Longitudinal assessment of prenatal, perinatal, and early-life aflatoxin B₁ exposure in 828 mother-child dyads from Bangladesh and Malawi

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**Abbreviations:** AFB₁, aflatoxin B₁; AFB₁-lysine, AFB₁-lysine albumin adduct; IDMS, isotope-dilution mass spectrometry; n.d., not detected; HAZ, height-for-age Z-score; HCZ, head circumference-for-age Z-score; LAZ, length-for-age Z-score; WAZ, weight-for-age Z-score; WLZ, weight-for-length Z-score; MMS, multiple micronutrient supplement; IFA, iron and folic acid supplement; LNS, lipid-based nutritional supplement; M₁-TM, maternal 1st trimester; M₂-TM, maternal 2nd trimester; M₃-TM, maternal 3rd trimester; M₃-mo, maternal 3 months postpartum; M-6mo, maternal 6 months postpartum; C-Cord, cord blood; C-6mo, child at 6 months of age; C-18mo, child at 18 months of age; C-24mo, child at 24 months of age; QC₀, blank quality control sample; QCₐ, low quality control sample; QCₐ, medium quality control sample; QCₐ, high quality control sample; UHPLC-MS/MS, ultra-high performance liquid chromatography-tandem mass spectrometry; CV, coefficient of variation as percent; LOD, limit of detection; LOWESS, locally weighted scatterplot smoothing; 95%CI, 95% confidence interval.
Abstract

Background: In utero or early life exposure to aflatoxin, which contaminates staple crops in disadvantaged settings, may compromise pregnancy and infant outcomes, but investigations into the extent, persistence, and determinants of aflatoxin exposure at these life stages have lacked longitudinal data collection and broad geographic representation.

Objective: Aflatoxin exposure and selected determinants thereof were characterized in mother-child dyads with serial plasma/serum samples in prenatal, perinatal, and early life in Malawi and Bangladesh.

Methods: Circulating aflatoxin B\(_1\) (AFB\(_1\))-lysine albumin adducts were measured in dyads from Bangladesh (n=573; maternal 1\(^{st}\) and 3\(^{rd}\) trimester, 3 months postpartum, cord blood, infant 24 months) and Malawi (n=255; maternal 2\(^{nd}\) and 3\(^{rd}\) trimester, 6 months postpartum, infant 6 and 18 months) with isotope dilution mass spectrometry. We examined AFB\(_1\)-lysine adduct magnitude, persistence, seasonality, associations with infant feeding, and estimated daily AFB\(_1\) intake.

Results: Maternal AFB\(_1\)-lysine was higher in Malawi (98% detectable; median, IQR: 0.469, 0.225–1.027 pg/µL) than Bangladesh (59%; 0.030, non-detectable (nd)–0.077 pg/µL). Whereas estimated dietary exposure in Malawi was temporally stable (648 ng AFB\(_1\)/day), estimated intake in Bangladesh was reduced 94% between rainy and winter seasons (98 to 6 ng/day). AFB\(_1\)-lysine was low in cord blood from Bangladesh (15% detectable; 0.045, 0.031–0.088 among detectable) and in Malawian infants at 6 months of age (0.072, nd–0.236), but reached maternal levels by 18 or 24 months (Bangladesh: 0.034, nd–0.063; Malawi: 0.370, 0.195–0.964). In Malawian infants, exclusive breastfeeding at 3 months was associated with 58% lower AFB\(_1\)-lysine levels at 6 months compared to other feeding modes (p=0.010).
Conclusions: Among pregnant women, aflatoxin exposure was persistently high in Malawi, while lower and seasonal in Bangladesh. Infants were partially protected from exposure in utero and with exclusive breastfeeding, but exposures reached adult levels by 18-24 months of age. Registered at clinicaltrials.gov: NCT00860470 and NCT01239693.

Keywords
Aflatoxin, mass spectrometry, diet, pregnancy, breastfeeding, infancy, cord blood, seasonality, toxicology

Subject Heading
Maternal and pediatric nutrition

Teaser Text: Aflatoxin B₁ exposure in rural Malawian and Bangladeshi mother-infant dyads differed in magnitude and seasonality, was reduced in utero and in infancy, but akin to maternal exposure by 18 months.
Introduction

Aflatoxins are mycotoxins produced by certain species of fungi that contaminate staple foods and proliferate in hot, humid environments, where crops may languish in fields or in poor storage facilities – conditions especially common in low- and middle-income countries (1–4). Aflatoxin contamination has been associated with specific staple crops, including maize, groundnuts, wheat, sorghum, and rice, but may occur in a variety of other foods as well (5). Levels of contamination can vary widely, resulting in human exposures that can range from nanograms to milligrams per day, depending upon the grain and amount consumed (6). Variation in dietary patterns and the wide heterogeneity in aflatoxin contamination in grains makes it difficult to estimate human exposure using dietary questionnaires and spot testing of staple foods, thus complicating exposure surveillance and primary prevention efforts. Moreover, just as the bioavailability of nutrients affects their absorption in the gut, only a fraction of an aflatoxin exposure is absorbed and reflected in the internal dose (the amount of aflatoxin that enters the body and may exert downstream biological effects). This variability cannot be captured by questionnaire or food survey exposure assessment approaches.

The development and validation of quantitative techniques for the measurement of biomarkers of aflatoxin internal dose in humans has been critical for their application to etiologic, intervention, and prevention studies in high-risk populations across the globe. Since the chemical characterization of the aflatoxin B₁-serum albumin lysine adduct (AFB₁-lysine) (7), a number of methods have been deployed to determine these adduct levels in humans, with the current gold-standard method of measuring aflatoxin internal dose being the measurement of serum AFB₁-lysine levels using isotope dilution mass spectrometry (IDMS) (8). The AFB₁-lysine adduct is particularly valuable, since the half-life of human serum albumin in circulation
allows a single AFB$_1$-lysine measurement to reflect a smoothed estimate of chronic aflatoxin exposure over the previous 1 to 3 months. Regardless of the approach used for biomonitoring, however, a finding of significant aflatoxin exposure in a population not only raises concerns of direct effects of aflatoxin toxicity and carcinogenicity, but in developing countries, often coincides with limited dietary diversity and micronutrient deficiencies. Thus, when aflatoxin exposure is determined to be substantial, these findings should be viewed as a sentinel for poor nutritional status and low quality of staple grains (11).

While much of the international focus on aflatoxin contamination has been framed within its carcinogenic properties (3,6), many of its other toxic effects can result in significant short- and long-term health consequences. In South Asia, Africa and Central America, where both maternal and child health status are tremendously compromised, potential effects of aflatoxin exposure may be additionally manifested in non-carcinogenic endpoints (12,13). Aside from its well-known role in hepatic carcinogenesis, aflatoxin induces many adverse local and systemic effects that impair normal organ and tissue function, resulting in inflammation, immune suppression, and growth retardation, all of which contribute to poor health (2,3,14). A role for aflatoxin in impairing reproductive outcomes and attenuating growth in childhood has been postulated primarily based on the experimental literature regarding aflatoxin exposure in poultry and livestock (4), but is additionally supported by observational cross-sectional reports and a limited number of longitudinal studies in humans. Several recent systematic reviews have focused on the available human studies (including a handful of randomized clinical trials) and aflatoxin biomarker measurements have been associated with adverse health outcomes during early life in some at-risk settings (14–19).
While prior studies have demonstrated the impact of seasonality on aflatoxin exposure and the potentially critical role of breastfeeding in mitigating exposure early in life, without longitudinal monitoring throughout pregnancy and early infancy (≤ 6 months of age), potentially important implications of timing of exposure may be obscured. Additionally, while many studies examining the relationship between aflatoxin exposure and child growth have been conducted in western sub-Saharan Africa, where groundnut and maize are predominant foods in the diet, fewer studies have investigated these questions in south or southeastern Asia, where stunting is common and often severe (20,21), but which features substantially different dietary patterns and staple foods, including rice.

Thus, the objective of the present study was to quantify longitudinal aflatoxin exposure in over 800 mother-child dyads across prenatal, perinatal, and early-life, at population research sites in eastern sub-Saharan Africa (Malawi) and southeast Asia (Bangladesh). These settings have broadly different dietary patterns, but common features including nutritional deficiencies among women and risk for poor growth and development among infants. Harnessing the strength of side-by-side analysis of samples collected within two community-based, randomized, controlled trials of nutritional interventions, and using the gold-standard IDMS method of AFB$_1$-lysine biomonitoring, we ascertained the prevalence, magnitude, and timing of aflatoxin exposure in relation to seasonality and breastfeeding practices. We also provide estimates of dietary aflatoxin intake at the population level across both settings.

**Methods**

*Subjects, study designs*

This study utilized serum or plasma samples and data previously collected from two completed trials: the cluster-randomized, controlled, double-blind JiVitA-3 trial in the Gaibandha
and Rangpur Districts of Bangladesh (clinicaltrials.gov registration NCT00860470) (22), and the individually randomized, controlled, partially double-blind iLiNS-DYAD-M trial in the Mangochi District of Malawi (clinicaltrials.gov registration NCT01239693) (23). The JiVitA-3 trial was approved by institutional review boards at the Johns Hopkins Bloomberg School of Public Health (Baltimore, MD, USA) and the Bangladesh Medical Research Council (Dhaka, Bangladesh). The iLiNS-DYAD-M trial was approved by the College of Medicine Research and Ethics Committee of the University of Malawi (Blantyre, Malawi) and the Ethics Committee of the Pirkanmaa Hospital District (Tampere, Finland). All participants confirmed their informed consent orally (JiVitA-3) or with signatures or thumb-prints on consent forms (iLiNS-DYAD-M). In both studies, consent was provided with the understanding that their samples or (anonymized) data may be used in future analysis. Local communities were given opportunities to provide input on study design and implementation. All data used in the present analysis was anonymized; only principal investigators and appropriate members of the respective research teams have access to the secured, non-anonymized data.

Among many other endpoints, both studies collected data for the assessment of pregnancy outcomes and child anthropometrics from birth until 24 months of age in the JiVitA-3 trial and until 18 months of age in iLiNS-DYAD-M. The JiVitA-3 trial was designed to test the effects of an antenatal multiple micronutrient supplement (MMS) vs. a standard-of-care control iron and folic acid (IFA) supplement. Similarly, the iLiNS-DYAD-M trial also examined the effects of MMS and IFA prenatal supplementation (although with slightly different formulations compared to JiVitA-3), but used these controls as active comparators against an experimental prenatal lipid-based nutrient supplement (LNS) arm. Details on the formulations of these supplements have been published elsewhere (22,23).
Newly married women aged 12 to 45 years old were recruited to the JiVitA-3 trial through a pregnancy surveillance program to identify and enroll women during the first trimester of pregnancy. A sub-study in a predetermined geographic subregion of the JiVitA-3 trial site was conducted from June 2008 to February 2011 for more intensive data collection, including blood samples in early pregnancy (~10 weeks gestation, here designated M-1TM) and late pregnancy (32 weeks gestation, M-3TM), and at 3 months postpartum in women (M-3mo), in cord blood (C-Cord) in a limited subset of sub-study participants, and in children at 2 years of age (C-24mo) in an even smaller subset. Among these available samples, a cohort of 1,526 women was identified in whom complete serial data collection was available either through the cord blood study or through 3 months postpartum; this cohort has been previously described (24), as has the group from which cord blood was collected (25,26), and forms the sampling frame for this study.

Pregnant Malawian women seeking prenatal care at clinics within the iLiNS-DYAD-M study catchment area were recruited for enrollment, provided they were > 15 years of age and ≤ 20 weeks of gestation. A total of 869 women were enrolled for complete follow-up in the study and followed until their children were 18 months of age; recruitment and follow-up occurred from October 2011 to April 2015. Blood samples were collected from mothers at baseline during the second trimester (M-2TM), 36 weeks gestation (M-3TM), and 6 months postpartum (M-6mo). Blood samples were also collected from children 6 months after birth (C-6mo) and at 18 months of age (C-18mo).

In both studies, extensive demographic, socioeconomic, dietary, anthropometric, and other data were collected to describe household, maternal, and infant characteristics. Infant feeding practices were assessed differently between sites, with the JiVitA project administering a questionnaire at 3 and 6 months of age regarding feeding practices occurring in the prior ~3
months, to ascertain usual feeding practices as exclusive, predominant, or partial breastfeeding. The ILiNS-DYAD-M study used the Infant and Young Child Feeding (IYCF) Indicators questionnaire (27) at 3 and 6 months of age to assess feeding practices in the last 24 hours. The JiVitA-3 trial used the Food Access Survey Tool (FAST) (28) to determine household food security for the period encompassing the first 6 months postpartum, while the iLiNS-DYAD-M study used the Household Food Insecurity Access Scale (HFIAS) (29) at maternal baseline enrollment. The Chronbach’s alpha value for the HFIAS instrument within the iLiNS-DYAD-M trial was 0.81, while the corresponding value for the FAST questionnaire was 0.85 in the JiVitA-3 trial.

Sample selection and representation

Supplemental Figure 1 depicts flow diagrams of sample selection for the current study, for each trial site. Aliquots from 1152 plasma samples from 230 mother-child dyads were selected for analysis from the iLiNS-DYAD-M trial. All of these 230 dyads had “complete” sets of plasma samples, i.e., plasma was available for each sample type within a dyad (M-2TM, M-3TM, M-6mo, C-6mo, C-18mo). Plasma was also available for analysis at mid-pregnancy (M-2TM) and late pregnancy (M-3TM) from an additional 25 mothers (without matched postpartum or child samples), bringing the total number of pregnancies included to n=255 and the total samples analyzed to n=1202.

Based on available samples and outcome data, serum samples from JiVitA-3 pregnancies were selected for analysis in groups comprised of the following combinations of maternal-child sample types: a) 1st and 3rd trimester of pregnancy, mother at 3 months postpartum, cord blood, child at 24 months of age (n= 58); b) 1st and 3rd trimester of pregnancy, mother at 3 months postpartum, child at 24 months of age (n=77); c) 1st and 3rd trimester of pregnancy, mother at 3
months postpartum, cord blood (n=235); d) 1<sup>st</sup> and 3<sup>rd</sup> trimester, mother at 3 months postpartum (n=203). Missing, low volume, or misattributed samples resulted in the combinations shown in Supplemental Figure 1. In total, n=573 maternal-infant dyads were represented, with 1<sup>st</sup> trimester n=569, 3<sup>rd</sup> trimester n=566, 3 month postpartum n=565, cord blood n=295, and 24-month-old n=138, for a total of n=2133 samples.

*Aflatoxin B<sub>1</sub> albumin adduct measurement*

*Sample processing.* AFB<sub>1</sub>-lysine levels were measured using modifications to the method reported by McCoy *et al* (8). Due to volumes available and requirements for detection, 70 µL of plasma was used for samples from Malawi, while 170 µL of serum was used in samples from Bangladesh. Phosphate-buffered saline (PBS, pH 7.2) was added to bring the total volume of all samples to 200 µL. Quality control (QC) samples were processed alongside unknowns for each batch, and prepared using AFB<sub>1</sub>-lysine-negative pooled human donor serum (Innovative Research Inc, Novi, MI) and AFB<sub>1</sub>-dosed rat serum (diluted with PBS to approximately 13 pg AFB<sub>1</sub>-lysine/µL), as follows: QC<sub>0</sub>, 200 µL human serum, 0 µL diluted rat serum; QC<sub>L</sub>, 195 µL human serum, 5 µL diluted rat serum; QC<sub>M</sub>, 190 µL human serum, 10 µL diluted rat serum; QC<sub>H</sub>, 180 µL human serum, 20 µL diluted rat serum. All samples were spiked with an isotopically labeled internal standard (IS, 100 µL at 5 pg AFB<sub>1</sub>-d<sub>4</sub>-lysine/µL), combined with 500 µL of a 6.5 mg/mL PBS solution of Pronase protease (537088, EMD Millipore, Billerica MA, USA), and incubated with agitation for 18 h at 37°C. After enzymatic digestion, samples were centrifuged (3 min at 14,000 x g) and the supernatant was processed on Oasis MAX solid-phase extraction 96-well plates (186000373, Waters, Milford, MA, USA), using a Positive Pressure-96 processor (186006961, Waters). Eluate (800 µL) was dried in a Speedvac (SPD120-15, Thermo Fisher Scientific) at 35°C for 4 hr and wells were washed with 100 µL methanol,
which was transferred to a V-well PCR plate. Samples were again dried in a Speedvac (35°C for 1 hr), reconstituted in 40 μL of 25% aqueous methanol, and transferred to an autosampler plate (60180-10217B, Thermo Fisher Scientific; Zone-Free sealing film, ZAF-PE-50, Excel Scientific) for UHPLC-MS/MS analysis.

**UHPLC-MS/MS analysis.** Analysis was performed on a Vanquish Flex Quaternary UHPLC system coupled to a TSQ Quantis triple quadrupole mass spectrometer (Thermo Fisher). 20 μL of sample was injected onto an Accucore Vanquish C18+ column (150 mm x 2.1 mm x 1.5 μm, Thermo Fisher) preceded by a HyperSil GOLD C18 guard column (10 mm x 2.1 mm x 5 μm, Thermo Fisher), which were held at 55 °C. Samples were separated with an 18-minute isocratic chromatography method, comprised of water (mobile phase A), acetonitrile (B), and 0.6% aqueous formic acid (C). Initial conditions were 80% A/10% B/10% C for 1 minute, stepped to 74% A/16% B/10% C and held for 7 minutes, followed by a step to 0% A/90% B/10% C for 3 minutes, and finally a step back to initial conditions, where the column was re-equilibrated for 7 minutes. Flow was diverted from the detector to waste from 10 – 14.5 minutes. The flow rate was held constant at 250 μL/minute.

Mass spectrometry electrospray ionization source conditions were: 3525 V in positive mode, sheath gas (nitrogen) 20 arbitrary units, auxiliary gas (nitrogen) 24 arbitrary units, sweep gas (nitrogen) 0 arbitrary units, ion transfer tube 350°C, vaporizer 350°C, cone voltage 10 V. Data acquisition was via selected reaction monitoring, using a collision gas pressure of 2 mTorr (argon) and the following transitions: 457.2 → 394.2 (AFB₁-lysine), 461.2 → 398.2 (AFB₁-d₄-lysine). Cycle time was set at 0.35 sec, resulting in a dwell time of 173 msec per transition. Collision energy and RF transmission voltages were set at 21 V and 138 V, respectively, for both
transitions. Q1 resolution was set at 0.7 full width at half-maximum (FWHM), and Q3 resolution was set at 1.2 FWHM.

Quantitation and assay performance. Peak areas were integrated automatically within TraceFinder 4.0 software (Thermo Fisher Scientific), followed by visual inspection and manual integration where necessary. Quantitation was performed using an 8-point, serially diluted, isotope dilution calibration curve in 25% aqueous methanol (v/v). Purified synthetic AFB\textsubscript{1}-lysine (200 pg/µL) was diluted with 25% aqueous methanol to 45 pg/µL (calibrator 1), followed by serial three-fold dilution to 0.021 pg/µL (calibrator 8). These calibrators were then each mixed with synthetic isotopically labeled AFB\textsubscript{1}-d\textsubscript{4}-lysine (5.0 pg/µL) in a 1:1 ratio, to create an 8-point isotope dilution calibration curve with a constant AFB\textsubscript{1}-d\textsubscript{4}-lysine concentration of 2.5 pg/µL and AFB\textsubscript{1}-lysine concentrations of 22.5 - 0.010 pg/µL. AFB\textsubscript{1}-lysine concentration was plotted against AFB\textsubscript{1}-lysine : AFB\textsubscript{1}-d\textsubscript{4}-lysine peak area ratios and a linear curve was fit with an intercept of 0 and 1/X weighted least-squares regression.

Samples were processed and analyzed in batches of 92 unknowns and 4 QCs (QC\textsubscript{0} [matrix blank], QC\textsubscript{L}, QC\textsubscript{M}, and QC\textsubscript{H}), with separate 8-point calibration curves run in duplicate for each batch. Samples within a mother-child dyad were processed together and analyzed sequentially. In total, 26 batches were run for the Bangladesh study and 14 for Malawi samples, amounting to approximately 4,500 total injections. Valid LC-MS data was available for 2,107/2,133 serum samples from Bangladesh (98.8%) and 1,164/1,202 plasma samples from Malawi (96.8%). QC sample performance across all batches was consistent: %CV for calculated AFB\textsubscript{1}-lysine concentrations in QC\textsubscript{L} (0.33 pg/µL), QC\textsubscript{M} (0.66 pg/µL), and QC\textsubscript{H} (1.31 pg/µL) samples was 11.3%, 10.8%, and 10.3%, respectively. Longitudinal quantitative accuracy was within 20% of the calculated concentration for each QC level (Supplemental Figure 2). Across
all batches, the average slope for the three dilutions of QC samples was 0.421 ± 0.009 (12.0% CV), while the average slope of the calibrators in 25% methanol was 0.424 ± 0.003 (4.5% CV), demonstrating that the presence of serum matrix did not alter quantitation (Supplemental Figure 3). Performance of the calibration curve was highly consistent across batches, with R² values ranging from 0.997 - 0.999. The limit of detection (LOD) for the assay (CV ≤20%) with 200 µL input serum was 0.01 pg/µL. Experiments using 100, 80, 60, or 40 µL of serum input demonstrated assay linearity down to 0.067 pg/µL (the lowest concentration tested) with all input volumes, but >20% inaccuracy at 0.067 pg/µL with serum input ≤ 100 µL (Supplemental Figure 4).

Data analysis

All statistical analyses were done separately by site. Demographic attributes of the participating populations are summarized as mean ± SD for continuous variables, or count and percent for categorical variables.

AFB₁-lysine values are presented in units of pg/µL serum/plasma and reported as median and interquartile range (IQR), unless indicated otherwise. Non-detectable samples were imputed as LOD/2 (0.005 pg AFB₁-lysine/µL). Medians and IQR values are inclusive of imputed non-detectable values, unless stated otherwise. AFB₁-lysine adduct levels have historically been reported as adduct concentration normalized to total serum albumin (e.g., pg adduct/mg albumin), requiring the use of a separate assay and serum aliquot for total albumin quantification. As noted above, available sample volumes were limited and were less than the typical assay volume of 200 µL (Bangladesh, 170 µL; Malawi, 70 µL). In order to maximize sample availability for AFB₁-lysine detection in the absence of prior exposure data (particularly in cord
blood and infants), we did not quantify total albumin levels and thus are not presenting AFB$_1$-lysine concentrations as normalized to total albumin.

Seasonality of sample collection in Bangladesh was defined as follows: winter, October 16 – February 15; dry, February 16 – June 15; rainy, June 16 – October 15. Seasons in Malawi were defined as: rainy, November 16 – April 15; dry, April 16 – November 15. These classifications are consistent with climate data reported by The World Bank (30,31). To explore associations of AFB$_1$-lysine with season within each study site, LOWESS curves were fit to AFB$_1$-lysine data separately for each sample type (e.g., M-1TM, M-3TM, etc.) using either 5-point (Malawi) or 10-point smoothing (Bangladesh), using date of sample collection as the independent variable. Differences in distributions of AFB$_1$-lysine concentrations between participant groups or between season of collection were assessed by the nonparametric Kruskal-Wallis test, using the Dwass, Steel, Critchlow-Fligner multiple comparison procedure in PROC NPAR1WAY of SAS. Tests were considered significant at $\alpha = 0.05$.

Post hoc regression analyses of seasonal adduct accumulation and clearance rates were conducted using tobit analysis (32,33) in PROC QLIM of SAS. The IDMS assay’s limit of detection (0.01 pg/μL) was used as the lower-bound censoring limit, day as the independent variable (as an integer relative to the day of the year defined as the beginning of accumulation or clearance), log$_{10}$-transformed AFB$_1$-lysine adduct concentrations as the dependent variable, and repeated measurements were grouped by mother to account for within-subject variance. Within the Bangladesh dataset, only maternal data were included in this analysis, due to the limited sample size for 24-month-old children and the high percentage of non-detectable values in cord blood samples. Data was pooled across all years of sample collection and aligned by date, irrespective of the year in which a sample was collected.
Estimates of daily intakes of AFB$_1$ were calculated as in **Equation 1** below, with the following variables and constants: W, body weight in kg; H, height in m; BV, blood volume in L as estimated in women by the Nadler equation \((34)\) or by body weight (estimated with WAZ) in prepubertal children \((35)\); hematocrit (Hct), estimated by hemoglobin/3 \((36,37)\); 2% conversion rate of ingested AFB$_1$ to AFB$_1$-lysine adduct \((38)\); 30-fold accumulation in AFB$_1$-lysine adduct concentration resulting from chronic exposure relative to an equivalent single dose, assuming a 28-day lifetime of albumin \((7)\).

**Equation 1:**

\[
\text{\( \mu g \) AFB$_1$ \( \text{day}^{-1} \) = \left( \frac{pg \text{ AFB$_1$-lysine}}{\mu L \text{ serum}} \right) \times \left( \frac{0.66 \ pg \ AFB$_1$}{pg \text{ AFB$_1$-lysine}} \right) \times BV \times Hct \times 0.02^{-1} \times 30^{-1} \]
\]

**Nadler equation:** \(BV = (0.3561 \times H^3) + (0.03308 \times W) + 0.1833\)

To ascertain persistence of exposure over the time course from early pregnancy to 18 or 24 months of child’s age within maternal and infants dyads, correlation analysis and partial correlation analysis were performed among data at all time points per study site. Partial correlations were calculated between log$_{10}$-transformed AFB$_1$-lysine levels at each sample collection time (including imputed non-detectable values), adjusting for seasonal variability in exposure to ascertain whether some dyads were perpetually at higher risk of exposure even as environmental aflatoxin may have changed. For samples from Malawi, which had few non-detectable samples, Spearman’s rank-order correlation is appropriate. Due to the higher percentage of non-detectable values in the Bangladesh data set, correlation analysis of AFB$_1$-lysine levels for samples from Bangladesh was also conducted using Kendall’s tau-b rank-order correlation \((39)\), which does not yield a p-value with partial correlation analyses \((40)\). Both correlation coefficients are presented for each site.

Finally, within the Malawi dataset, which uniquely contained data in 6 month old infants, regression analysis was done to ascertain the potential protective role of breastfeeding behaviors...
and other demographic factors on aflatoxin exposure in infancy. We report findings that followed
an extensive model fitting exercise (41) to generate a parsimonious model relating breastfeeding,
household food security, maternal parity, and season of sample collection to log$_{10}$-AFB$_1$-lysine
concentrations at 6 months of age, with β-coefficients expressed as geometric mean ratios with
95% confidence intervals.

Statistical analysis and data visualization were performed in SAS 9.4 (SAS Institute,
Cary, NC) and GraphPad Prism 8 (GraphPad Software, San Diego, CA).

**Results**

*Participant demographics, anthropometrics, and characteristics*

Features of each study sample are shown in Table 1 and have been published in detail
elsewhere (22–24,26,42). Within each study, participants were balanced across trial intervention
groups. Compared to participating mothers in Malawi, mothers from Bangladesh were younger,
shorter, weighed less, had lower BMI, had less education, and were nearly twice as likely to be
primiparous. As a result of the differing study designs, Bangladeshi mothers were enrolled at the
end of the first trimester (~11 weeks of gestation), while Malawian mothers’ baseline visit
occurred in the middle of the second trimester (~17 weeks gestation). Among infants, differences
could be seen in child anthropometry measures, as mean values of length-for-age Z-score (LAZ),
weight-for-length Z-score (WLZ), and weight-for-age Z-score (WAZ) in Bangladeshi children
were all lower than those of Malawian children at each time of assessment, particularly notable
for WLZ and WAZ. Patterns of breastfeeding practices were similar, although Bangladeshi
mothers reported higher rates of exclusive breastfeeding at 3 months postpartum (76% vs. 54%)
and at 6 months postpartum (14% vs. 7%). Homes in either setting were unlikely to have
electricity or piped water, although in Bangladesh water-sealed toilets were common. Food insecurity, albeit measured differently across studies, was considerably more severe in Malawi (82% of households reporting any level of food insecurity) than in Bangladesh (47%). These data reveal settings that challenge the well-being of mothers and infants in common and unique ways. However, descriptive data were not tested statistically between sites, given that variables were not consistently assessed in the same manner across the two trials.

**Aflatoxin exposure in Bangladeshi and Malawian mother-child dyads**

Aflatoxin exposure varied substantially by study site and sample type (Figure 1). In Bangladesh, 53% (1,124/2,107) of all samples had detectable levels of AFB$_1$-lysine, while 90% (1,053/1,164) of samples in Malawi had detectable AFB$_1$-lysine, such that it required analysis of nearly twice as many samples from the Bangladesh women to achieve the same number of samples with detectable AFB$_1$-lysine. In Bangladeshi women, the proportion of women with detectable AFB$_1$-lysine was consistent across time points (59%, 993/1,679), with 56% in mothers during early pregnancy (M-1TM, 315/561), 63% in mothers during late pregnancy (M-3TM, 352/558), and 58% in mothers at 3 months postpartum (M-3mo, 326/560). Malawian mothers experienced near universal aflatoxin exposure - 98% of maternal samples overall (712/728), 98% in mothers during mid-pregnancy (M-2TM, 243/249), 96% in mothers during late pregnancy (M-3TM, 239/249), and 100% in mothers at 6 months postpartum (M-6mo, 230/230). In both sites, samples collected from children 18-24 months of age revealed similar rates of AFB$_1$-lysine detection as in their mothers – in Bangladesh, 64% (86/135) of 24-month-old children had detectable levels, while in Malawi, nearly all samples collected from 18 month-old children were positive (95%, 211/221). In contrast, in Malawi, only 60% (130/215) of samples collected from
6-month-old children were positive for AFB\textsubscript{1}-lysine. Cord blood samples collected from Bangladesh pregnancies rarely contained detectable levels of AFB\textsubscript{1}-lysine (15%, 45/293).

Concentrations of AFB\textsubscript{1}-lysine were distributed log-normally and followed similar trends as those observed in AFB\textsubscript{1}-lysine detection prevalence – higher AFB\textsubscript{1}-lysine concentrations in Malawi than in Bangladesh, roughly equivalent concentrations in mothers and children at 18 or 24 months, but lower concentrations in the perinatal period (cord blood, Bangladesh) or infancy (6 months, Malawi) than later in childhood (Figure 2). Overall, mothers in Malawi had median AFB\textsubscript{1}-lysine concentrations of 0.469 pg/µL (IQR, 0.225 – 1.027), while median concentrations in Bangladeshi mothers were nearly 16-fold lower, at 0.030 pg/µL (n.d. – 0.077). Among Malawian mothers, AFB\textsubscript{1}-lysine concentrations were consistently high in the second trimester (0.466 pg/µL, 0.232 – 0.937), third trimester (0.439 pg/µL, 0.206 – 0.981), and 6 months post-partum (0.536 pg/µL, 0.241 – 1.135). Although substantially lower in magnitude than in Malawi, distributions in Bangladeshi mothers were also similar in the first trimester (0.027 pg/µL, n.d. – 0.066), third trimester (0.038 pg/µL, n.d. – 0.087), and at 3 months post-partum (0.025 pg/µL, n.d. – 0.076). By 18 and 24 months of age, AFB\textsubscript{1}-lysine concentrations in children from Bangladesh (24 months of age; 0.034 pg/µL, n.d. – 0.063) and Malawi (18 months; 0.370 pg/µL, 0.195 – 0.964) did not differ from maternal values. However, AFB\textsubscript{1}-lysine concentrations were lower in cord blood of Bangladeshi infants and 6-month-olds from Malawi (0.072 pg/µL, n.d. – 0.236) than at any other time point in each respective population (p<0.0001 for comparisons). In Bangladesh, AFB\textsubscript{1}-lysine was undetectable in 85% of cord blood samples, and among the 15% of cord blood samples that had detectable AFB\textsubscript{1}-lysine levels, the median (IQR) concentration was 0.045 pg/µL (0.031 – 0.088).
Examination of mother-specific AFB<sub>1</sub>-lysine trajectories revealed distinct patterns of exposure in Bangladesh and Malawi (Figure 3). Mothers in Bangladesh experienced substantial within-subject variability in AFB<sub>1</sub> internal dose across time (Figure 3A), as longitudinal sampling revealed groups of subjects alternating between non-detectable and detectable values (0.065, 0.038 – 0.130 pg/µL). In contrast, within Malawian mothers there was a greater persistence of AFB1-lysine at a higher magnitude over time (Figure 3B).

**Association of season with aflatoxin exposure**

Based on AFB<sub>1</sub>-lysine trajectories in Bangladeshi mothers (Figure 3A), we suspected that seasonal differences in aflatoxin exposure could explain the observed within-subject variance. As shown in Figure 4, we examined the seasonality of AFB<sub>1</sub>-lysine concentrations in both Bangladesh and Malawi. Regardless of maternal pregnancy status, the Bangladeshi dry season was associated with significantly lower serum AFB<sub>1</sub>-lysine concentrations (n.d., n.d. – 0.030 IQR; 37% detectable) than either the winter (0.041, n.d. – 0.097; p < 0.0001; 65% detectable) or rainy seasons (0.051, n.d. – 0.117; p < 0.0001; 74% detectable) (Figure 4A). Figure 4B depicts LOWESS analysis of serum AFB<sub>1</sub>-lysine concentration plotted against date of sample collection (spanning approximately 2.8 years from June 2008 to March 2011) and demonstrates a seasonal oscillation in AFB<sub>1</sub>-lysine concentrations, with an annual nadir shortly after the end of winter and an apex in mid-October, at the end of the rainy season.

In contrast to Bangladesh, we detected no significant differences between distributions of AFB<sub>1</sub>-lysine concentrations in maternal and child samples collected during Malawi’s rainy (0.368, 0.162 – 0.844; 98% detectable) vs. dry (0.330, 0.145 – 0.986; 91% detectable) seasons (Figure 4C). This was consistent with LOWESS analysis, which revealed no seasonal patterns in AFB<sub>1</sub> exposure among Malawian mother-child dyads across a period of approximately 2.7 years.
(September 2011 – April 2014; Figure 4D) and suggests that there is consistent exposure to aflatoxin throughout the year in Malawi.

We next sought to quantify the rates of AFB\(_1\)-lysine adduct accumulation and clearance in Bangladeshi mothers. Per our LOWESS analyses, (Figure 4A-B), we defined the period of accumulation to encompass the Bangladeshi dry (February 15 to June 15) and rainy (June 15 to October 15) seasons (242 days total), while adduct clearance occurred during the winter (October 15 to February 15; 123 days). Using censored regression analysis on log\(_{10}\)-transformed AFB\(_1\)-lysine values, which facilitated the estimation of adduct concentrations below the assay’s limit of detection (0.01 pg/μL), geometric mean AFB\(_1\)-lysine adduct levels in Bangladeshi mothers accumulated from an estimated nadir of 0.003 pg/μL on February 15 to a peak of 0.060 pg/μL on October 15 (Figure 5, left). Similarly, examining the clearance of adducts across three winter seasons revealed a peak of 0.099 pg/μL on October 15 and an estimated nadir of 0.007 pg/μL on February 15 (Figure 5, right). Averaged across both the accumulation and clearance periods, arithmetic mean peak (Oct 15) and nadir (Feb 15) concentrations were thus 0.080 and 0.005 pg/μL, respectively. Using these average values, the rates of accumulation and clearance were estimated as 0.310 and 0.610 fg/μL/day, respectively. Note that, based on albumin adduct clearance kinetics (43), only ~1% of AFB\(_1\)-adducted albumin resulting from exposures at the end of the rainy season (peak exposure) would be estimated to remain in circulation 123 days later (winter nadir).

Translating our measurements of AFB\(_1\) internal dose (reflected by the AFB\(_1\)-lysine biomarker) to group-level estimated dietary intake of AFB\(_1\) via Equation 1, plasma AFB\(_1\)-lysine concentrations in Malawian mothers (0.469 pg/μL) reflect a daily average dietary intake of approximately 648 ng AFB\(_1\) per day (12.2 ng/kg/day). This is contrasted with exposure in
Bangladesh, where year-round median AFB₁-lysine levels (0.030 pg/μL) result in estimated intakes of 37 ng AFB₁ per day and 0.86 ng/kg/d.

Accounting for the seasonality of exposure observed in Bangladesh, estimated AFB₁-lysine concentrations peaked at the end of the rainy season (0.080 pg/μL, per censored regression) and were at their lowest at the end of winter (0.005 pg/μL), translating to an estimated daily total intake of 98 and 6.14 ng AFB₁/day (2.3 and 0.14 ng/kg/day), respectively.

Estimated AFB₁ exposure in children (Bangladesh, 24 months of age, 0.034 pg/μL; Malawi, 18 months of age, 0.370 pg/μL) revealed a lower daily average total intake than in their mothers – 14 ng/day in Bangladeshi children (without accounting for seasonality) and 141 ng/day in Malawi. However, after normalizing to body weight, estimated child exposures were similar to maternal exposures at each site: 1.5 ng/kg/day in 24-month-old Bangladeshi children and 14.1 ng/kg/day in Malawian children at 18 months of age.

**Persistence and determinants of exposure**

Bivariate correlation analysis of AFB₁-lysine concentrations between times of sample collection within women and mother-infant dyads, without adjustment for seasonality, is shown in Supplemental Figure 5. Results were not substantially altered after adjustment for season of sample collection with partial correlation analysis, as seen in Table 2. Accounting for the variability contributed by season did not substantially alter the strength of associations in aflatoxin exposure observed between time points in Bangladesh or, as expected given the lack of seasonality in exposure, in Malawi. In Bangladesh, associations were weaker than those observed in Malawi, but that they were positive (as assessed by Kendall’s tau-b rank-order correlation test) in all but one case (M-3TM and M-3mo) suggests that certain dyads were at a persistently greater risk of exposure even as seasonal influences on exposure varied. Cord blood
AFB₁-lysine, despite being low in detection prevalence and concentration, was associated with maternal concentrations at the times surrounding its collection (τ=0.169 for M-3TM and τ=0.228 at M-3mo), while values in children at 24 mo showed modest but positive associations with prior values despite the extensive time interval between periods of sample collection. In mothers from Malawi, the strongest correlations were observed between samples collected during mid-pregnancy (M-2TM) and those collected either during the third trimester (M-3TM; ρ = 0.40, p < 0.0001) or 6 months post-partum (M-6mo; ρ = 0.30, p < 0.0001). Malawian maternal levels during the third trimester were less strongly correlated with concentrations measured 6 months after delivery (ρ = 0.26, p < 0.005). Additionally, a strong correlation was observed between maternal and child AFB₁-lysine concentrations measured in samples collected at the same study visit, 6 months after delivery (ρ = 0.63, p < 0.0001). Concentrations in samples collected from children at 18 months of age were less strongly correlated with levels at 6 months of age (ρ = 0.25, p < 0.005) and were not correlated with concentrations measured in their mothers at any point during or after pregnancy.

Given the dramatically lower AFB₁ exposure in 6-month-old children in Malawi than at any other sample collection time point, we sought to determine via multivariate regression analysis whether infant feeding mode could be a protective factor. In line with a hypothesized protective nature of exclusive breastfeeding, we demonstrated a 58% reduction (p = 0.010) in AFB₁-lysine concentrations among 6-month-old infants who were exclusively breastfed at 3 months of age, compared to other modes of feeding (Table 3). Additionally, children from households experiencing any degree of food insecurity (mild, moderate, or severe) exhibited 2.18-fold greater AFB₁-lysine levels (95% CI, 1.16 – 4.10) than children from food secure households.
Discussion

In this report, we present data from one of the largest longitudinal analyses of aflatoxin exposure throughout pregnancy and early childhood, representing over 800 mother-child dyads across two nutrition intervention trials, in settings where nutritional risks to mothers and infants are common, yet dietary patterns and food insecurity vary. We found striking and persistent levels of maternal aflatoxin exposure in rural Malawi, with nearly 100% prevalence and substantial plasma AFB₁-lysine concentrations, while exposure in Bangladeshi mothers was less prevalent, at approximately 60% of all maternal samples, and was up to one order of magnitude lower in concentration. However, these summary values belied significant seasonal variability in Bangladesh, with a nearly 3-fold annual dynamic range in AFB₁-lysine levels and large shifts in detection prevalence across the year. Finally, while offspring seemed to be relatively protected from aflatoxin exposure in utero and through breastfeeding early in infancy (at 6 months of age), children aged 18 (Malawi) or 24 months (Bangladesh) experienced rates and magnitudes of aflatoxin exposure that were comparable to those in their mothers.

Before comparing findings to those of previous studies, it is important to note that we report AFB₁-lysine concentrations in volumetric terms (e.g., pg/µL), rather than relative to total serum albumin concentrations (e.g., pg/mg albumin). Historically, expressing AFB₁-lysine values relative to serum albumin – which can be determined through various colorimetric assays or immunoassays in a serum aliquot separate from that used for AFB₁-lysine quantification – has been the conventional practice. However, while that approach was warranted to normalize AFB₁-lysine values in older methods (44,45) this is not necessary with IDMS (8), which is now the gold standard method for measuring AFB₁-albumin adducts and was the approach utilized in this report. Owing to the use of isotopically-labeled AFB₁-lysine internal standards and tandem mass
spectrometry quantification, the IDMS approach can directly quantify serum or plasma concentrations of AFB$_1$-lysine adducts, providing an accurate metric of AFB$_1$ internal dose (46). Furthermore, due to the variance and bias across albumin assay formats and manufacturers (47), presenting AFB$_1$-lysine values as normalized to serum albumin, rather than volumetrically, complicates harmonization and conversion of internal dose to exposure estimates as in Equation 1 above. Other groups have also questioned the utility of albumin normalization for AFB$_1$-lysine measurements (48). However, for the purposes of comparison to previously published, albumin-normalized results, a rough conversion to units of pg/mg albumin can be achieved through multiplication of values expressed in pg/µL by a factor of 23.8 (based on a population average albumin concentration of 42 mg/mL). We will discuss our data in the context of prior work by presenting estimated albumin-normalized values alongside our volume-normalized results, as well as with appropriate conversions to account for known quantitative biases of other AFB$_1$-lysine analytical methods (49). Using these conversions, the level of exposure we observed in Bangladesh is very similar to those reported recently from Nepal by Andrews-Trevino et al., with medians of 1.20 pg/mg in maternal samples during pregnancy (equivalent to approximately 0.053 pg/µL), 0.72 pg/mg in 3-month-old children (0.032 pg/µL), and 1.11 pg/mg at 18-22 months (0.049 pg/µL). Similarly, we report levels of exposure in Malawi mothers which, after conversion, are similar to maternal levels in other African populations, such as Ghana (mean, 10.9 pg/mg; equivalent to 0.481 pg/µL) (50) and Uganda (median, 5.83 pg/mg; equivalent to 0.256 pg/µL) (51), while being higher than levels recently reported in pregnant Rwandan women (median 1.7 pg/mg; equivalent to 0.071 pg/µL) (52).

In Latin America, where maize consumption is engrained within the culture and provides nearly 20% of energy intake, we have shown that tortillas account for over half of the total maize
intake and their consumption is dose-dependently associated with elevated AFB$_1$-lysine levels (53). Additionally, in Qidong, China, our group has shown that, driven by economic factors, a widespread dietary transition from maize to rice, which is less commonly contaminated, resulted in a dramatic drop in AFB$_1$-lysine concentrations and a precipitous decline in hepatocellular carcinoma incidence (54). In Malawi, maize comprises nearly two-thirds of energy intake among pregnant women (55), resulting in a diet with a very low degree of diversity and high contribution from one of the staple crops most commonly contaminated by aflatoxins (46). With complementary foods that mimic the adult diet, complementary feeding in Malawi places children at high risk of aflatoxin exposure, such that it is not surprising that children at 18 months of age experienced roughly the same level of aflatoxin exposure as their mothers. In a bivariate analysis among 6-month-old infants in the current study, consumption of nsima, a thick, maize-based porridge, was associated with 47% (95%CI: 1-114%) increased risk of AFB$_1$ exposure, while consumption of likuni, a commercially-produced, liquid maize-soy blended porridge, reduced exposure by 32% (95%CI: 8-50%). While these associations were no longer statistically significant after adjustment for other covariates (41), they suggest that replacing poor quality staples with higher quality alternatives might be necessary to reduce the burden of aflatoxin exposure during pregnancy and complementary feeding in settings like Malawi, where exposure is persistent. This approach is supported by results from interventions such as the one by Hoffman et al. in a community in eastern Kenya (56), in which the investigators facilitated the replacement of contaminated maize with clean maize, resulting in a reduced risk of stunting among children at ~1 year of age. This represents one strategy to reduce aflatoxin exposure in settings where monotonous diets rely on persistently contaminated foodstuffs.
We observed a statistically significant 2.18-fold increase in circulating AFB₁-lysine adduct levels among 6-month-old Malawian children in food insecure households vs. counterparts in food secure households. This is, to our knowledge, the first report of such an association between food insecurity and aflatoxin exposure. Moreover, given the very high rates of household food insecurity in this population (82%), this association is particularly troubling and suggests that food insecurity may play a significant role in driving aflatoxin exposure in Malawi. Previous research from several authors of this paper has shown an inverse association between food insecurity and dietary diversity in pregnant and lactating women in Malawi (57). Poor dietary diversity has been shown to be related to the magnitude of aflatoxin exposure and increasing dietary diversity is a valuable strategy for reducing exposure to aflatoxins (4). Thus, whether strategies focus on increasing dietary diversity or on reducing household food insecurity – such as by provision of lipid-based nutritional supplements (58) – a diminished dietary reliance on maize is likely to reduce aflatoxin exposure in Malawi.

In contrast to dietary patterns in Malawi, rice is the staple grain in Bangladesh. Additionally, food insecurity was lower in our sample of Bangladeshi women (47%) than in Malawi (82%), while dietary diversity was higher – foods such as fish were consumed frequently (~65% reported consuming at least 3 times weekly), while other meat, dairy, eggs, and yellow or green vegetables were consumed by at least 15% of respondents at least 3 times weekly (24). Work from Nguyen et al. supports the notion that Bangladeshi women have a relatively diverse diet, in that the majority of pregnant women reported consuming foods from 5 different food groups within the past 24 hours (59). Thus, while a monotonous maize-based diet likely explains the high, persistent exposure patterns observed in Malawi, greater dietary diversity in Bangladesh may explain the comparatively low overall exposure in Bangladesh. However,
previous work from Stevens *et al.* has shown that dietary diversity among pregnant women varies by season in Bangladesh, being lowest in the dry spring and summer, but highest during the late autumn and winter (60). In line with this, we found that aflatoxin exposure increases from February 15 through October 15, particularly during the rainy season (June 15 to October 15), while declining during the winter, from October 15 to February 15. A previous report from Bangladesh has shown a similar seasonal pattern of aflatoxin exposure, with the highest exposures at the end of the rainy season (61). Thus, aflatoxin exposure in Bangladesh may closely trail seasonal fluctuations in dietary diversity, such that aflatoxin exposure rises following periods of low dietary diversity and begins falling after dietary diversity reaches its yearly peak. In the present study, the rate of adduct clearance in Bangladeshi mothers during the winter season was 2-fold greater than the rate of accumulation during the dry and rainy seasons, while estimated maternal daily aflatoxin intake at the end of the winter was 94% lower than estimated peak intake after the rainy season. These data reveal a precipitous annual reduction in aflatoxin exposure in this population. The report on pregnant Bangladeshi women by Stevens *et al.* also revealed significant increases in the consumption of meat, poultry, and fish beginning in late autumn and continuing through the winter – aligning with the rapid decline we observe in aflatoxin internal dose (60). However, it is unclear whether these foods are directly displacing aflatoxin-contaminated foods from the diet or are surrogate indicators of more broad dietary changes. Future analyses will need to assess whether seasonal consumption of specific foods may enhance or mitigate aflatoxin exposure in this sample of Bangladeshi mothers. As in Malawi, interventions that encourage dietary diversity may present a feasible and economical strategy for primary exposure prevention in Bangladesh.
Patterns of exposure in Bangladesh and Malawi were used to ascertain estimates of aflatoxin intakes. In Malawian women, estimates of intake were ~650 ng/d year-round, with estimates in Bangladeshi women ranging from 98 ng in the season of highest exposure to 6 ng/d when exposure was lowest. Intakes in Malawi are consistent with a persistent, single source of aflatoxin in a food insecure environment, but identifying and mitigating transient sources of exposure in Bangladesh could be more challenging. Calculations also demonstrate that despite a lower total intake of aflatoxin in children than adults (~3- to 5-fold lower), after normalizing to body weight, estimated intakes in both Bangladeshi and Malawian children (1.5 ng/kg/d [annual] and 14.1 ng/kg/d, respectively) were slightly higher than those of women in the same setting (0.86 ng/kg/d [annual] and 12.2 ng/kg/d, respectively). It should be noted that these calculations represent group averages, while more refined estimates at the individual level could also be done to reveal the variance of intake in these environments.

As reported elsewhere (62), while AFB$_1$ is known to cross the human placenta, the fetus appears to be somewhat protected, with roughly 25% of the maternal dose reaching the fetal circulation. In Bangladesh, although aflatoxin exposure varied by season, maternal exposures during late pregnancy and at 3 months postpartum were modestly associated with cord blood aflatoxin adduct levels. Caution is warranted in interpreting the maternal-cord blood association due to the high rate of AFB$_1$-lysine assay detection-limit censoring in cord blood samples (39), but comparing AFB$_1$-lysine detection prevalence in maternal samples (60%, late pregnancy and 3 months postpartum combined) and cord blood (15%) does support the hypothesis that the fetus is protected from a proportion of the maternal AFB$_1$ exposure. Additionally, we found that while the median circulating AFB$_1$-lysine levels in mothers at 32 weeks of gestation was 0.038 pg/µL, the median concentration in cord blood samples was at least ~4-fold lower, below our assay’s
limit of detection of 0.010 pg/µL. These results are in line with data from western Africa, which showed a modest correlation (Spearman’s ρ = 0.383) between maternal AFB₁-lysine levels during pregnancy (100% detectable) and those in cord blood samples (49% detectable), as well as cord blood concentrations that were roughly 25% of those found in the maternal circulation during pregnancy (10.1 and 40.4 pg/mg, respectively; equivalent to ~0.085 and 0.34 pg/µL, respectively) (63). While cord blood samples were not collected from Malawian pregnancies in the present study, exposure in Malawian mothers at 36 weeks gestation (M-3TM; 96% detectable, 0.439 pg/µL) was comparable to values reported in western Africa, suggesting that fetal exposure in Malawi may follow similar trends. Assuming that only 25% of the maternal AFB₁ dose is transferred to the fetal circulation, it is likely that in utero exposure to significant levels of AFB₁ would be highly prevalent in the Malawi population.

While fetal protection from maternal aflatoxin exposure while in utero is governed by placental physiology, protection of infants from exposure after birth is in large part dependent upon breastfeeding and complementary feeding practices. Breastfeeding appeared to be protective against AFB₁ exposure in Benin and Togo (64,65) and Bangladesh (61), although not in Nepal (66). We found that exclusive breastfeeding at 3 months postpartum was associated with a 58% reduction in AFB₁-lysine levels in 6-month-old Malawian infants, relative to other modes of feeding. Notably, rates of exclusive or predominant breastfeeding among Malawian mothers were relatively high at 3 months postpartum (53.9% exclusive, 68.6% exclusive or predominant) but very low by 6 months postpartum (6.9% and 13.8%, respectively), suggesting that most children received protection from exposure during only a short window early in life. Coincidentally, these data highlight a significant benefit of the AFB₁-lysine biomarker for assessing exposure prevention or mitigation interventions – the long half-life of the albumin
adduct allows detection of the effects of a protective behavior 3 months after its occurrence, despite rates of the protective behavior having fallen to ineffective levels at the time of biomarker measurement. Use of biomarkers with shorter residence times, urinary metabolites (67,68), for example, would require much more frequent sampling to dissect the early vs. late trends.

It is important to note that aflatoxins can be transferred from mother to child through breastmilk. Aflatoxin M₁ (AFM₁) is a toxic and carcinogenic hydroxylated metabolite of AFB₁ found in breastmilk of aflatoxin-exposed women (46,69), which has been found to be inversely associated with HAZ and WAZ in several studies (16). Moreover, AFB₁ itself can be found in breastmilk, sometimes at levels higher than AFM₁ (70). While promoting recommended breastfeeding practices can be a valuable strategy to reduce AFB₁ exposure in infants (as well as for achieving the established nutritional and immunological benefits of breastmilk), it may not eliminate aflatoxin exposure even in exclusively breastfed infants, and it does not mitigate aflatoxin exposure and the attendant health risks within mothers. We did not assess breastmilk for aflatoxin content and therefore cannot ascertain whether infants in our study were exposed to AFM₁ through breastfeeding, or the proportion of infant AFB₁ exposure that may occur through the breastmilk.

This study was motivated by the growing body of evidence suggesting that aflatoxin exposure in utero or during critical windows early in life may lead to harmful and potentially irreversible impacts on child development (2,4). Aflatoxin exposure and its effects on child growth have been reported extensively in west African populations, with most investigations in this region demonstrating that either maternal or child exposures are associated with reduced birthweight (50), WAZ (63,71), LAZ/HAZ (63,65,71), and/or WLZ (71). In contrast, studies
exploring these questions in eastern Africa and southeast Asia have produced conflicting results both supporting (18,19,51) and not supporting (66,72–75) a role for aflatoxin in fetal growth restriction and childhood stunting. However, those studies that do not control for key determinants of aflatoxin exposure – such as seasonality and breastfeeding, as we show in the present report – may bias results towards the null (61,75), while other studies with similar sample sizes, populations, and exposures may reveal an effect of aflatoxin on growth parameters due to more complete statistical accounting of variance components (71). In fact, most of the studies that have failed to find an association between aflatoxin exposure and child growth outcomes have had small sample sizes, have not adjusted for key variables such as seasonality or breastfeeding practices, or both (66,72–75). Moreover, while some have suggested that null results may have been the result of low aflatoxin exposures, perhaps below a critical threshold for biological effect (75), the recently published study in Nepalese subjects from Andrews-Trevino and colleagues (18) revealed highly significant reductions in postnatal LAZ, WAZ, WLZ, length, and knee-heel length in association with aflatoxin exposures that were lower than those in many of the prior studies that have reported no association. Notably, the AFB₁-lysine levels that Andrews-Trevino et al. reported in Nepal are similar to those we measured in Bangladeshi mothers and children. The statistically significant results reported by Andrews-Trevino et al. are likely not only due to a larger sample size (n=1,675) than most prior investigations, but also through controlling for seasonal timing of sample collection, which we and others have shown to be a major factor influencing aflatoxin intake in southeast Asia. Of note, in the present study, growth characteristics of infants were poorer in the Bangladesh than Malawi setting, despite higher aflatoxin exposure in the latter. Thus, aflatoxin exposure is but one potential etiological contributor to poor growth, with a role that is likely context-specific and
challenging to disentangle from contributions of poor diet overall. Nonetheless, the findings here provide a foundation for further explorations of aflatoxin exposure on maternal and child outcomes, such as growth, which can be compared across settings.

This study has many strengths. The sample size, over 800 mother-child dyads across both sites, is among the largest longitudinal aflatoxin biomonitoring studies of its kind. Moreover, the diversity of maternal and child characteristics, magnitude and prevalence of exposures, and contextual factors presented by these two populations (region, climate, seasonality, diet, etc.) provides a unique juxtaposition of divergent exposure settings. Additionally, since both studies used the same gold-standard analytical method, in the same laboratory, within a short time span, comparison of aflatoxin exposures – and in future work, the influence of aflatoxin on growth outcomes – can be conducted across populations without concerns over methodological variance or harmonization. Moreover, the repeated measures design of both trials allows for longitudinal assessment of aflatoxin exposure within individuals and links maternal and child exposures within a dyad over time.

There are limitations to our study. While the dietary source of AFB$_1$ contamination is clear in Malawi (maize), we have not identified a primary source of exposure in Bangladesh. Additional work will be necessary to identify the source(s) of exposure in the Bangladeshi diet, as well as reveal low-risk foodstuffs that may be responsible for seasonally replacing contaminated dietary staples, leading to the annual and precipitous decline in AFB$_1$ exposure that we observed in this population. Secondly, while a unified analytical platform allows side-by-side quantitative comparison of aflatoxin exposure, many demographic variables in these two populations were not measured in both settings, or in the same manner. Additionally, the timing and type of sample collection differed by population, leaving some comparative gaps that cannot
be filled or that must be approached with caution (e.g., cord blood measurements allow direct assessment of in utero exposure in Bangladesh, but were not available in Malawi). Subgroup sample sizes within and between studies were unequal, and neither cohort was recruited as a representative sample of the population as a whole; both of these factors could present challenges when attempting to generalize our findings.

In summary, we report a large, detailed survey of longitudinal aflatoxin exposure across a reproductive event in mother-child pairs enrolled in two randomized nutrition intervention trials, conducted in substantially differing aflatoxin exposure contexts on two continents. Aflatoxin exposure was prevalent throughout the year and of high magnitude in Malawi, although exclusive breastfeeding may temporarily mitigate nearly 60% of exposure in infants. In Bangladesh, exposure magnitude was lower, but substantial, and subject to significant seasonal variability; censored regression analysis and dosimetry calculations suggest that after peak exposure at the end of the rainy season, Bangladeshi mothers eliminate 94% of their dietary AFB₁ exposure every winter. These results suggest that exposure mitigation or primary prevention in both settings is possible, although potentially through different strategies. Our findings not only provide scientists, interventionists, and policymakers with a detailed examination of aflatoxin exposure in two at-risk populations, but lay the foundation for follow-up studies examining the role of aflatoxin in child growth and development. Preventing aflatoxin exposure in the world’s most vulnerable communities will become even more pressing of an issue in the coming decades, as climate change is expected to substantially increase aflatoxin contamination of staple crops (76).
Author contributions

KGD, CPS, KPW Jr, PC, KJS, and JDG designed the research and conducted study oversight; AJM, KMM, PA, CPS, SS, HA, ABL, KPW Jr, KGD, and KJS conducted the clinical trials and collected data and biospecimens; JWS and SMB performed the mass spectrometry analysis; JWS, AJM, LS-FW, CDA, CPS, and KJS analyzed and interpreted the data; JWS, JDG, and KJS wrote the paper; KJS and JDG had primary responsibility for the final content of the paper. All authors have read and approved the final manuscript. The authors would like to acknowledge Gerald N. Wogan (1930-2021), who, with George H. Büchi, elucidated the structure of aflatoxin B₁ in 1963. Dr. Wogan contributed to much of our present knowledge on aflatoxin biochemistry and toxicology, was an elected member of the National Academy of Sciences, and was a beloved mentor to many scientists.

Data Availability

Data utilized to generate this manuscript will be made available via repositories at Johns Hopkins University and University of California-Davis for the JiVitA-3 and iLiNS-Dyad findings, respectively, and will be shared upon reasonable request.
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## Table 1. Participant characteristics

|                                | Bangladesh  | Malawi     |
|--------------------------------|-------------|------------|
| **Maternal**                   |             |            |
| Intervention group             |             |            |
| IFA                            | 292 (51.0%) | 84 (32.9%) |
| MMN                            | 281 (49.0%) | 85 (33.3%) |
| LNS                            | -           | 86 (33.7%) |
| Age, y                         | 23.2 (5.4)  | 25.1 (6.1) |
| Gestational age at first visit, wks | 11.1 (4.5) | 16.9 (2.3) |
| Height, cm                     | 148.9 (5.1) | 155.9 (5.6) |
| Weight, kg                     | 42.9 (6.1)  | 53.2 (7.2) |
| BMI                            | 19.4 (2.3)  | 21.5 (2.5) |
| Primiparous                    | 209 (36.5%) | 51 (20.0%) |
| Education, y                   |             |            |
| 0                              | 144 (25.2%) | 75 (30.0%) |
| 1 – 4                          | 79 (13.8%)  | 72 (28.7%) |
| 5 – 9                          | 305 (53.2%) | 89 (35.5%) |
| ≥ 10                           | 45 (7.8%)   | 15 (6.0%)  |
| **Child**                      |             |            |
| Sex, male                      | 311 (54.3%) | 117 (45.9%) |
| Gestational age at delivery, wks | 39.2 (2.7) | 40.0 (1.5) |
| **Anthropometry**              |             |            |
| LAZ Birth or 1 month           | -1.48 (1.08) | -0.89 (0.10) |
| 6 months                       | -1.37 (1.01) | -1.18 (1.12) |
| 18 or 24 months                | -2.16 (0.98) | -1.60 (1.05) |
| WLZ Birth or 1 month           | -0.84 (1.02) | 0.36 (1.10) |
| 6 months                       | -0.61 (0.99) | 0.35 (1.06) |
| 18 or 24 months                | -1.31 (0.91) | -0.13 (0.91) |
| WAZ Birth or 1 month           | -1.58 (0.96) | -0.30 (0.96) |
| 6 months                       | -1.37 (0.96) | -0.54 (1.13) |
| 18 or 24 months                | -2.08 (0.91) | -0.85 (0.97) |
| **Breastfeeding**              |             |            |
| 3 months of age                |             |            |
| Exclusive                      | 429 (76.2%) | 103 (53.9%) |
| Predominant                    | 65 (11.6%)  | 28 (14.7%) |
| Partial                        | 65 (11.4%)  | 60 (31.4%) |
| None                           | 4 (0.2%)    | 0 (0.0%)   |
| 6 months of age                |             |            |
| Exclusive                      | 68 (13.9%)  | 14 (6.9%)  |
| Predominant                    | 23 (4.7%)   | 14 (6.9%)  |
| Partial                        | 398 (81.1%) | 175 (86.2%) |
| None                           | 2 (0.4%)    | 0 (0.0%)   |
| **Household**                  |             |            |
| Electricity                    | 80 (14.0%)  | 20 (7.9%)  |
| Piped water access             | 1 (0.2%)    | 32 (12.6%) |
| Toilet facility                |             |            |
| None                           | 127 (22.2%) | 12 (4.7%)  |
| Pit latrine                    | 28 (4.9%)   | 207 (81.5%) |
| Ventilated pit latrine         | -           | 33 (13.0%) |
| Water sealed                   | 418 (72.9%) | -          |
| Flush                          | 0 (0.0%)    | 2 (0.8%)   |
| Food security                  |             |            |
| Food secure                    | 262 (53.4%) | 45 (17.9%) |
Abbreviations: IFA, iron-folic acid; LAZ, length-for-age Z-score; LNS, lipid-based nutritional supplement; MMN, multiple micronutrient; WAZ, weight-for-age Z-score; WLZ, weight-for-length Z-score

1 Mean (SD) or n (% of total).
2 In Malawi, n=4 missing educational attainment data.
3 Total number of respondents in Bangladesh is n=556 for gestational age at delivery, n=563 for LAZ at birth, n=482 for LAZ at 6 months, n=484 for LAZ at 24 months, n=480 for WLZ at birth, n=482 for WLZ at 6 months, n=483 for WLZ at 24 months, n=573 for WAZ at birth, n=486 for WAZ at 6 months, n=500 for WAZ at 24 months. In Malawi, n=255 for gestational age at delivery, n=238 for LAZ at birth, n=233 for LAZ at 6 months, n=248 for LAZ at 18 months, n=234 for WLZ at birth, n=233 for WLZ at 6 months, n=248 for WLZ at 18 months, n=239 for WAZ at birth, n=235 for WAZ at 6 months, n=248 for WAZ at 18 months.
4 Assessments at birth, 6, and 24 months in Bangladesh and 1, 6, and 18 months in Malawi.
5 Assessed in Bangladesh regarding feeding practices in the prior ~3-months; assessed in Malawi regarding feeding in past 24 hours. Total of n=563 respondents at 3 months and n=491 at 6 months in Bangladesh; n=191 at 3 months and n=203 at 6 months in Malawi.
6 In Malawi, n=1 missing toilet facility data.
7 Food insecure includes mild, moderate, or severe food insecurity. Assessed at 6 months postpartum in Bangladesh, n=491; assessed at maternal baseline enrollment in Malawi, n=251.
Table 2. Non-parametric partial correlation matrix for association of aflatoxin exposure between sample types among maternal-infant dyads from pregnancy through 24 or 18 months of age in Bangladesh and Malawi

| Bangladesh | Malawi |
|------------|--------|
| M-3TM      | M-3TM  |
| $r_s = 0.110$ | $r_s = 0.375$ |
| $p = 0.010$  | $p < 0.0001$ |
| $\tau = 0.075$ | $\tau = 0.263$ |
| n = 549     | n = 219 |
| M-3mo       | M-6mo  |
| $r_s = 0.131$ | $r_s = 0.293$ |
| $p = 0.002$  | $p < 0.0001$ |
| $\tau = 0.100$ | $\tau = 0.202$ |
| n = 549     | n = 224 |
| C-Cord     | C-6mo  |
| $r_s = 0.103$ | $r_s = 0.161$ |
| $p = 0.086$  | $p < 0.0001$ |
| $\tau = 0.090$ | $\tau = 0.111$ |
| n = 283     | n = 208 |
| C-24mo     | C-18mo |
| $r_s = 0.122$ | $r_s = 0.054$ |
| $p = 0.169$  | $p < 0.0001$ |
| $\tau = 0.104$ | $\tau = 0.037$ |
| n = 130     | n = 215 |
| M-1TM      | M-2TM  |
| M-3TM      | M-3TM  |
| M-3mo      | M-6mo  |
| C-Cord     | C-6mo  |
| M-1TM      | M-2TM  |
| M-3TM      | M-3TM  |
| M-3mo      | M-6mo  |
| C-Cord     | C-6mo  |

1 Data shown are the Spearman rho ($r_s$) or Kendall’s tau-b ($\tau$) partial correlation coefficients (adjusting for season of sample collection), p-value for the Spearman partial correlation ($p$), and the number of available samples for each comparison (n). As the partial Kendall distribution is unknown, p-values were not available for Kendall partial correlation analysis (40).
Table 3. Multiple regression of factors influencing aflatoxin B$_1$ exposure in 6-month-old Malawian children.

|                                                | Geometric mean ratio (95% CI) of AFB$_1$-lysine concentration | p-value |
|------------------------------------------------|---------------------------------------------------------------|---------|
| Exclusively breastfed at 3 months of age $^1$  | 0.42 (0.22, 0.82)                                             | 0.010   |
| Food insecure $^2$                              | 2.18 (1.16, 4.10)                                             | 0.016   |
| Mother primiparous $^3$                         | 0.52 (0.26, 1.03)                                             | 0.059   |
| Sample collected during dry season $^4$         | 0.56 (0.31, 1.01)                                             | 0.056   |

$^1$ vs. predominant, partial, or no breastfeeding at 3 months of age  
$^2$ Mild, moderate, or severe household food insecurity vs. food secure  
$^3$ vs. multiparous  
$^4$ vs. rainy season
**Figure 1.** Number and percentage of samples with detectable AFB$_1$-lysine adducts. A) Counts of samples with detectable and non-detectable AFB$_1$-lysine adducts. B) Percent of samples with detectable and non-detectable AFB$_1$-lysine adducts. M-1TM, mother at first trimester; M-2TM, mother at second trimester; M-3TM, mother at third trimester; M-3mo, mother at 3 months postpartum; M-6mo, mother at 6 months postpartum; C-Cord, child’s cord blood at delivery; C-6mo, child at 6 months old; C-18mo, child at 18 months old; C-24mo, child at 24 months old.
Figure 2. Distributions of AFB1-lysine adduct levels within Bangladeshi and Malawian mother-child dyads.

Box plots of AFB1-lysine adduct concentration with whiskers extended to 5th and 95th percentiles. Dotted red line indicates limit of detection (0.01 pg/µL); non-detectable values are imputed at 0.005 pg/µL. Horizontal bars indicate statistical significance in pairwise comparisons; ****p<0.0001 by Kruskal-Wallace test and Dwass-Steel, Critchlow-Fligner adjustment for multiple comparisons.
Figure 3. Subject-specific trajectories of AFB₁-lysine adduct levels within Bangladeshi and Malawian women through pregnancy and into the postpartum period. Data as spaghetti plots of AFB₁-lysine adduct concentration in (A) Bangladeshi and (B) Malawian women. Gray lines connect samples from a single individual. Dotted red lines indicates limit of detection (0.01 pg/µL); non-detectable values are imputed at 0.005 pg/µL.
Figure 4. Seasonality of AFB\textsubscript{1}-lysine adduct levels in Bangladesh and Malawi. A) Boxplot of AFB\textsubscript{1}-lysine adduct levels in Bangladeshi mothers, for samples collected during the winter, dry, or rainy seasons. Whiskers extend to the 5\textsuperscript{th} and 95\textsuperscript{th} percentiles. Dotted red line indicates limit of detection (0.01 pg/µL); non-detectable values are imputed at 0.005 pg/µL. B) AFB\textsubscript{1}-lysine adduct levels in Bangladeshi mothers, by date of sample collection. LOWESS curves were fit to data from each maternal sample type. C) Boxplot of AFB\textsubscript{1}-lysine adduct levels in Malawian mothers and children, for samples collected during either the rainy or dry seasons. Data are plotted as in A. D) AFB\textsubscript{1}-lysine adduct levels in Malawian mothers and children, by date of sample collection. LOWESS curves were fit to data from each sample type. Horizontal bars in boxplots indicate statistical
significance pairwise comparisons by Kruskal-Wallace test, using the Dwass, Steel, Critchlow-Fligner adjustment for multiple comparisons; **p<0.01, ****p<0.0001.
Figure 5. Rates of seasonal change in AFB$_1$-lysine internal dose in Bangladeshi women. Censored linear regression of AFB$_1$-lysine adduct concentration (log$_{10}$-transformed) vs. day of sample collection during the dry and rainy seasons (left) or winter season (right), across all maternal sample types (1TM, 3TM, 3mo) and years of sample collection (2008-2011). Blue shading indicates the timing of the rainy reason. Units of the independent variable was the number of elapsed days relative to February 15 in the dry and rainy seasons (242 days total) or relative to October 15 in the winter season (123 days total). Censored regression best-fit lines and equations are shown for dry+rainy and winter seasons separately. Both slopes are significantly different from 0 (p < 0.0001). Dotted red line indicates limit of detection (0.01 pg/µL); non-detectable values were imputed at 0.005 pg/µL. The daily proportion of samples with non-detectable AFB$_1$-lysine levels is shown in the band plots at the bottom of the graph; data represent LOWESS curves fit to daily values.