at which nutritional interference and immunosuppression occurs.

To make any impact on vaccine preventable diseases that continue to make up the bulk of morbidity and mortality in the paediatric population, the imperative to look further into the upstream causation pathway of immunosuppression cannot be overemphasized. This work was spurred largely by a desire to gather evidence that would enable clinicians to consider aflatoxin as a potentially significant stumbling block in addressing vaccine-preventable diseases that continue to remain a major cause of mortality in our set up.

CHAPTER 3: MATERIALS AND METHODS

Although this study set out to look at how chronic aflatoxin exposure impacts childhood immunity as exemplified by hepatitis B antibody levels, there are other independent factors like age, gender and the co-existing fumonisins which may also affect the measures of immunity. Socio-economic status, nutritional status as well as the conditions in which the food grain is stored affects immune response along the pathway. The figure below is an attempt at capturing the conceptual framework in a simplified way.

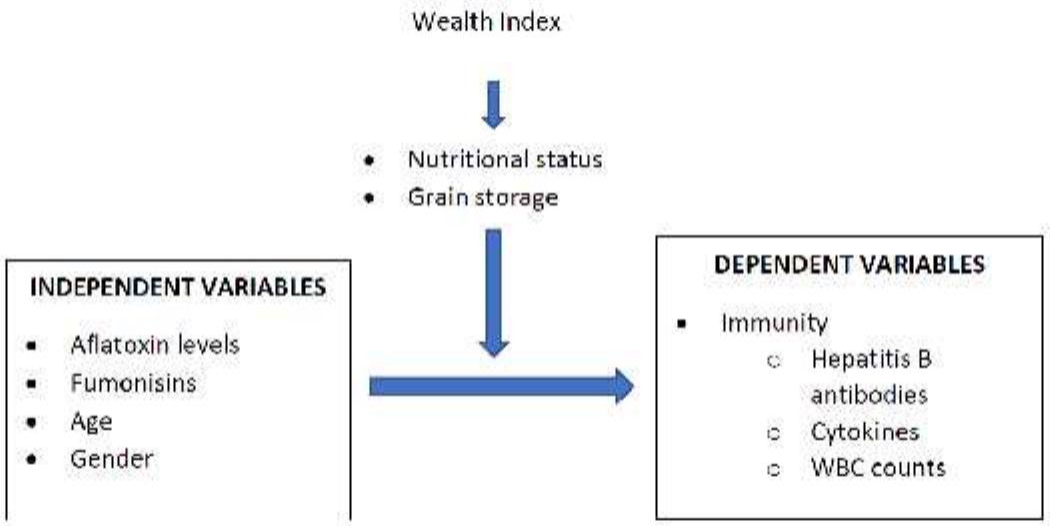


Figure 7: Conceptual Framework of the Study

3.1. Study Design

This was a cross-sectional study.

3.2. Study Area

Makueni County (formerly Makueni District) lies in the South-Eastern part of Kenya, approximately 180km from the capital city Nairobi. It covers an area of 8,009 km^2 and is located between latitude 1º 35´ and 30º0' South, and longitude 37º10´ and 38º 30´East. Elevation ranges between 600 and 1900 metres above sea level from Tsavo to Mbooni Hills respectively(99).

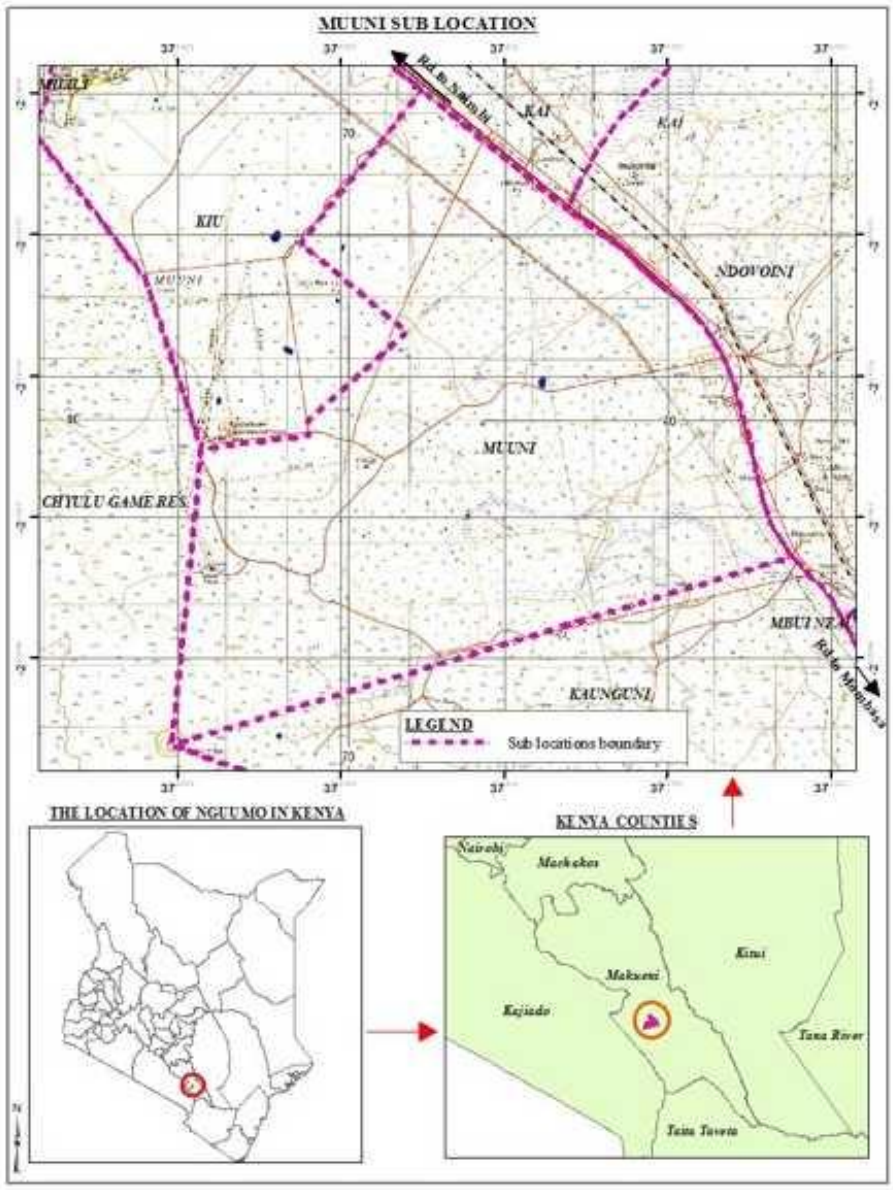


Figure 8: Map of Muuni Sub-Location. Bottom Right Panel: its Position within Makueni County. Bottom Left Panel: Makueni County in Kenya

Makueni is chiefly arid- and semi-arid. There are five forests with a total area of 153 km^2. Of these five, three contain wildlife, and are co-managed by the Makueni County. Due to the proximity to a river and some sparse rain, parts of the South support beekeeping, livestock, and even horticulture. In the more rain-prone North, there is increased farming including dairy, coffee, and food crop

production. However, sustainable agricultural production is limited by inadequate and unreliable rainfall (99).

There are two periods of rain: short rains which occur from October to December and long rains from March to May. The amount of precipitation differs by terrain; in the hilly North, rainfall is between 800mm and 1200mm annually, towards the Central areas <750mm, and the Southern low-lying grasslands receive about 500mm per annum of rain on average. Short rains favour crop production, but their inconsistency and sometimes delay result in drought, threatening food security (99).

Almost 80% of households rely on agriculture as the main source of income. A significant proportion of this population practices subsistence farming with 13% of arable land used for food crops, compared with 5% that is used for cash crops. The main subsistence crops include maize, sweet potatoes, cassava, peas, beans, sorghum and millet. Commercial crops available are coffee, sisal and cotton. Compared to the national average yield (2 tonnes per hectare), Makueni has very low maize productivity at an average of 0.8 tonnes per hectare (99).

Temperatures range from 18º - 33ºC with a mean of between 20ºC and 25ºC. There are three types of soil in Makueni: red clay, volcanic and sandy. The study area, Muuni sub-location, is centrally located in Makueni as shown in the figure below and has sandy soil.

3.3. Study Population

In 2009 population census, the population of Makueni County stood at 884,258. Of this population, about 56 per cent was below 20 years of age, and 29 per cent of the population comprised of children of below ten years old (99).

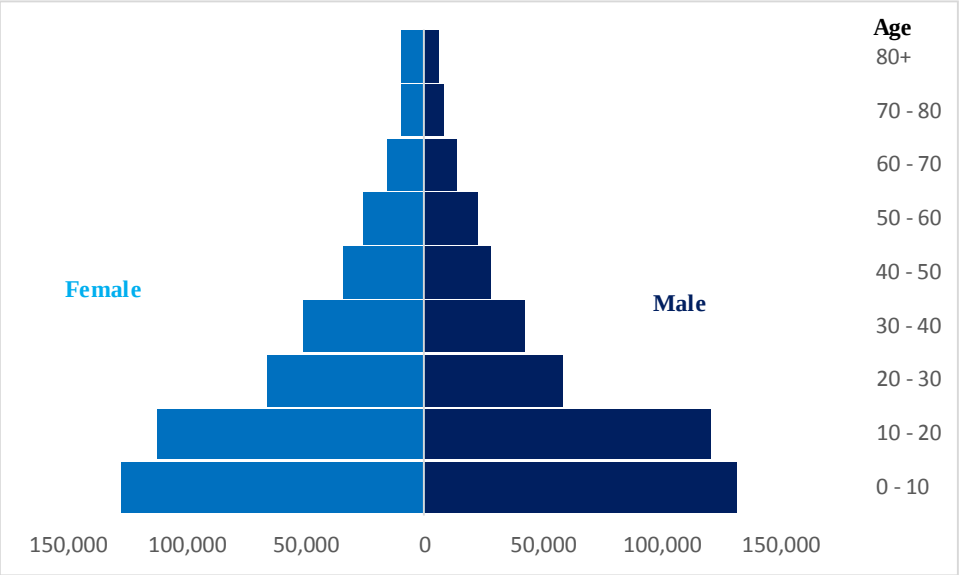


Figure 9: Makueni Population - 2009. Source: Kenya National Bureau of Statistics

During the 2009 census, it was projected that the population of the area would reach 939,879 by 2014, an annual population growth rate of about 1.23 per cent (99).

Makueni's population density is estimated at 110.4 people per km^2, against a country average of 65.3 people per km^2, making Makueni more populated on average. It also has high fertility, which is currently 5.1 children per woman, compared to a national average of 4.6 children per woman, which is attributed to low contraceptive use.

Makueni County performs poorly on most socio-economic indicators. The county scores a 0.48 on the Human Development Index (HDI)—a composite measure of development that combines indicators of life expectancy, educational attainment and income. This is against a national average of 0.52 (99). Poverty is prevalent in the county and manifests itself in other socio-economic outcomes such as poor nutrition, health, and education, as well as a lack of access to basic services. Unemployment is a major challenge in the county, especially among youth. The majority of the population is employed in agricultural activities, with limited opportunities in commercial ventures

and public service. The County ranks 9th out of 47 in poverty levels, with 60% of the residents living below poverty level.

Children aged 1 – 14 years in Muuni sub-location were the focus of this study due to their particular vulnerability and susceptibility to the effects of aflatoxin exposure.

The Enumeration Areas (EAs) used in this study were created during the 2009 Kenya Population and Housing Census and are shown below (Figure 11).

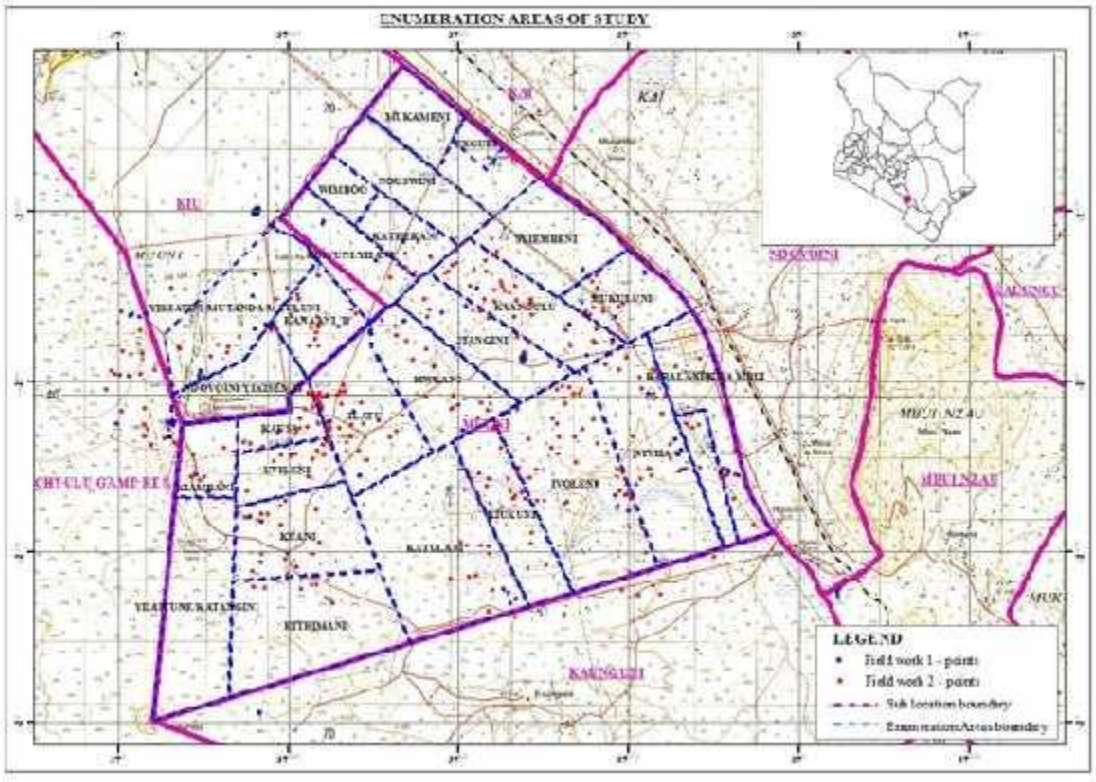


Figure 10: Study Enumeration Areas (EAs) in Muuni Sub-Location

The households selected from the EAs are mapped out in Figure 12.

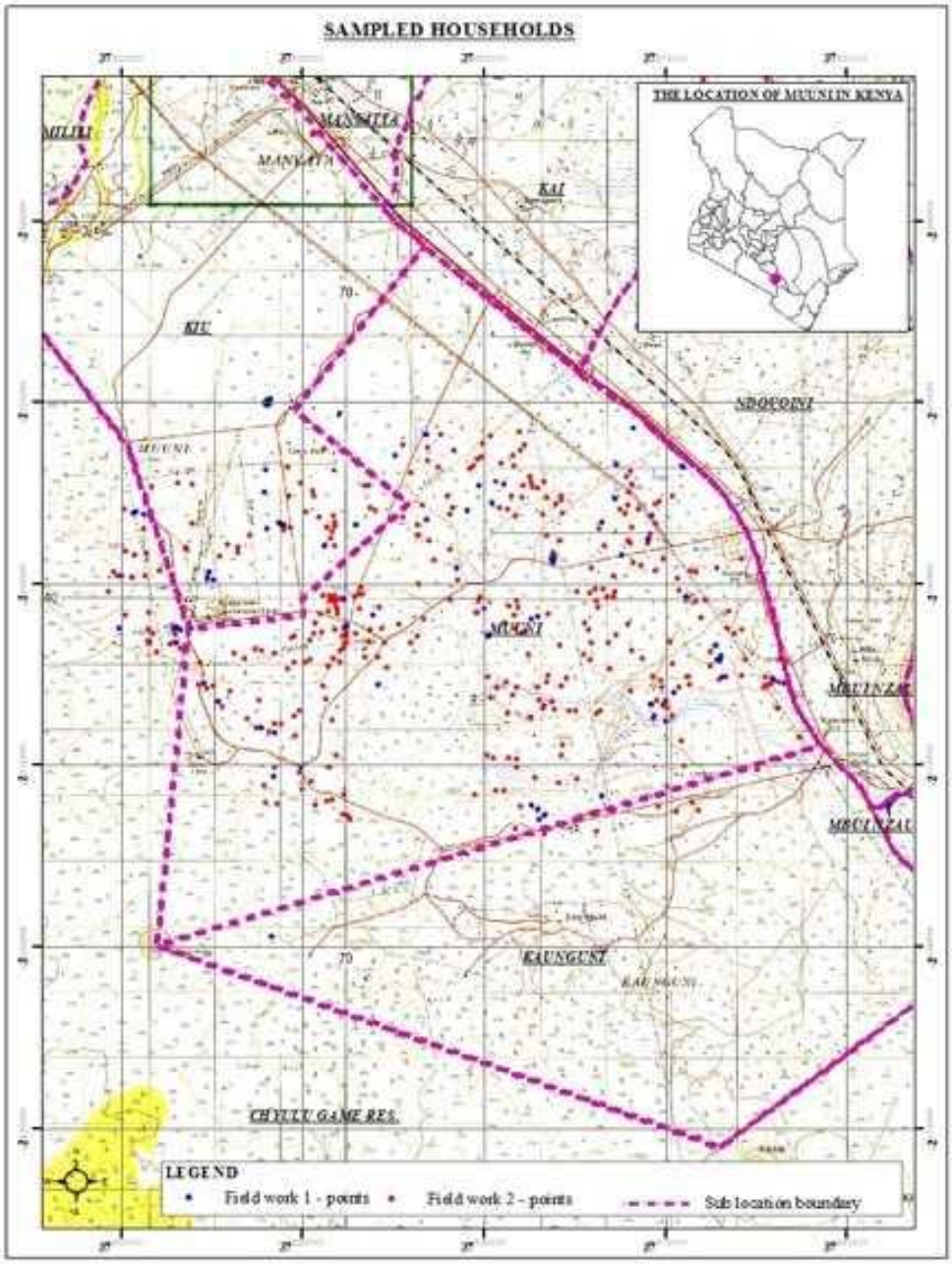


Figure 11: Sampled Households within the Enumeration Areas (EAs)

3.4. Sampling

The design of the study will utilize a two-stage cluster sampling design to produce representative estimates of Aflatoxin prevalence for the specified domain of interest. The 1st stage will involve

random selection of clusters from the database of Enumeration Areas (EAs) in Muuni sub-location. The 2nd stage will be simple random sampling households from the selected EAs. Therefore, the Primary Sampling Units (PSU) will be the Enumeration Areas (EA) as created during the 2009 Kenya Population and Housing Census.

Sampling Frame

For a sample to be drawn, a comprehensive sampling frame was put in place. A standalone statistical frame for Muuni Sub-Location based on the 2009 census Enumeration Areas (EAs) was developed by the Kenya National Bureau of Statistics for the purpose of this Aflatoxin Prevalence Study. To create the sampling frame, a complete listing of the selected EAs was done by identifying and mapping all existing structures and households within the EAs. The household listing process entailed visiting every household and getting information on household composition and numbering of structures. During this process, all the households within the selected EA were listed and stratified as shown in the table below:

Table 1: Stratification of Households in the Selected EA

Stratum	**Type**
SS-1:	Households having a child aged 1-14 years.
SS-2:	Other households

This made identification of the households of interest (those with children 1-14 years of age) easy and ensured that households that did not have the targeted children were not sampled for interviewing.

3.5. Sample Size Calculation

The target sample size for the study was calculated as 425 children aged 1-14 years. The key indicator for the sample size calculation was the prevalence of aflatoxin. The following formula was used to estimate the required sample size:

$$n = \frac{[\ Z\ (r)\ (1\text{-}r)\ (f)\ (\mathrm{k})\ ]}{[\ (\mathrm{e})^2\ (p)\ (n_h)\ ]}$$

Where

"n" is the required sample size, expressed as number of households

"Z" is a factor to achieve the 95 per cent level of confidence

"r" is the predicted or anticipated prevalence (coverage rate) of the prevalence of Aflatoxin indicator

"k" is the non-response factor necessary to raise the sample size for non-response

"f" is the deff (design effect)

"e" is the margin of error to be tolerated at the 95 per cent level of confidence

"p" is the proportion of the total population upon which the indicator, r, is based

"nh" is the average household size.

To estimate the sample the prevalence of aflatoxin in the area from a previous study would have been the ideal one to use. However, no study estimating the prevalence of aflatoxin among children aged 10-14 years in Makueni exists. Therefore, to calculate the sample, the default rate of 0.5, which gives the maximum sample size, was assumed. "Deff" (design effect) was taken at a default value of 1.7. To cater for an anticipated 10% non-response, "k" was put at 1.1. The margin of error was set at ± 6.5%.

The resulting number of children required for the study is **425** children. Given that only one child was to be selected for interview in every household, a total of 425 households drawn from 16EAs were visited for the study.

3.6. Sample Selection Criteria

The number of households selected in each EA was proportional to the number of households with eligible children (1-14 years) in the EA. Selection of these households was done using systematic random sampling method that consisted of selecting the sample households from the listing with a random start by the following criteria:

With "L" as the total number of households listed in the cluster, "Random" as a random number between zero and one (0-1), and "n" the number of households selected in the cluster:

I = L/n - the sampling interval.

(1) The first selected sample household was k (k is the serial number of the household in the listing) if and only if:

$(k-1)/L < Random \leq k/L$

(2) The subsequent selected households are those that had serial numbers:

$k + (j-1)*I$, (rounded to integers)

for $j = 2, 3, \ldots n$;

Random numbers were different and independent from cluster to cluster.

<u>Selection of children</u>

During data collection, all the children aged 1-14 years in each selected household were eligible for interview as long as they had documentary evidence of vaccinations. However only one eligible

child was selected randomly, from each sampled household, using the Kish grid method (see Appendix 2).

3.7. Description of Clinical Evaluations and Tests

In the field, the primary data was collected using the Household Questionnaire (Appendix 3) after the requisite consenting and accenting procedures. The anthropometric assessments were taken as detailed in the follow- up test. Then venous blood was drawn and samples of stool, urine and grain were collected. Only the total blood counts and stools were examined at the study site shortly after collection. The rest were placed in dry ice for transportation back to the Nairobi KAVI-ICR laboratories for analysis. The serum samples for aflatoxin levels and urine for urinary sphingolipid assessments as the measure of fumonisins were airfreighted in dry ice to Professor Jia Sheng Wang's laboratory at the University of Georgia at Athens USA, where they were analyzed.

a) Wealth Index

Makueni County has a poverty level of 60.6% that is higher than the national average of 45.2% (99). A general visual scan of Muuni sub-location gives an impression of a relatively homogenous community living on marginal land. Five variables were analyzed using multivariate statistical technique called Principle Components Analysis to generate an index. This was then used to categorize the households into five wealth quintiles that were merged into 3-poor, middle and rich categories following the small numbers in the categories. (Detailed in Appendix 4)

b) Dietary Diversity Score (DDS)

This score system complements anthropometric data in assessing nutritional status. Using the questionnaire where a listing of the various food types that are consumed the study participants in the previous 24 hours. The 9 food groups are scored from 0 to 9 moving from lowest to the highest. They consist of starches, dark leafy vegetables, other vitamin A-containing fruits or vegetables,

other fruits and vegetables, organ meat, flesh meat, eggs, legumes and milk. Food Agricultural Organization (FAO) classifies DDS into lowest (score of </=3), medium (score of 4-5) and high (score of >/=6). The higher the DDS, the higher the possibility that dietary requirements were being met adequately.

c) Anthropometry

We used the method prescribed by UNICEF for assessing the nutritional status of young children in household surveys (100).

Two research assistants after selecting the index child using the Kish Grid Method (Appendix 2) measured the height using a sliding board with a precision of 0.1cm with those younger than 2 years lying down. Measurements were taken in duplicate and the mean of the two measurements was used for analysis.

A Salter weighing scale with a precision of 100gm was used to measure to the nearest 0.01kg. The WHO reference population was used to derive standard deviation units (z-scores) from the median of the reference population. These were expressed as Weight-for age (WAZ), height-for-age (HAZ), Basal Mass Index for age (BAZ) and weight-for-height.

MUAC was done for all children using a MUAC measuring tape.

The special tape is divided with three different colours: red, yellow and green indicating severe acute, moderate and normal nutritional status respectively, was used for measuring the MUAC of each child (cut-off values according to WHO child growth standards).

The anthropometric information was recorded in the format below.

Childs name and number----------

Weight in kilograms----------

Length/height in cms------- Child <24 months, measure lying down

Child >24 months measure standing

MUAC in cms----------

Measurement/investigator identification code----------

Results of measurement

Measured----------

Not present----------

Refused----------

Others----------

d) Determination of aflatoxin B1 lysine adducts using HPLC with fluorescence detection

This test was done in Professor Jia-Sheng Wang's laboratory at the University of Georgia, Athens, USA and the detailed methodology was described by Qian *et al.*,(101).

The principle of the methodology

Aflatoxin B_1-lysine adduct bound to serum albumin is released after digestion with pronase. The Aflatoxin B_1-lysine is further concentrated through an Oasis Max cartridge. The concentrated Aflatoxin B_1-lysine is separated under High Performance Liquid Chromatography (HPLC) and detected in a fluorescence detector.

The methodology

Serum samples in micro-centrifuge tubes are placed in 56ºC water bath for 30 minutes to deactivate pathogens. Sample aliquots are used to determine albumin and total protein concentrations using a spectrophotometer under the UV wavelength of 630nm and 595nm respectively. Appropriate volumes of pronase are added to 150ul of serum samples for digestion. The samples are then loaded onto an oasis Max Cartridge [Milford, MA USA] after priming with 100% methanol. The cartridges

are then washed, and eluted with 2% formic acid in methanol. Eluents are evaporated to dryness using lab concentrator cold trapper and eventually reconstituted with 10% methanol. The samples are loaded on an Agilent 1200 High Performance Liquid Chromatography (Agilent Technologies, Wilmington, DE, USA) for separation. The solvents used in the mobile phase include 20mM ammonium phosphate monobasic (pH 7.2) and 100% methanol respectively. Chromatographic separation is performed on an Agilent C18 column. Injection volume is 100ul and the flow rate is maintained at 1ml/min. A single run is completed in 25 minutes. The excitation and emission wavelengths are 405nm and 470nm, which are set for the fluorescence detector online with HPLC software. Aflatoxin B_1 lysine adducts concentration is adjusted for albumin concentrations. The detection limit is 0.04pg/mg.

Calculation of AFB-1 lysine adducts and Results Interpretation

A standard curve is generated using pure commercially available aflatoxin B1.

Standard curve: $y = ax + b$ y=AFN-lys concentration; a and b generated from correlation analysis of standard curve with peak area, and x is the peak area for each sample

AFB-Lys content in 150µl: $y = x$ ng/ml $\times 150\mu l = 0.15X$ (ng)

After adjusting with recovery rate of approximately 75%, we got the final value. Then we achieved the value adjusted with corresponding serum albumin already measured. Final relative content is expressed in pg/mg albumin.

e) Determination of grain aflatoxin B1

This test was run locally in Kenya. In brief, the REVEAL Q+ FOR AFLATOXIN test method provided by the Neogen Corporation is a single-step lateral flow immunochromatographic assay based on a competitive immunoassay format. The test provides quantitative analysis for the

presence of Aflatoxin. Using an aflatoxin-antibody particle complex coated test strip and the Neogen AccuScan III and AccuScan Pro readers.

Sampling protocol

i. Estimate the number of homesteads from which to sample in a region.
ii. Ensure representativeness of the samples collected from every homestead by collecting samples from all sacks in each homestead.
iii. Collect samples using sampling probe (Figure 6)
iv. Sampling probe picks up small quantities of the sample from different portions of each sack
v. Combine all the incremental samples from every sack to form an aggregate sample of about 1-5kg/ from every homestead.

Figure 12: How to use a Sampling Probe

AccuScan Pro Reader setup

i. Select the QR code icon on the reader. Place the code (provided in the kit) into the cartridge and insert the cartridge into the reader.

ii. Return to the home screen and select the test strip icon. Touch the mycotoxin category and then select the Aflatoxin Q+ test type.

Sample preparation and extraction

i. Prepare 65% ethanol by mixing 6.5 parts of ethanol with 3.5 parts distilled or deionized water for each sample.

ii. Obtain a representative sample. Grind the sample so as at least 75% of the ground material passes through a 20 mm mesh sieve, about the particle size of instant coffee.

iii. Extract at ratio of 1 part sample to 5 parts 65% ethanol. For example, combine 10 g of ground sample with 50 ml of 65% ethanol. Vigorously shake for 3 minutes at 200 revolutions per minutes.

iv. Allow sample to settle and then filter with a filter paper.

Test procedure

i. Place the appropriate number of red sample dilution cups and clear cups (provided with kit). Label cups if necessary.

ii. Add 500 µl of sample diluent to each red sample dilution cup.

iii. Add 100 µl of sample extract to red dilution cup with sample diluent. Mix by pipetting up and down at least 5 times.

iv. Transfer 100 µl of diluted sample into a new clear sample cup.

v. Place a new Reveal Q+ for aflatoxin test strip with the sample end down into the sample cup and set timer for 6 minutes. Ensure the test strip comes into contact with liquid and begins to wick.

vi. Remove the strip from the sample cup after it has developed for 6 minutes.

Reading the test result

i. Select the assay type (e.g. aflatoxin) from the menu and ensure the device lot number matches the lot ID number selected on the reader. Failure to update the lot-specific QR code will cause inaccurate results.

ii. Fully insert the Reveal Q+ test strip into the black R labeled cartridge adapter with the sample end first and results facing out.

iii. Insert the cartridge with the test strip side up into the AccuScan Pro. The reader will automatically begin analyzing the cartridge.

iv. The AccuScan reader will analyze the test strip and results will be displayed and stored in the reader.

NB: The test strip should be read within 1 minute of completion of the 6 minute incubation.

f) Measurements of fumonisins in urine

Measurement of urinary fumonisins like serum aflatoxin levels was done in Professor Wang's laboratory in USA.

The principle of the methodology

Fumonisins are ubiquitous contaminants of cereal grains produced mainly by *Fusarium verticilliodes*. There are over 15 types of identified Fumonisins but only Fumonisin B_1 and B_2 have been confirmed to be biologically important. Fumonisins have structural similarity to the long chain base backbones of sphingolipids, specifically sphingosine and Sphinganine. Exposure to fumonisins disrupts the de novo ceramide synthesis process by altering the ratio of free sphinganine (Sa) and sphingosine (So). Riley *et al.,* 1996 determined that monitoring Sa So ratio in urine could be used as a biomarker for Fumonisin exposure. The methodology was validated by Cai *et al.,* 2010.

The Methodology

Homogenized urine is centrifuged at 1500×g for 10 min. The pellets are collected and washed once with 1.0ml of HPLC- grade water. 500ul of 1xPBS(pH7.4) and 20µl of 0.5 µM D-erythro-C20-sphingosine (C20So) is added to the pellets. The mixture is then briefly vortexed and 3 ml of ethyl acetate is added to each pellet samples. The samples are gently rotated for 30 minutes on a rugged rotator (Glascol, Terre Haute, IN, USA). The mixture is centrifuged again at 1500×g for 10 min for phase separation. 2ml of organic phase is transferred to a glass tube and evaporated to dryness in vacuum at 35ºC in a Labconco Centrivap (Kansas City, MO). The residues are then reconstituted in 275µl of 80% methanol. 121µl of the supernatant is transferred to a glass HPLC vial for analysis.

The fluorescent derivatives of So, Sa and C20So are resolved on an Agilent 1200 liquid chromatography system (Agilent Technologies, Wilmington, DE, USA). Chromatographic separations are performed on a Zorbax Eclipse XDB-C18 column (5 µm particle size, 250 × 4.6 mm, Agilent Technologies). The mobile phase consists of a linear gradient starting from 5 mM Triethylammonium Formate [TEAF] (pH 4.3)-methanol-acetonitrile (15/45/40, v/v/v) to methanol-acetonitrile (60/40, v/v) over 32 min at a flow rate of 1.0 ml/min. Excitation and Emission wavelengths are 340 nm and 455 nm, respectively. The peaks of Sa and So are identified by comparison with retention time of Sa and So standard. Recovery status is monitored using the relative areas of C20So in measured samples versus reference standard. Concentration of Sa and So is determined using external calibration curves generated from a serial of blank urine samples spiked with Sa and So standard.

The levels of urinary Sa and So are adjusted for creatinine levels. Creatinine in urine is analyzed using the 96-well Creatinine Assay Kit from Cayman Chemical (Ann Arbor, MI, USA). The analysis is based on a modified Jaffe colorimetric method that measures the difference in

absorbance (495 nm) of the creatinine-picrate complex before and after acidification. The assay was carried out according to manufacturer's instructions, and absorbances were measured using an ELx808 Absorbance Microplate Reader from BioTek Instruments, Inc. (Winooski, VT, USA).

g) Full Blood Counts

This test was performed in the field shortly after the blood samples had been collected. Humacount 3^R part machine haematology analyzer was the equipment used according to standard operating procedure.

Principle

For the determination of haematological measurements using Humacount 3-part haematology analyzer after the standard preparation of the samples for analysis. This was one of the test that was run in the field within 1-3 hours after collection.

For the determination of blood cell counts (white blood cells, red blood cells, and platelets), haemoglobin measurement and the calculation of haematological indexes (mean cell volume, mean corpuscular haemoglobin and mean cell haemoglobin concentration). Haematology measurements are obtained using Humacount 3 part machine.

Quality Control

Quality control (Q.C.) is a feature to monitor the operation and stability of the instrument. The HUMACOUNT offers 3 levels of separate Q.C. measuring capability, i.e. you can use 3 different blood controls in the same time to check stability. The manufacturers of blood control usually offer at least 3 different levels: 1 low abnormal, 1 normal (suitable for calibration as well), and 1 high abnormal blood controls.

If you program them to levels 1, 2 and 3, you can measure them every day before starting daily routine measurements to ensure stability of the system. If some of the results are out of the

permissible range, try another vial of blood control to see if the results are outside too on this control. If the second control confirms that the results are outside the permissible range, you can recalibrate the analyzer.

The stability of an open vial of blood control depends on the manufacturer, but it is usually 14 days only. The closed vials can be used longer (typically for 3 months).

Controls are stored at 2-8ºC and brought to room temperature on a roller mixer before use.

Routine Utilization

1. Sample Handling:

1.1. Anti-coagulant

Since some time will usually elapse between collection of samples and counting, it is necessary to preserve the sample with an anti-coagulant to prevent large groups of cells forming into clots or lumps of cell matter that will clog the cell counter. Choice of anti-coagulant is very important, as some anticoagulants will affect the shape and size of blood cells. In general EDTA, preferably sodium or potassium-based, is the only anti-coagulant recommended for use with electronic blood counters.

Care must be taken when using homemade containers pre-dosed with EDTA. If the container is not filled completely with blood, the ratio of EDTA to blood may reach a level, which results in osmotic transfer from the RBCs, shrinking them. The ratio of EDTA to blood should not exceed 3mglml. Generally, we recommend using manufactured sample tubes containing the necessary amount of EDTA.

1.2. Taking blood

After filling the sample tubes to the required level, do not forget to invert the sample tube – never shake several times to activate the anti-coagulant by correct mixing. It can be a real

problem especially when taking a small amount of blood from babies or small animals. Try to keep the 3 mg/ml maximum EDTA

1.3. Storage of samples

Anti-coagulant requires time to take its effect on the sample. On the other hand, it will destroy the cells by time. The fresh samples require at least 5-10 minutes to stabilize after taking, and they should be measured within 6 hours at standard room temperature (25ºC).

This rule basically applies for the 3-part WBC differential. The granulocytes are destroyed first, after 12-16 hours you get a much different histogram with much higher MID, because ageing will result in histogram looking over- lysed. The correct time of collapsing of white blood cells depends on the patient blood.

If you want to measure later, put the samples into refrigerator to extend their stability. Care must be taken in this case, because the samples must warm up to room temperature before measuring them. Roll the sample tubes between your palms to speed up warming. This will help mixing the sample, too. If the sample is not mixed correctly, PLTs and large WBCs can stuck together forming much bigger particles, which will result in distorted (lower) cell counts and the appearance of non-existing populations (e.g. in high region of WBC histogram).

2. Procedure

3. Parameters recorded

3.1. Measuring blood

You can make big mistakes if the sample is not correctly homogenized (all counted parameters will be higher), contains micro-bubbles (lower effective sample value, lower counted parameters), or cold (PLT count will be bad). At least 30 minutes is required for a sample tube to reach the room temperature if it was in refrigerator before.

It is recommended to use a rotary mixer to keep samples homogenous during the measuring session. If you do not have sample mixer, invert the tubes upside down several times before sampling. Never shake them to avoid formation of micro-bubbles inside; because small bubbles prevent correct sampling (air is sampled instead of blood).

Put your sample tube onto the sample holder and press the run key.

After measurement, check the results and histograms to identify any sort of disorder. Repeat the measurement in case of any doubt, you can adjust the lyse setting if necessary.

Validation (acceptance of the results) is a very important process. During validation the hematologist or clinician can sort out those reports, which require confirmation by manual counting under microscope. Do not forget, use the 3-part differential results to identify the abnormal cases, and make manual differential count on them if you need better WBC qualification.

3.2. Using pre-dilution Mode

Pre-dilution mode is used in two cases:

1. if capillary tube (e.g. 25 µl of volume) is used for taking blood from the patient,
2. In case of linearity error due to high cell concentration.

In both cases the sample must be prepared before measurement. Create the necessary dilution of whole blood (or capillary blood) sample according to the set-up pre-dilution mode (you can check it by selecting pre-dilution mode in Measurement menu, the ratio appears on the screen 1:3 or 1:10)

Prepare at least 100 µl of pre-diluted sample, because in pre-dilution mode the analyzer samples double volume (approx. 60 µl).

Sample preparation for 1:10 Pre-dilution Mode

I. Put 250µl* of clean diluent or isotonic solution into a clean cup or test tube.

II. Add 25µl* of whole or capillary blood and homogenize it by rotating the cup circularly the above sample is suitable for measurement in 1:10 predilution mode.

Sample preparation for 1:3 Pre-dilution Mode

I. Put 150µl* of clean diluent or isotonic solution into a clean cup or test tube.

II. Add 50µl* of whole or capillary blood and homogenize it by rotating the cup circularly the above sample is suitable for measurement in 1:3 pre-dilution mode.

Calibrating Pre-dilution Mode

i. Calibrate the analyzer in normal mode using blood control.

ii. Create the desired pre-dilution of blood control (1:10 or 1:3) with the method you wish to use for patient samples as well.

iii. Activate pre-dilution mode in Calibration menu.

iv. Perform calibration with the prepared sample.

v. After accepting the pre-dilution factors deactivate pre-dilution mode in Calibration menu to avoid mix-up of calibration factors.

vi. Make sure to use the same method and procedure (tools, settings of pipettes, dispenser volume, etc.) to creating samples for measurement, too. By doing so you will be able to compensate the inaccuracies of your dilution procedure.

***Exact volumes may vary. Keep the ratio: e.g. 1 unit of sample + 3 units of diluent (for 1:3 mode) constant**.

4. Parameters recorded

The following parameters will be seen on the printout:

- Total WBC x 10^9/L
- Neutrophils x 10^9/L
- Lymphocytes x 10^9/L
- Monocytes x 10^9/L
- Eosinophils x 10^9/L
- Basophils x 10^9/L
- Haemoglobin (g/dl
- RBC x 10^{12}/L
- Haematocrit
- MCV fl
- MCH pg
- MCHC
- Platelets x 10^9/L
- RDW

h) Measurement of Hepatitis B surface antibodies (anti-HBs) using Bioelisa anti-HBs kit

Measurement of Hepatitis B surface antibodies (anti-HBs) using Bioelisa anti-HBs kit in KAVI-ICR laboratory at The University of Nairobi.

Principle of the test

The use of Hepatitis B vaccine, has increased the usefulness of anti-HBs determination as a tool in pre-immunization "screening" (individuals should be negative for HBsAg, anti-HBc and anti-HBs)

and in monitoring seroconversion after immunization. An anti-HBs concentration greater than10 mIU/ml is considered as protective.

Bioelisa anti-HBs is a direct immunoenzymatic method of the "sandwich" type in which the samples to be analyzed are incubated in wells of a microplate that are coated with highly purified HBsAg (ad and ay subtypes). If a sample contains anti-HBs, it will bind specifically to the HBsAg in the well. After washing to remove residual sample, HBsAg conjugate to peroxidase is added and will bind to the antigen-antibody complex formed during the first incubation. After this second incubation and washing, an enzyme substrate containing a chromogen is added. This solution will develop a blue colour if the sample contains anti-HBs. The blue colour changes to yellow after blocking the reaction with sulphuric acid. The intensity of the colour is proportional to the concentration of anti-HBs present in the test specimens. The concentration of antibodies in the sample can be calculated by interpolation from a calibration curve.

Specimen collection and preparation

i. Use fresh serum. Samples can be stored at 2-8°C for 3 days. For longer periods, samples should be frozen (-20°C). Avoid repeated freezing and thawing.
ii. Samples showing visible particulate matter should be clarified by centrifugation.
iii. Serum samples should not be heat inactivated, since that may cause incorrect results.

Materials

i. Bioelisa Anti - HBs kit with microplate wells, conjugate, substrate, adhesive seals and controls.
ii. Multichannel pipettes and micropipettes (50 µl, 100 µl, 1000 µl) and disposable tips.
iii. Distilled or deionized water.

iv. Incubator at 37°C ± 1°C

v. Timer.

vi. Microplate reader with a 450 nm filter. Reference filter of 620 or 630 nm is advisable.

vii. Automated Elisa washer (Manual washing can be done).

viii. Disposable gloves and timer.

ix. Appropriate waste containers for potentially contaminated materials.

x. Disposable V-shaped troughs.

xi. Absorbent tissue or clean towel.

Precautions

i. Avoid contact of reagents with the eyes and skin. If that occurs, wash thoroughly with water.

ii. Wear gloves.

iii. Do not pipette by mouth.

iv. Do not smoke.

v. Dispose all used materials in a suitable biohazardous waste container. Remains of samples, controls, aspirated reagents and pipette tips should be collected in a container for this purpose and autoclaved 1-hour at 121°C or treated with 10% sodium hypochlorite (final concentration) for 30 min before disposal. (Remains containing acid must be neutralised prior addition of sodium hypochlorite).

vi. Certain reagents in this kit contain sodium azide as preservative. Sodium azide may react with lead or copper pipes and plumbing creating highly explosive metal azides. Flush drains with water thoroughly after disposing of the remains of reagents.

Handling instructions

i. Adjust washer to the plate used (flat bottom) in order to wash properly.

ii. Do not mix reagents from different lots.

iii. Do not use reagents after expiration date.

iv. Do not use the reagent if you observed any change in appearance of components included in the kit.

v. Extreme care should be taken to avoid microbial contamination and cross contamination of reagents.

vi. Use a new pipette tip for each specimen and each reagent.

vii. It is very important to prepare the substrate-TMB solution just 5-10 minutes before use. Keep it in a well-sealed container and avoid light exposure.

viii. Soaps and/or oxidising agents remaining in containers used for preparation of substrate-TMB solution can interfere with the reaction. If glass containers are used to prepare the solution, they should be washed with 1N sulphuric or hydrochloric acid, rinsed well with distilled water and dried before use. We recommend using disposable plastic containers.

Storage and stability of reagents

i. The components remain stable through the expiration date shown on the label if stored between 2-8°C.

ii. The bag containing the microplate should be brought to room temperature before opening to avoid condensation in the wells. Once opened the bag, microplate strips are stable for 3 months at 2-8°C in the plastic bag tightly sealed, with the silica gel.

iii. Once diluted, the washing solution is stable for two weeks if stored between 2-8°C.

iv. Store the chromogen in the dark. As the substrate-TMB solution is not stable once prepared, instructions for its use should be closely followed.

Reagent preparation

i. Allow all the reagents to reach room temperature (20-25°C) before running the assay.
ii. Gently mix all liquid reagents before use.
iii. Dilute the concentrate washing solution 1/10 with distilled or deionised water.
iv. For one plate, mix 50 ml of the concentrate solution with 450 ml of water. If less than a whole plate is used, prepare the proportional volume of solution.
v. Preparation of substrate chromogen solution (To be prepared not more than 5 minutes before its addition). If the entire plate is used add 280 µl of chromogen (TMB) to the bottle containing the substrate buffer (14 ml) and mix well. If the entire plate is not used, follow table 1. The final solution should be pink; discard if it becomes blue.

Table 2: Hepatitis B Antibodies Test (Reagent Preparation)

Strips required	1	2	4	6	8	10	12
Substrate buffer (ml)	1.0	2.0	4.0	6.0	8.0	10.0	12.0
Chromogen (TMB) µl	20	40	80	120	160	200	240

NOTE: The TMB is dissolved in DMSO. As the melting point of the DMSO is 18°C, the chromogen solution should be allowed to reach a temperature of 20-25°C, and be well mixed before use.

Sample Preparation

i. Qualitative assay (or quantitative until 100 mIU/ml) the sample is used undiluted.
ii. Quantitative assay: For quantitative determination of samples with a concentration of over 100mIU/ml they must be diluted as follows:

1) Prepare 200 µl of dilutions 1/10 and 1/100 from each sample to be evaluated:

2) Dilution 1/10: 20 µl of sample and add 180 µl of negative control.

3) Dilution 1/100: 20 µl of the dilution 1/10 of the sample and add 180 µl of negative control.

4) When the concentration of a sample is suspected to be higher than 10 000 mIU/ml, higher dilutions should be done from the 1/100 dilution following the same protocol (20 µl of diluted sample +180 µl of negative control).

Assay Procedure

1. Use only the number of strips required for the test. Reserve 8 wells for blank and controls. Transfer 100 µl of negative control to 2 wells and 100 µl of high positive calibrator to 2 wells and 100 µl of low positive calibrator to 3 wells. Leave a well empty for the substrate blank.
2. Qualitative assay: Transfer 100 µl of each sample to be tested into the assigned well. Quantitative assay: Transfer 100 µl of each sample undiluted, diluted 1/10 and diluted 1/100 to the appropriate wells. For each sample 3 wells are needed.
3. Cover the plate with the adhesive seal, mix gently and incubate for 1 hour at 37°C.
4. Remove and discard the adhesive seal. Aspirate the contents of the wells and fill them completely (approximately 350 µl) with the diluted washing solution. Repeat the process of aspiration and washing 3 more times. Ensure that each column of wells soaks for at least 15 seconds before the next aspiration cycle. After the last washing blot the inverted microplate on absorbent tissue to remove any excess liquid from the wells.
5. Add 100 µl of conjugate to all wells, except the blank. Avoid bubbles upon addition.
6. Cover the plate with the adhesive seal, mix gently and incubate for 30 minutes at 37°C.
7. During the last 5-10 minutes of this incubation prepare the substrate-chromogen solution as in the table above.

8. Remove and discard the adhesive seal. Wash the plate as in step 4.
9. Add 100 µl of substrate-TMB solution to each well, including the blank.
10. Incubate for 30 minutes at room temperature (20-25°C).
11. Stop the reaction by adding 100 µl of stopping solution in the same sequence and time intervals as for the substrate-TMB.
12. Blank the reader at 450 nm with the blank well and read the absorbance of each well, within 30 minutes. It is recommended to read in bichromatic mode using a 620 - 630 nm reference filter.
13. Since the anti-HBs is not for diagnosis, any positive samples will not be repeated for confirmation.

Quality Control

Results of an assay are valid if the following criteria are accomplished:

i. Substrate blank. Absorbance value must be less than or equal to 0.100.

ii. Negative control mean (NCx). Calculate the mean absorbance value of negative control after subtracting the blank. The mean value obtained should be lower than 0.100.

    i. NCx < 0.100.

iii. Low positive calibrator mean (LPCx). Calculate the mean absorbance value of low positive calibrator after subtracting the blank. The mean value obtained should be higher than 0.070 and besides, should be at least the double of negative control mean.

    i. LPCx > 0.070

    ii. LPCx ≥ 2 x NCx.

iv. High positive calibrator mean (HPCx). Calculate the mean absorbance value of high positive calibrator after subtracting the blank. The mean value obtained should be within 0.800 and 2.100.

   i. $0.800 \leq HPCx \leq 2.100$

v. If any value is outside the specified condition the assay should be repeated.

Results

Qualitative assay

- Calculate the mean absorbance of the low positive calibrator. This is the cut-off.

  Cut-off: LPCx

- Divide the sample absorbance by the cut-off value.

  Positive: ratio absorbance/cut-off ≥ 1.0

  Negative: ratio absorbance/cut-off < 0.9

  Equivocal: ratio absorbance/cut-off $\geq 0.9 < 1.0$

Quantitative assay

Plot in linear-linear graphic coordinates the anti-HBs concentrations of the negative control (0 mIU/ml), low positive calibrator (10 mIU/ml) and high positive calibrator (100 mIU/ml) on the abscissa (x axis) against their corresponding mean absorbance values on the ordinate (y axis). Draw a line through these three points. The concentration in mIU/ml of each sample can be derived from its absorbance using the calibration curve. If the sample was diluted the result should be multiplied by the dilution factor in order to obtain the actual anti-HBs concentration present in the serum.

NB: Refer to the kit insert for an example of the graph.

Interpretation of the results

A positive anti-HBs result indicates an immune response to hepatitis B virus infection, an immune response to vaccination or presence of passively acquired antibodies. Nevertheless, low levels of anti-HBs do not always mean protection against HBV. It is generally accepted that an anti-HBs concentration equal to or greater than 10 mIU/ml is indicative of resolution of a past infection or of protection in vaccinated people.

Limitations of the procedure

As with other serological tests, the results obtained with Bioelisa anti-HBs serve only as an aid to diagnosis and should be interpreted taking into consideration the patients' clinical history. Optimal assay performance requires strict adherence to the assay procedure described. Deviation from the procedure may lead to aberrant results. Although quantitative results show a good correlation with other assays for the majority of samples, there is some degree of variation, and even important variations for some individual samples. This is due to several factors such as the variability of HBsAg, which has many different antigenic subtypes, the characteristics of anti-HBs contained in the sample (specificity and affinity to different epitopes) and others related to the assay (manufacture method, calculation of results, etc.). Results higher than 100 mIU/ml should not be extrapolated from the calibration curve because there is saturation of the assay, with consequent loss of linearity and the extrapolated concentration would be incorrect. In this case the sample should be diluted or the result must be reported as higher than 100 mIU/ml.

The use of Hepatitis B vaccine has increased the usefulness of anti-HBs determination as a tool in monitoring seroconversion after immunization. Bioelisa anti-HBs is a direct immunoenzymatic method of the "sandwich" type in which the samples to be analyzed are incubated in wells of a

microplate that are coated with highly purified HBsAg (ad and ay subtypes). The first step is qualitative assay where one looks for the presence of antibodies. If this is positive, then the next step is the quantitative assay in three wells of undiluted, diluted 1/10 then 1/100 all in different wells. This is then read at 450nm within 30 minutes in each well.

Sample analysis

1. Remove reagents stored in the fridge and allow them to reach room temperature.
2. For reagents requiring reconstitution, reconstitute as per the manufacturer's instructions ensuring that you don't shake to prevent foam formation. Reagents not requiring reconstitution are ready for use (avoid shaking to prevent foam formation).
3. N/B: Refer to the kit inserts for stability of each reagent.
4. Samples for analysis are loaded using a work list that can be accessed directly from Routine menu → Work list setup or indirectly after Load on Fly from the System Monitor mask. Analysis samples should be clearly noted with their identification numbers. The work list is displayed with different colours; grey for rotors not configured, light green for rotors having at least one sample configured and dark green for the rotor selected by the user. The Methods list area displays all test available; tests in white are for methods available in the active reagent configuration while dark ones refers to methods with no reagents on board.
5. Select the next available position ensuring that you always tick on the low volume tab.
6. Enter the patient's details, click on the method tab to activate the tests and select all the tests required for the particular sample then click save.
7. Click on the SYS. MON and select the green button, enable the work list and select the relevant rotor (Work list 1 corresponds to Rotor A, Work list 2 Rotor B, Work list 3 Rotor C, Work list 4 Rotor D and then back to Rotor A) then click ok. A message is displayed on

the screen enquiring whether samples are already loaded or not. Click on "place rack". The rack is positioned showing the position to load samples then click ok for analysis to begin.

8. Results will be displayed on the workstation after analysis is complete. Select the results per patient and double click on display, print and sign.
9. NB: Patients samples were only analyzed after obtaining correct control results which had to be run daily before analysis of any patient samples.

i) Liver function test in KAVI-ICR laboratory at The University of Nairobi.

ILab Aries Analyzer system version 2.0 was used to measure AST, ALT, total protein, albumin, GGT as per the operators manual.

KENYA AIDS VACCINE INITIATIVE

LABORATORY

SOP Title: Clinical Chemistry by ILAB Aries				
SOP No: CL 65 Version: 2.0 **Supercedes:** **Effective Date:**				
Prepared By	**Name**	**Signature**	**Date (dd/mmm/yyyy)**	
Approved by				
SOP Periodic Review	**Name**	**Signature**	**Date Reviewed**	**Next review Date**

Revision History	**Version # [0.0]**	**Revision Date [dd/mmm/yyyy]**	**Description (notes)**
	2.0	08/Jun/2016	See summary of changes

1. INTRODUCTION

Chemistry tests will be performed using ILAB Aries instrument that has the following main parts:

1.1. THE SAMPLING ARM – It's the arm that performs all sampling operations. It reaches the aspiration points of Reagents plate, Samples plate, the delivery point of the Reactions plate or ISE module and the two Washing wells.

1.2. DILUTER - It allows performing of all the operations of aspiration and delivery of the liquid up till the capacity of 1000µl.

1.3. REACTION PLATES – It contains racks with reaction cuvettes. Involves aspiration and dispensing of the reagents and the samples by the sampling arm in the reaction cuvettes, optic reading of the cuvettes in incubation by the photometer, along with the relative positioning of the reaction plate, washing of the filled cuvettes by a washing station.

1.4. SAMPLES PLATE – The compartment where samples, calibrators and controls are loaded.

1.5. REAGENT PLATE – The compartment where reagents are loaded. It has cooler modules that ensure cooling of the reagents.

1.6. PHOTOMETER – Allows the direct readings of the Optic Density of the reaction that takes place in any cuvette of the Reaction plate. It has a sample channel for direct reading of OD value and a reference filter for its reference value. Light source is the halogen lamp. It also has Filter wheels for reading reactions at different wavelengths.

1.7. WASHING STATION – Allows washing operations of the reaction plate cuvettes. It's made up of five small probes i.e. Acid solution, alkaline solution, Water, Aspiration pad and a Dry pad.

1.8. WASTE – Connections found on the back of the instrument for waste discharge (Tubes with black fittings contain biological waste while those with white fittings are free of biological waste).

2. OBJECTIVE

2.1. The SOP describes how to perform Clinical Chemistry analysis using ILAB Aries analyzer.

3. RESPONSIBILITY

3.1. It's the responsibility of all laboratory technologists using the ILAB Aries analyzer to follow this SOP.

4. SPECIMEN

4.1. Serum

4.2. Standard/Calibrators

5. EQUIPMENT/MATERIAL/REAGENTS

5.1. **Equipment**

5.1.1. Computer (CPU and Monitor).

5.1.2. ILAB Aries Analyzer

5.1.3. Printer

5.2. **Reagents**

5.2.1. Probe Rinse

5.2.2. Cuvette cleaner

5.2.3. Acid Cuvette cleaner

5.2.4. Distilled water

5.2.5. Reagent specific to various parameters to be analyzed.

5.3. **Materials**

5.3.1. Sample cups

5.3.2. Sample cup adaptors

5.3.3. Reagent bottle adaptors

6. METHODOLOGY

6.1. Preparation of Controls and calibrators.

NB: Controls and Calibrators are obtained from the manufacturer in form of lyophilized sample.

6.1.1. Remove the lyophilized materials (respective controls and calibrators) from the fridge and allow them to warm up to room temperature. Calibrators are accompanied by their respective diluent for its reconstitution.

6.1.2. Prepare the Controls/Calibrators as per the manufacturer's instructions in the kit insert.

6.1.3. Aliquot the Controls/Calibrator into serum vials. The volume aliquot should be enough for daily use to avoid repeated freezing and thawing e.g. 500µl for controls and 300µl for the calibrator.

6.1.4. The aliquots with an exception of them to be used for the day should be stored at -80ºc freezer. Controls have a stability of two weeks while calibrator has a stability of 10 days at -80ºc for all analytes.

NB: As per the kit insert, reconstituted calibrator is stable for 1 day for Direct and Total bilirubin analytes but as per previous IQC results using a calibrator frozen at -80ºc, the analytes have been found to be stable with acceptable IQC results.

6.2. Analysis of Calibrators (Standards) and Control materials.

6.2.1. Remove the lyophilized calibrator and control materials from the fridge and allow them to warm up to room temperature.

6.2.2. On the system monitor, select Routine → Calibration & QC setup; the system displays the expired operations (reagent blanks and/or calibrations of methods that have been previously loaded which one can enable or disable as required). Enabling "Run" on the display screen means that RBL (reagent blank) and /or calibration and/or control will be required. If the Run is not enabled, no operations will be run for the relevant tests. When the calibrator's column displays a calibrator's name, it means that the relevant calibrator is enabled. The tab "controls" allows the enabling of QC analysis. The tab "materials" shows details of materials related to running of the operations defined through the methods and control list tabs. To confirm all selections (RBL, Calibration and Q.C) click "Assign/check" then verify the material positions, load the relevant calibrators and controls the click "ok". Select the "start" button: enable "Calibration &QC" function then select "ok" to start analysis. Controls can also be run using the worklist set up which can be accessed via the Routine tab menu → Worklist. Under worklist select position followed by the sample type that allows choosing "controls" as the sample type. Select also the control name depending on the level of controls that you want to run.

N/B: No calibration is required for enzymatic tests.

6.2.3. To review Calibration results, click on Routine → Calibration Results. In case there is a flag, place the mouse cursor on the flag field and a tool tip opens containing a description of the error. To see calibration plot, click on "calibration graph". The screen also displays control results showing the reference ranges and the mean ranges.

6.2.4. To review Quality control click Routine → Quality Control. The QC screen is divided into the Main area and the Details area. In the Main area, control data can be sorted by controls, methods and date or data range. After selecting a row in the Main area and pressing the "Levy-Jennings" button, a window will open displaying the L-J graph. **Control results will only be acceptable if at the two levels of controls are within +/-2SD**. All control print outs should be printed, signed, dated and filed.

6.2.5. In case control results are out of acceptable limits, a corrective action should be written detailing all the steps undertaken in troubleshooting the cause (Refer to a troubleshooting guide in the ILAB Aries user manual).

6.3. Sample Analysis

6.3.1. Remove reagents stored in the fridge and allow them to reach room temperature.

6.3.2. For reagents requiring reconstitution, reconstitute as per the manufacturer's instructions ensuring that you don't shake to prevent foam formation. Reagents not requiring reconstitution are ready for use (avoid shaking to prevent foam formation).

N/B: Refer to the kit inserts for stability of each reagent.

6.3.3. Samples for analysis are loaded using a work list that can be accessed directly from Routine menu → Work list setup or indirectly after Load on Fly from the System Monitor mask. Analysis samples should be clearly noted with their identification numbers. The work list is displayed with different colours; grey for rotors not configured, light green for rotors having at least one sample configured and dark green for the rotor selected by the user. The Methods list area displays all test available, tests in white are for methods available in the active reagent configuration while dark ones refers to methods with no reagents on board.

6.3.4. Select the next available position ensuring that you always tick on the low volume tab.

6.3.5. Enter the patient's details, click on the method tab to activate the tests and select all the tests required for the particular sample then click save.

6.3.6. Click on the SYS. MON and select the green button, enable the work list and select the relevant rotor (Work list 1 corresponds to Rotor A, Work list 2 Rotor B, Work list 3 Rotor C, Work list 4 Rotor D and then back to Rotor A) then click ok. A message is displayed on the screen enquiring whether samples are already loaded or not. Click on "place rack". The rack is positioned showing the position to load samples then click ok for analysis to begin.

6.3.7. Results will be displayed on the workstation after analysis is complete. Select the results per patient and double click on display, print and sign.

N: B Patients samples will only be analyzed after obtaining correct control results that must always be run daily before analysis of any patient samples.

7. MAINTENANCE

7.1. Daily maintenance

- Always wipe off dust from the surface of the instrument every morning.
- Check the levels of all the four liquid reservoirs at the beginning of each working day. The levels should be at least 50% mark for each reagent reservoir.
- Check the residual reagents level of each reagent and the residual number of tests it can analyze from the "reagent map" at beginning of each working day.
- Check the level of waste tanks before commencing analysis, emptying them as necessary.

- Carry out Water base Line (Water Blank) and Washing of Cuvettes prior to any daily analysis. On the system monitor click the start (green triangle on the system monitor display) button, select "Water Base Line" and "Cuvette Washing" on the display then click start for the water blanking cycle to start.
- Wipe the sample probe at the end of each working day.

NB: Refer to ILAB Aries Operator's Manual section 2.1 for a flow chart showing the sequential order for daily routine procedures. In the event that the machine is not in use for a lengthy period of time (more than one week), the above maintenance will be done twice a week.

7.2. Every Two weeks

- Clean the four liquid reservoirs *(Refer to ILAB Aries Operator's manual section 4. 3 for the procedure).*

7.3. Monthly

- Clean the hydraulic circuit *(Refer to ILAB Aries Operator's manual section 4.6 for the procedure).*

8. SAMPLES STABILITY

1.1. ALT/AST – Specimen should be analyzed after collection or stored for 2-8ºc for up to 24hrs.

1.2. Creatinine – Specimen may be stored for 7days at 2-8ºc and 1 month at -20ºc.

1.3. Bilirubin (Total and Direct)- Specimen are stable for 3 days at 2-8ºc out of direct sunlight or fluorescent light.

9. LIMITATIONS OF TEST

- Lipemic and haemolysed samples should not be used for any Chemistry analysis.

10. REFERENCE

- ILAB Aries Operator's Manual.
- Specific reagents, controls and calibrator kit inserts.

11. BACK-UP LAB

- The back-up laboratory will be PATHOLOGIST LANCET KENYA.

12. Summary of Changes

- *Added more notes on maintenance*
- *Added limitations of tests*
- *Reference Operator's manual for guidance on maintenance procedures*
- *Added preparation of controls and calibrator, their storage conditions and stability depending on the different parameters under test.*
- *Added References used to prepare this SOP.*
- *Changed the version of SOP from 1.0 to version 2.0*

ILAB ARIES CHEMISTRY ANALYZER DAILY MAINTENANCE LOG

MONTH_____ YEAR_____

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
DAILY																															
Dust off the surface of the equipment																															
Check level of all four liquid reservoirs (DH_2O, probe rinse, Acid cuvette cleaner, Cuvette cleaning before start of daily analysis)																															
Check levels of reagents, Calibrators and Controls(before start of daily analysis)																															
Check levels of waste tanks (before start of daily analysis)																															
Perform water blanking cycle(before start of daily analysis)																															
Clean sample probe(after shutdown)																															
EVERY TWO WEEKS																															
Clean of the four liquid reservoirs																															
MONTHLY																															
Clean of hydraulic circuit																															
OPERATOR INITIALS																															

j) 8 Plex Cytokines analysis

This was done at KAVI-ICR laboratory at The University of Nairobi with a view to explore the

inflammatory markers patterns using a combo of 8 cytokines. These mediators were selected following logistical considerations not least of this being the changes that may arise from stored serum. Additionally, we lacked baseline levels for cytokine in serum of asymptomatic children exposed chronically to aflatoxin. This is a relatively new test in the KAVI-ICR laboratory.

Cytokines, chemokines, and growth factors are a diverse group of cell signaling proteins expressed and secreted by virtually all cell types, including cells of endothelial, epithelial, and immune origin. These proteins interact with specific receptors on target cells to mediate important physiological responses such as growth, immunity, inflammation, and hematopoiesis. Dysregulation of expression is associated with pathological conditions ranging from cancer and diabetes to infection and autoimmune disease.

Bio-Plex Pro™ is a robust immunoassay test that enabled us to quantify 8 proteins in a single well in 3-4 hours from a small volume of serum. These were Interleukin 2, 4, 6, 8,10, TNF-alpha, GM-CSF and IFN-gamma. IL-4 and IL 10 are anti-inflammatory cytokines while the rest are pro-inflammatory. IL-2, IFN-gamma and TNF-alpha drive TH1 pathway while IL-4,6 and 10 drive TH2 pathway. The multiplex system uses xMAP technology on the MAGPIX™ system.

Bio-Plex Pro™ assays enable researchers to quantify multiple protein biomarkers in a single well of a 96-well plate in 3–4 hours. These robust immunoassays require as little as 12.5 µl serum or plasma or 50µl cell culture supernatant or other biological fluid. The use of magnetic (MagPlex) beads allows researchers to automate wash steps on a Bio-Plex Pro (or similar) wash station. Magnetic separation offers greater convenience and reproducibility compared to vacuum filtration.
Principle: The Bio-Plex® multiplex system is built upon the three core elements of xMAP technology: Fluorescently dyed microspheres (also called beads), each with a distinct color code or

spectral address to permit discrimination of individual tests within a multiplex suspension. This allows simultaneous detection of up to 500 different types of molecules in a single well of the 96-well microplate on the Bio-Plex® 3D system, up to 100 different types of molecules on the Bio-Plex® 200 system, and up to 50 different types of molecules on the Bio-Plex® MAGPIX™ system on the Bio-Plex 200 and Bio-Plex 3D systems, a dedicated flow cytometer with two lasers and associated optics to measure the different molecules bound to the surface of the beads. In the Bio-Plex MAGPIX, the entire sample load volume is injected into a chamber where the beads are imaged using LED and CCD technology a high-speed digital signal processor that efficiently manages the fluorescence data.

Assay Format

Bio-Plex Pro™ assays are essentially immunoassays formatted on magnetic beads. The assay principle is similar to that of a sandwich ELISA (Figure 1). Capture antibodies directed against the desired biomarker are covalently coupled to the beads. Coupled beads react with the sample containing the biomarker of interest. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a fluorescent indicator or reporter.

Data Acquisition and Analysis

Data from the reactions are acquired using a Bio-Plex system or similar Luminex-based reader. When a multiplex assay suspension is drawn into the Bio-Plex 200 reader, for example, a red (635 nm) laser illuminates the fluorescent dyes within each bead to provide bead classification and thus assay identification. At the same time, a green (532 nm) laser excites PE to generate a reporter signal, which is detected by a photomultiplier tube (PMT). A high-speed digital processor manages

data output, and Bio-Plex Manager™ software presents data as median fluorescence intensity (MFI) as well as concentration (pg/ml). The concentration of analyte bound to each bead is proportional to the MFI of reporter signal.

Using Bio-Plex Data Pro™ software, data from multiple instrument runs can be combined into a single project for easy data management, quick visualization of results, and simple statistical analysis.

IMPORTANT! Pay close attention to vortexing, shaking, and incubation instructions. Deviation from the protocol may result in low assay signal and assay variability.

Initial Preparation

i. Plan the plate layout.

ii. Start up/warm up the Bio-Plex® system (30 min). Bring assay buffer, wash buffer, and sample diluent to room temperature (RT). Keep other items on ice until needed. Begin to thaw frozen samples

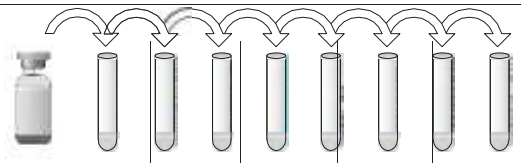
iii. Prime wash station for flat bottom plate or set vacuum manifold to–1 to –3" Hg for filter plate.

iv. Calibrate the Bio-Plex system by following the prompts within Bio-Plex Manager™ software. This can be done now or during an assay incubation step.

v. Reconstitute a single vial of standards in 500 µl of a diluent similar to the final sample type or matrix. Vortex for 5 sec and incubate on ice for 30 min.

vi. Prepare a fourfold standard dilution series and blank as shown below. Vortex for 5 sec between liquid transfers. If mixing diabetes assays with cytokine assays, refer to the diabetes instruction manual.

vii. After thawing samples, prepare as shown below.

128	50	50	50	50	50	50	50			Transfer Volume, µl
									150	
Reconstituted Standard		72	150	150	150	150	150	150	150	Diluent, µl
		S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**Blank**	

Data Entry and Analysis of Results

The questionnaire data entry was done using CSEntry for Android application. Data was then entered in password protected Microsoft Access database with built-in consistency checks. Verification of data already entered was conducted monthly. Frequencies and descriptive analysis were used to check potential outlying observations for further checking and identified suspected values extracted and rechecked to verify the validity of the values. The identified wrong values were corrected on a monthly basis and a report generated on data completeness/correctness. This continuous data cleaning process took place throughout the entire period of the study with input from a data manager.

Statistical Analysis

STATA software (StataCorp. 2017. Release 15. College Station, TX: StataCorp LLC) was used for data analysis. Descriptive analysis and graphical display were used to explore data patterns and correlation analyses performed between the different continuous variables such as immune variables and aflatoxin levels. Mean and standard deviation were used to summarize continuous variables and proportions were used to summarize categorical variables. The computed median level of serum aflatoxin (AFB1) was used to divide the considered as the cut-off point for 'high'

and ‘low’ aflatoxin levels for purposes of analysis of the various study variables. Aflatoxin levels were also studied as a continuous exploratory variable against the same variables to look for trends.

The immune indicators were summarized across the aflatoxin levels. The aflatoxin level was categorized based on the 25, 50, 75 percentiles. Logistic regression was used to assess the difference in immune profiles by the prevalence of aflatoxin exposure. The effect of aflatoxin exposure on the immune correlates in response to hepatitis B and Polio was assessed through logistic regression. Dummy variables were used to assess the effect of continuous variables where the relationships were not linear. All tests were assessed at 5% significant level.

The analytical framework below was the roadmap used to arrive to a multivariate logistic model to enable us to derive the variables that impacted on production of anti-HBs. This outcome variable was considered practical, reproducible and clinically useful measure of the immunosuppressive effect of aflatoxin for purposes of this study.

The Analytical Framework

Hypotheses

Aflatoxin —> Suboptimal immune response to Hep B vaccine

Aflatoxin —> Effects on cytokine levels

- Data quality
    - Range checks (inspect for unrealistic/extreme values)
    - Outliers
    - Missing data
- Descriptive analysis (Age, gender, anthropometric measures, wealth index, aflatoxin levels)
- Estimate of the primary dependent variable: Hep B antibody levels in the study population

- Table indicating the distribution of characteristics of study participants among whom Hep B antibody levels are reported versus those without data (to rule out bias)

  Select characteristics similar to those in the first results table above

- Univariate associations

Associations between independent variables in the first table and the primary outcome (Hep B antibody levels)

- Graphical presentations of correlation between aflatoxin level and individual cytokines (IL2, IL4…)
- Adjusted analyses

Multivariate logistic regression model with Hep B antibody level (binary variable) as dependent variable (outcome) and independent variables that were significant at $P<0.2$ in univariate analyses

- Tests for model fit
- AUC, AIC, VIF

3.8. Ethical Considerations

The study was approved by the joint Kenyatta National Hospital and University of Nairobi Ethical Review Committee (KNH/UON-ERC). Community as well as individual consenting/accenting was done having gone through the Local Government machinery.

CHAPTER 4: RESULTS

4.1 Summary of Tests and Variables Used in Study

Tables 3-7 below provide summary tests statistics for the variables used on the study. The frequencies across the groups are provided where variables are categorical in nature.

Table 3: Summary Test Statistics of Anti-HB Antibodies and Cytokine Levels

Immune Response		Frequency	Mean	Median
Anti-HB Levels mIU/L	Negative	107	1.5	1
	Positive	98		
	Total	**205**		
IL2 pg/ml		173	3.3	1.9
IL4_n "		146	0.3	0.2
IL6 "		173	8.8	1.9
IL8 "		173	1863.4	361.2
IL10_n "		156	3.2	2.4
IFNy1 "		123	0.8	0.7
GM-CSF_n "		37	0.4	0.1
TNFa "		173	37.2	27.6

Table 4: Summary Test Statistics for Aflatoxin and Fumonisins

Aflatoxin and Fumonisin Levels		Frequency	Mean	Median
Aflatoxin Level pg/mg albumin	high	203	1.5	2
	low	207		
	Total	**410**		
Fumonisins nM	Sphinganine	253	3.16E+15	2.14E+15
	Sphingosine	263	3.46E+15	2.74E+15

Table 5: Categories of Wealth Index

Wealth Index	Quintile	Frequency
	Poor	86
	Middle	260
	Rich	86
	Total	**432**

Table 6: Summary Statistics for Liver Function

Liver Function Tests	Frequency	Mean	Median
ggt (U/L)	390	16.0	15.0
alt "	390	18.2	17.0
ast "	390	37.8	37.0
Albumin (g/dl)	410	4.9	4.9

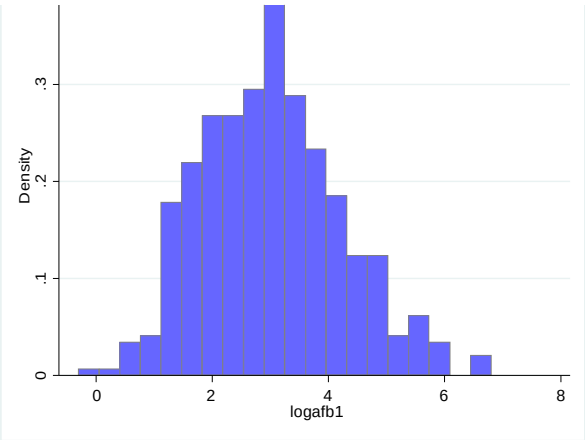
Table 7: Anthropometry and Dietary Diversity Scores

Anthropometry		Frequency	Mean	Median
Age Groups	Below 5 years	105	7.85	8.00
	Above 5	304		
	Total	**409**		
Weights for Age z-score (WAZ)	Underweight	32	-0.72	-0.78
	Normal weight	266		
	Total	**298**		
Height for Age z-score (HAZ)	Not stunted	353	-0.76	-0.79
	Stunted	67		
	Total	**420**		
BMI for Age z-score (BAZ)	Normal weight	343	-0.79	-0.78
	Overweight/obesity	16		
	Underweight	61		
	Total	**420**		
Mid Upper Arm Circumference (cm) (MUAC)	At Risk	36	16.69	16.50
	Normal	380		
	Sam	6		
	Total	**422**		
Dietary Diversity Score (DDS)	High	92	3.88	4.00
	Low	148		
	Medium	191		
	Total	**431**		

4.2 Serum Aflatoxin (AFB1)

Distributional Properties of Serum Aflatoxin

Plots 13-19 show the distribution of Aflatoxin Serum in general, and across grouped categories of Gender, Age Groups Wealth Index and Dietary Diversity Score.



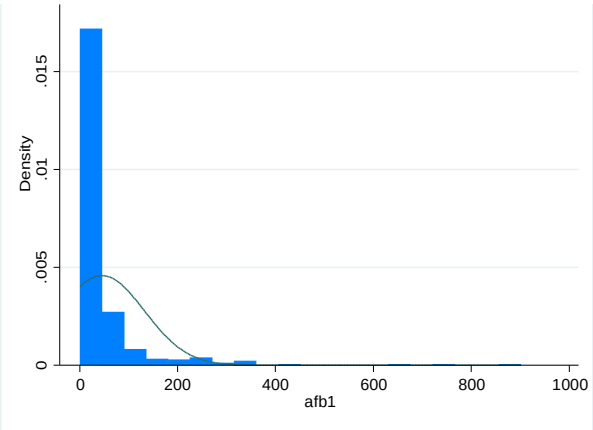


Figure 14: Distribution Plot of Serum Aflatoxin

Fi
Se

The grouped distribution plots below (by gender) shows that females tend to have higher levels of serum aflatoxin. The spread of their distribution is slightly larger than that for males, who seem to be more concentrated in the first bin.

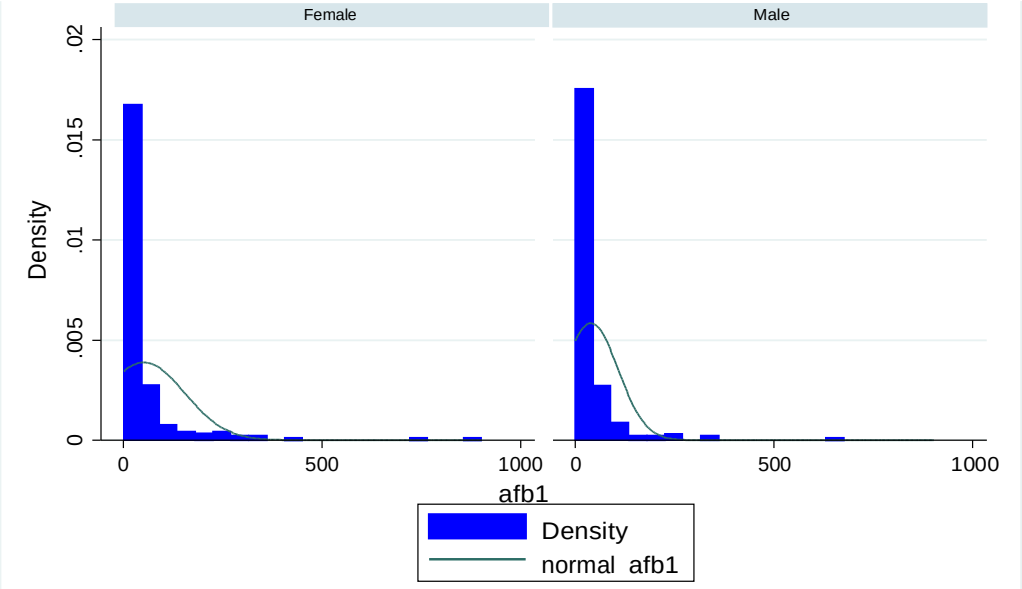


Figure 15: Distribution of Serum Aflatoxin Across Gender

Children above 5 years old tend to have higher levels of serum aflatoxin as shown in the plots below. The spread of their distribution is slightly larger than that for the children below 5 years old, whose concentration is in lower levels of serum aflatoxin.

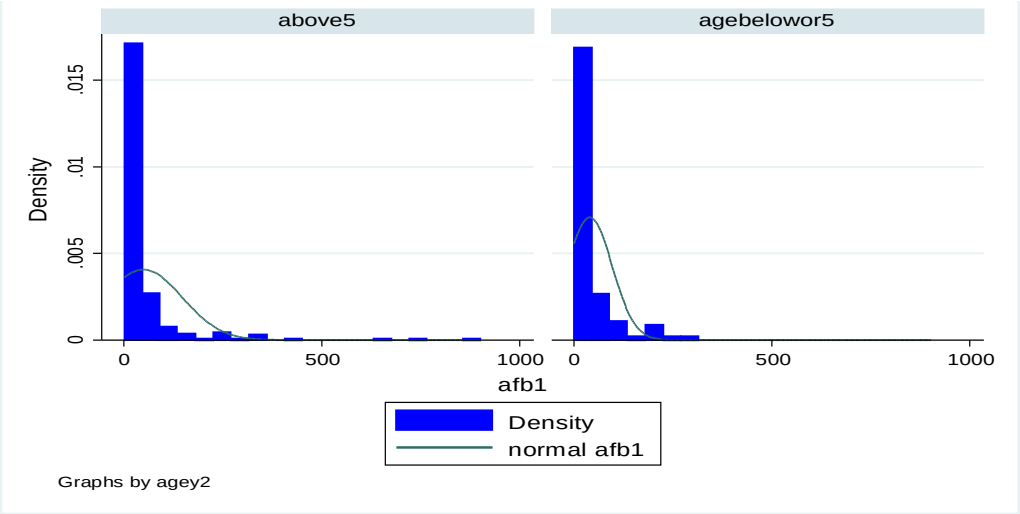


Figure 16: Distribution of AFB1 Across Age Groups

The plots below show distribution on the basis of Wealth Index. Children from poor households are seen to have higher level of serum aflatoxin, with those from rich households having lower levels of the serum (concentration is towards lower levels of serum Aflatoxin)

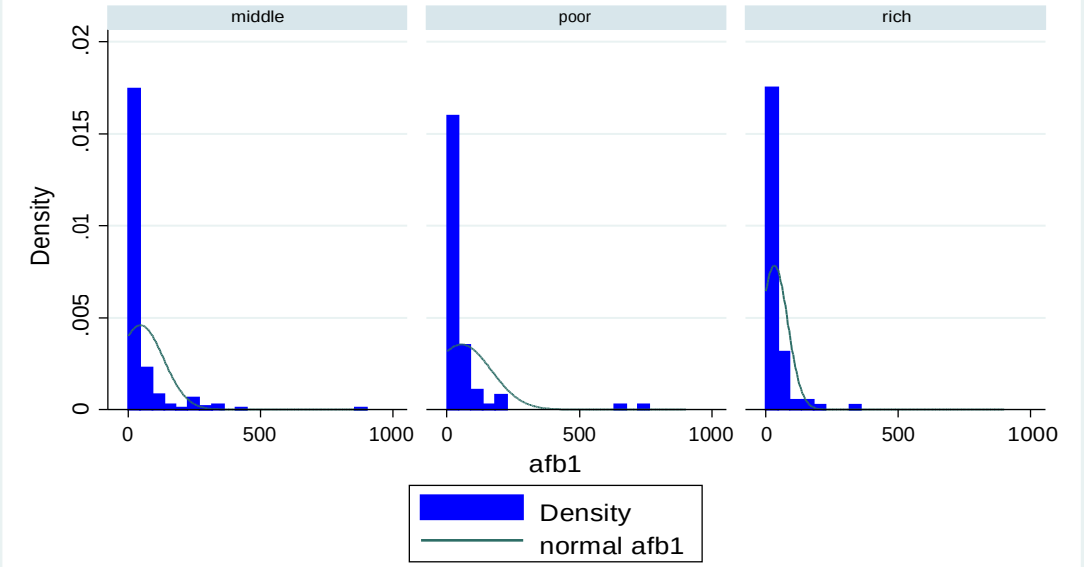


Figure 17: Distribution of Serum Aflatoxin by Wealth Index

The average level of serum aflatoxin based on Gender and Wealth Indices is shown in the bar graph below. Consistent with the inference from the distribution plots, the females consistently have higher levels of aflatoxin serum across the three wealth indices. The average level of serum

aflatoxin is highest in females from poor households. Interestingly, the average of level of serum aflatoxin in females from middle-income households is still higher than for males in poor households.

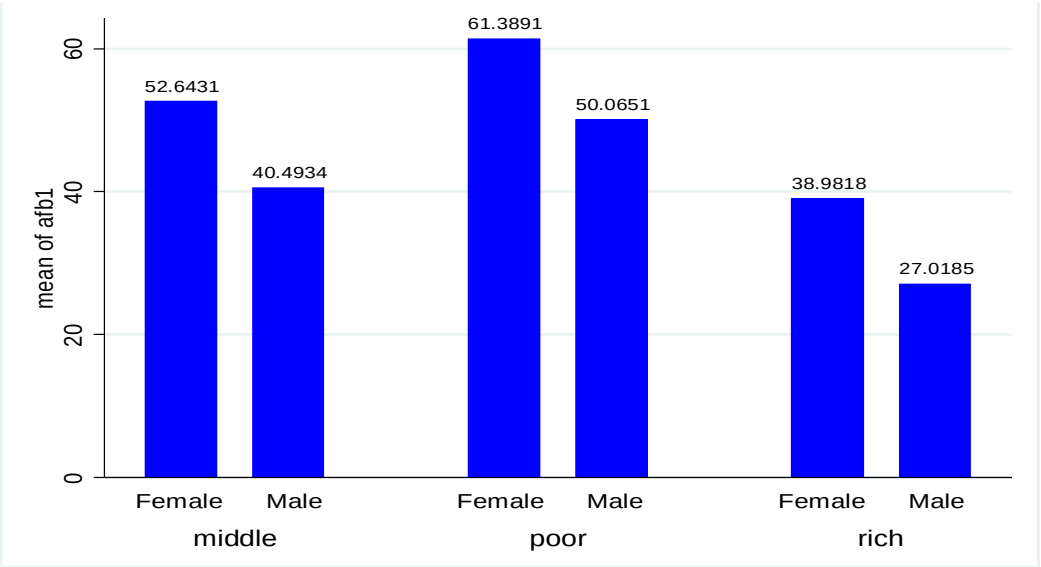


Figure 18: Average Serum Aflatoxin Across Wealth Index and Gender

Children with a low dietary diversity score have higher levels of serum aflatoxin as shown in the distribution below. The spread is slightly larger than that for the children with medium or high dietary diversity scores, whose concentration is in lower serum levels.

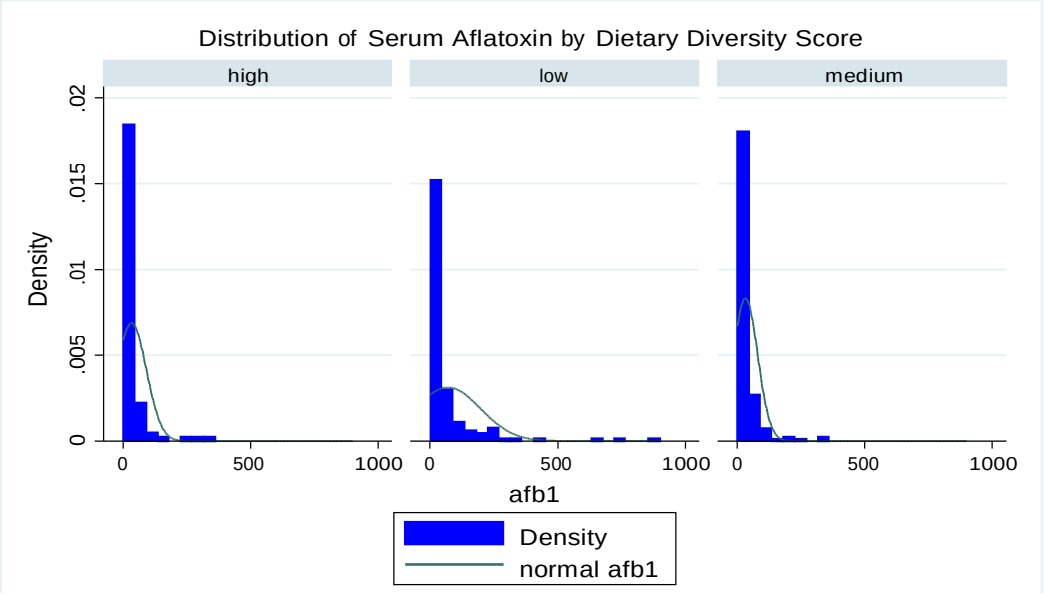


Figure 19: Distribution of Serum Aflatoxin by Dietary Diversity Score

Univariate Testing for Serum Aflatoxin

In this section, the objective is to investigate if there exists statistically significant relationships between the level of serum aflatoxin (AFB1, in its continuous form) and wealth index, age groups, gender, waz (weight for age z-score), haz, (height for age z-score), baz (BMI for age z-score), muac (Mid upper arm circumference), Albumin levels (for liver function) and Haemoglobin (normal vs. abnormal).

The consideration of the test of association to be used is made after analysis of the distributional properties of the variable of interest, Serum Aflatoxin Levels. If the variable is normally distributed, then an Analysis of variance (ANOVA) can be done to compare the means of groups of measurement data. It is used when you have a categorical independent variable (with two or more categories) and a normally distributed interval dependent variable. It tests for differences in the means of the dependent variable by the different categories of the independent variable.

If AFB1 is not normally distributed, a non-parametric version of the ANOVA- Kruskal Wallis Test- is used. Non-parametric tests make no assumptions about the distribution of the dataset (it is distribution free). This is therefore used when the normality assumption of the ANOVA is not met. Besides looking at the differences in the means and medians as well as the bar chart, it is important to formally test for normality of the assessed variables.

Tests of Normality of Aflatoxin Level

To formally test whether Serum Aflatoxin level is normally distributed, we use two tests of normality, namely, the Shapiro-Wilk Test and the Shapiro-Francia Test. The results are presented below:

Table 8: Testing for Normality of AFB1 Spread

Normality Test	z	p-value
Shapiro Wilk Test	11.9920	0.0000
Shapiro-Francia W' test	11.0840	0.0000

The tests above lead to a rejection of the null hypothesis of normality of the Serum aflatoxin variable. Therefore, the test used to associate aflatoxin levels to the other variables is the Kruskal Wallis and Dunn test. This is described further below

Description of the Kruskal Wallis Test and the Dunn Test

The Kruskal-Wallis test is a nonparametric (distribution free) test that makes no assumptions about the distribution of the dependent variable. The Kruskal-Wallis test investigates if it is likely that the observations in one group are statistically different from observations in another group. It is used for both continuous and ordinal-level dependent variables and is similar to the Mann–Whitney U test but can be applied to one-way data with more than two groups.

The analysis and results presented in this section enable inference on whether there are any *statistical differences* in the serum aflatoxin levels based on the wealth index, age groups, genders, and z-scores for weight for age, height for age, BMI for age and MUA

. The Kruskal-Wallis test gives a non-graphical description of the statistically significant differences and where they exist.

The null and alternative hypothesis of the Kruskal Wallis Test is shown below:

- Null hypothesis: The groups are sampled from populations with identical distributions.
- Alternative hypothesis: The groups are sampled from populations with different distributions.

If the Kruskal Wallis Test reports significance, a post-hoc analysis is performed to determine which groups differ from each other. If no significant difference across each group is noted, no post hoc

analysis is carried out. *The most widely used post-hoc test for the Kruskal–Wallis test is the Dunn test* and it is the test that was used in this study. The Dunn Test reports the results among multiple pairwise comparisons after a Kruskal-Wallis test for stochastic dominance among k groups. The null hypothesis of the Dunn test in each pairwise comparison is that it is equally likely to observe a random value in the first group that is larger than a random value in the second group. This implies that there is no statistical difference in the different groups.

Table 9: Differences in the serum aflatoxin levels depending on a number of variables

Test	Statistics	Conclusion
Serum Aflatoxin Levels by Gender	chi-squared = 0.124 probability = 0.7243	NO statistically significant difference in aflatoxin levels across the 2 genders.
Serum Aflatoxin Levels by Wealth Index	chi-squared = 11.501. probability = 0.0032	The results indicate that there is a statistically significant difference in aflatoxin levels across the three wealth indices. A further analysis is done to assess where the differences lie (below).
Serum Aflatoxin by Dietary Diversity Score	chi-squared = 19.021 probability = 0.0001	The results indicate that there is a statistically significant difference in aflatoxin levels across the three dietary diversity scores. A further analysis is done to assess where the differences lie (below).
Serum Aflatoxin Levels by Age Groups	chi-squared = 0.097 probability = 0.7550	NO statistically significant difference in aflatoxin levels across the age groups.
Serum Aflatoxin Levels by Weight for Age z-score	chi-squared = 0.006 probability = 0.9395	NO statistically significant difference in aflatoxin levels across the different weight for age categories.
Serum Aflatoxin Levels by Height for Age z-score	chi-squared = 0.006 probability = 0.9395	NO statistically significant difference in aflatoxin levels across the different Height for age categories
Serum Aflatoxin Levels by BMI for Age z-score	chi-squared = 1.351 probability = 0.5090	NO statistically significant difference in aflatoxin levels across the different BMI for age categories.
Serum Aflatoxin Levels by MUAC	chi-squared = 0.228 probability = 0.8923	NO statistically significant difference in aflatoxin levels across the different MUAC categories.
Serum Aflatoxin Levels by Albumin (Liver Function Test)	chi-squared = 1.352 probability = 0.2450	NO statistically significant difference in aflatoxin levels across the different Albumin categories (High vs Low).
Serum Aflatoxin Levels by Haemoglobin	chi-squared = 0.182 probability = 0.6700	NO statistically significant difference in aflatoxin levels across the different Haemoglobin categories (normal vs abnormal).

Serum Aflatoxin and Wealth Levels (Dunn Test Results)

The results from the Kruskal Wallis test above indicate that there is a statistically significant difference in Serum aflatoxin levels across the three wealth indices. A further analysis is done to assess where the differences lie. This is done using the Dunn test, a test for stochastic dominance among multiple pairwise comparisons following a Kruskal-Wallis test:

Table 10: Results of Dunn test to interrogate the differences in AFB1 across wealth indices

	Comparison	*Z*	*P.unadj*	*P.adj*
1	middle--poor	-1.233	0.218	0.218
2	middle—rich	2.739	0.006	0.006
3	poor—rich	3.247	0.001	0.001

The reported p-values are significant for comparison 2 and 3. This shows that there is a statistically significant difference between the middle class and rich class, as well as between the poor class and the rich class. However, there is no statistical difference between the middle class and the poor class. The aflatoxin exposures are higher in both the middle and poor class relative to the rich class, as indicated by the positive z-scores.

Corresponding to the results presented above, the table of monoletters presented below indicate which groups are similar and which are different from each other: (those with similar letters are statistically similar).

Table 11: Monoletters to explore similarities and differences between wealth indices groups

	Group	*Letter*	*MonoLetter*
1	middle	a	a
2	poor	a	a
3	rich	b	b

This shows that the rich class is statistically different from the remaining two classes.

A univariate regression analysis is carried out between the log-transformed level of aflatoxin exposure and wealth index; the results presented below are in support of the Kruskal Wallis and Dunn test carried out above:

Table 12: Univariate regression analysis for AFB1 and wealth index

logafb1	*Coef.*	*Std. Error*	*t*	*P>|t|*
Poor	0.17	0.15	1.11	0.2660
Rich	-0.42	0.15	-2.82	0.0050
_cons	3.07	0.08	40.12	0.0000

The comparison group (eliminated) is the middle wealth index. The results indicate that all other factors held constant, if the individual is RICH, then their level of serum aflatoxin is lower than that of an individual in middle class by 42% (100*-0.42). If the individual is POOR, then their level of aflatoxin is higher than that of an individual in middle class by 17% (100*0.17).

Serum Aflatoxin and Dietary Diversity Score (Dunn Test Results)

The results from the Kruskal Wallis test above also indicate a statistically significant difference in aflatoxin levels across the three dietary diversity score categories (high, low, medium). A further analysis is done to assess where the differences lie. This is also done using the Dunn test:

Table 13: Dunn test results to explore where the difference in AFB1 levels lie in the wealth indices categories

	Comparison	*Z*	*P.unadj*	*P.adj*
1	High vs Low	-3.90	0.0001	0.0001
2	High vs Medium	-1.00	0.3179	0.3179
3	Low vs Medium	3.55	0.0004	0.0004

The reported p-values are significant for comparison 1 and 3. This shows that there is a statistically significant difference in aflatoxin exposure between children with High dietary diversity score and those with Low diversity score, as well as between the Low diversity score and Medium diversity score. However, there is no statistical difference between the High and Medium diversity score

categories. The serum aflatoxin levels are higher for children with low dietary diversity scores. The level of aflatoxin is also higher for children with medium diversity scores as compared to high (though insignificant)

Corresponding to the results presented above, the table of monoletters presented below indicate which groups are similar and which are different from each other: (those with similar letters are statistically similar)

Table 14: Monoletters to explore differences between Dietary diversity scores as regards AFB1 levels

	Group	*Letter*	*MonoLetter*
1	High	A	a
2	Low	B	b
3	Medium	A	a

This shows that children with low dietary diversity score are statistically different from the remaining two classes.

A univariate regression analysis is carried out between the log-transformed level of aflatoxin exposure and dietary diversity score; the results presented below are in support of the Kruskal Wallis and Dunn test carried out above:

Table 15: Univariate Regression Results to Explore differences between Dietary diversity Categories and AFB1 Levels

logafb1	*Coef.*	*Std. Error*	*t*	*P>|t|*
DDS==High	-0.14	0.15	-0.9400	0.3480
DDS==Low	0.53	0.13	3.9700	0.0000
_cons	2.86	0.09	32.7700	0.0000

The comparison group (eliminated) is the medium dietary diversity score group. The results indicate that all other factors held constant, if the child has a low dietary diversity score, then their level of serum aflatoxin is significantly higher than that of a child with a medium score by 53%. A

child with a low score also has a 67% higher level of serum aflatoxin compared child with a high dietary diversity score [(0.53- -0.14) * 100]

4.3 Fumonisins and Serum Aflatoxin

The regression analysis results indicate that Sphinganine (sa) and Sphingosine (so) are statistically significant in their effect on immunity. It is known that the ratio of sa to so is a better indicator of fumonisin level due to low variability of the ratio as opposed to measuring sa and so the individually(102). A granular analysis of the relationship between sa/so and AFB1 is shown in figures 20-24. Fumonisins are known to co-occur with aflatoxin and has similar immunomodulatory effects. They cause their effects by disrupting ceramide synthesis.

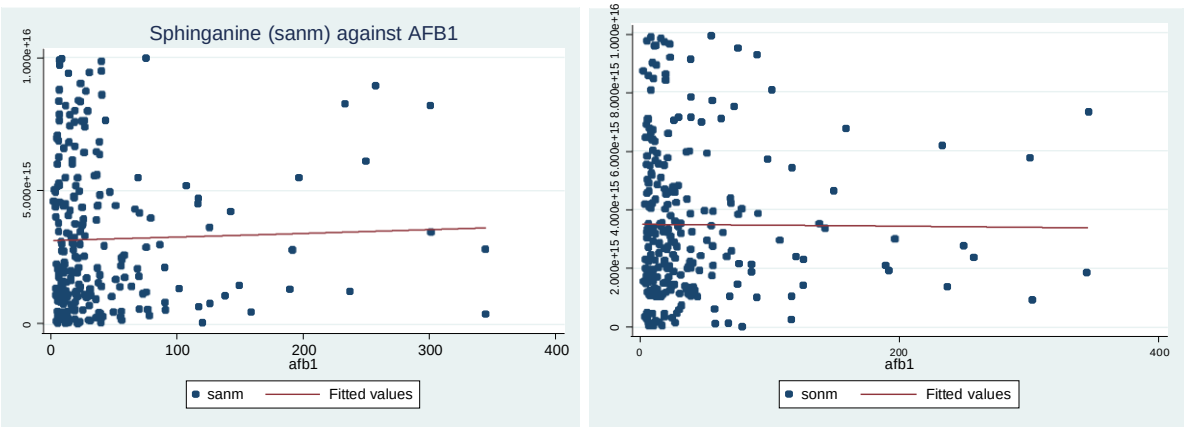
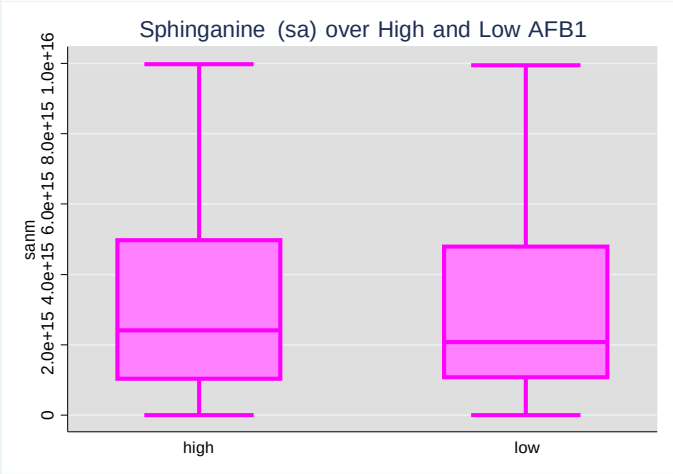


Figure 20: Sphinganine (sanm) and Sphingosine (sonm) against AFB1

The scatter plots and the corresponding line of best fit show a weak positive relationship between the two Fumonisins and Serum Aflatoxin. An analysis of the distribution of the sphinganine and sphingosine (saso) across High and Low Serum Aflatoxin is shown in the box plots 21 and 22.



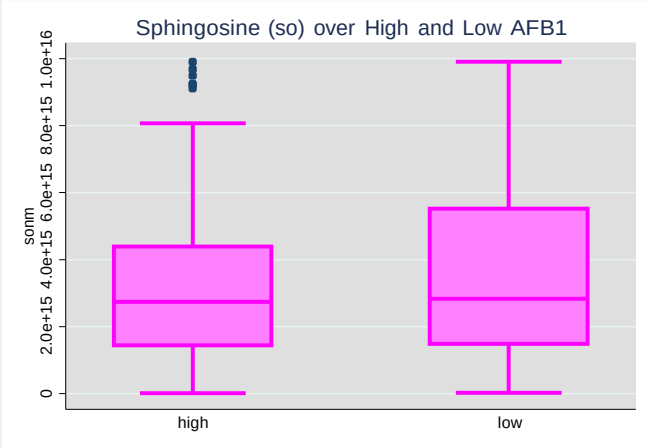
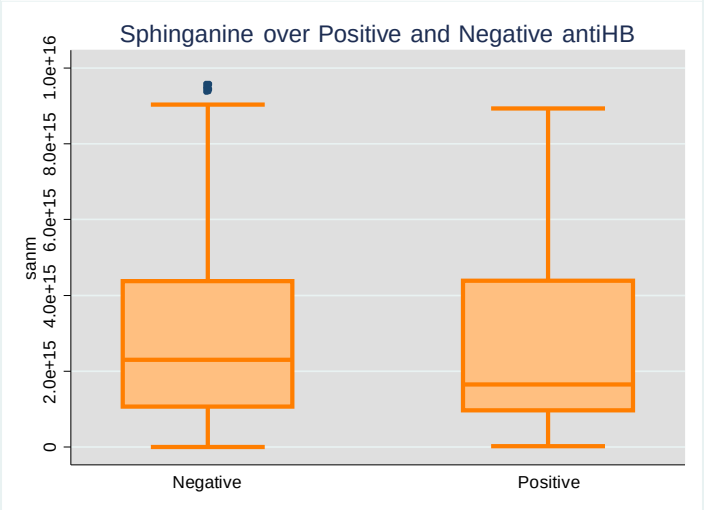


Figure 21: Sphinganine (sa) over High and Low AFB1

Figure 22: Sphingosine (so) Over High and Low AFB1

There is a slight noticeable difference in the level of Sphingosine across high and low aflatoxin levels. Though the median is the same across the two classifications, the level of Sphingosine in children with low serum aflatoxin category exhibits a larger spread.

Fumonisins and Anti-HBs antibodies



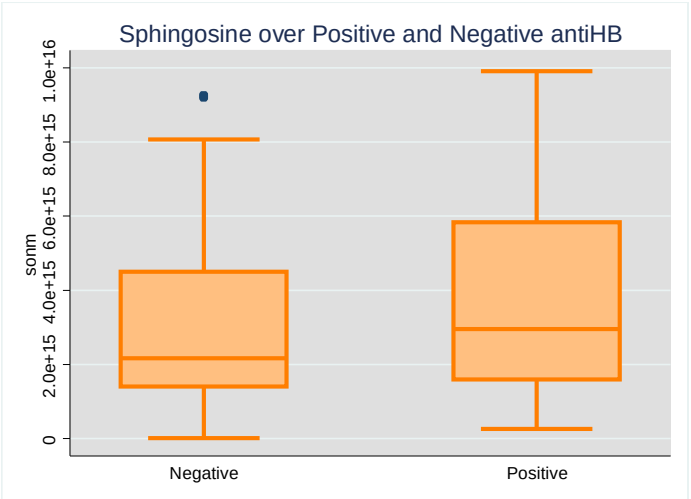


Figure 23: Sphinganine Over Positive and Negative Anti HB

Figure 24: Sphingosine Over Positive and Negative Anti HB

There is a slight difference in the level of Sphingosine across Negative and Positive Anti-HBs antibodies levels. The median in the children with negative Anti-HBs is slightly lower. Similarly, the spread of sphingosine levels in the box plot of Positive Anti-HBs category is larger. A further analysis into the relationship/association between the two is carried out using the multivariate logistic regression. A ratio of the sphinganine and Sphingosine is used in the multivariate model.

4.4 Grain Aflatoxin

This section seeks to describe the statistical properties of Grain Aflatoxin and investigate if there is any relationship between Grain Aflatoxin, Serum Aflatoxin and Fumonisins (Ratio: sa//so).

Statistical Properties of Grain Aflatoxin

The Distribution of grain aflatoxin (aflatoxin in contaminated grains) is shown in figures 25 and 26.

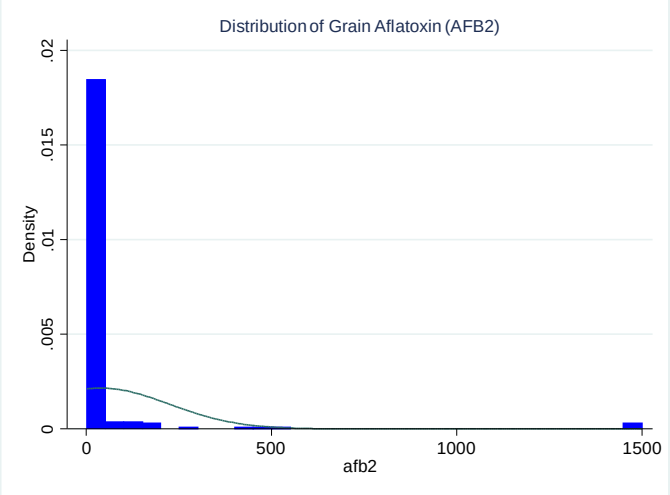


Figure 25: Distribution of Grain Aflatoxin (AFB2)

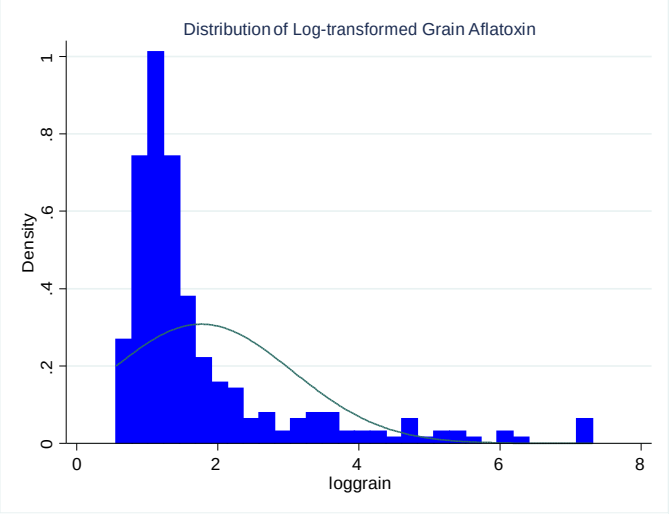


Figure 26: Distribution of Log-Transformed Grain Aflatoxin

There is evidence of skewness to the right, similar to what was observed for the Serum Aflatoxin in the previous section. This is an indication of deviation from the normal distribution.

More summary statistics are shown below: The mean is much larger than the median value. This is a result of outlier data points in the variable, as shown in the distribution above.

Table 16: Categorization of AFB2 Levels

	count	*mean*	*median*	*skewness*	*kurtosis*	*min*	*max*
Grain Aflatoxin	281.0	39.1	3.6	7.2	56.1	1.8	1500.0

Table 17: Summary Statistics of Grain Aflatoxin (AFB2) Levels

AFB2	*Freq.*	*Percent*	*Cum.*
<= 5	195	69.4%	69.4%
> 5 & <= 10	36	12.8%	82.2%
> 10 & <= 500	45	16.0%	98.2%
> 500	5	1.8%	100%
Total	281	100%	

The highest proportion of grain aflatoxin (69%) had contamination of less than 5 ppb. Roughly 2% had levels greater than 500.

To formally test whether Grain Aflatoxin level is normally distributed, we use two tests of normality, namely, the Shapiro-Wilk Test and the Shapiro-Francia Test. The results are presented in table 18 below:

Table 18: Testing Normality of Grain Aflatoxin Levels Using 2 Tests

Normality Test	*z*	*p-value*
Shapiro Wilk Test	11.9330	0.0000
Shapiro-Francia W' test	10.9660	0.0000

The tests above lead to a rejection of the null hypothesis of normality of Grain aflatoxin.

Relationship of Grain Aflatoxin with Serum Aflatoxin

The scatter plot above shows the association between Serum Aflatoxin and Grain Aflatoxin. The axis values have been truncated for visibility of a clearer pattern. A positive relationship is observed. A further analysis of the relationship is carried out using a simple regression analysis whose results are shown below.

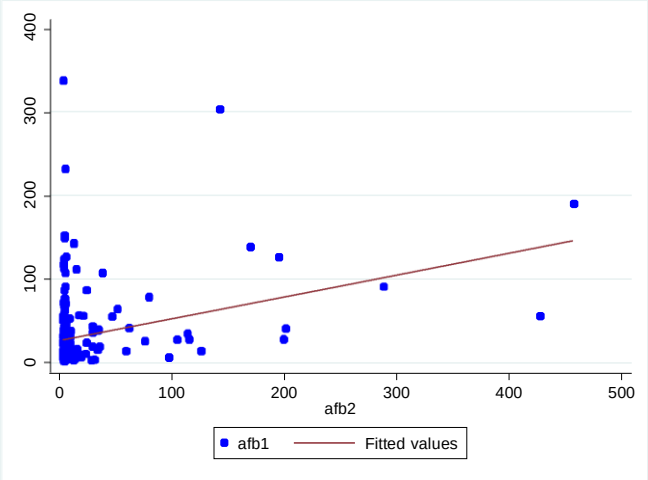


Figure 27: Serum Aflatoxin (AFB1) against Grain Aflatoxin (AFB2)

Holding all other factors constant, there is a positive and statistically significant relationship between Grain aflatoxin and Serum Aflatoxin (p-value is 0.00) as confirmed in table 19. Increase in grain aflatoxin increases serum aflatoxin.

Table 19: Testing for Relationship Between AFB2 and AFB1

Testing for relationship between grain aflatoxin and serum aflatoxin

F (1,270)	104.5200			
Prob>F (model p-value)	0.0000			
R-squared	27.91%			
Adjusted R-squared	27.64%			
afb1 (Serum Aflatoxin)	Coef.	Std. Error	t	P>|t|
afb2 (Grain Aflatoxin)	0.24	0.02	10.22	0.00
_cons	29.43	4.46	6.60	0.00

Relationship of Grain Aflatoxin with Fumonisins

The scatter plot in figure 27 shows the association between saso (ratio of Sphinganine to Sphingosine) and grain aflatoxin (AFB2). There is an almost imperceptible negative relationship depicted by the line of best fit.

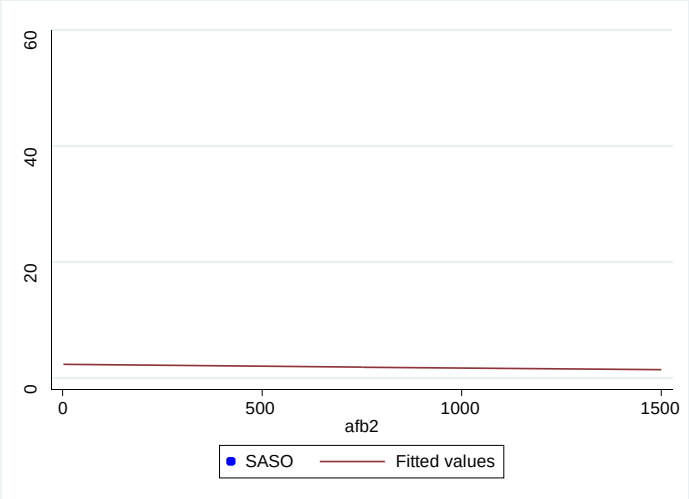


Figure 28: Sphinganine/Sphingosine against AFB2

A further analysis using a simple regression analysis was carried out as shown in table 20. There is NO statistically significant relationship between the two variables. The goodness of fit of the model as indicated by the R-squared of the model is almost zero.

Table 20: Results of Testing the differences between AFB2 and Fumonisins (saso)

F(1,172)	0.0900			
Prob>F (model p-value)	0.7618			
R-squared	0.05%			
Adjusted R-squared	-0.53%			
SASO	Coef.	Std. Error	t	P>|t|
afb2	-0.0015	0.0050	-0.3000	0.7620
_cons	3.3106	1.0382	3.1900	0.0020

4.5 Anti-HBs Antibodies

Univariate Analysis for Anti-HBs Antibodies

Anti-Hepatitis B has been chosen as the practical parameter to assess how immunity is possibly affected by aflatoxin. Before proceeding to the multivariate analysis, a univariate analysis of how the anti-HB antibodies relate to characteristic variables including grouped categories of the wealth index, age groups, gender, waz (weight for age z-score), haz, (height for age z-score), baz (BMI for age z-score), muac and dietary diversity score is done. In addition, we assess whether there is a univariate relationship between anti-HB antibodies and Aflatoxin exposure.

Two hundred and five samples were randomly selected for measurement of the anti-HBs. 47.8% of this sample tested positive for anti-HBs, with approximately 52.2% testing negative (table 21).

Table 21: Results of Samples Tested for anti-HBs Antibodies

Hp_anti	*Freq.*	*Percent*	*Cum.*
Negative	107	52.2	52.2
Positive	98	47.8	100
Total	205	100	

Since anti-HBs is captured as a binary variable (Positive or Negative), we used a Chi-Square test (or its correction, the Fisher's Exact Test) to test for its statistical relationship with the characteristic categorical variables. The Fisher's exact test is used when you want to conduct a chi-square test, but one or more of the cells has an expected frequency of five or less. It does not have a test statistic, but instead computes the p-value directly (table 22).

Table 22: Results of Testing Anti-HBs Against Various Categorical Variables

Anti-Hepatitis B antibodies (Positive or Negative)	*Pearson Chi2 or Fisher's Exact*	*P-value*
Gender (Male vs Female)	Pearson chi2(1) = 0.7988	0.3710
Wealth Index (Poor, Rich or Middle)	Pearson chi2(2) = 4.3657	0.1130
Age Groups (Above 5 or below 5)	Pearson chi2(1) = 13.9917	0.0000
Age Groups (Under 5 years, 5-8 years, Above 8)	Pearson chi2(2) = 14.4394	0.0010
BAZ (Normal Weight, Overweight or Underweight)	Fisher's exact = 0.067	
HAZ (Stunted or Not Stunted)	Pearson chi2(1) = 1.2085	0.2720
WAZ (Underweight or Normal weight)	Pearson chi2(1) = 0.2023	0.6530
MUAC (at risk, normal or sam)	Fisher's exact = 0.336	
Dietary Diversity Score	Pearson chi2(2) = 0.2557	0.8800

The results show a statistically significant association between the level of anti-HB antibodies and the age groups. A further analysis into the nature of the statistical relationship is done at the multivariate level to understand which age group has a significantly higher level of immune response. From the same univariate analysis, no other statistically significant association is noted at the univariate level.

A second set of tests is done to assess the relationship between anti-Hepatitis B antibodies and the following continuous variables: i) aflatoxin serum ii) Fumonisins and iii) Liver Function tests (Albumin, ast, alt, ggt, tprotein). Since one of the variables is categorical (anti-HB) and the others are continuous, a Kruskal Wallis test is done, where the assumption of normality is not met. Where normality is met, then a One-way ANOVA is done.

Table 23: Results of Testing Anti-HBs Against Various Continuous Variables

	Variable	*Normally Distributed*	*Kruskal Wallis/One Way ANOVA*	*P-value*
Aflatoxin	Aflatoxin Serum	No	chi-squared = 0.428	0.5129
Fumonisins	Sanm	No	chi-squared = 0.718	0.3967
	Sonm	No	chi-squared = 2.620	0.1055
	Saso	No	chi-squared = 1.002	0.3169
Liver Function Tests	Albumin	No	chi-squared = 0.145	0.7031
	Ast	No	chi-squared = 1.348	0.2456
	Alt	No	chi-squared = 1.886	0.1696
	Ggt	No	chi-squared = 0.962	0.3266
	Tprotein	No	chi-squared = 1.045	0.3066

The results presented above show that there is no statistically significant association between the anti-HB antibodies and the variables at univariate level.

Multivariate Analysis for Anti-HBs Antibodies

To build a multivariate regression model that seeks to investigate how immunity (measured by anti-HB antibodies) is affected by the aflatoxin serum, we use a logistic regression model since our dependent variable is binary in nature. The logistic regression contains other variables, considered to be control variables. These control variables will include the anthropometry tests, the fumonisins, the liver function tests and the dietary diversity score. The dependent variable, Anti-HB, takes the form of a log-odds ratio that is expressed as follows:

$$Log\left[\frac{P(AntiHBs==NEGAtive)}{P(AntiHBs==POSItive)}\right] = \alpha_1 + \boldsymbol{X}\beta_1^{'}$$

Where:

$\frac{P(AntiHBs==NEGAtive)}{P(AntiHBs==POSItive)}$ are the odds of an outcome of Negative Anti-HBs rather than Positive Anti-HBs. An odd is the ratio of the probability one outcome over another.

α_1 is the model intercept, $\boldsymbol{X}$ is the matrix of independent variables considered for the model. $\beta_1^{'}$ is the vectors of beta coefficients for each of the independent variables used in the model. The results are presented in the table below:

Table 24: Logistic Regression Results (Dependent Variable: Anti-Hepatitis Bs)

Wald chi-square	15.8400			
p-value (chi-square)	0.4640			
Pseudo R-Squared	19.21%			
ANTIHEPB==Negative	Odds Ratio	Std. Error	z	P>|z|
Saso	1.18	0.0944	1.7400	0.0810
Log Aflatoxin	1.35	0.2695	1.1100	0.2670
GENDER==Female	0.26	0.6811	-1.9500	0.0510
AGE==Above 5	4.24	0.6814	2.1200	0.0340
WAZ==Underweight	0.52	1.1900	-0.5600	0.5780
BAZ==Underweight	0.83	0.9233	-0.2000	0.8420
HAZ==Stunted	1.10	1.0678	0.0900	0.9310
MUAC==At Risk	3.42	0.6920	1.7800	0.0750
Wealth Index==Poor	1.59	0.7538	0.6100	0.5400
Wealth Index==Rich	0.78	0.8319	-0.3000	0.7640
Alt	1.03	0.0507	0.5700	0.5720
Ggt	0.92	0.0742	-1.0800	0.2810
Albumin	0.77	0.5547	-0.4800	0.6350
Tprotein	565.32	3.2634	1.9400	0.0520
DDS (High)	0.66	0.9690	-0.4400	0.6630
DDS (Low)	2.01	0.7714	0.9000	0.3670
_cons	0.04	3.4849	-0.9500	0.3400

The results above indicate how each of the independent variables affects the immune response of the child through the odds ratio. This response that is captured by the Anti-HBs level can either be Positive (=/> 10mIU/L) or Negative (<10mIU/L). When the Odds ratio is less than 1, this implies a decrease in the likelihood of observing Negative Anti-HBs (***Favorable***). When the Odds Ratio is greater than 1, there is an increase in the likelihood of observing Negative Anti-HBs (***Adverse***).

Serum Aflatoxin is seen to reduce the dampen immune response in the study sample. The likelihood of observing negative Anti-HBs is 1.35 times higher when the level of Serum Aflatoxin increases. The noted effect is insignificant at a 95% confidence interval. This, however, can be attributed to the sample size used for the study (205).

The ratio of the 2 fumonisins (saso) is used in the regression above (Sphinganine over Sphingosine). The odds ratio indicates that the probability of observing negative level of Anti-HBs is 1.18 times higher when saso increases. There is a decrease in the immune response of the children if the ratio is high.

The age group dummy variable significantly affects the immunity response of the child. Children above 5 years old are more likely to have a lower immune response as compared to children below 5 years old. Specifically, the odds ratio indicates that children above 5 years are up to 4 times more likely to have negative Anti-HBs as compared to the base group (children below 5 years old).

It is seen that females are more likely to have a better immune response as compared to males. The odds ratio indicates females are 74% less likely to have negative anti-HBs. The effect is however marginally significant (p-value is 0.051)

The odds ratio on "Poor" is 1.6, indicating a higher likelihood (odds) of having negative Anti-HB. Children from poor backgrounds are 1.6 times more likely to have lower immune responses as compared to children from rich backgrounds.

For children who are stunted (according to the Height for Age score), they are 1.10 times more likely to have Negative Anti-HBs than children who are not stunted. Similarly, children who are at risk, according to their Mid Upper Arm circumference are 3.42 times more likely to have lower immunity response (i.e. more likely to have Negative Anti-HBs.)

The results, however, show that Children who are Underweight (according to the BMI for Age and Weight for Age Scores) are less likely to have low (negative) immunity response.

Children with a high dietary diversity score have better immune response as compared to children with low dietary diversity score. Those with Low DDS are 2 times more likely to have negative anti-HBs than those with high DDS.

Albumin and ggt levels are associated with increase the immune response of the child. The odds ratios indicate that when Albumin and ggt levels are high, there is a lower likelihood of observing negative anti-HBs.

Assessment of Model Performance

The signal detection ability of the model is assessed using a ROC curve (Receiver Operating Characteristic Curve depicted by figure 20). A model with no predictive power would have a straight 45 degrees line from the origin. The ROC curve generates a graphic that shows the trade-off between the rate at which you can correctly predict something with the rate of incorrectly predicting something.

The greater the predictive power, the more bowed the curve, and hence the Area under the Curve (AUC) is often used as a measure of the predictive power. A model with NO predictive power has an AUC (Area under Curve) of 0.5 (50%); a perfect model has an AUC of 1 (100%)(103).

For the logistic regression estimated above, the area under the curve below of approximately 0.77 (77%) which indicates acceptable separability power of the model. This means the model is able to accurately classify Positive vs. Negative immunity response given the data provided.

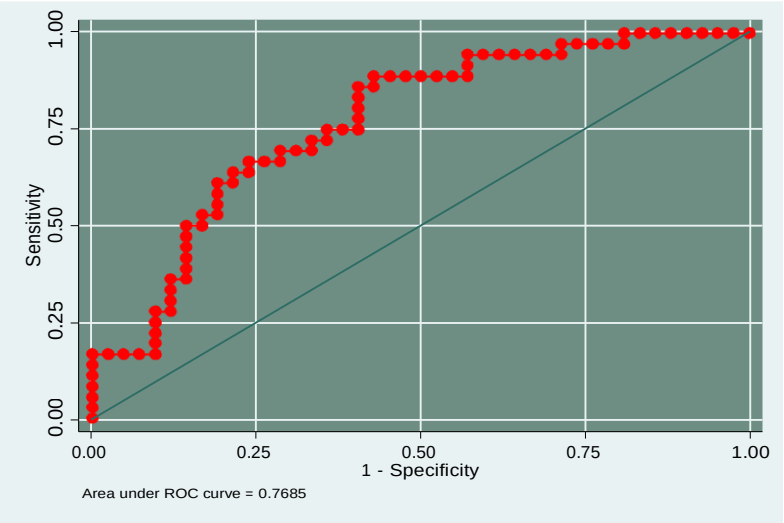


Figure 29: Assessment of Model Performance Using Receiver Operating Characteristic (ROC) Curve.

4.6 Cytokines Analysis

The objective of measuring cytokine levels in serum, notwithstanding the fact that the serum had been in storage for at least one month, was to allow us to explore if there is an association between Aflatoxin blood levels with selected immune markers (IL-2, 4, 6, 8, 10, GM-CSF, TNF-alpha and interferon -gamma). Other association tests between each of the 8 Cytokines against dichotomous variables like gender, age, wealth index, WAZ (normal or underweight), HAZ (normal or stunted), BAZ (normal or wasted), hemoglobin (normal or abnormal) and albumin (low or high) are also be provided.

Aflatoxin and Cytokines: Linear Association

The analysis of cytokines was done for a total of 173 samples. Cytokines are known to be low to undetectable in the absence of an appropriate trigger. The linear association of the Cytokines to Serum Aflatoxin levels (log transformed) is shown in the scatter plots in figure 29 below. The

association seems generally negative for 6 out of the 8 cytokines, with the exception of IL-10 and GM-CSF.

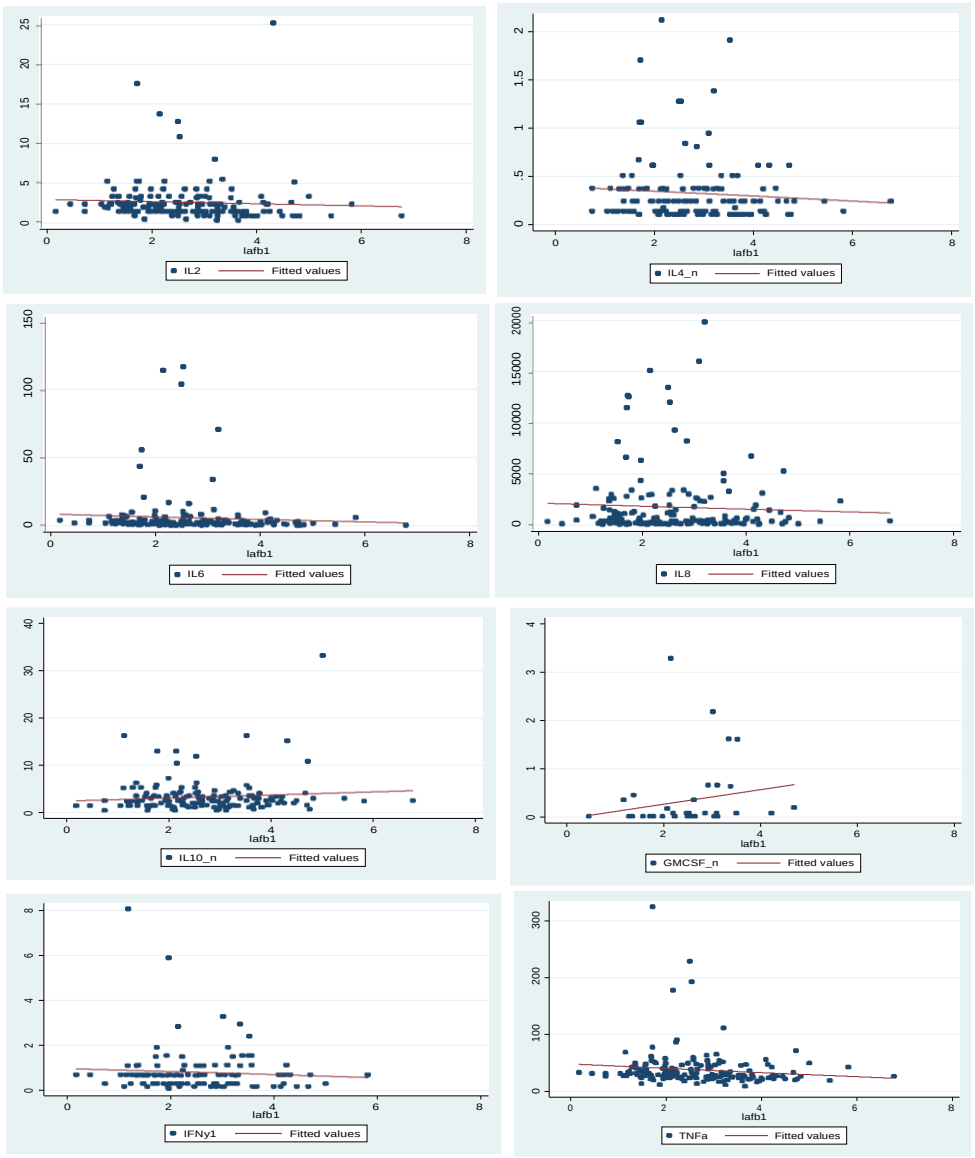


Figure 30: Scatter Plots of the 8 Studied Cytokines

Distribution of Cytokines Across High and Low Aflatoxin Categorization

The box plots in figures 30-37 below give the distributional properties of the Cytokines with categorized exposures to Aflatoxin. There appears to be a slight difference in the level of IL2, IL6

and TNFa when there are high aflatoxin levels. These cytokines appears slightly depressed compared to their median levels when aflatoxin is Low.

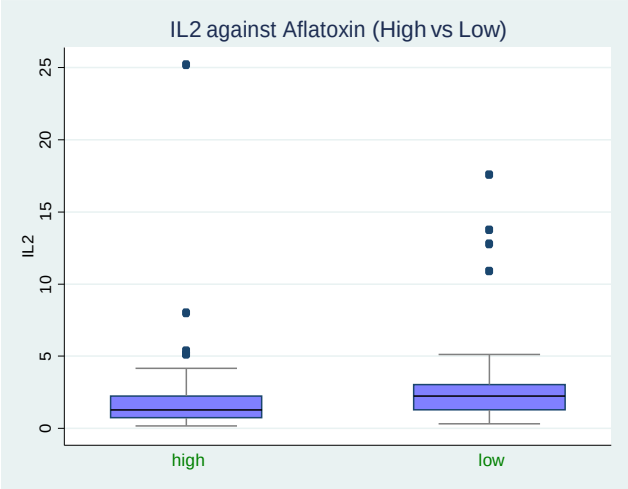


Figure 31: IL2 Against Aflatoxin (High vs Low)

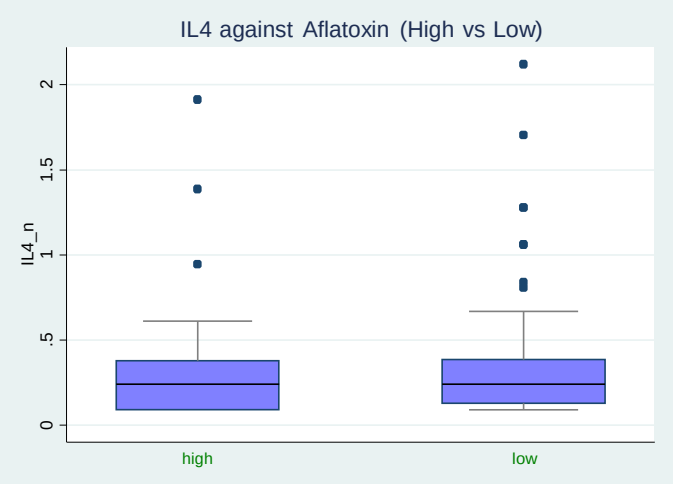


Figure 32: IL4 Against Aflatoxin (High vs Low)

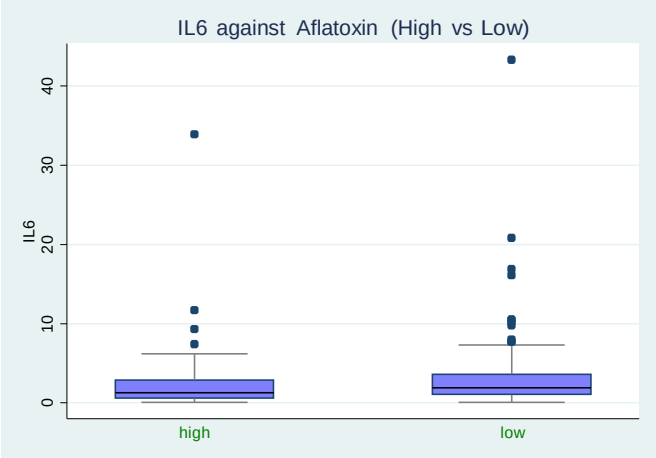


Figure 33: IL6 Against Aflatoxin (High vs Low)

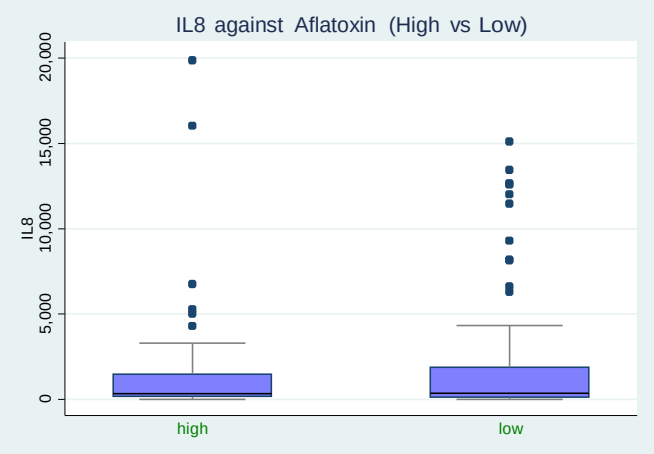
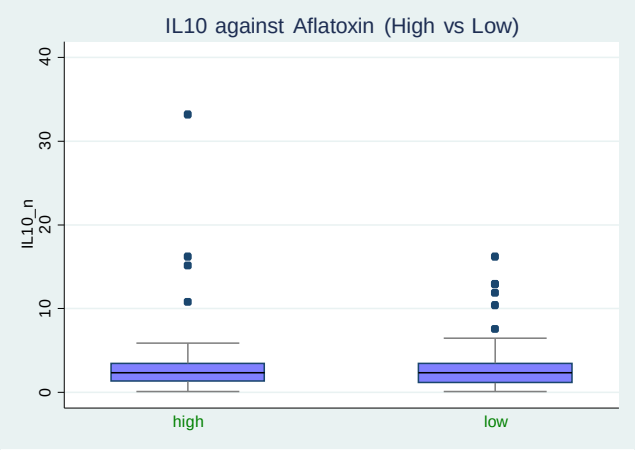


Figure 34: IL8 Against Aflatoxin (High vs Low)



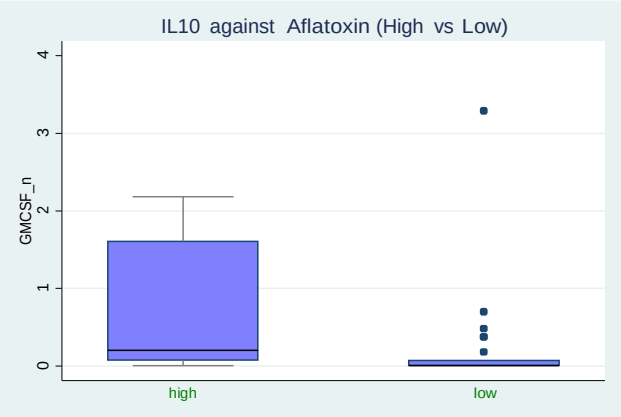


Figure 35: IL10 Against Aflatoxin (High vs Low)

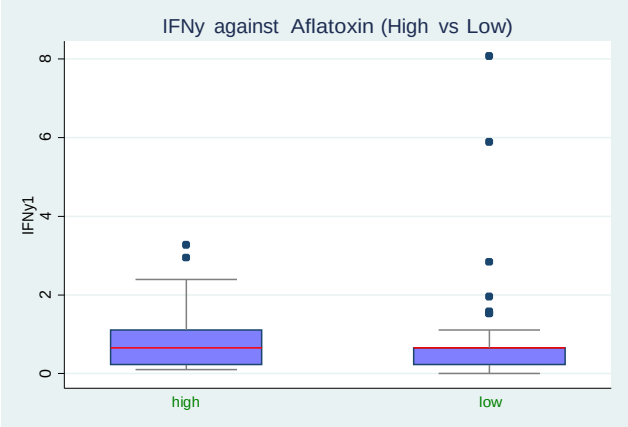


Figure 36: IL10 Against Aflatoxin (High vs. Low)

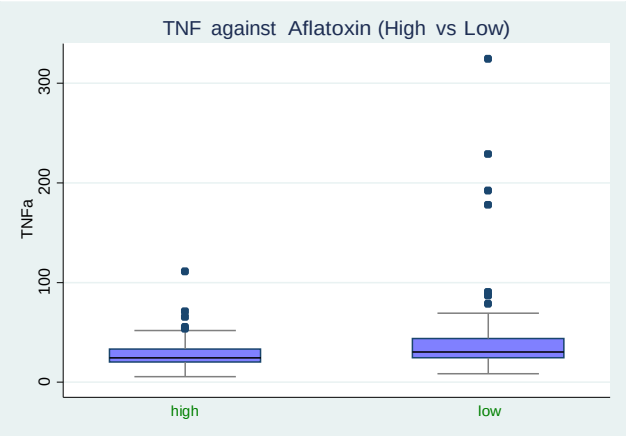


Figure 37: IFNy Against Aflatoxin (High vs. Low)

Figure 38: TNF Against Aflatoxin (High vs. Low)

Cytokines and Characteristic Variables: Association Tests

In this section, the objective was to assess if there are any statistically significant associations between the Cytokines and grouped categories of the Aflatoxin Levels, wealth index, age groups, gender, waz (weight for age z-score), haz, (height for age z-score), baz (BMI for age z-score) and muac (Mid upper arm circumference).

The consideration of the test of association to be used was made after analysis of the distributional properties of the different cytokines. If the cytokine was normally distributed, then an Analysis of variance (ANOVA) was done. Analysis of variance (ANOVA) is the most commonly used technique for comparing the means of groups of measurement data. It is used when you have a categorical independent variable (with two or more categories) and a normally distributed interval dependent variable and the main objective is to test for differences in the means of the dependent variable by the different categories of the independent variable.

If the Cytokine is not normally distributed, a non-parametric version of the ANOVA was used, the Kruskal Wallis Test. Non-parametric tests make no assumptions about the distribution of the dataset (it is distribution free). This is therefore used when the normality assumption of the ANOVA is not met.

Tests of Normality of Cytokines

To test whether the cytokines were normally distributed, we used the Shapiro Wilk Test whose results are presented in table 25 below:

Table 25: Testing if the Cytokines are Normally Distributed using Shapiro Wilk's Test

Variable	*Obs*	*z*	*Prob>z*
IL2	173	10.8	0.0000
IL4_n	146	8.0	0.0000
IL6	173	10.5	0.0000
IL8	173	9.5	0.0000
IL10_n	156	8.9	0.0000
TNFa	173	9.6	0.0000
IFNy1	123	8.4	0.0000
GMCSF_n	37	5.6	0.0000

None of the Cytokines was normally distributed, based on the p-values observed (less than the 5% level of significance). Therefore, Kruskal Wallis test of association (non-parametric) was used.

Kruskal Wallis Test Between Cytokines and Aflatoxin Level (High vs. Low)

The Kruskal Wallis results show a significant statistical difference in the levels of IL2, TNFa and GMCSF across the two Aflatoxin Categories as indicated in table 26.

Table 26: Results of Kruskal Wallis Test between Cytokines and High/low AFB1

Cytokines and Aflatoxin Level	Chi-Sq Stat	P-value
IL2	7.611	0.0058
IL4_n	0.871	0.3507
IL6	2.617	0.1057
IL8	0.002	0.9635

IL10_n	0.004	0.9519
TNFa	9.605	0.0019
IFNy1	0.341	0.5595
GMCSF_n	5.645	0.0175

A Dunn test (table 27) on the three Cytokines was carried out to assess the nature of the differences and the results indicated that:

1) IL2 levels are significantly lower when there is high Aflatoxin Exposure
2) TNFa levels are significantly lower when there is high Aflatoxin Exposure
3) However, GMCSF levels are significantly higher when there is high Aflatoxin Exposure

Table 27: Dunn Test Results to Assess the Nature of the difference between IL2, TNF-Alpha and GM-CSF Cytokines

Cytokine	*Comparison*	*Z*	*P.unadj*	*P.adj*
IL2	High vs Low Aflatoxin	-2.7802	0.0054	0.0054
TNFa	High vs Low Aflatoxin	-3.1005	0.0019	0.0019
GMCSF	High vs Low Aflatoxin	2.5351	0.0112	0.0112

Kruskal Wallis Test Between Cytokines and Other Variables

Table 28: IL2 and Categorical Variables

IL2 and Categorical Variables	*Chi-Sq Stat*	*P-value*
Gender (Male vs Female)	0.008	0.9309
Wealth Index (Poor, Rich or Middle)	2.783	0.2487
Age Groups (Above 5 or below 5)	1.072	0.3005
Age Groups (Under 5 years, 5-8 years, Above 8)	1.854	0.3957
BAZ (Normal Weight, Overweight or Underweight)	3.351	0.1872
HAZ (Stunted or Not Stunted)	1.073	0.3003
WAZ (Underweight or Normal weight)	1.423	0.2329
MUAC (at risk, normal or sam)	0.730	0.6943

There are no statistically significant associations between the level of IL2 Cytokines and the characteristic variables.

Table 29: IL4 and Categorical Variables

IL4 and Categorical Variables	*Chi-Sq Stat*	*P-value*
Gender (Male vs Female)	0.004	0.9497
Wealth Index (Poor, Rich or Middle)	0.280	0.8692
Age Groups (Above 5 or below 5)	2.755	0.0969
Age Groups (Under 5 years, 5-8 years, Above 8)	2.384	0.3037
BAZ (Normal Weight, Overweight or Underweight)	1.186	0.5528
HAZ (Stunted or Not Stunted)	0.892	0.3450
WAZ (Underweight or Normal weight)	0.705	0.4010
MUAC (at risk, normal or sam)	1.362	0.5061

There are no statistically significant associations between the level of IL4 Cytokines and the characteristic variables.

Table 30: IL6 and Categorical Variables

IL6 and Categorical Variables	*Chi-Sq Stat*	*P-value*
Gender (Male vs Female)	0.164	0.6855
Wealth Index (Poor, Rich or Middle)	5.265	0.0719
Age Groups (Above 5 or below 5)	0.062	0.8034
Age Groups (Under 5 years, 5-8 years, Above 8)	0.214	0.8987
BAZ (Normal Weight, Overweight or Underweight)	2.548	0.2797
HAZ (Stunted or Not Stunted)	1.622	0.2028
WAZ (Underweight or Normal weight)	1.606	0.2050
MUAC (at risk, normal or sam)	1.172	0.5565

There is a marginal significance of IL6's association with the Wealth Index Classifications. However, this is only noted at the 90% confidence level. All other variables for not report any statistically significant associations between the level of IL6 Cytokines and the characteristic variables.

Table 31: IL8 and Categorical Variables

IL8 and Categorical Variables	*Chi-Sq Stat*	*P-value*
Gender (Male vs Female)	0.065	0.7981
Wealth Index (Poor, Rich or Middle)	3.163	0.2056

Age Groups (Above 5 or below 5)	7.163	0.0074
Age Groups (Under 5 years, 5-8 years, Above 8)	10.065	0.0065
BAZ (Normal Weight, Overweight or Underweight)	1.060	0.5885
HAZ (Stunted or Not Stunted)	0.257	0.6121
WAZ (Underweight or Normal weight)	3.371	0.0664
MUAC (at risk, normal or sam)	3.941	0.1394

Table 32: Dunn Test Results to Interrogate Differences in the Age Groups as Regards IL8

Comparison	*Z*	*P.unadj*	*P.adj*
9 to 14 years vs Below 5	2.5609	0.0104	0.0104
9 to 14 years vs 5 to 8	2.3013	0.0214	0.0214
Below 5 years vs 5 to 8 years	-0.8150	0.4151	0.4151

The analysis shows there is a statistical difference in IL8 for sampled children between 9 to 14 years vs. those Below 5 years and those between 9 to 14 years vs. 5 to 8 years. It is clear that the 9 to 14 age-bracket has statistically higher IL8 levels as compared to the younger age brackets.

Table 33: Monoletters to Explore differences between Age Groups and IL8

Group	*Letter*	*MonoLetter*
9 to 14 years	a	a
Below 5 years	b	b
5 to 8 years	b	b

Table 34: IL10 and Categorical Variables

IL10 and Categorical Variables	*Chi-Sq Stat*	*P-value*
Gender (Male vs Female)	0.030	0.8630
Wealth Index (Poor, Rich or Middle)	0.646	0.7240
Age Groups (Above 5 or below 5)	2.192	0.1387
Age Groups (Under 5 years, 5-8 years, Above 8)	0.632	0.7291
BAZ (Normal Weight, Overweight or Underweight)	1.765	0.4138
HAZ (Stunted or Not Stunted)	0.598	0.4392
WAZ (Underweight or Normal weight)	5.241	0.0221
MUAC (at risk, normal or sam)	0.616	0.7351

There is a noted significance of IL10's association with the WAZ (Weight for age z-score). All remaining variables for not report any statistically significant associations between the level of IL10 Cytokines and the characteristic variables. A further analysis of the differences between the two

groups (Underweight or Normal weight) is done to assess how the groups differ from each other. This is done using a Dunn test. The results are shown below:

Table 35: Results of Dunn Test to Explore Nature of the difference in WAZ as Regards Underweight and Normal Weight

Comparison	*Z*	*P.unadj*	*P.adj*
Normal Weight vs Underweight	-2.2959	0.0217	0.0217

The results indicate that the sampled children with normal weight have statistically lower IL10 levels as compared their underweight counterparts

Table 36: IFNy and Categorical Variables

IFNy and Categorical Variables	*Chi-Sq Stat*	*P-value*
Gender (Male vs Female)	1.832	0.1759
Wealth Index (Poor, Rich or Middle)	3.400	0.1827
Age Groups (Above 5 or below 5)	0.020	0.8868
Age Groups (Under 5 years, 5-8 years, Above 8)	0.030	0.9853
BAZ (Normal Weight, Overweight or Underweight)	1.253	0.5345
HAZ (Stunted or Not Stunted)	0.794	0.3728
WAZ (Underweight or Normal weight)	0.088	0.7663
MUAC (at risk, normal or sam)	2.103	0.3493

There are no statistically significant associations between the level of IFN-y Cytokines and the characteristic variables

Table 37: TNFa and Categorical Variables

TNFa and Categorical Variables	*Chi-Sq Stat*	*P-value*
Gender (Male vs Female)	0.018	0.8946
Wealth Index (Poor, Rich or Middle)	1.376	0.5026
Age Groups (Above 5 or below 5)	3.402	0.0651
Age Groups (Under 5 years, 5-8 years, Above 8)	5.577	0.0615
BAZ (Normal Weight, Overweight or Underweight)	2.604	0.2720
HAZ (Stunted or Not Stunted)	0.782	0.3765
WAZ (Underweight or Normal weight)	0.022	0.8814
MUAC (at risk, normal or sam)	1.415	0.4930

There is a marginal significance of TFNa's association with the Age Group Classifications. However, this is only noted at the 90% confidence level. All remaining variables for not report any statistically significant associations with the Cytokine

Regression Analysis for the Cytokines

A multivariate analysis of how the level of serum aflatoxin affects the Cytokine levels is done using a regression analysis, with the remaining characteristic variables appearing as control variables. These will include wealth index, age groups, gender, waz (weight for age z-score), haz, (height for age z-score), baz (BMI for age z-score) and muac (Mid upper arm circumference) and liver function tests (Albumin, tprotein, ast, alt, ggt) and dds (dietary diversity score). The coefficients from the regression analysis are presented below, with the corresponding p-values in brackets beneath them. P-values less than 0.05 indicate significance of the coefficient. These are highlighted in red.

Table 38: Regression Analysis Results for Cytokines with other Study Variables

	IL2	*IL4*	*IL6*	*IL8*	*IL10*	*TNFa*	*IFNy1*	*GMCSF*
Log Aflatoxin	-1.26	-0.04	-0.82	-80.04	-0.05	-2.50	0.02	-0.09
	(0.128)	(0.228)	(0.444)	(0.724)	(0.871)	(0.183)	(0.871)	(0.882)
Female	0.07	0.02	-0.50	31.17	-0.30	2.03	-0.24	1.96
	(0.966)	(0.784)	(0.816)	(0.945)	(0.631)	(0.589)	(0.31)	(0.478)
AGE==Above 5 years	-1.99	0.02	-3.87	149.53	-0.61	-8.08	0.25	-0.43
	(0.283)	(0.709)	(0.113)	(0.77)	(0.386)	(0.058)	(0.323)	(0.746)
WAZ==Underweight	0.22	-0.13	-6.18	-677.35	1.82	-2.15	-0.42	-5.47
	(0.945)	(0.205)	(0.14)	(0.441)	(0.125)	(0.766)	(0.339)	(0.528)
BAZ==Normal Weight	-0.38	0.09	1.50	652.79	1.68	2.45	-0.39	0.37
	(0.933)	(0.586)	(0.801)	(0.602)	(0.309)	(0.812)	(0.538)	(0.861)
BAZ==Underweight	0.92	0.35	15.45	2218.14	2.58	18.33	-0.26	0.96
	(0.856)	(0.065)	(0.022)	(0.116)	(0.169)	(0.115)	(0.722)	(0.478)
HAZ==Not Stunted	-0.93	-0.08	2.77	140.57	-1.03	5.89	0.36	0.67
	(0.776)	(0.463)	(0.520)	(0.877)	(0.392)	(0.432)	(0.363)	(0.461)
MUAC==Normal	2.93	0.09	2.99	728.95	1.38	0.16	0.48	-3.38
	(0.274)	(0.329)	(0.394)	(0.325)	(0.181)	(0.980)	(0.264)	(0.192)
MUAC==Sam	2.84	0.02	2.67	110.13	-0.71	-4.72	1.57	0.00
	(0.664)	(0.923)	(0.755)	(0.951)	(0.766)	(0.751)	(0.15)	(0.674)
WEALTH==Middle	-3.24	-0.12	-5.34	-645.37	-1.09	-6.14	0.47	-0.75
	(0.132)	(0.133)	(0.06)	(0.277)	(0.192)	(0.210)	(0.083)	(0.606)
WEALTH==Poor	-6.82	-0.06	-2.52	-214.00	-1.91	-4.52	0.15	-1.00
	(0.01)	(0.542)	(0.464)	(0.768)	(0.066)	(0.451)	(0.671)	(0.687)
Albumin	1.08	0.15	2.84	1039.95	1.38	3.54	-0.29	-2.74
	(0.613)	(0.046)	(0.313)	(0.082)	(0.083)	(0.469)	(0.378)	(0.261)
tprotein	-10.84	-0.09	-26.35	-438.98	0.70	-74.80	-2.05	-6.40
	(0.272)	(0.818)	(0.044)	(0.872)	(0.848)	(0.001)	(0.11)	(0.106)
Ast	0.27	0.00	0.04	11.43	0.08	0.37	0.02	0.21
	(0.038)	(0.380)	(0.827)	(0.745)	(0.079)	(0.203)	(0.347)	(0.147)
Alt	0.82	0.00	-0.08	2.31	-0.03	-0.05	-0.01	-0.11
	(0.000)	(0.662)	(0.583)	(0.942)	(0.518)	(0.854)	(0.622)	(0.336)
Ggt	0.15	0.00	0.06	-31.40	-0.10	-0.03	0.02	0.09
	(0.484)	(0.921)	(0.834)	(0.585)	(0.225)	(0.953)	(0.414)	(0.471)
Dds	0.52	-0.04	-0.97	-173.18	-0.55	-1.89	-0.06	0.07
	(0.391)	(0.096)	(0.224)	(0.303)	(0.022)	(0.173)	(0.465)	(0.775)
_cons	-19.58	-0.33	7.33	-3967.16	-3.16	56.41	1.72	12.25
	(0.105)	(0.416)	(0.642)	(0.234)	(0.472)	(0.042)	(0.364)	(0.293)

For the Cytokines, 7 out of the 8 Cytokines show a decline when the level of aflatoxin increases. The only exception is IFNy. The effect of Aflatoxin exposure on the 7 Cytokines is however insignificant. Since the model used log of Aflatoxin, then if we change the level of Aflatoxin

exposure by 1%, we expect our y variable to change by $\beta_1/_{100}$ units. Specifically, IL8 levels decline by up to 8.004 when the level of Alfatoxin increases by 1%. The reduction in TNFa levels is by 0.025 when Aflatoxin levels increase by 1%. This followed closely behind by a decline in IL2 levels by 0.0126.

The highlighted cells indicate which variables significantly affect the specific Cytokines. It is noted that IL2 is positively and significantly affected by the liver function tests results, particularly, ast (+0.27) and alt (+0.82). There is also significant decline in the level of IL2 for the sampled children who are from poor households (-6.82).

Six out of the 8 Cytokines are positively affected by Albumin levels (with the exception of IFNY and GM-CSF). Specifically, IL4 is positively and significantly affected by Albumin levels. 7 out the 8 Cytokines (except IL10) are negatively affected by t-protein levels. The effect of t protein is significant on TNFa and IL6.

It is noted that IL6 levels are significantly higher in children with a lower BMI for Age Score (Underweight). IL10 is significantly reduced in children who have a higher DDS (dietary diversity score).

Seven out the 8 Cytokines record a decline when the child is from a poor background. The only exception is IFNy once again. To further explore which cytokines drove the cytokine data, we looked at the Principal Component Analysis of the same. This is discussed fully in the following pages.

Principal Component Analysis for the Cytokines

Principal component analysis is a method of extracting important variables (in form of components) from a large set of variables available in a data set. It extracts low dimensional set of features from a high dimensional data set with a motive to capture as much information as possible. With fewer variables, visualization also becomes much more meaningful.

PCA gets some linearly independent components (Correlation between each is zero). These principal components are normalized linear combination of the original predictors in a data set. Each component captures some level of variance in the data set, with the first one capturing the most.

How it works

Principal Component analysis achieves its objective by producing Eigen Vectors and Eigen Values. Every eigenvector has a corresponding eigenvalue. Simply put, an eigenvector is a direction, such as "vertical" or "45 degrees", while an eigenvalue is a number telling you how much variance there is in the data in that direction

The eigenvector with the highest eigenvalue is, therefore, the first principal component. The number of eigenvalues and eigenvectors that exits are equal to the number of dimensions the data set has.

In the analysis, we will consider three results presented below:

1) Eigen Values of the Principal Components
2) Co-ordinates (Correlations between the Cytokines and the principal components (PCs))
3) Quality of representation of the Cytokines: Given by Cosine-Squared

Table 39: Eigen Values of the Principal Components

Eigen Value Results for the Principal Component Analysis

Dimension/Principal Component	Eigenvalue	Variance (%)	Cumulative Variance (%)
Dim.1	3.6164	45.20	45.20
Dim.2	1.1940	14.93	60.13
Dim.3	0.9857	12.32	72.45
Dim.4	0.8626	10.78	83.23
Dim.5	0.6085	7.61	90.84
Dim.6	0.4375	5.47	96.31
Dim.7	0.2215	2.77	99.08
Dim.8	0.0737	0.92	100.00

The eigenvalues measure the amount of variation retained by each principal component/dimension. Eigenvalues are large for the first PCs and small for the subsequent PCs. The extracted eigen values above show that the Cytokine Data has eight dimensions/ principal components.

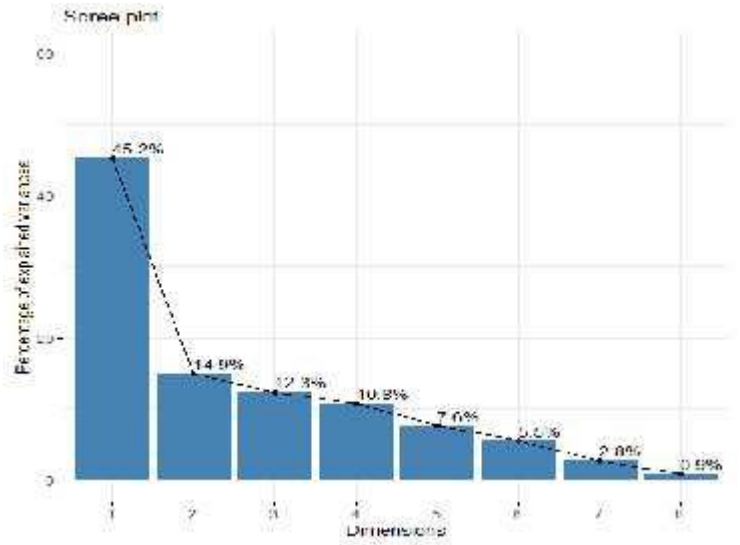


Figure 39: Bar chart of eigenvalues against the 8 principal components

The amount of variance explained by each principal component is visually represented in the scree plot above.

Eigenvalues can be used to determine the number of principal components to retain after PCA (104). If the *eigenvalue* > 1 indicates that the Principal Component accounts for more variance than accounted by one of the original variables in standardized data.

Therefore, based on the above, we will consider Principal Component 1 and 2. The two components have eigen values greater than 1, and cumulatively they explain up to 60% of the variation within the data set.

2) Co-ordinates

The goal of PCA is to summarize the correlations among a set of observed variables with the principal components. The correlation between a variable and a principal component (PC) is used as the coordinates of the variable on the PC. The plot shown below is also known as a variable correlation plot.

Positively correlated observations are grouped together. Negatively correlated variables are positioned on opposite sides of the origin (opposed quadrants). The distance between variables and the origin measures the quality of the variables on the factor map

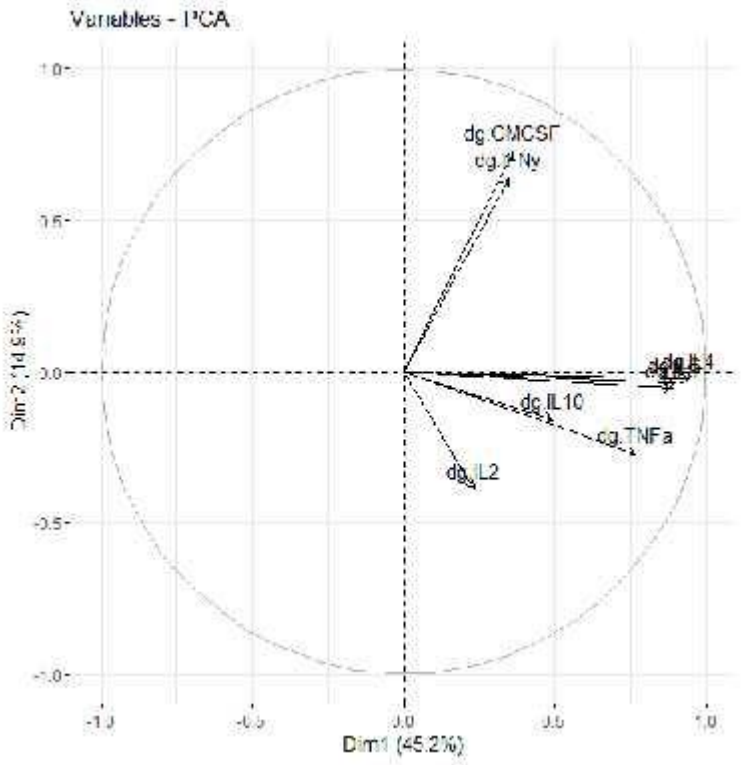


Figure 40: Correlations of Cytokines and the 1st and 2nd Principal Components

The chart alongside plots correlations of the Cytokines and the 1ST and 2ND Principal Components. Cytokines that appear in the same quadrant have similar relationships with the Principal Components. The x-axis represents correlations with Principal Component 1 (Dim1) while the y-axis represents correlations with Principal Component 2 (Dim2)

For example, GMCSF and IFNy are positively correlated with both Principal Component 1 and 2. The remaining 6 cytokines (IL2, IL4, IL6, IL8, IL10 and TNFa) are positively correlated to Principal Component 1, but negatively correlated to Principal Component 2.

3) Quality of Representation: Cosine-Squared

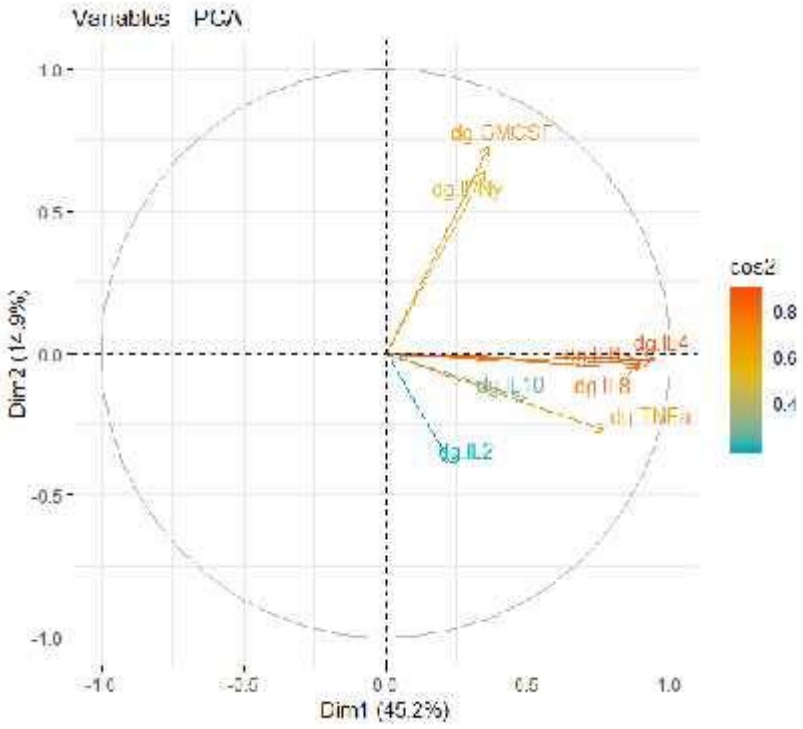


Figure 41: Squared Cosine Showing the Quality of the Variables that Make Up Principal Components

The quality of representation of the variables on the factor map is also known as squared cosine. It is estimated by taking the square of the correlations (all of the squared cosines are therefore positive).

The squared cosine shows the importance of a principal component for a given observation. The squared cosine (squared factor loading) is the percent of variance in that observation explained by the principal component. A high cos2 indicates a high contribution of the principal component on the Cytokine. In this case, the Cytokine is positioned close to the circumference of the correlation circle. A low cos2 indicates that the observation (Cytokine) is not perfectly represented by the PCs. In this case the Cytokine is close to the centre of the circle. Components with a large value of cos2 contribute a relatively large portion and therefore these components are important for that

observation. In the plot below, the closer a variable is to the circumference, the better we can reconstruct this variable from the first two principal components (and therefore this makes it more important to interpret these components).

The results show that IL4, IL6, IL8 and TNFa are well represented by the first principal component. The contribution of the first principal component to these four Cytokines is high. They are close to the circumference of the circle. The variation in GM-CSF, IFNy, IL2 and IL10 is not well represented by the first Principal Component. The squared cosines are relatively lower.

The second principal component separates two Cytokines (GM-CSF and IFNy) from the remaining 6 Cytokines. This is shown by how highly the second component contributes highly to GMCSF and IFNy but does not do the same for the remaining Cytokines.

The objective of PCA is to select a subset of variables from a larger set, based on which original variables have the highest correlations with the principal component. It appears from the analysis above, the variation in the Cytokine Data is driven by four Cytokines; namely IL4, IL6, IL8 and TNFa, based on their how well they are represented by the first principal component. A further analysis can be done to understand the factors responsible for the differing behaviour between the groups of Cytokines, especially between the first four well explained by PC1 (IL4, IL6, IL8 and TNFa) and the remaining four (IL2, IL10, GMCSF and IFNY)

4.7 Conclusion

The purpose of this cross-sectional study was to determine the prevalence and clinical immunological effects of aflatoxin exposure in children below 14 years of age from Makueni County, Kenya. The clinical effects were represented by anthropometric measurements. Serum aflatoxin was the measure of exposure, while the Hepatitis B surface antibodies were the outcome

measure that represented immune response. The 8 inflammatory markers (cytokines) were exploratory in nature to assist us look for possible patterns of inflammation in children exposed to immune modulating aflatoxin. The Wealth index, dietary diversity scores, anthropometry tests, liver function tests and Fumonisin levels were used in the analysis.

Due to the close interactions of the measured variables, conclusions were drawn following univariate then multivariate analysis as indicated below.

Univariate Analysis

Preliminary results from the Univariate analysis indicated that there was a statistically significant difference in aflatoxin levels across the three wealth indices. The aflatoxin exposures were significantly higher in both middle and poor class relative to the rich class. There was no statistical difference between the middle class and the poor class. A simple regression analysis indicated that all other factors held constant, if the individual was RICH, then their level of aflatoxin exposure was 42% lower than that of an individual in middle class. If the individual was POOR, then their level of aflatoxin exposure was 17% higher than that of an individual in middle class.

A statistically significant difference in aflatoxin levels across the three dietary diversity score categories (high, low, medium) was also seen. A further analysis showed that the difference in aflatoxin exposure lay between children with high dietary diversity score and those with low diversity score, as well as between the low diversity score and medium diversity score. However, there was no statistical difference between the high and medium diversity score categories. The aflatoxin exposures were higher for children with low dietary diversity scores. A simple regression proved that if the child had a low dietary diversity score, then their level of aflatoxin exposure was 53% higher than that of a child with a medium score. A child with a low score also had a 67% higher level of aflatoxin exposure compared child with a high dietary diversity score.

The results showed a statistically significant association between the level of anti-HB antibodies and age groups at the univariate testing level. Further analysis carried out at the multivariate stage (below) was able to decipher where the differences lay, particularly detailing which age group had higher anti-HB antibodies. At this stage of the analysis, no significant relationships were observed between the Aflatoxin exposure and the level of Anti-HB antibodies.

A univariate analysis of the Cytokines revealed that IL2 and TNFa levels were significantly lower when there was high Aflatoxin Exposure. However, GMCSF levels were significantly higher when there was high Aflatoxin Exposure. There was a noted significance of IL8's association with the Age Group Classifications. The 9-14 age-bracket had statistically higher IL8 levels as compared to the younger age brackets. The findings also revealed that children with normal weight had statistically lower IL10 levels as compared their underweight counterparts.

Multivariate Analysis

The multivariate logistic regression used in the study assessed how each of the independent variables affected the immune response of the child. The key independent variable here was the level of Aflatoxin Exposure. The immune response (as captured by the Anti-HBs level) was either Positive or Negative.

Aflatoxin Serum was found to reduce the immune response in children below 14 years. The likelihood of observing negative Anti-HBs was 1.35 times higher when the level of Aflatoxin exposure increased. Similarly, the ratio of sphingasine (sa) to sphingosine (so) -subsequently denoted as saso- was the variable that was used in regression analysis to represent fumonisins, confirmed that they depressed immune response of the children. The probability of observing negative level of Anti-HBs was 1.18 times higher when the ratio -saso -increased.

The age group dummy variable significantly affected the immune response of the child. Children above 5 years old had a significantly lower immune response as compared to children below 5 years old. Specifically, the odds ratio indicated that children above 5 years were up to 4 times more likely to have negative Anti-HBs as compared to the base group (children below 5 years old).

Females were more likely to have a better immune response compared to males. The odds ratio indicated that females had up to 74% reduced odds of having negative anti-HBs (OR = 0.26). The effect was however marginally significant (p-value is 0.051)

The odds ratio on "Poor" was 1.6, indicated that children from poor backgrounds were 1.6 times more likely to have lower immune responses as compared to children from rich backgrounds.

For children who were stunted (according to the Height for Age score), they were 1.10 times more likely to have Negative Anti-HBs than children who were not stunted. Similarly, children who were at risk, according to their Mid Upper Arm circumference were 3.42 times more likely to have lower immune response (i.e. more likely to have Negative Anti-HBs.)

The results, however, showed that Children who were Underweight (according to the BMI for Age and Weight for Age Scores) were less likely to have low (negative) immune response.

Children with a high dietary diversity score had better immune response as compared to children with low dietary diversity score. Those with Low DDS were 2 times more likely to have negative anti-HBs compared to those with high DDS.

Albumin and ggt levels were associated with increased immune response of the child. The odds ratios were less than 1, an indication that when Albumin and ggt levels were high, there was a lower likelihood of observing negative anti-HBs.

From the Cytokines analysis, it was found that 7 out of the 8 Cytokines showed a decline when the level of aflatoxin increased. Since the model used log of Aflatoxin, then if we changed the level of Aflatoxin exposure by 1%, we expected that our y variable would change by $\beta_1/_{100}$ units. Specifically, IL8 levels declined by up to 8.004 when the level of Aflatoxin increased by 1%. The reduction in TNF-a levels was by 0.025 when Aflatoxin levels increased by 1%. This was followed closely behind by a decline in IL2 levels by 0.0126.

The principal component analysis of the Cytokines showed that GM-CSF and IFN-y had similar behavior in that they were both positively correlated with both Principal Component 1 and 2. 4 cytokines (IL4, IL6, IL8 and TNFa) behaved similarly in that they were strongly correlated to Principal Component 1, but negatively correlated to Principal Component 2.

CHAPTER 5: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Although literature exists that suggest that aflatoxin has immune modifying effect that possibly affects vaccine effectiveness, there have been few childhood specific data that have sought to further explore this. This is in spite of the continued high morbidity and mortality largely from vaccine preventable infections as discussed in Chapter 1.

Of the 433 children whose serum samples were tested for AFB1, there detection rate of 100% with the highest levels being 901.15 pg/mg albumin while the lowest was 0.74 pg/mg albumin with a median of 19.62 with a skewing to the right. This rate of exposure is higher than in previously quoted studies. Leroy *et al.*, study (105) reported a median level of 7.47pg/mg albumin, while Yard and colleagues reported a median level of 1.78pg/mg albumin(106). Turner, working in the Gambia, reported a geometric mean adduct level of 22.3pg/mg (107), while a study in Nepal had median levels of 3.62pg/mg albumin (108). The Nepalese study reported that aflatoxin exposure during the first 36 months of life was not associated with impaired growth in their children (108). This may perhaps be due to the low median levels. Yard's paper showed a big difference in the median levels of aflatoxin across Kenyan counties as depicted by Figure 10. Our study took place between June and December 2016 when the area was experiencing scattered light rains. It may well be that some of the analyzed grains may have been purchased from elsewhere and not necessarily grown in the homestead from which they were collected.

This study confirmed a trend of higher level of aflatoxin in the females across wealth index compared with the males. This was not statistically significant. It remains unclear whether this is a biological effect in gender differentiation in the handling of aflatoxin in the diet. It is recognized that there is a difference in the way the different sexes react to vaccines even before the onset of hormonal differences and this may also be true for aflatoxin handling between the sexes(109).

Innate detection of viruses by pattern recognition receptors in rats has been known to differ between the males and females (110). It has also been known that some genes that encode for immunological proteins are in X chromosomes which may result in higher amounts of expression in females compared to males. Besides, inflammatory immune response are higher in females in both humans and rodents as is differences in cytokine responses in infections. Future studies may wish to consider exploring if indeed there exists sex differences as regards aflatoxin exposure in pre-pubertal children.

This study showed that females had better immune response compared to the males (OR 0.26). This effect was however only marginally significant (P-value 0.05). Further, children above 5 years were up to 4 times more likely to have no protective antibodies. Poor children and those stunted also experienced poor immune response. This effect was not demonstrated in underweight children however.

There was a significant association between the level of serum aflatoxin and the socio-economic class. Wealth index statistically predicted the likelihood of a child having high serum aflatoxin. All other factors held constant, if the individual is RICH, then their level of aflatoxin exposure was 42% lower than that of an individual in middle class. If the individual was POOR, then their level of aflatoxin exposure was 17% higher than that of an individual in the middle class.

Dietary diversity score and wealth score index was strongly associated. A child with low dietary diversity score experienced a level of aflatoxin exposure that was 53% higher than that of a child with a medium score. A child with a low dietary diversity score had 67% higher level of aflatoxin exposure compared child with a high dietary diversity score. Not surprisingly, there was also a strong association between serum aflatoxin levels and grain contamination with aflatoxin. Whereas

these findings may seem obvious, they clearly point to the centrality of poverty in aflatoxin exposure and directing us to an important starting point, certainly at a policy level, in aflatoxin mitigations measures. Leroy *et al.,* 2015 showed a similar trend in Meru Kenya (105).

Malnutrition is said to occur when there is one or more of the following situations; wasting (<-2sd WHZ), underweight (<-2sd WAZ) or stunting (<-2sd HAZ). Sixteen (16) per cent of the population in this study was stunted compared to the national figure 26% in Kenya Demographic Health Survey of 2014 that assessed a younger age range of 18-23 months. Stunting indicates the effects of chronic food shortage and has serious implications especially for children one thousand days old. The 2014 KDHS anthropometric measurements used data obtained 5 years previously(44). Our 2016 study had only 25% of studied population falling in the under 5 years' category while the rest were older. Any comparisons of malnutrition rate in the two groups may not be appropriate due to the highly variable populations used in arriving at the malnutrition rate levels. The possibility that food supplies may have improved by the time the 2016 study took place hence the lower rate of stunting cannot be ruled out.

Wasting generally suggests recent illness especially after diarrhoeal illness or following a rapid deterioration in food supplies. Wasting rates in this study was 5% against a national rate of 4% (KDHS), with a higher prevalence among under one-year-olds(44). This study excluded children under one-year-old making a direct comparison inaccurate. It should be noted that underweight reflects both effects of acute and chronic malnutrition status. The rate of underweight in our study was 11% similar to the 2014 KDHS national rate that showed that, Makueni County had registered a slightly lower underweight rate of 10.2%.

Aflatoxin impairs the intestinal barrier function resulting in mal-absorption and micronutrient deficiency (111). Systemic immune activation following increased permeability of the enterocytes (111) and inhibition of protein synthesis (13) are other postulated mechanisms that lead to malnutrition. This study, like others, showed a direct correlation between stunted children and the poor and an inverse correlation with gross domestic product (112). That the stunting rate of 16% found in this study is lower than the 26% of the national rate may suggest that Makueni County may have introduced positive intervention measures in the last 5 years.

Taken together, malnutrition as an independent factor, is the biggest risk factor for global burden of disease as previously indicated (68,69). By extension, one could infer that malnutrition has significant contribution to immunomodulation. In the presence of high prevalence of serum aflatoxin in vulnerable populations, infectious diseases are likely to continue contributing to our excess morbidity and mortality if we fail to intervene on upstream factors like widespread chronic aflatoxin consumption.

5.1 Sero-Protection Assessment

This study set out to measure effects of aflatoxin on immunity by measuring the levels of hepatitis Bs antibodies. While recognizing that not all measured antibodies are functional, quantifying hepatitis Bs antibodies is both practical and inexpensive compared to cell -mediated based tests. The technology to do so is more likely to be available in medium-sized laboratories in the county hospitals. The measures of IgG subclass and antibody avidity are known to give a better indication of quality of the humoral response again barring the costs and complexity of the procedure. 'In-house' functional assays- such as the neutralization assays used in assessing polio and measles antibody responses- can provide additional information. These tests are however both laborious and difficult to standardize rendering them less useful to the clinician in a county hospital.

Three doses of Hepatitis B vaccines achieve a protection rate of >90% (113). Anti –HBs concentration have been reported to rapidly decrease in the first year and more slowly thereafter but immune memory persists longer (114). We were however, unable to find any recent local epidemiological studies to determine the actual levels of hepatitis Bs antibodies in children except a yet to be published cross-sectional dissertation for University of Nairobi study by Wacheke-Nganga in 2009 (64).

Extended Programs of Immunizations (EPI) have been in place for a long time in countries that contribute the highest and mortalities. Vaccine-preventable conditions continue to cause unacceptably high mortality rates compared to countries with similar programs in the Northern hemisphere. There is urgent need for well-designed, adequately powered prospective studies to determine the effects of mycotoxins on robust clinical and immunological outcomes like IgG subclass, antibody avidity and cellular immune responses. This need is particularly urgent in the context of discussions on Sustainable Development Goals, Universal Health Coverage and the role of high impact interventions such as vaccines, in achieving defined targets. The researchers of these studies must synthesize and present the results of these studies specifically for the political leadership who are pivotal in ensuring policy formulation and operationalization.

The outcome of such research may well spur the scientific community with interest in vaccinology to re-look at our current vaccine scheduling to ensure it is informed by contextual local data.

Many of the schedules included in the Expanded Program of Immunization were developed empirically with little understanding of the cellular immune responses in those early days. Malnutrition, poor sanitation and personal hygiene, overcrowding, gut microbiota, sex, genetic factors, contaminated food and water are non-vaccine factors that impact on effectiveness of

vaccinations. In addition, gut dysbiosis is increasingly being recognized as a possible cause of the suboptimal immune responses to oral vaccines in developing countries (115). Our study has shown the inverse relationship between aflatoxin and anti-HBs antibodies where the likelihood of observing no protective antibodies was 1.35% higher as AFB1 increased. Fumonisins levels (saso) similarly reduced the likelihood of observing no protective hepatitis B antibodies 1.18 times higher as fumonisins increased. We used only one vaccine to demonstrate that aflatoxin is not only highly prevalent in the study population but that it significantly depresses antibody response. There is need undertake national sero-prevalence studies for the other routinely administered vaccines to ascertain if a similar trend exists and if so, whether the continued mortality is not related to vaccine failure.

Having confirmed a strong association between high serum aflatoxin levels and low hepatitis Bs antibodies, our data revealed that only 48% (98/205) tested positive for HBs screening tests. Four of the samples did not achieve the protective threshold of 10 mIU/ml - the level considered to give protection (116). This means that only 46% of the studied children can be considered to have sero-protection antibodies against hepatitis B infection. It must be remembered that this study took anti-HBs as our primary outcome measure of immune response with a dichotomized exploratory variable aflatoxin for purposes of analysis of the data set. There may well have been loss of data in using the median level of aflatoxin in order to derive ‘high’ and ‘low’ levels of aflatoxin for the sake of statistical analysis.

There is a shortcoming in the use antibodies as the only measure of vaccine sero-protection. In a study by Liao *et al.*,(117), 46% of children vaccinated 5-7 years previously had titres below 10mIU/ml. When these were given a booster dose, 90% showed evidence of an anamnestic response. The long incubation period of the disease has been reported to allow the anamnestic

response to be highly protective (109). In that study Plotkin went further to suggest that $CD4^+$ responses are better correlates of protection than antibody titres.

Passively acquired maternal antibodies and subclinical hepatitis B infections are other two reasons that may affect levels of anti-HBs. The asymptomatic children in this study were drawn from the community at the time of testing. It is known that vaccine antibody responses elicited before 12 months of age rapidly decline (118,119).

None of the children in our study were less than a year therefore minimizing the likelihood of having maternal antibodies. Van Damme *et al.*, have reported low to undetectable levels of Hepatitis B antibodies in 15-50% of fully vaccinated patients 5-15 years post vaccination (120). This study selected children who had been vaccinated within 1-13years previously. That 54% of them did not have protective antibodies in an area of high aflatoxin exposure is a matter of public health concern given the compounding effect of hepatitis B infections and chronic low dose exposure to aflatoxin. Hence, the findings of this study are broadly in line with those of other researchers in this area.

Vaccine delivery-related issues like site and dose of vaccine, the cold chain integrity, timelines of vaccinations, were not considered in this study.

Literature has suggested that aflatoxin depresses cell-mediated immunity even as it induces an inflammatory response that may lead to failure of immunizations (121). The baseline cytokine data in this study was more difficult to interpret. Most of the cytokines showed negative correlation with aflatoxin except for IL-10, TNF-alpha and perhaps GM-CSF. Principal Component Analysis confirms that the variation in the cytokine data is driven by IL4 (anti-inflammatory), IL-6 and IL 8 both of which are pro-inflammatory. A study using Peripheral Blood Mononuclear Cells (PBMCs)

that are stimulated with titrated levels of aflatoxin would greatly augment the study of relationships between cytokine response and serum aflatoxin levels. Taken together, the results we have from this study will form a basis of such studies in future.

5.2 Study Limitations

Fieldwork Challenges & Limitations

There was considerable underestimation of the repetitive process of fieldwork especially in community and individual consenting activities. The logistics of travel to the study site that was over 200km away from my workstation combined with the need to use motorcycles to access the sparsely spread out homes with poor access roads was not only cumbersome it also added significantly to the cost of the study. The community and individual consent processes were necessarily lengthy and repetitive to build trust as community entry is a complex process. This involved working with the local administration, local village leadership, The Senior Management of the County Government as well as the ministries of agriculture, health and education.

Drawing of blood from children, some as young as one-year in a rural home set up and occasionally out in the open elicited many emotions for both the children and the adult alike. This on a number of occasions resulted in spillage, inadequate sampling volumes, unclear/lost labeling and some vehicular accidents that led to loss of some of the data. Luckily the sample size had factored in this and we had 409 fully studied cases for most variables.

Sporadic local beliefs, often not verbalized, relating to suspicion that the blood may be used for purposes other than the stated research, contributed to lengthy consenting processes. On the positive side, I received overwhelming assistance from community gate-keepers, colleagues in the field, County Government Officers that included the Minister of Health and Governor of Makueni County to surmount this setback of community entry.

Laboratory and analysis limitations

The cross-sectional design of this study did not make it possible to infer temporal association between exposure and outcome as these were assessed simultaneously. We collected these data over 5 months and we know that there may be seasonal variations in aflatoxin contamination. We failed to run robust testing of the freshly collected stool samples while in the field. This may have enabled us to explore the contribution of helminthes to the nutritional status of the children besides interrogating the TH2 cytokines that are associated with helminthiasis. Combining these with the testing of a smaller number of samples of PBMCs stimulated with varying levels of aflatoxin to assess the responses of the TH1 and TH2 cytokines would be as conclusive as can get with in-vitro studies.

HIV testing that has significant impact on immunity was not done in this study due to challenges by ethical approval processes. For completeness, follow-up studies would do well to combine quantification of antibodies of a number of the other vaccines, immune memory cells and quality components like avidity, specificity and neutralizing capacity.

On the positive side, this cross-sectional study enabled us to prove that aflatoxin exposure in asymptomatic children-a vulnerable group- is perhaps higher than previously thought. There have been few purposely-designed community-based studies that have focused on children, immune response and aflatoxin exposure in an area known to have high prevalence of aflatoxin. Many previous studies of aflatoxin mainly focused on adults and a few children if at all. Use of scavenged blood held in storage informed the important study of Yards *et al.,* that showed the prevalence of aflatoxin in the different Kenyan counties. The aflatoxin and nutrition studies concentrated on children under five years but left out the age band of 9-14 years that includes adolescents.

We obtained significant and varied findings and trends, some failed to hit the threshold of statistical significance but the baseline information will enrich subsequent scientific research as well as open new areas of study.

My contribution in this research is the demonstration that aflatoxin exposure in children is high in the study population. Less than half of the studied population did not have protective antibodies to hepatitis B antigens. That should spur a renewed drive to explore if this trend holds true for the other vaccines even in other parts of the country.

5.3 Conclusion

- There is high prevalence of aflatoxin in children of Makueni County aged 1-14 years
- More than half of the studied population did not have protective anti-HBs antibodies possibly associated with aflatoxin exposure.
- Although there seemed to have been a declined rate of stunting of children in Makueni County, the malnourished children remained more at risk of having high aflatoxin.
- This study showed a direct relationship between poverty, dietary diversity and aflatoxin exposure. Focus on poverty reduction actions is central to intervention efforts.

Co-occurrence of fumonisins with aflatoxin is high in this community and fumonisins also independently depressed immune response in this study.

5.4 Recommendations

1. Long-term follow-up studies that will seek to find out the critical threshold for risk of exposure is highly recommended.
2. Booster vaccinations of Hepatitis B and perhaps other vaccines following appropriate investigations of the paediatric age group is a prudent public health intervention. Even in

the absence of economic data, it is obvious that it is cheaper to vaccinate the vulnerable population compared to the cost of coping with the alternative.

3. Ensure regular random check of anti-HBs in the various communities known to be at risk of high aflatoxin exposure to reduce risks of chronic liver diseases. The other mitigation measures for aflatoxin control like pre- and post- harvest interventions should be in place. Future studies will do well to include sero-surveillance in the vaccines as a way of ensuring heightened effectiveness of the vaccination programs.
4. Advocacy: The practitioners of public health should be the first advocates of upstream measures (dietary diversity, school feeding programs with safe foods, poverty reduction strategies) that are geared towards reducing contamination of foodstuff and subsequent exposure to the children. Agitating for inclusion of child environmental health into the teaching curricular of Medical Training Centres and Medical Schools will develop clinicians that are from very early on in their training, aware of environmental factors like aflatoxin that have serious effects on child health. Knowledge acquisition is the first step towards concerted mitigation measures and earlier recognition of environmentally induced diseases including by aflatoxin. Medical voices in trade and food standards regulatory authorities is to be encouraged so that food safety is not just maintained for purposes of the export market only but also for the local consumers of the foods. Spurring the scientific community to explore alternative ways of using heavily contaminated grains to allow the peasant farmers to have some money in the pocket for the unsafe grain that they would otherwise have been destroyed.
5. For those already diagnosed to have high levels of aflatoxin, joint robust clinical trials would perhaps allow aflatoxin binders to become an acceptable 'treatment' option if conclusively shown to be beneficial especially in outbreak situations.

6. Availing of diagnostic tests including those for serum aflatoxin levels in at least a national or a regional laboratory with support from well-established international laboratories for standardization is long overdue.
7. Medical personnel should be central in pushing for One Health Approach for keeping the populations healthy. It is time that we worked together with professionals in agriculture, veterinary medicines, basic science researchers, entrepreneurs as well as sit on boards that ensure that health considerations are not overridden by short term profiteering.
8. This study underscores the role of sorting out upstream issues like aflatoxin to respond to issues of food security and nutrition as well as effective affordable health for all. These are 2 important areas that contribute to The Big Four Agenda of 2018-2022 in Kenya.

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APPENDICES

APPENDIX I: HOUSEHOLD LISTING FORM

F1

Household Listing Form

L1. Cluster Number & Name

L2. No.of HHs listed

L3. Total No. of HHs with child(ren) between 1-14 years

L4 Total No. of HHs with at least 1 child between 1-14 years who has a vaccination card

L5 Total No. of HHs with no child aged 1-14 years

S_No	Structure Number	Resi-dential (Yes/No)	**HH Serial Number**	Name of the head of the household	Is there any child aged between 1-14 years in this HH? (Yes/No)	***If yes in L11*** Is there atleast 1 child aged between 1-14 who has a vaccination card	**Serial No. of HHs with child aged 1-14 years**	**Serial No. of HHs with at least 1 child aged 1-14 years who has a vaccination card**	**Number of all children aged between 1-14 in the household**	Remarks
L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16

APPENDIX 2: CHILD-SELECTION – KISH GRID METHOD

TABLE FOR SELECTION OF RESPONDENT

Look at the last digit of the household number (HH03). This is the number of the row you should go to. Check the total number of eligible persons (from **BS03).** This is the number of the column you should go to. Find the box where the row and the column meet and circle the number that appears in the box. This is the line number of the child who will be interviewed. Then go to **BS04 below** and record the line number of the selected person.

For example, if the household number is '26; go to row '6'. If there are three children aged 1-14 years in the household, go to column '3'. Follow the row and column and find the number in the box ("2"). The eligible child is the second one i.e, the one in line '02'.

Last digit of the household number	*Total number of 9-59 months persons in the household*							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
0	1	2	2	4	3	6	5	4
1	1	1	3	1	4	1	6	5
2	1	2	1	2	5	2	7	6
3	1	1	2	3	1	3	1	7
4	1	2	3	4	2	4	2	8
5	1	1	1	1	3	5	3	1
6	1	2	2	2	4	6	4	2
7	1	1	3	3	5	1	5	3
8	1	2	1	4	1	2	6	4
9	1	1	2	1	2	3	7	5

BS04**.** Record the line number of the selected person here ☐

BS05. Name of selected child _____

BS06. Child ID ___ ___/___ ___ ___/___
Cluster / Household / Line

BS07. Blood Drawn from child? (1=Yes 2=No) ☐

APPENDIX 3: HOUSEHOLD QUESTIONNAIRE

IDENTIFICATION	
HH01. EA NAME……	HH02. EA NUMBER: ___ ___ ___ ___
HH03. HOUSEHOLD NUMBER: ___ ___ ___	
HH04. INTERVIEWER _____ : ___ ___ NAME CODE	HH05. SUPERVISOR _____ : ___ ___ NAME CODE
_____ /_____ /_____ DD MM YY	_____ /_____ /_____ DD MM YY

Blood Sample: Child selection

BS01. Name of Household head: _____

BS02. List all Eligible children aged 1 - 14 years who live in the household.

1.	2.	3.		4.
Line No	Name	Is (name) male Or female? *1= male 2= female (CIRCLE APPROPRIATELY)*		Age in years *99=dk**
LINE	NAME	M	F	AGE
01		1	2	___
02		1	2	___
03		1	2	___
04		1	2	___
05		1	2	___
06		1	2	___
07		1	2	___
08		1	2	___

BS03. How many eligible children in the household? ☐

HOUSEHOLD CHARACTERISTICS		HC
HC2. HOW MANY ROOMS IN THIS HOUSEHOLD ARE USED FOR SLEEPING?	Number of rooms..... __ __	
HC3. *Main material of the dwelling floor.* *Record observation.*	Natural floor Earth / Sand..... 11 Dung..... 12 Rudimentary floor Wood planks..... 21 Palm / Bamboo..... 22 Finished floor Parquet or polished wood..... 31 Vinyl or asphalt strips..... 32 Ceramic tiles..... 33 Cement..... 34 Carpet..... 35 Other (*specify*) _____ 96	
HC4. *Main material of the roof.* *Record observation.*	Natural roofing No Roof..... 11 Thatch / Palm leaf..... 12 Sod..... 13 Rudimentary roofing Rustic mat..... 21 Palm / Bamboo..... 22 Wood planks..... 23 Cardboard..... 24 Finished roofing Metal/Tin..... 31 Wood..... 32 Calamine / Cement fibre..... 33 Ceramic tiles..... 34 Cement..... 35 Roofing shingles..... 36 Other (*specify*) _____ 96	
HC5. *Main material of the exterior walls.* *Record observation.*	Natural walls No walls..... 11 Cane / Palm / Trunks..... 12 Dirt..... 13 Rudimentary walls Bamboo with mud..... 21 Stone with mud..... 22 Uncovered adobe..... 23 Plywood..... 24 Cardboard..... 25 Reused wood..... 26 Finished walls Cement..... 31 Stone with lime / cement..... 32 Bricks..... 33 Cement blocks..... 34 Covered adobe..... 35 Wood planks / shingles..... 36	

	Other (*specify*) _____ 96	
HC6. WHAT TYPE OF FUEL DOES YOUR HOUSEHOLD MAINLY USE FOR COOKING?	Electricity ..... 01 Liquefied Petroleum Gas (LPG) ..... 02 Natural gas ..... 03 Biogas ..... 04 Kerosene ..... 05 Coal / Lignite ..... 06 Charcoal ..... 07 Wood ..... 08 Straw / Shrubs / Grass ..... 09 Animal dung ..... 10 Agricultural crop residue ..... 11 No food cooked in household ..... 95 Other (*specify*) _____ 96	01⇨HC8 02⇨HC8 03⇨HC8 04⇨HC8 05⇨HC8 95⇨HC8
HC7. IS THE COOKING USUALLY DONE IN THE HOUSE, IN A SEPARATE BUILDING, OR OUTDOORS? *If 'In the house', probe*: IS IT DONE IN A SEPARATE ROOM USED AS A KITCHEN?	In the house In a separate room used as kitchen ..... 1 Elsewhere in the house ..... 2 In a separate building ..... 3 Outdoors ..... 4 Other (*specify*) _____ 6	
HC8. DOES YOUR HOUSEHOLD HAVE: [A] ELECTRICITY? [B] A RADIO? [C] A TELEVISION? [D] A NON-MOBILE TELEPHONE? [E] A REFRIGERATOR? [F]*Country Specific Items* *(Add as necessary)*	Yes No Electricity ..... 1 2 Radio ..... 1 2 Television ..... 1 2 Non-mobile telephone ..... 1 2 Refrigerator ..... 1 2 *Country Specific Item* 1 2	
HC9. DOES ANY MEMBER OF YOUR HOUSEHOLD OWN: [A] A WATCH? [B] A MOBILE TELEPHONE? [C] A BICYCLE? [D] A MOTORCYCLE OR SCOOTER? [E] AN ANIMAL-DRAWN CART? [F] A CAR OR TRUCK? [G] A BOAT WITH A MOTOR [H]*Country Specific Items* *(Add as necessary)*	Yes No Watch ..... 1 2 Mobile telephone ..... 1 2 Bicycle ..... 1 2 Motorcycle / Scooter ..... 1 2 Animal drawn-cart ..... 1 2 Car / Truck ..... 1 2 Boat with motor ..... 1 2 *Country Specific Item* 1 2	
HC10. DO YOU OR SOMEONE LIVING IN THIS HOUSEHOLD OWN THIS DWELLING? *If "No", then ask:* DO YOU RENT THIS DWELLING FROM SOMEONE NOT LIVING IN THIS HOUSEHOLD? *If "Rented from someone else", circle "2". For other responses, circle "6".*	Own ..... 1 Rent ..... 2 Other (*specify*) _____ 6	

HC11. DOES ANY MEMBER OF THIS HOUSEHOLD OWN ANY LAND THAT CAN BE USED FOR AGRICULTURE?	Yes ..... 1 No 2	2⇨HC13
HC12. HOW MANY HECTARES OF AGRICULTURAL LAND DO MEMBERS OF THIS HOUSEHOLD OWN? *If less than 1, record "00". If 95 or more, record '95'. If unknown, record '98'.*	Hectares ..... ___ ___	
HC13. DOES THIS HOUSEHOLD OWN ANY LIVESTOCK, HERDS, OTHER FARM ANIMALS, OR POULTRY?	Yes ..... 1 No 2	2⇨HC15
HC14. HOW MANY OF THE FOLLOWING ANIMALS DOES THIS HOUSEHOLD HAVE? [A] CATTLE, MILK COWS, OR BULLS? [B] HORSES, DONKEYS, OR MULES? [C] GOATS? [D] SHEEP? [E] CHICKEN? [F] PIGS? [G]*Country Specific Additions (Add as necessary)* *If none, record '00'.If 95 or more, record '95'. If unknown, record '98'.*	Cattle, milk cows, or bulls ..... ___ ___ Horses, donkeys, or mules ..... ___ ___ Goats ..... ___ ___ Sheep ..... ___ ___ Chicken ..... ___ ___ Pigs ..... ___ ___ *Country Specific Addition* ..... ___ ___	
HC15. DOES ANY MEMBER OF THIS HOUSEHOLD HAVE A BANK ACCOUNT?	Yes ..... 1 No 2	

APPENDIX 4: HOUSEHOLD WEALTH INDEX QUINTILES

Wealth Index Quintiles are used to determine the relative economic status of the households surveyed. In order to measure it, a proxy index was created based on the survey responses from several household variables.

These variables fall in the following categories:

a) Water and sanitation

b) Nutrition

c) Economy

d) Agriculture and livestock

e) Household assets and amenities

The wealth index was then generated using the multivariate statistical technique (Principal Components Analysis). Principal components are weighted averages of the variables used to construct them. Among all weighted averages, the first principal component is usually the one that has the greatest ability to predict the individual variables that make it up, where prediction is measured by the variance of the index. The wealth index was therefore the first principal component of the indicated variables.

The generated index was then used to categorize the HHs into five quintiles:

1. Poorest

2. Second

3. Middle

4. Fourth

5. Richest

Statistics

		Albumin (g/dL)	AFB1-Lysine (pg/mg albumin)
N	Valid	396	396
	Missing	37	37
Mean		4.8852	44.5828
Median		4.8751	19.8804
Mode		4.89[a]	.74[a]

a. Multiple modes exist. The smallest value is shown

Wealth Index Quintiles by AFB1-Lysine

		AFB1-Lysine (pg/mg albumin)			
		Low level of AFB1-Lysine (pg/mg albumin)		High level of AFB1-Lysine (pg/mg albumin)	
		Count	per cent	Count	per cent
	Poorest	34	17.2	44	22.2
	Second	29	14.6	48	24.2
	Middle	37	18.7	40	20.2
	Fourth	45	22.7	35	17.7
	Richest	53	26.8	31	15.7
Total		198	100.0	198	100.0

Wealth index quintiles * AFB1-Lysine (pg/mg albumin) Crosstabulation

Count

		AFB1-Lysine (pg/mg albumin)		
		Low level of AFB1-Lysine (pg/mg albumin)	High level of AFB1-Lysine (pg/mg albumin)	Total
Wealth index quintiles	Poorest	34	44	78
	Second	29	48	77
	Middle	37	40	77
	Fourth	45	35	80
	Richest	53	31	84
Total		198	198	396

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	13.099[a]	4	0.011
Likelihood Ratio	13.223	4	0.010
Linear-by-Linear Association	10.725	1	0.001
N of Valid Cases	396		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 38.50.

Wealth Index Quintiles by Albumin					
		Albumin (g/dL)			
		Low level of Albumin (g/dL)		High level of Albumin (g/dL)	
		Count	per cent	Count	per cent
	Poorest	41	20.7	37	18.7
	Second	36	18.2	41	20.7
	Middle	40	20.2	37	18.7
	Fourth	42	21.2	38	19.2
	Richest	39	19.7	45	22.7
Total		198	100.0	198	100.0

Wealth index quintiles * Albumin (g/dL) Crosstabulation

Count

		Albumin (g/dL)		
		Low level of Albumin (g/dL)	High level of Albumin (g/dL)	Total
Wealth index quintiles	Poorest	41	37	78
	Second	36	41	77
	Middle	40	37	77
	Fourth	42	38	80
	Richest	39	45	84
Total		198	198	396

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	1.275[a]	4	0.866
Likelihood Ratio	1.276	4	0.865
Linear-by-Linear Association	0.150	1	0.699
N of Valid Cases	396		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 38.50.

APPENDIX 5: INFORMED CONSENT FORM

Title of study: The prevalence and impact of chronic aflatoxin exposure among children in Makueni District, Kenya.

Researcher: Dr David Githanga
Paediatrician
PhD Student, Nairobi University

Researcher's Statement

An Enrolment questionnaire will be administered during the selection of the households. If the screening results show that you are eligible to continue with the study, we will ask you to complete a survey. You may refuse to answer any questions.

What is a consent form?

I am asking you and your child/children to take part in a research study. The purpose of this consent form is to give you the information you will need to help you decide whether you and your child agree to be in the study or not. Please read this form carefully. You may ask questions about the purpose of the research, what we will ask you to do, the possible risks and benefits, your rights as a volunteer, and anything else about the research or any in this form that is not clear. When we have answered all of your questions, you can decide if you want to be in the study or not. This process is called "informed consent." We will give you a copy of this form for your records.

This consent form may have words that you do not understand. Please ask the researcher or the study staff to explain any words or information that you do not clearly understand. You may take home an unsigned copy of this consent form to think about or discuss with family or friends before making your decision.

What is the study all about/ Introduction

The ground on which we grow our crops has some naturally-occurring moulds called fungus. These produce a by-product called aflatoxin that is found in most of the foods we eat. To a large extent, aflatoxin is found to affect grains especially maize during the harvesting and storage period.

In the recent years, research has indicated that aflatoxin affects health in a number of ways. These include acute diarrhoea and vomiting, malnutrition, suppression of immunity leading to the children getting more frequent or severe infections. Chronic liver disease and cancer of the liver are well understood long term effects of aflatoxin. This is particularly so in people who have hepatitis B or C. If very high levels of aflatoxin- laden foods are consumed, death from acute liver failure can occur as was experienced in a large scale in Makueni in 2004 where 317 cases were reported and 125 people died. The minimum level of aflatoxin exposure required to cause a problem in children is unknown.

Aflatoxin in an expectant mother can cross the placental barrier and affect the growing baby in the mother's womb. It is found in the milk as well as baby weaning foods and the normal home diet. Levels can vary from home to home, season to season.

What is aflatoxin?

This is a naturally occurring by product of a mould found in the ground. It cannot be removed by mere cooking of food. You cannot see, smell, feel or taste aflatoxin in grains. Only laboratory testing can discover its presence. Level of aflatoxin production is affected by the local climate and the storage processes of especially the grains like maize. Aflatoxin is also toxic to animals and can contaminate meat, milk and eggs.

What is the purpose of the study?

This proposed research is meant to look at how common aflatoxin problem is at various childhood ages, how this affects growth and immunity of the children among other things. The answers to these questions will help in the plans of reducing these common problems not just locally but in the country as a whole.

How you can help

You have three options to consider concerning your participating in this study. You may choose to accept to allow your child to participate in the study or you could decline.

If you decide to participate, you will be asked a few questions confidentially, the child will be examined by the doctor in the study. The weight, height and size of the upper arm will be taken and recorded. Then a small blood sample of not more than 5mls will be removed by experienced

health care providers as painlessly as possible. A bottle of stool collection will be left with you for collection the following day. These will be transported to Nairobi for analysis. There are no physical risks to you or your child in this exercise.

The decision to either authorize or not authorize the use of your child's stool and blood samples for research purposes will not in any way impact on the quality of service that your child may receive.

Risks and Discomfort

There are no health risks to you or your child baby from taking the samples of blood or stools specimens.

Benefits of the Study

Research is needed to define levels of aflatoxin in children aged 1 to 14 years. By participating in the study, neither you nor your child will benefit directly. You will be contributing toward advancing our knowledge on aflatoxin. Research carried out on the samples by researchers at KAVI-ICR or their collaborators may lead to the development of marketable strategies to minimize the widespread problems. Any such benefits will remain with the research team at the University of Nairobi and their research partners.

Other Information

Costs to You

There is no cost to you in taking part in this study.

Confidentiality

Efforts will be made to keep all personal information related to you and your child confidential. We will create a random study identification number for each person in the study and will label all study data with this number and not your name. The files of the individuals in this study will be stored in a room that is accessible only to the study researchers and is under lock and key. The link between your child's identifying information (like name and address) and study identification number will be destroyed after the data has been analyzed. Any publication of this study will not use your name or your child's name or identify you or your child personally.

In addition to the researchers, the following groups may have access to study data which may include identifiable health information from your medical records:

- Kenyatta National Hospital/University of Nairobi ethics and research committee (the board that oversees research at KNH and at University of Nairobi);
- Sponsor study monitors and Auditors

Any reviewer from one of these organizations will protect your privacy if your records are examined. Although we will make every effort to keep your information confidential, no system for protecting your confidentiality can be completely secure. It is possible that unauthorized persons might discover that you are in this study, or might obtain information about you.

Withdrawal

At any time, you are free to withdraw or refuse to participate in the research study. Additionally, we may withdraw you from this study without your consent if we believe it is in your best interest or if you are unable to follow study procedures,

Problems or Questions

If you ever have any questions about the study you should contact the Principal Investigator, Dr David Githanga at KAVI-Institute of Clinical Research, University of Nairobi. If you have questions about your rights as a research participant, you should contact Professor Guantai, the Chair of the Kenyatta National Hospital/University of Nairobi Ethics and Research Committee, at 2726300-9 ext. 44102.

Specimen Storage and Use of your Samples for Future Studies

We may require to save some blood specimens at KAVI-ICR laboratories and other collaborating Institutions.

If you agree, we will keep them in unidentifiable form (not linked to your name) indefinitely or for as long as there is sample that can be used for future research. If you do not want to have your samples stored for future research, you can still be in this study and your samples will be destroyed once testing for the study is completed.

If you agree to the storage of your samples now, but change your mind before the end of the study, let the study staff know and we will make sure that your samples do not get stored for future research. We will not sell your samples or hand them over to unauthorized personnel.

Please mark, initial and date one option:

_____I DO agree to have my samples stored for future research

_____(signature/date)

_____I DO NOT agree to have samples from my infant stored for future research

_____(signature/date)

_____	_____	_____
Witness Name (printed)	Witness Signature	Date

APPENDIX 6: THE STUDY IN PICTURES

Picture 1: Training data collectors in the administration of study questionnaire

Picture 2: Training Data collectors on use GPS to map households

Picture 3: Community entry: Meeting with Chief (4th left)

Picture 4: Community Engagement: meeting Community Health Volunteers

Picture 5: The fixed facility (Ilatu Dispensary) where the study was centred

Picture 6: Illustrating the common mode of transport during the study

Picture 7: The Investigator at field lab at Ilatu Dispensary

Picture 8: Running serum aflatoxin samples on HPLC with Ruth Wangia at University of Georgia, Athens, USA.

Picture 9: The Investigator meeting with Supervisor Prof Anzala, (second right)

Picture 10: Meeting with IITA staff in Katumani Labs, Kenya where analysis of grain aflatoxin was performed. Dr. Charity Mutegi (2nd Left), Dr. Ranajit (kneeling 1st left) and Dr. Peter Cotty of United States Department of Agriculture (back right)

Picture 11: PI (2nd left) providing study feedback report to Makueni Governor Kivutha Kibwana (3rd left) and other County Officers

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De Simone, C., G. Caldarola, V. Coco, S. Palumbo, K. Fogimo, A. Sgambato, A. Malerino, M. Corb, M.T. Sandri, F. Vecchitelli, and E. Capolongo. "Circulating endothelial cell levels in psoriatic patients and their modification after anti-TNF-alpha (Etanercept) treatment". Journal of the European Academy of Dermatology and Venereology, 2013

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Submitted to Liverpool School of Tropical Medicine on 2017-08-15

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http://id.yahooapis.com

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https://geoenvironmental-disasters.springeropen.com/articles/10.1186/s40677-018-0096-2

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Grund, Liliane Zito, Monica Lopes-Ferreira, and Carla Lima. "The Hierarchical Process of Differentiation of Long-Lived Antibody-Secreting Cells Is Dependent on Integrated Signals Derived from Antigen and IL-17A". PLoS ONE, 2013.

<1% match (Internet from 09-Mar-2013)
http://www.vitagil.com.tr

<1% match (student papers from 06-May-2015)
Submitted to University of Mustansiriyah on 2015-05-06

SUBJECT'S STATEMENT

This study has been explained to me. I and my child volunteer to take part in this research, subject to the granting of relevant approvals. I have had a chance to ask questions. If I have questions later about the research, I can ask one of the researchers listed above. If I have questions about my rights as a research subject, I can ask Kenyatta National Hospital/University of Nairobi Ethics & Research committee.

I give the permission to the researchers to use my medical records as described in this consent form. I will receive a signed and dated copy of this consent form.

This study will not commence prior to obtaining the relevant local and international approvals.

_____	_____	_____
Mother's Name (printed)	Mother's Signature	Date
_____	_____	_____
Father's Name (If applicable) (printed)	Father's Signature	Date
_____	_____	_____
Name of Researcher Obtaining consent (printed)	Signature of Researcher obtaining consent	Date
_____	_____	_____
Witness Name (printed)	Witness Signature	Date

Copies to: Parents, Researcher's file, Medical record (if applicable)

KISWAHILI TRANSLATION

Vipimo vya kulinganisha sehemu tofauti za mwili.

WHO itatoa idadi ya watu watakao tumika kama kiashiria kupimwa uzito kulingana na umri, urefu kulingana na umri na uzito kulingana na urefu .Vipimo hivi vitahesabiwa kwa kuangalia kiwango ambacho vinaenda kinyume au nje ya (alama ya z) kipimo cha katikati cha kiashiria ya idadi ya watu.

Mkanda spesheli utagawanyishwa kwa rangi tatu tofauti:Nyekundu, Manjano na Kijani kibichi kuonyesha ukali mkubwa wa papo hapo,ukali wa kadri na ukali wa kawaida wa hali ya lishe kisha itatumika kupima mduara wa sehemu ya mkono ya kati na ya juu kwa kila mtoto.(viashiria vya maadili vitalinganishwa na viwango vya WHO vya ukuaji wa mtoto).ratili dijitali itatumika kupima uzito na itasomwa kwa kila kilo 0.1 itakayoongezeka.Urefu utapimwa na stadiometer inayoweza kubebwa au kusongeshwa kwa urahisi na iliyo ambatanishwa na kibao cha kichwa .Vipimo vitachukuliwa mara mbili ,viongezwe pamoja kisha alama yao ya kati itatumiwa kwa uchambuzi.

Habari itarekodiwa kwa mpangilio iliyoodhoreshwa hapo chini:

Jina la mtoto na nambari…….

Uzito kwa Kilo……

Urefu kwa sentimita……..Mtoto chini ya miezi 24, pima akiwa amalala chini

Mtoto zaidi ya miezi 24,pima akiwa amesimama.

Mduara wa mkono wa sehemu ya kati na juu kwa sentimita…….

Kipimo/nambari ya usiri ya mtafiti…….

Matokeo ya kupimwa

Alipimwa……

Hayupo…….

Alikataa……

Mengine…….

Kiambatanishi cha nne

FOMU YA IDHINI

Kichwa Cha Utafiti: Kuenea/maambukizi na athari ya sumu ya chakula miongoni mwa watato katika wilaya ya Makueni, nchini Kenya.

Mtafiti: Dr.David Githanga
Daktari wa watoto
Chuo Kikuu Cha Nairobi

Taarifa ya mtafiti

Maswali ya kujiunga yataulizwa wakati wa kuchagua kaya au nyumba. Matokeo ya ukaguzi yakionyesha kwamba umehitimu kuendela na utafiti tutakuuliza ukamilishe maswali ya utafiti huu. Unaweza kataa kujibu swali lolote.

Je fomu ya idhini ni nini?

Ninakuuliza wewe na mtoto au watoto wako mshiriki katika utafiti. Lengo la fomu hii ya idhini ni kukupa habari utakayo hitaji kukusaidia kuamua kama wewe na mtoto wako mnakubali kujiunga na utafiti huu au la.Tafadhali soma fomu hii kwa makini.Unaweza uliza maswali kuhusu lengo la utafiti, nini tutakuuliza ufanye, uwezekano wa hatari na faida, haki yako kama anayejitolea, na kitu kingine chochote kuhusu utafiti au kitu chochote katika fomu hii ambacho hakieleweki.Tukishayajibu maswali yako yote,unaweza amua kama unataka kushiriki katika utafiti au la. Utaratibu huu unaitwa utoaji wa idhini.Tutakupa nakala moja ya fomu hii ya rekodi zako.Fomu hii ya idhini yaweza kuwa na maneno ambayo huelewi.Tafadhali uliza mtafiti au wafanyikazi wa utafiti wakueleze maneno au habari huelewi vizuri. Unaweza chukua nakala moja ya fomu ya idhini ambayo haijatiwa saini uende nayo nyumbani ili ufikirie au ujadiliane na familia au marafiki kabla ya kuamua.

Je utafiti ni juu ya nini? Utangulizi

Ardhi ambayo tuna panda mimea kwa kawaida hutokwa na ukungu (mould) uitwayo kuvu (fungus).Hizi huzalisha sumu itwayo aflatoxin inayopatikana katika vyakula vingi tunavyo kula. Sumu hii ya chakula kwa kiwango kikubwa hupatikana kuathiri nafaka haswa mahindi wakati wa kuvuna na huhifadhi

Kwa miaka ambayo imepita hivi karibuni utafiti umeonyesha yakwamba sumu ya chakula (aflatoxin) huathiri afya kwa njia nyingi. Hii inajumuisha kuhara papo hapo na kutapika, ukosefu wa lishe bora kwa mwili, kukandamiza kinga na kusababisha watoto kupata maambukizi mara kwa mara au maambukizi makubwa makali. Ugonjwa sugu wa maini na saratani ya maini vinafahamika zaidi kuwa madhara ya muda mrefu ya aflatoxin (sumu ya chakula).Hii haswa ni kwa watu walio na homa ya manjano B au C.

Kama vyakula vyenye kiwango cha juu sana ya aflatoxin vikiliwa, kifo inaweza tokea kutokana na maini kushindwa kazi kama ilivyo fanyika kwa kiwango kubwa kule Makueni mwaka wa 2004 ambapo kesi 317 ziliripotiwa na watu 125 kufariki. Kiwango cha chini cha aflatoxin kinachoweza sababisha kifo kwa watoto hakijulikani.

Aflatoxin kwa mwanamke aliye na mimba inaweza kuvuka kizuizi cha kondo la nyuma (placenta) na kuathiri mtoto anayekua katika tumbo ya mama.Inapatikana kwa maziwa na pia kwa chakula cha kumwachisha mtoto maziwa na kwa chakula cha kawaida. Kiwango kinaweza tofautiana kutoka boma kwa boma, msimu kwa msimu.

Je aflatoxin ni nini?

Hiki ni kizao cha kiasili kinacho tokana na ukungu ipatikanayo kwenye ardhi. Kinaweza ondolewa kwa kupika chakula.Huwezi iona, nusa,gusa au onja ladha ya aflatoxin katika nafaka.Upimaji katika maabara tu ndio inaweza gundua uwepo wake.Hali ya hewa na njia inayotumika kuhifadhi haswa nafaka kama mahindi huathiri Kiwango cha uzalishaji wa aflatoxin . Aflatoxin pia ni sumu kwa wanyama na inaweza kuenea na kuchafua nyama, maziwa na mayai.

Je lengo la utafiti ni nini?

Utafiti huu uliopendekezwa una lengo la kuangalia jinsi tatizo la kawaida la aflatoxin liko katika miaka tofaiti ya utotoni, jinsi tatizo hili linaathiri ukuaji na kinga ya watoto miongoni mwa vitu vingine. Majibu ya maswali haya yatasaidia katika mipango ya kupunguza haya matatizo ya kawaida sio tuu mtaani bali pia nchini kote.

Jinsi unaweza saidia

Una chaguo tatu za kuzingatia kuhusu kushiriki kwako katika utafiti huu.Unaweza chagua kukubalia mtoto wako kushiriki katika utafiti au unaweza kataa.

Ukiamua kushiriki utaulizwa maswali chache kwa usiri, mtoto atachunguzwa na daktari wa utafiti.Uzito , urefu na ukubwa wa mkono wa juu utachukuliwa na kurekodiwa.Kisha sampuli ndogo ya damu isiyo zidisha mililita 5 itatolewa na wahudumu wa afya waliohitimu bila uchungu wowote iwezekanavyo.Utaachiwa chupa cha kukusanya kinyesi siku ifuatayo.Hii itasafirishwa Nairobi ichambuliwe.Hakuna hatari ya kimwili kwako au mtoto wako katika zoezi hili.

Uamuzi wa aitha kuwapa ruhusa au kutowapa ruhusa kutumia kinyesi cha mtoto wako na sampuli ya damu kwa lengo la utafiti hakuwezi athiri kwa njia yoyote ubora wa huduma ambayo mtoto wako anaweza pokea.

Hatari na Usumbufu

Hakuna hatari zozote za afya kwako au mtoto wako kwa kuchukua sampuli za damu au sampuli za kinyesi.

Faida ya utafiti

Utafiti unahitajika kufafanua kiwango cha aflatoxin katika watoto wenye miaka 1 hadi 14.Kwa kushiriki katika utafiti,wewe wala mtoto wako hamtapata faida kwa njia ya moja kwa moja.Utakuwa unachangia katika kuendeleza elimu yetu ya aflatoxin.Utafiti utakao fanyiwa sampuli hizi na watifiti katika kituo cha utafiti cha KAVI-ICR au washirika wao huenda ukasababisha maendeleo ya mikakati ya soko ili kupunguza upana wa matatizo iliyo enea.Faida yoyote kama hiyo itabakia na timu ya watafiti katika Chuo Kikuu Cha Nairobi na washirika wao wa utafiti.

Habari Nyingine

Gharama kwako

Hakuna gharama kwako kwa kushiki katika utafiti huu.

Usiri

Juhudi itafanywa kuweka habari yote ya kibinafsi inayokuhusu wewe na mtoto wako kwa usiri.Tutatengeneza nambari spesheli ya kibahati nasibu ya kila mtu katika utafiti na habari yote ya utafiti itatambuliwa na nambari hii wala sio jina lako.Faili za watu binafsi wa utafiti huu zitahifadhiwa katika chumba ambacho kinafikiwa na watafiti pekee na kinafungwa kwa kufuli na ufunguo.Uhusiano baina ya habari inayotambulisha mtoto wako(kama jina na anwani) na nambari spesheli ya utafiti utaharibiwa baada ya habari yote kuchambuliwa. Uchapishaji wowote wa utafiti huu hautatumia jina lako au la mtoto wako au kukutambua wewe au mtoto wako kibinafsi.Mbali na watafiti,kundi zifuatazo zinaweza pewa habari ya utafiti ambayo inaweza jumuisha habari kuhusu afya kutoka kwa rekodi zako za kimatibabu.

- Kamati ya maadili na utafiti ya Hospitali Kuu ya Kenyatta/Chuo Kikuu cha Nairobi (Bodi ambalo linasimamia utafiti katika KNH na Chuo Kikuu Cha Nairobi);
- Mfadhili, wano fuatilia utafiti na wakaguzi.

Mtathmini yeyote kutoka kwa moja ya shirika hizi ataweka usiri wako salama kama rekodi zako zitachunguzwa. Ingawa tutafanya juhudi yote kuweka habari yako kwa usiri, hakuna mfumo wowote unaweza kuweka salama usiri wako kwa ukamilifu.Kuna uwezekano ya kwamba

watu ambao hawana ruhusa wanaweza gundua yakwamba uko katika utafiti huu au wanaweza pata habari kukuhusu.

Kujiondoa

Wakati wowote uko huru kujiondoa au kukataa kushiriki katika utafiti. Kuongezea, tunaweza tukakuondoa kutoka kwa utafiti huu bila idhini yako kama tunaamini ni kwa ubora wa masilahi yako au ukishindwa kufuata taratibu za utafiti.

Matatizo au maswali

Ukiwahi kuwa na swali lolote kuhusu utafiti unapaswa kuwasiliana na mtafiti mkuu Dr.David Githanga katika kituo cha utafiti cha KAVI,Chuo Kikuu Cha Nairobi.Ukiwa na maswali kuhusu haki yako kama mshiriki wa utafiti unapaswa kuwasiliana na Profesa Guantai,Mwekiti wa kamati ya maadili na utafiti ya Hospitali Kuu ya Kenyatta/Chuo Kikuu cha Nairobi kwa nambari 2726300-9 ext 44102.

Uhifadhi wa sampuli zako na matumizi ya sampuli zako kwa utafiti ya siku zijazo.

Tunaweza hitajika kuweka akiba au kuhifadhi baadhi ya sampuli za damu katika maabara ya KAVI-ICR na mashirika mengine yanayo shiriki na KAVI-ICR.

Ukikubali tutaziweka kwa njia ambayo hautatambuliwa (yaani bila uhusiano na jina lako) kwa muda usiojulikana au muda mrefu bora kuna sampuli ambazo zinaweza tumika kwa utafiti wa siku za usoni.Kama hutaki sampuli zako zihifadhiwe kwa ajili ya utafiti ya siku zijazo ,bado unaweza kuwa katika utafiti huu na sampuli zako zita haribiwa baada ya kupimwa utafiti ukishakamilika.

Ukikubali sampuli zako zihifadhiwe sasa hivi,lakini ubadilishe uamuzi wako kabla ya mwisho wa utafiti,julisha wafanyikazi na tutahakikisha ya kwamba sampuli zako hazitahifadhiwa kwa ajili ya utafiti wa siku zijazo. Hatutauza Sampuli zako au kupeana kwa watu ambao hawana ruhusa.

Tafadhali weka alama ya jina lako na tarehe katika moja utakayo chaguo hapo chini:

----------Mimi ninakubali sampuli zangu zihifadhiwe kwa ajili ya utafiti ya siku za usoni

---------- ----------

Saini Tarehe

----------Mimi sikubali sampuli kutoka kwa mtoto wangu mchanga kuhifadhiwa kwa ajili ya utafiti wa siku za usoni

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Saini Tarehe