Published in final edited form as:

Mol Nutr Food Res. 2015 March ; 59(3): 574–581. doi:10.1002/mnfr.201300619.

Aflatoxin exposure is inversely associated with IGF1 and IGFBP3 levels *in vitro* and in Kenyan school children

Jovita M. Castelino¹, Michael N. Routledge¹, Shona Wilson², David W. Dunne², Joseph K. Mwatha³, Kimani Gachuhi³, Christopher P. Wild⁴, and Yun Yun Gong^{1,5,*}

¹Division of Epidemiology, School of Medicine, University of Leeds, Leeds, UK

²Department of Pathology, University of Cambridge, Cambridge, UK

³Kenya Medical Research Institute, Nairobi, Kenya

⁴International Agency for Research on Cancer, 150 Cours Albert-Thomas, Lyon, 69372 Cedex 08, France

⁵Institute of Global Food Security, School of Biological Sciences, Queen's University Belfast, Stranmillis Road, Belfast BT9 5AG, UK

Abstract

Scope—This study explores the relationship between aflatoxin and the insulin-like growth factor (IGF) axis and its potential effect on child growth.

Methods and results—199 Kenyan schoolchildren were studied for aflatoxin-albumin adduct (AF-alb), IGF1 and IGFBP3 levels using ELISA. AF-alb was inversely associated with IGF1 and IGFBP3 ($P < 0.05$). Both IGF1 and IGFBP3 were significantly associated with child height and weight ($P < 0.01$). Children in the highest tertile of AF-alb exposure (> 198.5 pg/mg) were shorter than children in the lowest tertile (< 74.5 pg/mg), after adjusting for confounders ($P = 0.043$). Path analysis suggested that IGF1 levels explained ~16% of the impact of aflatoxin exposure on child height ($P = 0.052$). To further investigate this putative mechanistic pathway, HHL-16 liver cells were treated with AFB₁ (0.5, 5 and 20 μ g/ml for 24–48 hours). IGF1 and IGFBP3 gene expression measured by qPCR and protein in culture media showed a significant down-regulation of IGF genes and reduced IGF protein levels.

Conclusion—Aflatoxin treatment resulted in a significant decrease in IGF gene and protein expression *in vitro*. IGF protein levels were also lower in children with the highest levels of AFB-alb adducts. The data suggest that aflatoxin-induced changes in IGF protein levels could contribute to growth impairment where aflatoxin exposure is high.

Keywords

Aflatoxins; exposure biomarker; child growth; insulin-like growth factor; IGF1; IGFBP3

*Dr Yun Yun Gong, Institute of Global Food Security, School of Biological Sciences, Queen's University Belfast, Stranmillis Road, Belfast, BT9 5AG, UK, y.gong@qub.ac.uk, Fax number: +44 (0) 2890976513.

The authors declare no conflict of interest.

Introduction

Staple diets in some eastern regions of Kenya consist mainly of locally grown maize, which is frequently contaminated with aflatoxin. In a recent survey it was reported that the percentage of maize having levels of contamination above regulatory limits varied from 16% to 51% from year to year [1]. Proliferation of aflatoxin-producing *Aspergillus spp.* on grains is increased by warm temperatures and high humidity together with poor storage conditions. Chronic exposure to aflatoxin is associated with hepatocellular carcinoma, whilst acute aflatoxin poisoning can be fatal [2]. In a toxicosis incident in Kenya in 2004, over 100 deaths were attributed to aflatoxin exposure at contamination levels up to 220-times the acceptable maximum level in maize in Kenya of 20 ppb [3].

Aflatoxin exposure, as measured by blood aflatoxin-albumin adduct (AF-alb) biomarker levels, has also been associated with marked growth retardation in children in Benin and Togo in a cross-sectional study [4], as well as in a cohort study over an 8-month period [5]. In a Kenyan based study it was observed that significantly more children with wasting had been fed aflatoxin-contaminated flour than had children without wasting [6]. Moreover, a study in a Gambian population has shown that exposure to aflatoxin *in utero* is associated with longitudinal growth faltering in the first year from birth [7], and a study in Ghana showed an association between reduced body weight and height in infants at birth [8]. Whilst these studies consistently indicate an association between dietary aflatoxin exposure and impaired growth, evidence of a plausible mechanism would contribute significantly to establishing a causal association.

One hypothesis for the mechanism by which aflatoxin induces child growth retardation is through effects on insulin-like growth factor (IGF). IGF facilitates the growth promoting effects of growth hormone (GH), and hence is a key factor influencing child growth [9]. The liver is both the main site for aflatoxin metabolism in the body and for biosynthesis of IGF. Liver-derived IGF1 in serum is mostly bound to a family of binding proteins, in particular to IGF binding protein-3 (IGFBP3) [10]. The complex of IGF1 with binding proteins serves as an IGF1 reserve in the circulation, thus extending the half-life of the growth factor [10]. IGF1 is crucial for post-natal growth [11]. The influence of IGF1 on growth is substantiated by the significant association between low birth weight and short stature, and polymorphisms in the *IGF1* promoter region, which reduce IGF1 levels in circulation [12, 13].

Aflatoxin exposure in male broiler chicks (in the form of *A. parasiticus* culture material in the diet) resulted in a significant reduction in body weight through the down regulation of the *IGF1* gene [14]. This finding provides some preliminary support for the hypothesis that aflatoxin may act to impair child growth through a disruption of the IGF system, either by directly lowering IGF1 levels or affecting other components in the IGF axis.

In this report we have investigated this hypothesis by analysing IGF levels and biomarkers of aflatoxin exposure in serum samples collected from school-aged children in the Makueni District in Kenya, a high aflatoxin exposure region. We have also studied the effects of

aflatoxin B₁ (AFB₁) on gene and protein expression of key components of the IGF axis in human liver cells exposed to AFB₁ *in vitro*.

Materials and Methods

1. Population study

Study participants—The study site, which has been described in a previous publication [15], was the Makueni District in Kenya, which is characterised by biannual seasonal rains. Maize based dishes, githeri and ugali, constitute the main dietary staple and thus are a potential source of aflatoxin exposure.

School-aged children were recruited from two neighbouring schools, Yumbuni in the west and Matangini (Lower Mangalet) in the east. These children were a sub-set from a cross-sectional study, in which 56% of children were stunted and had a high prevalence of both aflatoxin exposure and *Schistosomiasis* infection [15, 16]. Serum samples collected in May/June 2002, 119 from Yumbuni and 61 from Matangini, were used in the current study and have been previously analysed [16]. Height and weight were measured using standard methods [17]. The male to female ratio for these children was 1:1 and the age range was 6–17 years. Ethical clearance was obtained from The Kenya Medical Research Institute Ethical Committee (SSC NO 501).

Serology—Serum samples were analysed for aflatoxin-albumin adduct (AF-alb) levels by a competitive ELISA, as described previously [18]. Briefly, albumin was extracted, digested and purified from serum samples followed by quantification of adduct levels by ELISA. Final concentrations are presented as picogram (pg) aflatoxin per milligram (mg) albumin. The detection limit of AF-alb for this assay was 3 pg/mg albumin. Each sample was analysed in triplicate on at least two days. One negative and three positive controls with known AF-alb levels were run with each batch of the samples. Intra-assay coefficient of variation (CV) was < 15 % and inter-assay CV was 25 %.

Serum IGF1 and IGFBP3 were measured using the IGF1 and IGFBP3 Quantikine ELISA kits (R&D Systems, Abingdon, UK) following the manufacturer's instructions. For the IGF1 ELISA, serum samples were pre-treated prior to analysis to dissociate the IGF1 from its binding proteins. A serum sample with a known concentration (96.7 ng/ml for IGF1 and 2336.5 ng/ml for IGFBP3) was used for quality control. The inter-assay coefficient of variance (CV) was below 10% and intra-assay CV was below 3% for both assays.

HHL-16 cell-based study—HHL-16, which are transformed non-tumourigenic human liver cells, immortalised with Moloney's mouse leukaemia virus [19] were kindly provided by Dr. Arvind H. Patel (MRC Virology Unit, Glasgow). Cells were maintained in Advanced Modified Eagle's Medium (Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin and 1% non-essential amino acids (Sigma, Dorset, UK). The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ and were split every 2–3 days when they reached ~80% confluence.

AFB₁ treatment for gene expression and protein analysis—Triplicate cultures of HHL-16 cells grown in 25 cm² flasks (Corning, NY, USA) to approximately 80% confluence were treated for either 24 or 48 hours with 0.5, 5 and 20 µg/ml AFB₁/ml dissolved in DMSO. The doses were determined based on a minimum level of cell toxicity induced at the highest dose as measured by a cytotoxicity assay using CellTiter 96[®] AQueous One Solution Reagent (Promega, Southampton, UK) (data not shown). DMSO concentration for cells treated with 0.5 and 5 µg/ml were at 0.0025% and those for cells treated with 20 µg/ml were at 0.1% DMSO. Cells treated with DMSO corresponding to treatment concentrations served as controls to exclude potential toxicity from DMSO alone. RNA was harvested using an RNeasy Mini kit (Qiagen Ltd, West Sussex, UK) following the manufacturer's instructions. Quality and quantity of extracted RNA was determined using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, DE, USA).

Reverse transcription real-time quantitative PCR (qPCR)—One µg total RNA was reverse-transcribed to cDNA using SuperScript II First-Strand Synthesis Mix (Invitrogen, Paisley, UK), following the manufacturer's instructions. Primers for *GAPDH* and *IGF1* and *IGFBP3* were designed using the Primer3 software (<http://frodo.wi.mit.edu/primer3/>) and are shown in Table 1. The cDNA was amplified using SYBR Green (Applied Biosystems, CA, USA). The qPCR reactions were run on an ABI 7900HT real-time PCR analyser (Applied Biosystems, CA, USA) using absolute quantification and results were determined using the Sequence Detection System (SDS) software. Each reaction was performed in duplicate and a negative control was routinely included. The expression of IGF was normalised against endogenous GAPDH gene expression. Each PCR reaction was run for 40 cycles and included a denaturation step at 95°C for 15 seconds and annealing at 60°C for 1 minute. After 40 cycles, a dissociation curve was produced for each PCR product at 95°C for 15 seconds and 60°C for 15 seconds.

Cell supernatant protein analysis—Following AFB₁ treatment of cells, conditioned cell media were collected and centrifuged at 1000 g for 5 minutes to separate cell debris. The supernatant was enriched using Vivaspın 2 ultrafiltration columns with 3000 Molecular Weight Cut Off (MWCO) polyethersulfone (PES) membrane (Sigma-Aldrich, Dorset, England, UK). The columns were centrifuged at 4000 g for 50 minutes at room temperature to obtain approximately 30-times concentrated media, which were analysed immediately.

IGF1 and IGFBP3 protein levels in concentrated cell supernatants were measured using the above mentioned IGF1 and IGFBP3 Quantikine ELISA kits (R&D Systems, Abingdon, UK) following the manufacturer's instructions. Cell supernatants were not pre-treated for IGF1 protein analysis and were diluted 2-fold for IGFBP3 protein analysis. The measured protein concentrations were corrected for the concentration factor. All samples were analysed in triplicate on two separate occasions.

Statistical Analyses—As AF-alb, IGF1 and IGFBP3 levels were not normally distributed, all three parameters were natural-log transformed before statistical analysis. Geometric means and 95% confidence intervals (CI) for AF-alb, IGF1 and IGFBP3 are presented unless otherwise stated. A scatter plot with a fractional polynomial fit was analysed to determine the association between AF-alb levels and child height. Subsequently,

children were divided into AF-alb exposure tertiles: low- < 74.5 pg/mg, medium- 74.6–198.4 pg/mg and high- > 198.5 pg/mg. A multivariate regression model was used to examine body height changes in the three groups after adjusting confounders for age, sex, school, liver disease state and infection status. Stata software was used for the above analysis (version 10, StataCorp, College Station, TX, USA). Regression and path model analyses were constructed to determine if AF-alb was associated with impaired child height through lower IGF1/IGFBP3 levels using Amos Graphics (version 5, Amos Development Corporation, IBM Corp, NY, USA). Non-standardised and standardised regression coefficients were calculated based on the path model involving AF-alb, IGF1, height and age. Confidence intervals and *P* values were tested employing a bootstrap approach. For the *in vitro* normal HHL-16 liver cell model, student's *t*-test was used to compare each treatment with their respective controls and *P* values <0.05 were considered statistically significant.

Results

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

A description of the study population, including measured AF-alb, IGF1 and IGFBP3 levels is summarised in Table 2. There were no significant differences in the mean age, height and weight between children from the two schools. There were no significant differences in IGF1 and IGFBP3 levels in children from the two schools but both parameters were significantly higher in boys compared to girls [geometric mean: 123 ng/ml (95% confidence interval (CI): 110, 138) vs 87 ng/ml (79, 96) for IGF1 and 2065 ng/ml (1913, 2229) vs 1776 ng/ml (1654, 1908) for IGFBP3] and increased with age (regression coefficient β : 0.07, *P* < 0.001 for IGF1 and 0.02, *P* = 0.02 for IGFBP3).

AF-alb levels were significantly higher in children from Matangini compared to those from Yumbuni (207.7 pg/mg vs. 72.2 pg/mg; *P* < 0.001). AF-alb mean levels did not differ by sex or age (*P* > 0.05).

AF-alb, IGF1 and IGFBP3 and growth

Examining the correlations at the individual level between AF-alb and the two IGF parameters revealed an inverse association with both IGF1 (β : -0.27, *P* = 0.039) and with IGFBP3 (β : -0.39, *P* = 0.046) (Figure 1). Also at an individual level, both IGF1 and IGFBP3 were positively associated with child height (β : 11.7, *P* < 0.001 for IGF1 and 7.9, *P* = 0.001 for IGFBP3) and weight (β : 8.1, *P* < 0.001 for IGF1 and 5.5, *P* = 0.001 for IGFBP3). A scatter plot with a fractional polynomial fit between AF-alb levels and child height revealed a near linear relationship between the two parameters (Figure 2). Subsequently the AF-alb data was divided into low, medium and high. Children with higher AF-alb levels (> 198.5 pg/mg albumin) were significantly shorter than children with lower exposure (< 74.5 pg/mg albumin), after adjusting for age, sex, school, disease state and infection status (overall adjusted R^2 of model = 0.628, *P* < 0.001).

Path analysis was introduced to establish the path and directionality of the relationship between AF-alb, height and IGF1 or IGFBP3 levels with age as an effect mediator, as both

IGF1 levels and height are influenced by age. The model shown in Figure 3 had an acceptable fit suggesting that the available data were explained well by the hypothesised model ($\chi^2 = 0.343$, $P = 0.558$). The non-standardised and standardised regression coefficients as well as the P values were obtained using the statistical software Amos Graphics with these results being summarised in Figure 3. The path model including age as an effect mediator showed a significant ($P = 0.048$) inverse association between the child height and AF-alb. Approximately 16% of the effect of AF-alb on child height can be attributed to lower IGF1 levels (estimated using standardised coefficients of the direct and indirect effects) ($P = 0.052$). A similar path diagram was tested for IGFBP3 but an indirect effect of AF-alb on height through IGFBP3 was not significant.

IGF gene expression in AFB₁ treated HHL-16

IGF gene expression in control and treatment groups, measured using reverse transcription qPCR, is shown in Figure 4A. Expression of both genes increased between 24 and 48 hours under almost all conditions, including control. After 24 hours of treatment with AFB₁, IGF gene expression was reduced but the changes were not statistically significant. However, at the highest AFB₁ dose, a significantly lower expression was observed for *IGFBP3* (2.4 fold change compared to control, $P = 0.005$). In contrast, expression of both IGF genes was significantly reduced following a 48-hour treatment with 5 and 20 $\mu\text{g/ml}$ AFB₁ with an average 2 fold change in expression compared to controls ($P < 0.05$).

IGF1 and IGFBP3 protein levels in AFB₁ treated HHL-16

IGF protein levels in cell culture supernatants were measured by ELISA, following 24 and 48 hour AFB₁ treatment. As with the trend observed in gene expression, overall IGF protein expression increased with incubation time. Protein levels were reduced with increasing AFB₁ dose (see Figure 4B). IGF1 levels were significantly lower in cells treated with 20 $\mu\text{g/ml}$ AFB₁ after 24 hours ($P < 0.05$) and at 5 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ AFB₁ after 48 hours ($P < 0.001$), when compared to the control cells. IGFBP3 protein levels were significantly lower at 5 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ AFB₁ following 24 hours treatment and at all three doses after 48 hours of treatment. Protein levels were most markedly reduced at the highest AFB₁ dose with a 2-fold average decrease ($P < 0.001$) (see Figure 4B).

Discussion

This study provides preliminary support for the hypothesis that the IGF growth axis plays a role in aflatoxin-associated child growth impairment. In this study we have shown that aflatoxin exposure is associated with reduced levels of IGF1 and IGFBP3 in children aged between six and seventeen years. We have also provided evidence for an inverse association between aflatoxin exposure and IGF gene and protein expression in cultured liver cells. In the current study, multivariate analysis revealed that children with higher AF-alb levels were shorter than the children in the lower tertile of AF-alb after adjusting for confounders, although the correlation was not significant when analysing data as continuous variables. It should be noted that earlier studies found a correlation between aflatoxin exposure and impaired growth in much younger children [4, 5]. In this study the children are older, thus, the effects of aflatoxin on growth may be less pronounced. Nevertheless, the observations

here begin to provide data in support of the potential mechanisms underlying aflatoxin-associated growth impairment in African children, building on well-established evidence for aflatoxin-induced severe growth retardation in a number of species including ducks, pigs and broiler chicks [20, 21].

Additionally, serum IGF1 and IGFBP3 levels were lower in the children exposed to higher levels of aflatoxin. Results from the path analysis suggest that ~16% of the effect on child height could be attributable to aflatoxin-associated reduction in IGF levels, thus indicating a potential pathway between aflatoxin and child stunting. The contribution may have been underestimated because the children were at school age and IGF1 may be modulated by a more complicated growth hormone pathway [22, 23]. This may have resulted in a less significant impact on the IGF axis and child growth by aflatoxin exposure when compared to children under five. Other aflatoxin-mediated contributions to child stunting may include immune modulation and susceptibility to infections as well as possible damage to gut function through aflatoxin exposure, leading to poor nutrient absorption [24]. Indeed, it is possible that there is a multi-component path effect responsible for the total effect of aflatoxin exposure on impairment in child height gain.

The importance of the IGF axis in growth is well established [11, 25]. The presence of binding proteins, particularly IGFBP3, regulates the growth axis by controlling the levels of free IGF1 in circulation and prolonging the half-life of IGF1 [25]. As both IGF1 and IGFBP3 are primarily produced in the liver, the major site of aflatoxin metabolism and toxicity, the growth retardation associated with aflatoxin exposure may be related to changes in the expression of these key members of the IGF growth axis.

To examine experimentally the effects of AFB₁ on IGF1 and IGFBP3, an *in vitro* model of cultured non-tumourigenic HHL-16 liver cells was used. More commonly studied liver cell lines such as HepG2 were not utilised in this study to avoid any bias towards gene expression and protein profile caused by cancer-related changes. Derived from a healthy human liver, HHL-16 cells have been immortalised with Moloney's mouse leukaemia virus and retain primary hepatocyte characteristics, expressing hepatocyte-specific markers as well as cytochrome P450 enzymes, which are necessary for aflatoxin bio-activation [19]. In the present study, these cells were treated with three AFB₁ doses for one to two days. Results from both reverse transcription qPCR and ELISA analyses showed that AFB₁ treatment resulted in reduced gene and protein expression of IGFs in liver cells, particularly following 48 hours treatment with the higher AFB₁ doses. These observations are consistent with the hypothesis that AFB₁ exposure is able to alter expression of IGF axis components that play a key role in growth.

Recently, it was reported that primary human hepatocytes exposed to 0.02 µg/ml and 0.78 µg/ml of AFB₁ for 24 hours had suppressed *IGFBP3* and *IGF1* expression, respectively [26]. These AFB₁ doses are much lower than those used in the present study, which could be an indication of the variable sensitivity of the different liver cell models. Interestingly, exposure to other mycotoxins has also been associated with a decrease in IGF gene expression or IGF1 protein levels. For instance, ochratoxin A was found to down regulate *IGFBP1* and *IGF2* gene expression in the human hepatocellular cell line, HepG2 [27], while

it has been proposed that another mycotoxin, deoxynivalenol (DON) causes growth retardation in mice by lowering plasma IGF1 and insulin-like growth factor acid-labile subunit (IGF-ALS) levels [28]. These changes suggest that mycotoxin-associated growth impairment may follow a similar pathway involving the disruption of the IGF growth axis, and as these mycotoxins exert toxicity through different mechanisms, this suggests that the events leading to effects on the IGF1 growth axis may be non-specific. However, further work is required in this area.

Through the combination of a population-based study and an *in vitro* liver cell-based model, the current report provides evidence that aflatoxin-induced disruption of the IGF growth axis may contribute to growth impairment. Additional factors may also be involved in the mechanism and require further research. Further enquiry into the mechanisms by which aflatoxin impinges on child growth is needed both *in vitro* and in a purpose-designed population study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIEHS, USA programme grant no. ES06052. Jovita M. Castelino acknowledges support for her PhD study from the International Agency for Research on Cancer.

The authors thank Mrs Anne E. Sutcliffe for laboratory assistance in aflatoxin biomarker analysis and Dr. Yu-Kang Tu for his advice and assistance with the statistical analyses. We also thank all the field workers and the participants without whom the study would not be possible.

Abbreviations

AF-alb	aflatoxin-albumin adducts
AFB₁	Aflatoxin B ₁
ALS	acid-labile subunit
CI	confidence intervals
DON	deoxynivalenol
HCC	hepatocellular carcinoma
HHL-16	human hepatocyte line 16 cells
IGF	insulin-like growth factor
IGF1R	insulin-like growth factor 1 receptor
IGFBP	insulin-like growth factor binding protein
MWCO	molecular weight cut-off
PES	polyethersulfone
qPCR	quantitative PCR

References

1. Daniel JH, Lewis LW, Redwood YA, Kieszak S, Breiman RF, Flanders WD, Bell C, Mwihi J, Ogana G, Likimani S, Straetemans M, McGeehin MA. Comprehensive assessment of maize aflatoxin levels in Eastern Kenya, 2005–2007. *Environ Health Perspect.* 2011; 119(12):1794–9. [PubMed: 21843999]
2. IARC. IARC monographs on the evaluation of carcinogenic risks to humans. Vol. 82. World Health Organization, International Agency for Research on Cancer; 2002. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene; p. 1-556.
3. Probst C, Njapau H, Cotty PJ. Outbreak of an Acute Aflatoxicosis in Kenya in 2004: Identification of the Causal Agent. *Applied and Environmental Microbiology.* 2007; 73(8):2762–2764. [PubMed: 17308181]
4. Gong YY, Cardwell K, Hounsa A, Egal S, et al. Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: cross sectional study. *BMJ.* 2002; 325(7354):20–21. [PubMed: 12098724]
5. Gong YY, Hounsa A, Egal S, Turner PC, et al. Postweaning exposure to aflatoxin results in impaired child growth: A longitudinal study in Benin, west Africa. *Environmental Health Perspectives.* 2004; 112(13):1334–1338. [PubMed: 15345349]
6. Okoth SA, Ohingo M. Dietary aflatoxin exposure and impaired growth in young children from Kisumu District, Kenya: Cross sectional study. *Afr J Health Sci.* 2004; 11(1–2):43–54. [PubMed: 17298116]
7. Turner PC, Collinson AC, Cheung YB, Gong Y, et al. Aflatoxin exposure in utero causes growth faltering in Gambian infants. *International Journal of Epidemiology.* 2007; 36(5):1119–1125. [PubMed: 17576701]
8. Shuaib FM, Jolly PE, Ehiri JE, Yatich N, et al. Association between birth outcomes and aflatoxin B1 biomarker blood levels in pregnant women in Kumasi, Ghana. *Tropical Medicine & International Health.* 2010; 15(2):160–7. [PubMed: 20003033]
9. Woods KA, Camacho-Hübner C, Savage MO, Clark AJL. Intrauterine Growth Retardation and Postnatal Growth Failure Associated with Deletion of the Insulin-Like Growth Factor I Gene. *New England Journal of Medicine.* 1996; 335(18):1363–1367. [PubMed: 8857020]
10. Ohlsson C, Mohan S, Sjögren K, Tivesten Å, et al. The Role of Liver-Derived Insulin-Like Growth Factor-I. *Endocrine Reviews.* 2009; 30(5):494–535. [PubMed: 19589948]
11. Le Roith D, Bondy C, Yakar S, Liu JL, et al. The somatomedin hypothesis: 2001. *Endocrine Reviews.* 2001; 22(1):53–74. [PubMed: 11159816]
12. Arends N, Johnston L, Hokken-Koelega A, Duijn Cv, et al. Polymorphism in the IGF-I Gene: Clinical Relevance for Short Children Born Small for Gestational Age (SGA). *Journal of Clinical Endocrinology and Metabolism.* 2002; 87(6):2720. [PubMed: 12050240]
13. Vaessen N, Janssen JA, Heutink P, Hofman A, et al. Association between genetic variation in the gene for insulin-like growth factor-I and low birthweight. *The Lancet.* 2002; 359(9311):1036–1037.
14. Yarru LP, Settivari RS, Antoniou E, Ledoux DR, et al. Toxicological and gene expression analysis of the impact of aflatoxin B1 on hepatic function of male broiler chicks. *Poultry Science.* 2009; 88(2):360–371.
15. Wilson S, Booth M, Jones FM, Mwatha JK, et al. Age-adjusted Plasmodium falciparum antibody levels in school-aged children are a stable marker of microgeographical variations in exposure to Plasmodium infection. *BMC Infectious Diseases.* 2007; 7(1):67. [PubMed: 17603885]
16. Gong YY, Wilson S, Mwatha JK, Routledge MN, et al. Aflatoxin Exposure May Contribute to Chronic Hepatomegaly in Kenyan School Children. *Environmental Health Perspectives.* 2012:893–896. [PubMed: 22370114]
17. Wilson S, Vennervald BJ, Kadzo H, Ireri E, et al. Health implications of chronic hepatosplenomegaly in Kenyan school-aged children chronically exposed to malarial infections and Schistosoma mansoni. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 2010; 104(2):110–116. [PubMed: 19818465]

18. Chapot, B.; Wild, CP. ELISA for quantification of aflatoxin-albumin adducts and their application to human exposure assessment. In: Warhol, M.; van Velzen, D.; Bullock, GR., editors. *Techniques in Diagnostic Pathology*. Vol. 2. San Diego, CA: Academic Press; 1991. p. 135-155.
19. Clayton RF, Rinaldi A, Kandyba EE, Edward M, et al. Liver cell lines for the study of hepatocyte functions and immunological response. *Liver International*. 2005; 25 (2):389–402. [PubMed: 15780065]
20. Dersjant-Li Y, Versteegen MWA, Gerrits WJJ. The impact of low concentrations of aflatoxin, deoxynivalenol or fumonisin in diets on growing pigs and poultry. *Nutrition research reviews*. 2003; 16(2):223–239. [PubMed: 19087391]
21. Han XY, Huang QC, Li WF, Jiang JF, et al. Changes in growth performance, digestive enzyme activities and nutrient digestibility of cherry valley ducks in response to aflatoxin B1 levels. *Livestock Science*. 2008; 119(1–3):216–220.
22. Lupu F, Terwilliger JD, Lee K, Segre GV, et al. Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. *Developmental Biology*. 2001; 229(1):141–162. [PubMed: 11133160]
23. Herrington J, Carter-Su C. Signaling pathways activated by the growth hormone receptor. *Trends in Endocrinology & Metabolism*. 2001; 12(6):252–257. [PubMed: 11445442]
24. Wild CP, Gong YY. Mycotoxins and human disease: a largely ignored global health issue. *Carcinogenesis*. 2010; 31(1):71–82. [PubMed: 19875698]
25. Grimberg A, Cohen P. Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. *Journal of Cellular Physiology*. 2000; 183(1):1–9. [PubMed: 10699960]
26. Josse R, Dumont J, Fautrel A, Robin MA, et al. Identification of early target genes of aflatoxin B1 in human hepatocytes, inter-individual variability and comparison with other genotoxic compounds. *Toxicology and Applied Pharmacology*. 2012; 258(2):176–87. [PubMed: 22100608]
27. Hundhausen C, Boesch-Saadatmandi C, Matzner N, Lang F, et al. Ochratoxin A Lowers mRNA Levels of Genes Encoding for Key Proteins of Liver Cell Metabolism. *Cancer Genomics & Proteomics*. 2009; 5(6):319–332. [PubMed: 19287073]
28. Amuzie CJ, Pestka JJ. Suppression of insulin-like growth factor acid-labile subunit expression—A novel mechanism for deoxynivalenol-induced growth retardation. *Toxicological Sciences*. 2010; 113(2):412–421. [PubMed: 19805407]

Figure 1.

Regression analysis plots comparing log of AF-alb adduct with (A) log of IGF1 concentrations and (B) log of IGFBP3 concentrations with respective regression coefficients presented in each graph. An inverse linear relationship is observed between AF-alb and both IGF1 and IGFBP3.

Figure 2.

Scatter plot depicting the association between AF-alb adduct levels and child height. Fractional polynomial regression with 95% confidence intervals is also presented, showing the near linear relationship between the two parameters at AF-alb levels higher than ~50 pg/mg albumin.

Figure 3.

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

Figure 4.

Graphs depicting (A) gene expression and (B) protein expression relative to untreated cells (NT) in HHL-16 cells for IGF1 and IGFBP3 across treatment groups- Untreated (NT), 0.0025% DMSO (D5), 0.1% DMSO (D20), 0.5 $\mu\text{g/ml}$ AFB₁ (A0.5), 5 $\mu\text{g/ml}$ AFB₁ (A5) and 20 $\mu\text{g/ml}$ AFB₁ (A20) at two time points- 24 hours (dark bars) and 48 hours (white bars) * represents $P < 0.05$, ** represents $P < 0.001$ when comparing treatments with appropriate controls i.e. A0.5 and A5 compared to D5 and A20 compared to D20. Error bars represent standard deviation.

Table 1

Primer sequences for GAPDH, IGF1 and IGFBP3

Gene	Accession Number	Forward primer (5' to 3')	Reverse primer (3' to 5')	Product Size
GAPDH	NM_002046	TTGTCAAGCTCAATTCCCTGGTAT	TCTCTCTTCCTCTTGTGCTCTTG	150 bp
IGF1	NM_001111283	CCCAACCCAGCCCTTATTAT	CCCCATCTCAGAAAAAGGAA	146 bp
IGFBP3	NM_001013398	GTGGATCCCTCAACCAAGAA	TAGGTTCCCAAGAGTGCCCTA	139 bp

Table 2

Characteristics of the children in this study

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

^a geometric mean (95% CI) presented for these parameters* $P < 0.001$