Early Life Exposure to Mycotoxins and Child Linear Growth in Nepal: Methods and Design of a Prospective Birth Cohort Study

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Abstract A growing body of mainly cross-sectional evidence suggests an association between mycotoxins, particularly aflatoxin exposure, and poor linear growth in children. We describe the design and methods of a rigorous longitudinal birth cohort study aimed to deepen our understanding of this hypothesized relationship and to validate dried blood spots as a less invasive, low-cost collection method for venous blood samples. The AflaCohort study was conducted in Banke district of Nepal from 2015 to 2019. A total of 1,675 pregnant women ages 16-49 were recruited from 17 Village Development Committees of the district. The research team collected maternal and child anthropometry data at birth and every 3 months from birth through the first year of life. Children were revisited at 18-22 months and 24-26 months of age. Questionnaires administered at the household level assessed risk factors for aflatoxin exposure and poor linear growth. One maternal venous blood sample was collected during gestation and child blood samples were collected at 3, 6, 12 and 18-22 months of age to assess concentrations of aflatoxin B_1 (AFB_1)-lysine adduct. One breast milk sample was collected from mothers when the child was 3 months of age to assess levels of aflatoxin M_1. Serum ochratoxin A and urinary levels of fumonisin B_1 and deoxynivalenol were measured at 18-22 months of age. Environmental enteric dysfunction was assessed using a lactulose:mannitol (L:M) test at 18-22 months of age. The study collected dried blood spots from a subset ($n \approx 1200$) of mothers and children to compare AFB_1 concentrations with those found in matching venous blood samples. Biomarker assessments were conducted using a high-performance liquid chromatograph method. Findings from the study will help identify certain factors that warrant interventions to reduce aflatoxin-related stunting in Nepal. This study was registered at ClinicalTrials.gov as NCT03312049.

Keywords: aflatoxins, children, dried blood spots, gestation, growth, Nepal, mycotoxins

1. Introduction

Stunting among children under 5 years of age is a complex condition with multiple underlying etiologies [1] and represents a persistent public health challenge. A research prioritization meeting on food-borne toxins, organized in 2012 by the International Food Policy Research Institute and the Bill & Melinda Gates Foundation, concluded that, "While there is solid association of stunting with exposure to mycotoxins, the causality has not been proven and the percentage of stunting attributable to mycotoxins in general or to specific mycotoxins is not known." [2] As noted during that meeting, typically only 35% of stunting in children can be attributed to known factors, underscoring the need to identify other potential contributors to poor linear growth.

This widely suspected aflatoxin-growth problem remains poorly understood, particularly in terms of nutritional implications and variability of the relationship across populations. Research has been limited on this topic in Asia, particularly in regions of Nepal like Banke, where the hot, humid climate is favorable to aflatoxin

production. A study examining aflatoxin contamination levels in food in Nepal [3] found 18% of samples to be heavily contaminated with aflatoxins particularly in the Far West region of the country, which also has some of the highest rates of stunting in Nepal (MoHP 2012). Thus, a better understanding of the association between maternal and/or early life mycotoxin exposure (levels in serum, urine and breast milk) and infant and young child growth is essential to more fully understand the extent to which mycotoxin exposure contributes towards stunting in Asia.

In this paper, we describe the purpose, methods and design of a USAID-funded longitudinal birth cohort study implemented in Banke, Nepal from 2015-2019. The AflaCohort Study aimed to ascertain the associations of the exposure to different mycotoxins at different time periods in early life, neonatal outcomes and subsequent linear growth and stunting within the context of specific socio-demographic, agricultural and health risk factors. Additionally, this study serves as a platform to validate data collection methods such as dried blood spots (DBS) as a less invasive, low-cost collection method in lieu of venous blood samples for the assessment of common mycotoxins such as aflatoxin.

2. Methods

2.1. Study Design and Setting

The AflaCohort Study was conducted in the Banke District of Nepal, a tropical region in the mid-west Terai region known for high agricultural productivity. The AflaCohort Study followed mother-child dyads longitudinally until the child reached two years of age. A prospective longitudinal birth cohort design is particularly useful for evaluating the relationship between risk factors and the development of certain health outcomes [4]. This design allowed us to identify presence, timing and chronicity of aflatoxin exposure in young children.

The study was conducted in two phases. The aim of Phase 1 was to assess maternal and infant aflatoxin exposure and its association with linear growth, using length-for-age (LAZ) *z*-score, up to one year of age. The primary exposure and outcomes were serum aflatoxin B1-lysine adduct levels, aflatoxin M1 in breast milk, and linear growth of children up to 12 months of age. Phase 2 was added on to follow the children up to two years of age. The aim of Phase II was to measure exposure to multiple mycotoxins and test associations between these mycotoxins and linear growth (LAZ) at 24-26 months of age. The second phase also provided the opportunity to test associations between exposure to various mycotoxins and environmental enteric dysfunction (EED) and to quantify aflatoxin levels in a variety of commonly consumed foods in a subset of AflaCohort households.

The specific aims of Phase I of the study were to: a) Examine the relationship of gestational aflatoxin exposure and birth outcomes, including infant birth weight, b) Examine the relationship of exposure to mycotoxin of infants through breast milk and their linear growth, c) Examine the relationship of exposure to mycotoxin through complementary feeding and linear growth, and d) Enumerate the relative contributions of maternal and infant aflatoxin exposure in impairing linear growth at one year of age. Phase II of the study was designed to: a) Enumerate the relative contributions of maternal and infant aflatoxin and other mycotoxin (OTA, DON and FB_1) exposure in impairing linear growth and cognitive development at two years of age and b) Assess the link between aflatoxin exposure, gut inflammation and poor linear growth.

The selection of Banke as a district is based on several criteria including nutritional characteristics, sufficient population to sample, and accessibility with respect to blood sampling and cold chain management. A total of 17 of the 46 eligible Village Development Committees (VDC) were selected for the study. Key selection criteria of the VDCs included sufficient population (>5000/VDC), proximity and accessibility to major roads and cities, ability to maintain a cold chain and transportation access. The 17 VDCs selected for the study include Basudevpur, Bageswari, Bankatawa, Belahari, Ganapur, Khaskarkando, Khajurakhurda, Kohalpur, Manikapur, Puraina, Puraini, Rajhena, Samsherganj, Sonpur, Tithiria, Udharapur and Udayapur (Figure 1). Most of the research was conducted before the 2017 restructuring of VDCs into rural municipalities, therefore Nepal's former administrative structure is used throughout the paper.

2.1.1. Study Timeline

Field activities commenced with the first pregnancy census in June 2015 and recruitment began in July of 2015. Due to political nation-wide strikes hampering data collection efforts, recruitment was put on hold from August-November 2015. Full field activities resumed with a second pregnancy census between November-December 2015. Recruitment was complete within an 8-month period (December 2015-July 2016). Data collection for all time points was finalized in March 2019.

Figure 1. Map of study sites

2.1.2. Power Calculation

The AflaCohort Study was powered to study the relationship between maternal aflatoxin (i.e. serum aflatoxin B1-lysine adducts) during pregnancy and rate of change in length and length-for-age z-score in the offspring. The sample size computation was at 80% power, 5% confidence, 1.5 design effect and an attrition/loss to follow up of 20%. Based on the reduction of -0.207 SD length-for-age (-0.409 to -0.006) for every one unit increase in log average serum aflatoxin B1-lysine adduct levels as reported by Turner et al. [5] this results in a sample size of 785 mother-infant dyads.

Furthermore, according to Turner et al. [5], mothers with aflatoxin levels higher than the median had children (at week 52 post birth) significantly shorter than mothers with aflatoxin levels lower than the median. Considering this, the need to compare women at a higher than the median versus those lower than the median (low aflatoxin exposure) led to a sample size of 1675 for a two group comparison.

2.1.3. Focus Groups and Pre-Testing of Quantitative Study Tools

Formative focus groups were conducted in 4 study sites in Banke (Bankatawa, Sonpur, Kohalpur and Manikapur) before pre-testing the birth cohort study questionnaires. Seven semi-structured focus groups (4 female, 3 male) were conducted in Nepali, Awadhi and Tharu. A total of 40 women and 34 men, aged 18 and up, representing a mix of ethnic groups (Brahmin, Muslim, Madhesi, Dalit, Magar, Janjati, Chhetri and Tharu) participated in the focus groups. All participants either were farmers or had some training in farming. Participants were asked to define food safety, factors influencing food safety in their home and community and perceptions and effects of mold/fungus infestations. Inductive methods were used to code and identify themes in the study transcripts. Knowledge generated during this qualitative study was used to refine the birth cohort study tools.

The research manager and a select group of supervisors pre-tested quantitative questionnaires in both paper and electronic format in a non-study VDC in Banke. Study team members were able to identify gaps and refine the tools. Pre-testing also helped the research team understand how respondents interpreted the questions.

2.1.4. Pregnancy Census

Before data collection commenced, locally hired and trained enumerators and field guides conducted a month-long pregnancy census to identify pregnant women in the 17 VDCs. Female Community Health Volunteers (FCHVs), whom already worked closely with mothers' groups, provided their most recent monthly mothers' group lists to help identify new and existing pregnancies and potential participants for the study. Women were approached for participation at their homes (accompanied by the FCHVs and local hired field guides) or during their health facility visits.

2.1.5. Eligibility, Consent and Recruitment

Women were eligible to participate if they were: 16-49 years old; less than 30 weeks of gestation (by the woman's estimate); had no plans to move out of the study area throughout the study period; planned to deliver in the study area; provided written informed consent herself or through a legal guardian; and had a singleton, live birth. Women were excluded if they were severely malnourished (mid-upper arm circumference (MUAC) <17.5 cm), had severe anemia (hemoglobin <7.0 g/dL), or had pregnancy-induced hypertension. Children were excluded if they were severely malnourished (defined as weight-for-length (WLZ) z-score ≤-3 at three months of age, MUAC <11.5 cm at 6-26 months, or bilateral pitting edema)), had severe anemia (hemoglobin <7.0 g/dL), were born with congenital anomalies, were born very low birth weight (<1500 g) or suffered from sepsis or respiratory distress syndrome.

Written informed consent was obtained from all participants. A trained data collector read and explained the consent form to the potential participant at the initial visit and the participant was given time to talk to her family before agreeing to participate. Consent forms were available in both Nepali and Awadhi. The participant was then asked to sign (or stamp with thumb prints if the participant was unable to read or write) the document if she agreed to participate. All eligible pregnant women providing informed consent themselves, of legal age (16 years old) and/or are married, or through a legal guardian were enrolled in the study.

2.1.6. Field Research Team, Training and Quality Control Procedures

Since loss to follow-up needs to be minimized and is always of concern in cohort studies, efforts were made to establish rapport and a close, trust-based relationship with the participant and her family. All field research team members were locally hired from the Banke district. Seventeen female enumerators, one assigned to each study site, administered surveys in Nepali or in Awadhi, depending on the participant's language preference. Also, locally hired field guides (one per VDC) worked closely with study enumerators and supervisors to facilitate and maintain open communication channels with participating families. Six supervisors and 4 nurses were each assigned 3-5 VDCs. Since loss to follow-up needs to be minimized and is always of concern in cohort studies, efforts were made to establish rapport and a close, trust-based relationship with the participant and her family. All field research team members were locally hired from the Banke district. Seventeen female enumerators, one assigned to each study site, administered surveys in Nepali or in Awadhi, depending on the participant's language preference. Also, locally hired field guides (one per VDC) worked closely with study enumerators and supervisors to facilitate and maintain open communication channels with participating families. Six supervisors and 4 nurses were each assigned 3-5 VDCs.

The Nutrition Innovation Lab's Tufts University/Helen Keller in-country team, with support from Patan Academy of Health Sciences research staff and faculty, conducted a month long training with the research team before the study commenced. The field research team was composed of a research manager, 6 supervisors, 17 enumerators, head nurse, 4 field nurses, 1 lab technician, 2 ultrasonologists, 17 field guides and 3 logistics/office support personnel.

The training was designed to help the research team understand the overall structure of the study, introduce the team to the complexities of the implementation of the study, the logistics and operations, the roles and responsibilities, study tools and equipment, biological/anthropometric measurements being collected and understand the basic research principles of ethics governing all studies involving human subjects. Refresher trainings were scheduled throughout the study period. The research manager, head nurse and supervisors conducted periodic refresher trainings on data entry, anthropometric and biological sample collection techniques.

Table 1. Anthropometric and developmental data collected during the AflaCohort Study

Parameter/metric	Data collector	Gestation/Age of child
Mother		
Survey	Enumerator	Gestation, birth, 3, 6, 9, 12, one year after the gestational visit, 18-22, 24-26 months
Weight	Enumerator	Gestation, 3, 9, 12 and 18-22 months
Height	Enumerator	Gestation
Mid-upper arm circumference	Enumerator	Gestation, 3, 9, 12 and 18-22 months
Serum sample	Nurse	Gestation
Dried blood spot	Nurse	Gestation (sub-sample)
Breast milk sample	Nurse	3 months
Child		
Weight	Enumerator	Birth, 3, 6, 9, 12, 18-22, 24-26 months
Length	Enumerator	Birth, 3, 6, 9, 12, 18-22, 24-26 months
Mid-upper arm circumference	Enumerator	3, 6, 9, 12, 18-22, 24-26 months
Head circumference	Enumerator	3, 6, 9, 12, 18-22, 24-26 months
Knee-heel length	Enumerator	3, 6, 9, 12, 18-22, 24-26 months
Serum sample	Nurse	3, 6, 12, 18-22 months
Dried blood spot	Nurse	3, 6, 12 months (sub-sample)
Urine sample	Nurse	18-22 months
Cognitive Function (ASQ)	Enumerator	18-22 months

ASQ: Ages and Stages Questionnaire (ASQ-3)

Six field supervisors and a head nurse performed daily crosschecking of electronic forms for consistency and completeness. Supervisors and enumerators regularly calibrated scales, height-length boards, and knemometers with standard weights (2 and 5 kg) and length rods (130 cm and 170 cm). Calibrations were done after every 50th mother/child was measured. Lost or damaged MUAC tapes were replaced. The field research team, including enumerators, supervisors and nurses, met on a weekly basis to report on progress, resolve questions, and plan follow-ups for the upcoming week.

2.1.7. Data Collection and Analyses

Women were first visited during gestation and again at birth. Women and their children were visited at predetermined intervals - i.e. 3, 6, 9,12, 18-22 and 24-26 months after delivery. During the visit, enumerators collected in-depth survey data on diet, health, vaccinations, household characteristics and agricultural practices (Table 1). Enumerators also assessed both maternal and child anthropometry. Enumerators revisited participants one year after the gestational visit to collect a second round of yearly agricultural data.

Enumerators visited participants to administer in-depth survey questionnaires and collect anthropometric data. Once survey data collection was completed, enumerators worked closely with field nurses to schedule a time with the participant for the biological sample collection, with the goal of minimizing the window between survey data and biological sample collection (maximum one week gap).

Questionnaires

All data were collected using handheld Android tablets on which an electronic, pre-coded data collection form was programmed. Enumerators administered time point specific questionnaires to the participants. Questionnaires were used to collect information on household characteristics, socio-economics and demographics, diet intake and diversity through gestation and post-partum, current pregnancy event and prenatal care, pregnancy outcome, sanitation and hygiene, diet intake of infant, breastfeeding frequency, duration and type of complementary feeding, immunization and morbidity status of the infant, food security and agricultural production. Enumerators were also trained to assess child development using an age-appropriate Ages and Stages Questionnaire (ASQ-3™) previously pilot tested in Nepal [6]. The ASQ-3 screens for developmental performance of children in the areas of communication, gross motor skills, fine motor skills, problem solving, and personal-social skills. With the exception of the 18-22-month visit, which took place at selected community sites (e.g. health centers, local halls, FCHVs homes), enumerators collected data face-to-face in the participant's home.

Anthropometry

In addition to the collection of survey data, enumerators measured maternal and infant anthropometry at all biological time points (i.e. gestation, birth, 3, 6, 9, 12, 18-22 and 24-26 months of age). Maternal weight and height were collected during the gestational visit and maternal weight measurements were repeated when the child was 3, 6, 9, 12, 18-22 and 24-26 months of age. Maternal height and weight were measured using extendable height-length ShorrBoard® Measuring Boards and Seca scales, respectively; while adult mid-upper arm circumference standard insertion tapes were used to measure circumference of the upper left arm.

Infant/child (tared) birth weight and supine length were collected within 72 hours of birth. Enumerators again collected child supine length and tared weight plus MUAC, head circumference and knee heel length measurements during follow-up visits at 3, 6, 9, 12, 18-22 and 24-26 months of age. Shorr Boards and a Seca scales were used to collect child length and weight, respectively. Shorr knemometers/knee length calipers were used to measure the distance between the child's knee and heel. Color-coded pediatric mid-upper arm circumference measuring tapes were used to measure circumference of the upper left arm. Pediatric circumference bands were used to measure child head circumference.

Height/length, weight, knee heel, head circumference and MUAC were measured to the nearest 0.1 cm or 0.1 kg. Measurements were collected in triplicate and means were used for analysis. Except for the 18-22-month visit which took place in selected community sites (e.g. health center, local halls, FCHVs homes), enumerators collected anthropometric data in the participant's home. Anthropometric indexes (weight-for-length, weight-for-age, length-for-age and head circumference-for-age Z-scores) were based on the WHO 2006 Child Growth Standards [7].

Clinical Measures and Biological Sample Collection

A team of 4 nurses, led by an additional head nurse, trained in adult and pediatric phlebotomy collected all biological samples in the participant's home, with the exception of the 18-22 month child urine samples and blood samples which were collected at designated community sites.

Gestational Age. Three methods for determining gestational age (GA) were used at enrollment. First, enumerators visited women in their homes and asked them to recall their last menstrual period (LMP). GA was then determined by estimating the number of weeks between her LMP and the enrollment date. Within a few days of the visit by the enumerator, nurses visited the women in their homes for symphysis-fundal height (SFH) measurement. Women were asked to empty their bladder and lie down in a supine position. A non-elastic measuring tape was used to measure SFH, which is the distance between the top of the mother's uterus and the top of her pubic symphysis. Fundal height in centimeters was then used to calculate GA in weeks. The third measurement for GA was an ultrasound scan performed by a physician from the Nepalgunj Medical College either in the woman's home or at a health clinic where women were scheduled to meet. Women were asked to lie down in a supine position. A portable, battery operated, real-time KX5600 full digital B mode ultrasound scanner (XUZHOU KAIXIN Electronic Instrument Co., LTD) was then used to measure biparietal diameter, gestational sac, femur length and crown-rump length.

Hemoglobin. First, hemoglobin tests were administered to mothers and children using a portable hemoglobinometer (HemoCue Hb 301; HemoCue, Inc. Brea, CA) during gestation, 3, 6, 9, 12 and 18-22 months of age. The

HemoCue was used following a standard protocol. In short, the nurse cleaned the puncture site (i.e. finger stick for pregnant women, heel stick for children who were not yet walking, or finger stick for children who were walking) with an alcohol swab, pierced with a sterile safety lancet and used the microcuvette to collect the blood. The HemoCue 301 analyzers determined the hemoglobin level in the samples. Any mother or infant found to have anemia was recorded as anemic and the individual was referred to the appropriate health facility or personnel for treatment. Neither blood draws nor dried blood spots were collected if women or infants were found to be either severely anemic (<7.0 g/dL) or malnourished (maternal MUAC <17.5 cm, ≤-3 WFH z-score at 3 months, <11.5 cm infant 6-12 months or edema).

Dried Blood Spot. Following the hemoglobin tests, a sub-sample of mothers (n≈300) and children (n≈900) were randomly selected for a DBS. Blood from the fingerprick was spotted onto Whatman ™ Qualitative Filter Paper and dried at room temperature using Whatman Dry Raks®. To randomize the selection of households for this part of the study, one in every 5 consenting households was selected for the DBS collection. For the gestational and 3 months visits, the first and sixth household in each VDC was chosen as the first DBS sample. This sampling design was repeated with the children at 6 and 12 months of age but starting with the second and seventh household at 6 months and the third and eighth household at 12 months. Child DBS samples were collected from each 5th participant in the VDC until 300 samples were collected for that each age group.

Venipuncture. Nurses collected one 3-5 ml maternal blood sample from pregnant women's antecubital veins. Nurses performed pediatric dorsal venipuncture to collect 1-3 mL of blood from the children at 3, 6, 12 and 18-22 months of age. Five mL BD Vacutainer® blood collection tubes and 21 gauge needles were used to collect blood samples from mothers; while 23 gauge butterfly needles were used to collect samples from the children. Blood samples were collected for aflatoxin B1, ochratoxin A, inflammatory marker and growth hormones testing.

Breast Milk. In addition to blood samples, 25-50 ml of breast milk were collected from mothers when the child reached 3 months of age for the purpose of testing for aflatoxin M_1 (AFM$_1$), a major metabolite of aflatoxin B1 frequently found in milk.

Urine. Environmental enteric dysfunction was measured using a urinary lactulose:mannitol (L:M) dual sugar absorption test. This noninvasive proxy marker is commonly used as an alternative to histopathological methods, such as small intestinal biopsies, used to diagnose EED [8]. At the AflaCohort laboratory in Kohalpur, the lab technician prepared batches of 1000 mL of lactulose:mannitol solution consisting of 625 mL of sterile distilled water, 375 mL of Lactulose (concentration of 10 g/15 mL; Lactulose Solution; Mckesson, San Francisco, CA) and 50 grams of mannitol (D-mannitol powder; Sigma-Aldrich, St. Louis, MO). Mothers and children were transported to the closest health facility for data collection.

Children fasted for at least two hours (with at least 1 hour of observed fasting) prior to and 30 minutes following the administration of the disaccharide solution.

A maximum of 20 mL was administered. Drinking water and breast milk were allowed ad libitum throughout the test after the 2-hour mark. At around ten minutes before the 4-hour mark, children were given a wholesome lunch. The urine was collected for a total of 5 hours. Children were encouraged to void prior to administration of the solution and were carefully monitored to ensure that the solution was not spilled, spit out, or vomited. If any of these events did occur, nurses attempted to reschedule for a different day.

A sterile adhesive pediatric urine collection bag (PDC Healthcare UR-Assure™ Pediatric Urine Collectors) was placed and changed as needed during a 5-hour collection period. Study nurses collected and measured urine volumes. Thimerosal (Sigma-Aldrich) was added to the urine collection containers to avoid bacterial growth. In the community sites where collection took place, nurses aliquoted 2 mL of urine for L:M testing, and 50 mL for DON and FB$_1$ testing. Leftover urine was aliquoted to serve as a back-up for mycotoxin or L:M testing. Samples were transported to the lab on ice to limit bacterial growth. Table 2 summarizes the timing of sample collection for biomarkers measuring mycotoxin exposure and environmental enteric dysfunction in women and children enrolled in the AflaCohort Study.

Table 2. Biological samples collected to measure mycotoxin exposure and environmental enteric dysfunction during the AflaCohort Study

	Sample	Gestation/Age of child
Aflatoxin B$_1$	Serum	Gestation, 3, 6, 12, 18-22 months
Aflatoxin M$_1$	Breast milk	3 months
Ochratoxin A	Serum	18-22 months
Fumonisin B$_1$	Urine	18-22 months
Deoxynivalenol	Urine	18-22 months
Lactulose:mannitol dual sugar absorption test	Urine	18-22 months

Sample Transportation, Storage and Lab Processing

Immediately after the biological samples were collected, coded and de-identified samples were placed in cool boxes filled with icepacks and a thermometer, and transported on scooters within 5 hours of sample collection to a central laboratory for processing and storage. Samples were delivered to a laboratory technician at the AflaCohort Laboratory located in the Nepalgunj Medical College Kohalpur Hospital. In the laboratory, the blood samples were allowed to clot for a half hour at room temperature and subsequently centrifuged at less than 5000 RPM for 10 minutes. The serum was divided into 3 aliquots and stored in a -20 ℃ (or lower) freezer in the Nepalgunj Medical College Hospital in Kohalpur. DBS samples were left out to dry at room temperature for several additional hours to ensure proper drying. Biological samples were air-shipped on a weekly basis to the PAHS for storage in a -80 ℃ freezer.

Biomarker Analyses

Mycotoxins. Serum, breast milk and urine samples were air-shipped on dry ice to the University of Georgia for mycotoxin analysis. Concentrations of mycotoxins in blood, breast milk and urine samples were analyzed using high-performance liquid chromatography (HPLC) according to methods previously described [9,10]. All

serum samples and DBS were tested for aflatoxin B_1-lysine adduct concentrations. Serum samples from the 18-22-month visit were tested for both aflatoxin B1 and ochratoxin A. Breast milk samples were tested for concentrations of aflatoxin M_1. Urine samples were tested for DON and fumonisin B1. For the DBS samples, an additional elution step was performed, where two 1cm-diameter spots were punched off blood cards for each sample and washed twice with 500 µL of 1x PBS to elute out the contents.

Serum samples. After deactivation in a 56 °C water bath for 30 minutes, approximately 150 µL of each serum sample, or 500 µL of each DBS eluants, were digested by pronase (pronase:total protein, 1:4, w:w) at 37 °C for 3 hours to release AFB_1-lys adducts. The digests were passed through and washed in a Waters mixed-mode strong anion-exchange (MAX) solid-phase extraction cartridge (SPE), eluted with 2% formic acid in methanol, vacuum-dried with a Labconco Centrivap concentrator (Kansas City, MO), and reconstituted with 25% methanol water for HPLC-fluorescence detection. An Agilent 1200 HPLC-fluorescence system (Santa Clara, CA) was used to quantify AFB_1-lys adduct concentrations. The mobile phases consisted of buffer A (20 mM $NH_4H_2PO_4$, pH 7.2) and buffer B (100% Methanol), running at a gradient to allow separation within 25 minutes of injection. The typical retention time for AFB_1-lys adduct was approximately 13 minutes. Separation was achieved using Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 x 250 mm) equipped with a guard column, maintained at 25 °C and a flow rate of 1 mL/min during analysis. Sample injection volume was 100 µL. Excitation and emission wavelengths for detection were 405 nm and 470 nm, respectively. Calibration curves of an authentic standard were generated weekly. Quality assurance and quality control procedures included simultaneous analysis of one authentic standard for every 10 samples, and two daily quality control samples. The limit of detection (LOD) is 0.4pg/mg albumin. The average recovery rate was 90% for the report. The AFB_1-lys adduct concentration was adjusted by albumin concentration, measured via UV/Visible spectrophotometry.

Serum OTA extraction was performed using an immunoaffinity column OchraTest (VICAM) in conjunction with the AFB_1-lys extraction. For this step, the sample digest was passed three times through an immunoaffinity column, and then drained directly into the pre-conditioned SPE cartridge that was used for the AFB_1-lys extraction to allow for OTA extraction of the same sample. The immunoaffinity column was then washed with 1mL of HPLC water, which was also collected into the SPE column. Extraction then followed the protocol as provided by the manufacturer. For OTA analysis, the same HPLC conditions and mobile phases were used as for AFB_1-lys concentration analysis, with the exception of excitation and emission wavelengths set to 333 and 477 nm. The gradient was also shortened to 10 minutes to allow the analyte to be eluted out earlier. Calibration was performed with an authentic standard and was evaluated weekly. The final concentration was obtained by adjusting with serum volume. The average recovery was approximately 85%. We did not test for an LOD, but all sample results were higher than the minimum concentration used for a standard curve, which was 2pg/injection or 0.02 ng/mL blood.

Breast milk samples. For the AFM_1 analysis, 10 mL breast milk samples were warmed to 37 °C, followed by centrifugation at 5000 rpm, and then to remove the fat content using a plastic spatula. The liquid portion was diluted 1:1 with 1x phosphate buffer (PBS) and loaded onto an immunoaffinity column (AflaM1 HPLC, VICAM), at a rate of about 1-2 drops per second. Extraction followed the manufacturer's protocol, to a final volume of contents reconstituted in 0.25mL of 25% methanol in water.

Reverse-phase ultra - high - performance liquid chromatography (UHPLC) with florescence detection method in a Dionex Ultimate 3000 system (ThermoFisher Scientific, West Palm Beach, FL) was used to determine the level of AFM_1 in breast milk extracts. Ten µL of each reconstituted sample was injected for LC analysis. Buffer A was 10% methanol in HPLC water while Buffer B was pure methanol. Separation was achieved via a gradient of mobile phase, starting from 95:5 A:B to 50:50 A:B in 6 minutes, then to 5:95 A:B in 4 minutes, maintenance for 5 minutes followed by re-conditioning to 95:5 A:B for subsequent runs. Gradient flow rate was 0.4 ml min^{-1}, and temperature was maintained at 40 °C. For detection, the excitation and emission wavelengths were set to 360 and 440 nm. The concentrations of AFM_1 in milk were estimated from an external standard calibration curve 0.04-10 ng mL^{-1} 25% methanol, prepared weekly from AFM_1 in chloroform (9.93 mg mL^{-1}).

Urine samples. For the FB1 analysis, 10 mL urine was centrifuged, and the supernatant was passed through an immunoaffinity column (FumoniTest HPLC, VICAM), cleaned-up following the manufacturer's protocol, then further extracted with an Oasis HLB SPE column. The final contents were eluted with 2 mL of 2% formic acid in methanol, dried under a gentle stream of nitrogen at 35 °C, and reconstituted with 200 µL of 25% methanol in water.

Quantification was performed using Agilent 1200 HPLC-fluorescence system (Santa Clara, CA). On-line derivatization of analytes with o-phthaldialdehyde (OPA) was performed via an injection program, which mixes 10 µL of the reagent with the sample for 1 minute prior to injection to allow fluorescent detection. The injection volume was 100uL. Separation was achieved using a C18 reverse phase column maintained at flow rate of 1.0 mL/min and temperature of 35 °C. Mobile consist of a gradient from 35:65 A (100mM NaH2PO4, pH 3.35):B (100% methanol) to 20:80 A:B to allow isolation of OPA derivatives of FB_1 within 13 minutes, to be monitored at excitation/emission wavelengths of 300/440 nm. The concentration was then estimated using an external calibration curve generated via injecting FB_1 standards of known concentration, and then adjusted by creatinine concentration, measured using creatinine kits (Cayman), to obtain the exposure parameters in pg/mg creatinine. The limit of detection is 20pg/injection (estimated to be approximately 4pg/mL urine), with an average recovery of 83.4%

The analysis of urinary excretion of DON will be performed according to previous published methods [11,12] with modifications. Briefly, 10 mL of urine

samples will be aliquoted and centrifuged to remove the pellets. The supernatants will be digested with beta-glucuronidase (Type IXA from *Escherichia coli*; Sigma-Aldrich) to release the conjugated DON. The digests will then be extracted using Multisep #227 column (Romer Labs), following manufacturers' protocol. The extracts will then be dried using a Labconco vacuum drier and reconstituted with 250 μL of 10% ethanol. A final volume of 100 μL will be injected to HPLC for analysis. Quantification will be made using an Agilent HPLC 1200 system coupled with a quaternary pump, an autosampler, a diode array UV/Vis detector, a fluorescent detector, and a thermostat column compartment which keeps the C18-reverse phase column (4.6 mm x 15 cm) at 20 °C. The isocratic mobile phase of 5% methanol will be used for chromatographic separation at the flow rate of 0.5mL/min. The peak of free DON will be detected at UV 220 nm wavelength around 7 minutes. A calibration curve with DON standard will be generated based on peak areas to quantitate concentration of DON. The final values will be adjusted by the urinary creatinine concentration. The LOD is 0.6 ng DON/mL urine with an average recovery of approximately 82.8%.

Inflammatory Markers and Growth Hormones. If additional funding is secured, a subset of blood samples (n=500 from the gestational, 3, 6, and 12 month visits) will be tested for inflammatory markers and growth hormones, specifically C-reactive protein, Alpha-glycoprotein, chemokines and cytokines (e.g. interleukin 6), insulin-like growth hormone factor 1 and insulin-like growth factor-binding protein 3. A concurrent assessment of inflammation markers and growth hormones will provide an opportunity to understand if there is a link between maternal and child aflatoxin exposure, inflammation status and poor linear growth. Serum samples will be sent to Tufts University for analysis and the aforementioned biomarkers will be measured using either HPLC or an enzyme-linked immunosorbent assay (ELISA) test.

Lactulose-Mannitol Ratio. A urine sample (2 mL) from 18-22 month-old children were air-shipped on dry ice to Baylor University in Houston, Texas for L:M ratio analysis. Lactulose-Mannitol concentrations were measured using high-performance liquid chromatography using previously described methods [13]. An assessment of EED will provide an opportunity to understand if there is a link between maternal and child aflatoxin exposure, gut inflammation and poor linear growth.

Data Management and Statistical Analyses

Questionnaires were programmed in electronic form using Open Data Kit (ODK) to enable administration of the tool using hand-held Android devices. Data were stored on the tablet and transferred daily to a secure central database either by wireless technology directly or by the transfer of SD storage cards to laptop computers with a wireless connection. The data manager, located in Kathmandu, downloaded and reviewed data from all time points for errors on a daily basis. Any data collection issues raised by the data manager were brought to the immediate attention of the research manager and supervisors. Any missing data required the enumerator to return to the household to re-obtain the data. Data from biological samples will be combined with the field electronic data to carry out the specific objectives of the AflaCohort Study.

Data analyses are led by Tufts University in close collaboration with local co-investigators. The World Health Organization (WHO) standards were used to calculate anthropometric z scores. Z score outliers (less than −6 or >5 for weight-for-age (WAZ), less than −5 or >5 for WLZ, and less than −6 or >6 for LAZ) are excluded from all analyses. Differences in growth in children relative to aflatoxin exposure in mothers and children across different time points and different sites will be examined using linear and logistical statistical procedures including (but not limited to) linear, logistical and quantile regression analysis, independent and paired sample t-tests, chi-square tests as well as generalized estimating equations (repeated measures test).

Ethics and Dissemination

The Nepal Health Research Council Ethical Review Board and the Tufts Health Sciences Institutional Review Board approved this study. The research team also obtained permission from District Public Health Officers before commencing research activities and provided updates throughout the study period. The individually identifiable private information for participants was stripped from datasets and destroyed. All datasets were de-identified using study ID numbers and are available only to study investigators.

Table 3. Sample sizes and data collection dates

Visit	Start date	End date	n	Child age (mean ±SD)
Gestation	5-Jul-15	17-Jul-16	1664	-
Birth Live births Measured within 72 hours of birth	24-Aug-15	8-Mar-17	1569 1490	0.06 ±0.15 0.04 ±0.03
3 months	22-Nov-15	7-Jun-17	1469	2.98 ±0.18
6 months	22-Feb-16	7-Sep-17	1428	5.96 ±0.19
9 months	29-May-16	3-Dec-17	1448	8.92 ±0.21
12 months	16-Aug-16	26-Feb-18	1440	12.02 ±0.20
18-22 months	24-May-18	05-Oct-18	736	21.28 ±1.03
24-26 months	04-Jun-18	15-Mar-19	1184	25.58 ±0.87

n:sample size; SD:standard deviation

Table 4. Mean and standard deviations for weight-for-age (WAZ), length-for-age (LAZ) and weight-for-length (WLZ) *z*-scores

Visit	n	WAZ	n	LAZ	n	WLZ
Birth [a]	1484	-1.01 ± 1.05	1483	-1.01 ± 1.05	1389	-0.78 ± 1.02
3 months	1464	-0.87 ± 1.16	1463	-0.87 ± 1.16	1462	-0.30 ± 1.17
6 months	1410	-0.90 ± 1.18	1425	-0.90 ± 1.18	1409	-0.36 ± 1.17
9 months	1403	-1.22 ± 1.15	1442	-1.22 ± 1.15	1404	-0.76 ± 1.14
12 months	1386	-1.44 ± 1.11	1422	-1.44 ± 1.11	1386	-0.98 ± 1.07
18-22 months	732	-1.63 ± 1.02	732	-1.63 ± 1.02	733	-1.03 ± 0.94
24-26 months	1177	-1.65 ± 1.03	1162	-1.65 ± 1.03	1176	-0.99 ± 0.94

LAZ: length-for-age; n:sample size; WAZ: weight-for-age; WLZ: weight-for-length
[a]Measured within 72 hours of birth

Descriptive findings will be reported back to the participants at the district/provincial level during local dissemination meetings a few months after the study is completed. Following the completion of data analysis the team will prepare research briefs and submit publications to peer-reviewed journals. The team will present the findings to the Government of Nepal, the academic community in Nepal, USAID Nepal and globally. It is anticipated that the findings will have important national and global resonance. If it is shown to be true that chronic exposure to aflatoxins in the food supply can impair birth outcomes and child growth (controlling for other potential factors), this will have a significant impact on the focus of nutrition, agriculture and food systems policies and programming.

3. Results

Data collection was completed in March of 2019. Data were collected from 86% and 70% of children at their 12- and 24-26-month visits, respectively.

Table 3 shows the start and end dates for data collection for each of the time points. It also shows participation rates in each of the visit and the mean age for the children for each visit. Child visits were scheduled with a ± 2 week window from the target age (e.g. ± 2 weeks from the child's first birthday). The age range for the children at the 18-22- and 24-26-month visit was wider than that of the visits during the first year of life. The smaller sample size seen in the 18-22-month visit is not a reflection of high drop-out rates. Rather, delays in commencing data collection for that follow-up visit resulted in many of the children aging out of the target age window. This delay in data collection also resulted in a modified design with a wider age range during these last two visits, attempting to maximize the window between the two visits while keeping the final visit as close to the two year mark as possible.

Table 4 shows mean and standard deviations for weight-for-age (WAZ), length-for-age (LAZ) and weight-for-length (WLZ). Results revealed decreases z-scores across the board as children got older. Mean length-for-age z-scores decreased from -0.77 ± 1.16 at three months to -1.75 ± 1.08 at 24-26 months. Mean weight-for-age and weight-for-length z-scores also decreased during that time, from -0.87 ± 1.16 to -1.65 ± 1.03 and -0.30 ± 1.17 to -0.99 ± 0.94, respectively.

4. Discussion

Here we described the design of a prospective birth cohort study in Banke, Nepal, which is the first longitudinal research study designed to empirically study the associations between aflatoxin and poor linear growth in an area of Nepal with high stunting rates. Our longitudinal birth cohort study recruited and repeatedly visited 1675 mother-infant dyads from gestation through 2 years of age (2015-2019). Empirical evidence to date showing an association between aflatoxin exposure and poor child growth has come mostly from observational studies [5,14] and recently from a cluster randomized control trial that indicated aflatoxin may affect linear growth at younger ages [15].

One of the main strengths of the AflaCohort Study is that it is a prospective birth cohort. Because aflatoxin is a known carcinogen, we chose an observational prospective birth cohort design as a robust alternative to a RCT to answer our research question. This longitudinal design offered a rare opportunity to observe a large group of pregnant women and their children during the first 1000 days of the child's life. Data were collected on a rolling basis during gestation and follow-ups were planned and conducted until the child turned one. The study was extended for a second year to allow follow-up throughout the first 1000 days of the child's life. This long-term follow-up allowed us to properly examine associations between our main toxin of interest, aflatoxin, and child growth. Furthermore, the rich complex dataset from this study allowed us to control for other aspects that affect growth and nutrition (e.g. genetics, inflammation, diet quality). This is also the first study to collect blood samples during gestation, at three months of age when most infants were still exclusively breast fed and three additional times as the children's diets gradually began to resemble adult diets. No studies, to our knowledge, have examined such a broad range of inflammatory markers, growth hormones or mycotoxins. This will allow us to understand co-exposure to multiple mycotoxins and be one of the first studies to examine the association between exposure to aflatoxin and inflammation patterns.

As with any complex study design, various challenges arose during the implementation of this research. Maintaining cold chain for serum, breast milk and urine samples was a challenge in our study site. Maximum temperatures in Banke can reach 40 ℃ or higher, making cold chain management even more challenging. During monsoon

season, road blockages made it difficult for study nurses to transport samples. Efforts, such as allowing for extra travel time, were made to maintain cold chain throughout transportation, processing and storage. Equipment was tested multiple times before launching the study and items were replaced with different brands if necessary. Some materials were not available locally and were imported from abroad, which added time and cost constraints to the study planning. Moreover, the biomarker analyses were costly. Samples had to be exported to the United States for analysis resulting in high transportation costs, particularly since it was a priority to ensure the integrity of the overall study linking biomarker levels with child anthropometry.

Lack of trust in research, especially due to the blood draw component of the study, was also an obstacle to research participation. Efforts had to be made to ensure community leaders and influential individuals understood the safety precautions and rigorous training undertaken by study personnel. Members of the study management team routinely met with community leaders such as teachers, community Maulanas (local Muslim scholar), community health volunteers, etc. to provide study information and answer any questions on the study protocol. Study team members also held open meetings for participants who had questions. Efforts were made to keep attrition as low as possible, by regularly sharing results of the study and having the same study personnel visit each household over time. However, because of the long duration of the study, loss to follow-up was inevitable. Data were collected from 86% and 70% of children at their 12 and 24-26-month visits, respectively. A 20% attrition was taken into account in the power calculations for the original study design (i.e. following-up until child's first birthday). Once funding for the second phase of the study was awarded, some of the children had aged out of the 18-22- and/or the 24-26-month visit. This resulted in Phase II visits having smaller sample sizes than the Phase I follow-up visits.

This study will be the first to document the cumulative effects of aflatoxin exposure and its association with child growth. Findings from the study will also provide information on alternative, less invasive aflatoxin testing methods as well as identify certain factors that warrant interventions to reduce aflatoxin-related stunting in Nepal. Multivariate data analyses are underway, and results will be submitted for publication in 2020.

Acknowledgements

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