**Aflatoxin exposure in Nigerian children with severe acute malnutrition**

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A B S T R A C T

Aflatoxin exposure is an important public health concern in sub-Saharan Africa as well as parts of Latin America and Asia. In addition to hepatocellular carcinoma, chronic aflatoxin exposure is believed to play a role in childhood growth impairment. The most reliable biomarker of chronic aflatoxin exposure is the aflatoxin-albumin adduct, as measured by ELISA or isotope dilution mass spectrometry (IDMS). In this report, we have used high resolution LC-MS/MS with IDMS to quantitate AFB1-lysine in an extremely vulnerable population of Nigerian children suffering from severe acute malnutrition. To increase the sensitivity and reliability of the analyses, a labelled AFB1-13C615N2-lysine internal standard was synthesized. AFB1-lysine concentrations in this population ranged between 0.2 - 59.2 pg/mg albumin, with a median value of 2.6 pg/mg albumin. AFB1-lysine concentrations were significantly higher in stunted children (median = 4.6 pg/mg) compared to non-stunted (1.2 pg/mg), as well as in children with severe acute malnutrition (4.3 pg/mg) compared to controls (0.8 pg/mg). The median concentrations were also higher in children with kwashiorkor (6.3 pg/mg) compared to those suffering from marasmus (0.9 pg/mg). This is the first report of the use of high-resolution mass spectrometry to quantitate AFB1-lysine in humans.

*Keywords:*

Aflatoxin

Severe acute malnutrition

Aflatoxin-lysine adduct

High resolution LC-MS/MS

Isotope dilution mass spectrometry

Kwashiorkor

**1. Introduction**

Human exposure to aflatoxin mainly arises from consumption of staple crops including maize and groundnuts damaged by *Aspergillus flavus* and/or *A. parasiticus* (JECFA, 2017; Pitt et al., 2012; Wild et al., 2015). More than 500 million people mainly in sub-Saharan Africa as well as in parts of Latin America, and Asia are exposed to aflatoxin B1 at concentrations that substantially increase mortality and morbidity (Pitt et al., 2012). Exposures between 20 and 120 µg/kg body weight per day results in acute aflatoxicosis when consumed over a period of 1–3 weeks. Symptoms include vomiting and abdominal pain, typically resulting in death, especially in children (Azziz-Baumgartner et al., 2005; Wild and Gong, 2009). Chronic exposure causes liver cancer in humans, and individuals with concurrent hepatitis B infection are at much greater risk (JECFA, 2017; Wild et al., 2015; Wild and Gong, 2009).



**Figure 1.** Chemical structures of the four major aflatoxins found in maize, peanuts, and other food crops. AFB1 is the most carcinogenic and commonly reported aflatoxin.

Of the four main aflatoxins: AFB1, AFB2, AFG1 and AFG2 (Figure 1), AFB1 is the most toxic and commonly occurring (JECFA, 2017; Pitt et al., 2012; Wild et al., 2015). The genotoxic and mutagenic effects of AFB1 arise following P450 (CYP3A4, CYP1A2, and CYP3A5 in some populations) epoxidation of AFB1 to the highly reactive *exo*-8,9-epoxide form that results in intercalation between DNA base pairs and reacts with the guanyl N7 atom (Wojnowski et al., 2004). Subsequent depurination releases an AFB1–N7-guanine adduct that is eliminated in urine. Urinary screening for this adduct as well as the AFM1 metabolite produced following hydroxylation of AFB1 by CYP1A2 are effective biomarkers for short-term aflatoxin exposure (Groopman et al., 1993; JECFA, 2017). In addition to covalent modification of DNA, AFB1 8,9-epoxides can form covalent adducts with serum albumin following rapid hydrolysis in blood to AFB1-dihydrodiol in equilibrium with the lysine reactive AFB1-dialdehyde (Johnson et al., 1996). Owing to the half-life of serum albumin (**~**20 days), the AFB1-albumin adduct is the best available indicator of long-term exposure to AFB1 (JECFA, 2017; Wild and Turner, 2002).

Growth faltering in developing nations has not been entirely explained by inadequate nutrition alone as dietary intervention schemes have failed to fully recover growth performance (Bhutta et al., 2013; Dewey and Adu‐Afarwuah, 2008; Wild et al., 2015). Although the general mechanism of growth faltering is unknown, poor digestive and barrier functions caused by chronic inflammatory enteropathy of the mucosa of the small intestine is believed to contribute to growth retardation in children (Prendergast and Kelly, 2016). Aflatoxin exposure is known to cause growth retardation in animals (JECFA, 2017; Pitt et al., 2012) and may also induce enterocyte damage and contribute to ‘leaky gut’. Aflatoxin has also been shown to be a potent immunosuppressant in relevant animal models (Mohsenzadeh et al., 2016; Pitt et al., 2012; Raisuddin et al., 1993; Wild et al., 2015).

Relatively few studies have examined the relationship between aflatoxin exposure and growth faltering (JECFA, 2017; Wild et al., 2015). A recent critical analysis found six studies with well-defined sample sizes, aflatoxin exposure or dose assessments, outcome measures, and appropriate multivariate analyses. Taken together, these studies indicate that aflatoxin exposure contributes to child growth impairment, independent of and together with other risk factors that may cause stunting (Wild et al., 2015). Based on information from relevant animal models and limited human data, a number of mechanisms for the epidemiological findings were suggested. Enteropathy may be partly attributable to aflatoxin related toxic damage on the intestine epithelium, resulting in poor uptake of nutrients. Aflatoxin associated immune suppression could also increase susceptibility to infections and incidence of diarrhea in children. Finally, liver toxicity of aflatoxin may damage the production of insulin like growth factor pathway proteins resulting in an adverse impact on child growth (Ubagai et al., 2010; Wild et al., 2015).

In a 2002 study of 479 children from Benin and Togo, Gong *et al.* found a significant dose response association between AFB1-albumin concentrations and height-for-age (HAZ), weight-for-height (WHZ), and weight-for-age scores (WAZ) (Gong et al., 2002). This study was followed by an 8 month longitudinal study of children in Benin that found a strong negative correlation between concentrations of AFB1-albumin adducts and growth velocity (Gong et al., 2004). A study of aflatoxin exposure *in utero* also demonstrated a negative association between aflatoxin and growth increases in the first years of life (Turner et al., 2007). While many studies employ an established ELISA based method to quantitate the aflatoxin-albumin adduct concentrations, Mitchell et al. (2017) used isotope dilution mass spectrometry (IDMS) to quantitate AFB1-lysine (AFB1-Lys) in order to examine associations between aflatoxin exposure and child growth impairment. They found no significant association in their cohort of Nepalese children, however there was a low prevalence of stunting in this population (19%) (Mitchell et al., 2017)

Recently, we undertook a non-targeted cross-sectional metabolomics study of 58 Nigerian children, of which 47 had severe acute malnutrition (SAM). Although there were no significant differences in the stool microbial or metabolite composition, we found that 15% of the plasma metabolome was significantly altered in cases of SAM (McMillan et al., 2017). In the present work, we expanded upon our metabolomic analysis of this cohort by measuring the AFB1-Lys concentrations by LC-IDMS.

**2. Materials and Methods**

*2.1 Study design*

The plasma samples used for AFB1-Lys quantitation originated from a previously published study designed to identify metabolic perturbations associated with SAM (McMillan et al., 2017). SAM was defined as a WHZ < - 3 or MUAC < 11.5 cm and/or nutritional oedema according to the World Health Organization (WHO) guidelines (World Health Organization and Unicef, 2009). Some 21 of the children were suffering from marasmus (caloric deficiency) and 26 of the children were suffering from kwashiorkor (protein deficiency). Briefly, children from both rural and urban populations aged 6 – 48 months with SAM that were admitted to the Federal Medical Centre, Gusau, Zamfara State, Nigeria during the summer of 2012, were asked to participate. Consent was obtained from individual patients and ethical approval was provided by the Joint Ethical Review Committees of the University of Ibadan / University College Hospital, Ibadan, Nigeria. Control patients (MUAC >12.5 cm or WHZ score ≥ -1 and no nutritional oedema) were recruited from the paediatric ward or outpatient clinics. Baseline demographic and clinical information was collected from all patients.

*2.2 Chemicals*

Aflatoxin B1 was obtained from Toronto Research Chemicals (Toronto, Canada). HPLC grade acetone, Pronase® (nuclease-free), L-lysine, Oxone® monopersulfate and copper carbonate basic (Cu2CO3(OH)2) were obtained from Sigma (Oakville, Canada). 13C615N2-L-lysine was purchased from Cambridge Isotopes Laboratories, Inc. (Andover, MA, USA). LC–MS grade acetonitrile, water and formic acid were purchased from Fisher Scientific (Ottawa, Canada).

*2.3 Synthesis of internal standards*

AFB1-L-Lys as well as AFB1-13C615N2-L-Lys were synthesized according to previous methods (Guengerich et al., 2002; Scholl and Groopman, 2004) with some modifications as described below. Dimethyldioxirane (DMDO) used for the epoxidation of AFB1 was synthesized based on a reported procedure (DeMatteo and Hassan, 2013). Distilled water (20 mL), acetone (30 mL) and NaHCO3 (24 g) were added to a round bottom flask and stirred for 20 minutes in an ice bath. Oxone® monopersulfate (12.5 g) was slowly added to the mixture and stirred for an additional 15 minutes. The pale yellow DMDO/acetone solution was collected by rotary evaporation in the ‘bump trap’ that was submerged in a dry ice/acetone cooling bath. Approximately 10 mL of DMDO/acetone solution was collected and dried with anhydrous Na2SO4(s). The solution was stable up to one week at –20 °C before degradation was observed.

The AFB1 8,9-epoxide intermediate was prepared by dissolving 5 mg of AFB1 in 1 mL of dry dichloromethane (CH2Cl2) and 600 µL of the freshly prepared DMDO solution. This mixture was stirred for 15 minutes and evaporated to dryness under N2. This procedure was repeated 3 times in order to ensure full conversion of AFB1. The resulting AFB1 8,9-epoxide was hydrolyzed to its dialdehyde form by dissolving the residue in 800 µL CHES buffer (20 mM, pH 10.0) and 200 µL of acetonitrile and stirred for 30 minutes.

A Cu-Lys solution was prepared by dissolving 36.4 mg of either L-Lys.HCl or 13C615N2-L-Lys.HCl with 42.5 mg Cu2CO3(OH)2 in 1.6 mL deionized H2O (M. Bodanszky, 2002). This Cu(Lys)2 solution was mixed with 4 mL of a potassium phosphate buffer (0.1 M, pH 7.4) and refluxed for two hours.

The copper solution was filtered, and the blue-colored filtrate was added to the AFB1 dialdehyde solution. The reaction between AFB1 dialdehyde and Cu(Lys)2 was stirred overnight at room temperature and filtered through a 0.45-µm PTFE syringe ﬁlter. The resulting mixture was purified by reversed-phased flash column chromatography using an automated Büchi system (12 g C18 Sepacore column) and gradient method starting at 25/75 acetonitrile/H2O and increasing to 100/0 over 20 mins. Fractions were screened by LC-MS and those containing the desired product were combined and evaporated to dryness and the mixture was further purified by semi-preparative HPLC (Agilent 1200 HPLC) using an acetonitrile/H2O gradient that started at 5% acetonitrile and increased to 100% over 13 minutes (Agilent Zorbax Eclipse 5 µm C18 250 x 9.4 mm column). The fraction containing the AFB1-L-Lys was evaporated under N2 and freeze-dried.

*2.4 AFB1-Lys quantitation*

AFB1-Lys was quantitated in human serum following established IDMS methodology with some additional steps to allow for simultaneous, plasma metabolomic analysis. Firstly, plasma proteins were precipitated and small molecules extracted by the addition of 805 μL of 8:2 methanol:H2O to 230 μL of plasma (Dunn et al., 2011). Samples were vortexed for 15 s and centrifuged at 20 000 rcf for 15 min to pellet precipitated proteins. The supernatant was transferred to a separate vial and dried for metabolomic analysis and the remaining protein pellet stored at -80 °C for AFB1-Lys analysis. AFB1-Lys is stable under these conditions and can be fully recovered following protein precipitation (Scholl and Groopman, 2008). Thawed samples were re-constituted in 230 µL PBS and mixed with a Teflon pestle (Sigma, Oakville, Canada). For albumin quantification, 10 µL of the re-suspended protein pellet was removed and mixed with 10 µL of 2% SDS in MilliQ water to fully dissolve the protein precipitate. Quantitation of albumin was accomplished using a Human Serum Albumin ELISA kit (ab179887, Abcam Inc. Toronto, Canada) according to the manufactures instructions. 75 µL of 1.0 ng/mL 13C615N2-labelled internal standard was added to the remaining suspension along with 230 µL of 6.5 mg/mL Pronase® dissolved in MilliQ water. Samples were vortexed for 15 s and incubated overnight at 37 °C with agitation at 1400 rpm in thermomixer (Eppendorf). After incubation, 460 µL of deionized water was added and samples were processed using strong anion mixed mode-solid phase extraction (SPE) following established methods without modification (McCoy et al., 2005). Briefly, 30 mg Oasis® MAX cartridges were activated with 2 additions of 1 mL methanol, and conditioned with 2 additions of 1 mL MilliQ water. Samples were added to the cartridges and allowed to pass through over 5 minutes. In order, the cartridges were treated with 1 mL of MilliQ water, 1 mL of 70% methanol, 1 mL of 1% NH4OH, and 0.5 mL of methanol. Samples were eluted from the vacuum dried cartridges with 800 µL of 2% formic acid (FA) in methanol, dried down in a speedvac and reconstituted in 100 µL of 25% methanol and transferred to polypropylene 250 µL HPLC vials.

All analyses were performed using an Easy-nLC 1000 nano-flow system coupled to a Q-Exactive Orbitrap mass spectrometer. 10 µL of each sample was injected onto a 100 µm x 2 cm Acclaim C18 PepMap™ trap column and washed with 20 µL of 0.1% FA. Bound compounds were then eluted onto a 75 µm x 15 cm Acclaim C18 PepMap™ analytical column (Thermo Scientific, Waltham USA). A flow rate of 300 nL/min was used throughout the entirety of the run. The gradient started at 5% B (acetonitrile + 0.1% FA) and increasing to 35% over 20 minutes, it then increased to 90% B over 2 minutes and held for 5 minutes. The trap and analytical columns were equilibrated with 6 µL of 0.1% FA in water between each run. The injection needle and loop system were washed during analysis with isopropanol, acetonitrile:water (50:50) and finally with 0.1% FA in water. The nanospray voltage was set at 1.90 kV, capillary temperature 275 °C, and S-lens RF level 60. Each sample was analyzed by a combination of full MS and parallel reaction monitoring (PRM). Full MS was acquired between mass range 150-900 *m/z* at 35,000 resolution, automatic gain control (AGC) target of 5×106 and maximum injection time (IT) of 256 ms. PRM analysis monitoring the AFB1-Lys and AFB1-13C615N2-L-Lys transitions to *m/z* 394.1268 and 400.1413 respectively was accomplished using a 1.0 *m/z* isolation window, 17, 500 resolution, AGC target 5×106 and 25 NCE. Peak areas were integrated in the Xcalibur software package at < 5 ppm mass accuracy. A calibration curve consisting of seven concentrations of unlabelled AFB1-Lys: 30, 200, 400, 1000, 2000, 3000 and 4000 pg/mL each with 500 pg/mL AFB1-13C615N2 lysine was constructed with a linear fit. Using high resolution LC-MS/MS, the limit of detection (LOD) was defined as the lowest concentration of AFB1-Lys spiked into blank, processed plasma that could be detected by five consecutive injections. The limit of quantitation was determined as the lowest concentration where the relative standard deviation of the peak area ratio (AFB1-Lys/AFB1-13C615N2-Lys) was below 20% (n=5). With this approach, both the LOD and LOQ were 22 pg/ml plasma.

*2.5 Statistical analysis*

The concentrations of AFB1-Lys in samples were heavily left-skewed and therefore non-parametric statistical tests were used. Wilcoxon tests were applied for discrete variables with two groups and Spearman’s correlations for continuous variables. For comparisons with more than two groups (Residence, SAM type), the Kruskal-Wallis test (non-parametric ANOVA) was applied followed by the Dunn’s post-hoc test with a Benjamini-Hochberg correction to account for multiple comparisons (Benjamini and Hochberg, 1995). values less than 0.05 were considered statistically significant. To account for potential confounding variables such as age, logistic regressions were performed using stunting as a categorical dependant variable and AFB1-Lys as the independent variable (predictor). Odds ratios (OR) with confidence intervals that did not cross 1 were considered statistically significant. Samples with values below the LOD were replaced with the LOD/2 prior to statistical analyses (Hornung and Reed, 1990). All analyses and figures were compiled using these LOQ-imputed values.

**3. Results**

*3.1 Demographics of patient population*

The demographics of the patient population are described in detail elsewhere (McMillan et al., 2017). Briefly, 58 children ranging from 6-48 months of age were recruited for this study. All were weaned at the time of sample collection. Of these children, 37(64%) were male and 47(81%) were diagnosed with severe acute malnutrition (SAM), defined as a weight-for-height z-score (WHZ) < -3 or middle upper arm circumference (MUAC) < 11.5 cm and/or oedema. The number of stunted children, defined as having a height-for-age z-score (HAZ) < -2 was 43(74%). There was no correlation between HAZ and WHZ (Spearman’s rho =0.20, P =0.13), however children with SAM were significantly more likely to be stunted (Fisher’s Exact Test P = 0.00037). Age did not differ significantly between children with kwashiorkor, marasmus or controls (Kruskal Wallace test P=0.079), while age was marginally significant between stunted and non-stunted children (Wilcoxon Rank Sum P = 0.05). The type of residence (rural, urban or peri-urban) differed significantly between SAM and controls with more children with SAM originating from a rural residence.

*3.2 Measurement of AFB1-Lys adduct*

Previously established methods for the quantitation of the AFB1-Lys adduct involve the addition of a tetradeuterated AFB1-Lys internal standard followed by enzymatic digestion of the plasma sample and solid phase extraction (SPE) clean-up. Due to the potential for chromatographic shifts observed with deuterated compounds relative to the unlabelled form, we used 13C615N2-L-lysine for synthesis of our internal standard (Figure 2) (Wang et al., 2007). Fragmentation (MS/MS) of the synthesized AFB1-13C615N2-Lys internal standard gave product ions that were in agreement with the fragmentation pattern previously shown for AFB1-13Cε,15Nε-Lys (Scholl and Groopman, 2004).



**Figure 2**. High resolution MS/MS spectrum of synthesized AFB1-13C615N2-Lys internal standard showing fragmentation pattern.

To allow concurrent quantitation of both small molecules and peptides in a single plasma sample, a modified extraction procedure was used. This involved the addition of a protein precipitation step prior to Pronase® digestion and SPE clean-up, which others have demonstrated does not affect AFB1-Lys quantitation (Scholl et al., 2006). AFB1-Lys was resolved by nano-LC and detected by parallel reaction monitoring (PRM) using a high-resolution Q-Exactive Orbitrap mass spectrometer (Figure 3 b,c). This is the first reported use of high-resolution MS for AFB1-Lys analysis in humans, and allowed for simultaneous non-targeted screening of additional components present in the pooled digested sample (Figure 3a). This revealed the presence of high intensity multiply charged ions that are the result of incomplete protein digestion.



**Figure 3**. Digested pooled plasma samples from 5 random individuals were analyzed simultaneously by (a) full MS, and (b) PRM AFB1-Lys and (c) PRM AFB1-13C615N2-Lys.

*3.3 Association between AFB1-Lys, SAM and Stunting*

Among all children studied, 81% had AFB1-Lys adduct concentrations above the limit of detection, ranging from 0.2 - 59.2 pg/mg albumin, with a median value of 2.6 pg/mg albumin(Table 1). The concentration of AFB1-Lys was significantly higher in children with SAM (P = 0.0083) and in stunted children (P = 0.032) as compared to controls (Figure 4A, C). AFB1-Lys concentrations differed significantly between children with edematous malnutrition (kwashiorkor) and non-edematous malnutrition (marasmus), with higher AFB1-Lys concentrations reported in kwashiorkor (Figure 4B). A weak but significant correlation between AFB1-Lys and HAZ was also identified (Spearman’s rho = -0.34, P = 0.0093, Figure 4D), while there was no correlation between AFB1-Lys and WHZ (Spearman’s rho = -0.17, P = 0.20). There was no significant effect of urban or rural residence on AFB1-Lys concentrations (P > 0.05).

There was a significant positive correlation between AFB1-Lys and age (Spearman’s rho = 0.41, P = 0.0016), prompting us to assess age as a potential confounder. While age was marginally significant between stunted and non-stunted children (Wilcoxon Rank Sum P = 0.05), the association between AFB1-Lys and stunting remained significant after adjusting for age (odds ratio tertile 3, 9.11;95% confidence interval [CI], 1.28-186.56). As expected, albumin was also significantly associated with both stunting and malnutrition status (P = 3.94E-5, 0.022 for stunting and malnutrition type respectively). AFB1-Lys remained significantly associated with malnutrition type when values were not normalized to albumin (P = 0.028). This was not the case with stunting (P = 0.144).

To assess the interaction between SAM, stunting and AFB1-Lys, logistic regressions between stunting and AFB1-Lys were performed. The association between stunting and AFB1-Lys was no longer significant after adjustment for malnutrition status (odds ratio quartile 3, 1.21;95% confidence interval [CI], 0.086-31.45), suggesting the association between AFB1-Lys and stunting is not totally independent from malnutrition type (marasmus, kwashiorkor or control).

**Table 1**: Plasma AFB1-Lys concentrations according to growth impairment type.

|  |  |  |
| --- | --- | --- |
|  | n (%) | AFB1-Lys  median (range)  pg/mg albumin |
| All children | 58 (100) | 2.6 (0.2-59.2) |
| SAM | 47 (81) | 4.3 (0.2-59.2) |
| Kwashiorkor | 26 (55) | 6.3 (0.3-27.6) |
| Marasmus | 21 (45) | 0.9 (0.2-15.6) |
| Control | 11 (19) | 0.8 (0.2-2.9) |
| Stunted | 43 (74) | 4.6 (0.2-27.6) |
| Non-stunted | 15 (26) | 1.2 (0.2-59.2) |

Where the value was below the limit of detection, half the LOD was used.



**Figure 4**. Comparison of plasma AFB1-Lys concentrations in (a) control and SAM individuals, (b) control, edematous malnutrition (kwashiorkor), and non-edematous malnutrition (marasmus), (c) non-stunted and stunted individuals and (d) Spearman’s correlation (rs) with HAZ. For boxplots in a-c, the boxes represent the 25th and 75th quartiles, and the line the median value. Circles extending beyond the lines are outliers defined as values greater or less than 1.5 times the interquartile range.

**4. Discussion**

This work describes a modified method for quantitation of AFB1-Lys in plasma using IDMS and its application to a cohort of Nigerian children with growth impairments. The addition of a protein precipitation step prior to Pronase® digestion and SPE cleanup was a crucial modification that allowed us to use one sample for both AFB1-Lys quantitation and metabolomic profiling. To improve the reliability of the aflatoxin adduct analysis, fully labelled 13C615N2-Lys was used in the synthesis of the internal standard instead of the previously reported tetradeuterated lysine (Scholl and Groopman, 2004). 13C stable isotope standards are generally preferred as they do not introduce retention time shifts relative to the unlabelled form as can be the case for deuterium labels (Wang et al., 2007). The use of a high-resolution mass spectrometer with nanospray ionization in full scan mode revealed the presence of other compounds present in the pooled digested sample (Figure 3a), many of which were at intensities 100,000 times greater than the targeted AFB1-Lys. Some of these peaks were multiply charged and are likely peptides resulting from incomplete Pronase® digestion. This agrees with a previous study with serum albumin (Delatour et al., 2007).

To demonstrate the applicability of our revised method to a clinically-relevant cohort, we quantitated AFB1-Lys in plasma from a group Nigerian children with growth impairments. This population is particularly at risk of aflatoxin exposure due to the widespread consumption of maize(Maziya-Dixon, 2004). This is highlighted by surveys reporting detectable AFB1 in 67.1 % of stored Nigerian maize, with 64.1 % of samples found to exceed the European Union maximum acceptable limit (Adetunji et al., 2014). The detection rate for AFB1-Lys among all children in this study was 81%, with concentrations ranging from 0.2-59.2 pg/mg albumin and a geometric mean of 2.4 pg/mg albumin. Consistent with previous studies (Gong et al., 2002; Gong et al., 2003; Gong et al., 2004), the stunted children we examined had significantly higher concentrations of AFB1-Lys and there was a weak but significant correlation between HAZ and AFB1-Lys. AFB1-Lys concentrations did not differ between children with marasmus and controls but were significantly higher in children with kwashiorkor, again consistent with previous findings (Coulter et al., 1986; Lamplugh and Hendrickse, 1982; Ramjee et al., 1992). Aflatoxin exposure is not likely the causative agent of kwashiorkor but it is believed that it leads to liver impairments that increase the activity of the P450 enzyme responsible for AFB1 epoxidation, resulting in DNA damage and protein adduct formation (Coulter et al., 1986). Although there was not a significant correlation between WHZ and HAZ in this cohort, children with SAM were significantly more likely to be stunted. Further, the association between stunting and AFB1-Lys was no longer significant after adjusting for malnutrition type. This suggests that the interaction between stunting and AFB1-Lys may be due in part to the effects of kwashiorkor, but further studies are needed to clarify these complex interactions. As noted, a number of mechanisms have been proposed as to how aflatoxin may contribute to growth impairments in children, including immune modulation, reduced insulin like growth factor 1 (IGF-1), and aflatoxin-induced enteropathy (JECFA, 2017; Wild et al., 2015). The significant correlation between HAZ and AFB1-Lys provides further epidemiological support for a causal role of aflatoxin and stunting.

Most of the studies that focused on the relationship between aflatoxin exposure and growth impairment have used ELISA based aflatoxin-albumin measurements. One study used IDMS (Mitchell et al., 2017) and examined a cohort of Nepalese children below the age of 36 months. They found no significant associations between AFB1-Lys concentrations and growth impairment, but it should be noted that the children were much healthier than those in our current study. The mean concentration of AFB1-Lys was relatively low for our cohort (geometric mean 3.6 pg/mg albumin). Mitchell et al. (2017) suggested that chronic aflatoxin exposure above a threshold is necessary for stunting to occur. However, it is likely more complicated. Improved diets containing nutrients that upregulate p450 enzymes, favour the detoxification pathways as well as increasing circulating GST, leading to more rapid excretion of aflatoxin (Wild et al., 2015). This would reduce the toxicity of the diet.

Robust comparison between IDMS values in the present study and those using the various ELISA-based methods are urgently needed. ELISA measurements tend to yield higher concentrations than IDMS(Scholl et al., 2006), although this was not tested directly in our study. With little direct evidence from human samples, it has been proposed that this could be caused by incomplete proteolytic digestion of albumin, the formation of additional amino acid-aflatoxin adducts or other aflatoxin-lysine adducts such as AFG1-Lys(Scholl et al., 2006). Based on our analysis by nanoLC-HRMS it is clear that incomplete digestion was occurring (Figure 3), however, we cannot be certain that it is a major cause of the measurement discrepancy.

To date, the only approach to compare IDMS values with ELISA measurements is to increase IDMS values by a factor of 2.6(Scholl et al., 2006). This results in a mean of 6.16 pg/mg albumin within this study when expressed as ELISA equivalents. Shirima et al. (2015) measured AFB1-albumin concentrations by ELISA in a cohort of 166 Tanzanian children at recruitment (age 6-14 months), 6 and 12 months after recruitment and found geometric mean concentrations of 4.7, 12.9 and 23.5 pg/mg albumin, respectively, but did not find an association between aflatoxin exposure and growth impairment (Shirima et al., 2015). Conversely, a cross-sectional study of children in Benin and Togo (age 9 months-5 years) observed a significant association between aflatoxin exposure and HAZ, but measured a higher mean concentration of 32.8 pg/mg albumin (Gong et al., 2002). Generally, the aflatoxin-albumin concentrations in studies that observed significant correlations between aflatoxin exposure and HAZ were higher than studies that did not. The range of AFB1-Lys concentrations in the present study were higher than those reported in Nepalese children (Mitchell et al. 2017) and similar to the Tanzanian cohort (Shirima et al., 2015).

A major analytical challenge that hinders evaluation of results between studies is the uncertainty of comparing ELISA and IDMS based measurements or even between IDMS methods given that no certified reference material or commercially available standards are yet available. Differences in albumin quantitation methods (ELISA vs colorimetric methods) may also contribute to variation between studies.

In conclusion, we have developed a highly sensitive and selective method for quantitation of AFB**1**-Lys in human plasma and applied it to an important, yet poorly understood condition, childhood growth impairment. Although this study was of modest size, it comprised a population with a high prevalence of stunting and aflatoxin exposure. Detectable population level increases in liver cancer appear to begin at exposures above 1 ng/kg BW/day and some 40% of liver cancer on a global basis due to aflatoxin exposure occurs in Africa (JECFA, 2017; Liu and Wu, 2010 ). The range of adduct concentrations measured corresponded to chronic exposure of 400 to 1000 ng/kg BW/day of the average child in this study (see Groopman et al., 2014). Exposures over this range are not uncommon in sub-Saharan Africa (JECFA, 2017; Wild et al., 2015). As noted, child fatalities have been reported after 1-3 weeks exposure of 20 µg/kg BW/day (Wild and Gong, 2009). This study further highlights the prevalence of aflatoxin in children and emphasizes the need to develop strategies to reduce aflatoxin exposure in this vulnerable population.

**Authors’ contributions**

AM and JBR performed the experiments and wrote the manuscript. KMNB was responsible for AFB1-Lys synthesis. MWS, SJA, JDM and GR helped conceive the experiments and prepare the manuscript. SJA, AEO and OOA conceived the original study and AEO and OOA led the study in Nigeria. All authors read and approved the final manuscript.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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