Entebbe Mother and Baby Study

PROTOCOL AMENDMENT

Amendment title: Investigation of asthma, eczema and allergy among school-aged children in the Entebbe Mother and Baby Study

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Collaborating institutions in Uganda:

MRC/UVRI Uganda Research Unit on AIDS
Uganda Virus Research Institute
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Overseas collaborating institutions and collaborators:

University of Cambridge, UK	Prof. David Dunne	Expertise in immunology of helminths and assistance with assays for IgE responses to allergens
Leiden University Medical Centre, The Netherlands	Dr Marielle Haks	Development of the Reverse transcription Multiplex Ligation-dependent Probe Amplification (RT-MLPA) assay
London School of Hygiene & Tropical Medicine, UK	Dr Emily Webb	Expertise in statistics
	Prof. Neil Pearce	Expertise in asthma epidemiology
University of Nottingham, UK	Prof. Ian Hall	Expertise on asthma; training of study physicians in modern asthma management
	Prof. Hywel Williams	Expertise on eczema; training of study physicians in eczema diagnosis and management. Review of photographs for validation of eczema diagnosis

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SUMMARY

This protocol amendment will extend follow up of the Entebbe Mother and Baby Study, and will allow further investigation of asthma, eczema and atopy outcomes in children at school age (5 to 13 years). We will address the hypothesis that pre-natal and pre-school exposure to infections, particularly helminth infections, provides long-term protection against asthma, eczema and allergy, and that treatment of helminths during pregnancy and early childhood results in increased susceptibility to these conditions.

A summary of the additional procedures proposed is presented in Table 1, below.

Table 1. Summary of new activities to be conducted as part of the planned work

1	**General Activities**	
	Follow up:	Extended from 10 years to 15 years (or to the end of funding, if earlier)
	Annual routine visits:	Extended from 10 years to 15 years (or to the end of funding, if earlier)
	Home visit:	Home visit at age 9 years to determine location by GIS, and environmental exposures; GIS coordinates for schools in the study area at which study children attend will also be obtained.
2	**Nine-year-old assessment for asthma, eczema and atopy**	
	Consent and assent for special procedures	
	ISAAC-based questionnaire and examination	
	Lung function tests and Exercise Induced Bronchospasm testing	
	Skin prick testing for responses to common environmental allergens	
	Blood sample, 15 ml, for full blood count, *Mansonella perstans*, malaria, immunological assays, storage	
	Nasal lavage (asthmatics, and a sample of non-asthmatics)	
3	**Assays on stored samples**	
	Blood	
	Total and allergen-specific IgE :	Cord blood, 1 to 5 and 9 year old samples
	Hepatitis A serology:	1, 2, 3, 4 and 5 year old samples as required to determine age of infection
	PCR for malaria:	If funds permit, on samples obtained at age 9 years
	Stool	
	PCR for helminths and protozoa:	If funds permit, on stool samples obtained at 5 and 9 year old samples

BACKGROUND

The Entebbe Mother and Baby Study was designed to investigate the hypothesis that helminth infections modulate the host immune response both to themselves and to unrelated immunogens, pathogens and allergens.[1] The study, which was a randomised, double-blind, placebo-controlled trial of anthelminthic treatment during pregnancy and early childhood, has been completed.

The trial found no major effect of maternal helminths, or of anthelminthic treatment during pregnancy, on the primary outcomes of infant responses to vaccines, or on susceptibility to infectious diseases.[2] Among secondary outcomes, there was a possible benefit of albendazole for maternal anaemia among mothers with heavy hookworm infections, but there were none of the expected benefits for birth weight or perinatal mortality.[3] By contrast, observational analyses suggested a protective effect of maternal helminths against eczema in infancy, and there were significant adverse effects of treating pregnant women for helminths on eczema incidence in their offspring: albendazole treatment during pregnancy resulted in increased incidence of eczema in infancy across all participants, and praziquantel treatment during pregnancy resulted in increased incidence of eczema among infants of women with schistosomiasis.[4]

Previous studies had shown that anthelminthic treatment could influence atopy (defined by skin prick test positivity to common environmental allergens)[5-6] but this trial was the first to show an effect on an allergy-related disease outcome. Moreover, the result was hailed by the international allergy research community as showing, for the first time, that intervention during pregnancy can influence susceptibility to an allergy related condition in the offspring.[7]

Infantile eczema is a distressing condition, characterised by a recurrent itchy rash that may be dry and scaling, or wet and weeping. Secondary infection can sometimes have severe consequences. However, a much greater concern is the association between eczema and atopy in infancy, and subsequent onset of asthma. Asthma now affects about 300 million people globally,[8] and is the most common chronic respiratory condition in young people. If poorly managed, as is often the case in resource poor settings, asthma can be fatal.[9-10] Asthma cannot be diagnosed with any confidence in early childhood and commonly presents for the first time at age five and above – although early wheezing may persist into asthma, especially if associated with atopy in infancy.[11]

We now therefore wish to determine whether the effects that we have observed on eczema in infancy translate into a long-term impact of early-life exposures on susceptibility to eczema, asthma and atopy.

RATIONALE FOR THE PROPOSED ADDITIONAL WORK

Helminth infections are widespread in Uganda, but recent and on-going mass treatment programmes are resulting in a decline in their prevalence in some settings.[12] There is evidence to suggest that helminth infections may protect against allergy-related conditions, including asthma, eczema and atopy.

This means that mass treatment campaigns against helminths may result in an epidemic of such conditions in Uganda, which the country is currently ill-equipped to manage. Epidemiological data on the interactions between helminths and asthma, eczema and atopy will help the Ministry of Health to anticipate and plan for this changing disease dynamic.

Immunological investigations designed to understand the interaction between helminths and asthma, eczema and atopy may guide the development of new interventions against these conditions.

Tropical developing countries, such as Uganda, in which interventions against helminths are only now becoming established, provide opportunities to investigate these interactions in ways that are no longer possible in resource rich settings, and to provide information of both local and global importance.

HYPOTHESIS

Our hypothesis is that pre-natal and pre-school exposure to infections, particularly helminth infections, provides long-term protection against asthma, eczema and allergy, and that treatment of helminths during pregnancy and early childhood results in increased susceptibility to these conditions.

OBJECTIVES

Through long-term follow up of the Entebbe Mother and Baby Study cohort, within which we randomised treatment for helminth infections during pregnancy and in early childhood, we plan to address the following objectives

1. To describe the phenotypes of asthma and eczema in EMaBS
2. To describe risk-factors for asthma, eczema and atopy in EMaBS
3. To determine the effect of anthelminthic interventions *in utero* and in the first five years of life on asthma, eczema and atopy outcomes at school age.
4. To determine the effects of current and early-life exposure to helminths and their treatment on the expression of immunological pathways in response to allergens

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METHODOLOGY

Study design

The Entebbe Mother and Baby Study (EMaBS), to which participants were recruited between 2003 and 2005, was a trial of anthelminthic treatment during pregnancy and early childhood.[13] The trial interventions, both of which have been completed, were as follows:

> During the second or third trimester of pregnancy, expectant mothers were randomised to receive single-dose albendazole (400 mg) or matching placebo and single-dose praziquantel (40 mg/kg) or matching placebo in a 2x2 factorial design.
>
> From age 15 months to five years the offspring were randomised to receive quarterly albendazole or matching placebo. The dose was 200 mg while aged 15 to 21 months and 400 mg while aged two to five years.

The additional work proposed here will comprise an observational follow up of the trial cohort.

Study population

Women were recruited into the EMaBS at the Entebbe Hospital Antenatal Clinic if they were resident in the study area (Entebbe Municipality and the two neighbouring parishes of Katabi sub-county), planning to deliver in the hospital, willing to know their HIV status, and were in the second or third trimester of pregnancy. They were excluded if they had possible helminth-induced disease (haemoglobin <8.0 g/dL, clinically apparent severe liver disease, or diarrhoea with blood in stool) or a history of an adverse reaction to anthelmintics, if they had already been enrolled in the trial during an earlier pregnancy, or if the pregnancy was deemed abnormal by a midwife.

All the children of the pregnancies were eligible for inclusion into the study of anthelminthic treatment in early childhood when they reached 15 months of age.

Follow-up of all children to five years was completed in 2011. Between 2012 and 2016 children will be school-age: six to 13 years old. All EMaBS children still under follow up will be eligible for inclusion in this further study.

General data collection methods

In accordance with the already-approved follow up procedures for EMaBS children, clinical follow up will continue at the EMaBS study clinic.

> Routine follow up visits: EMaBS children are asked to attend for follow up visits once per year, at, or as soon as possible after, each birthday. Participating children who fail to attend are followed up by the field team.
>
> Informed consent: Informed consent for continued follow up from age five to 10 years has been obtained from parents or guardians of participating children at age five (or the first subsequent routine attendance) and assent is being obtained from children at their first routine visit aged eight or above, if they are capable of providing it. **For this new work** additional consent from the parent or guardian and assent from the child will be sought at age nine years for additional investigations to be undertaken at age nine years, and for follow up to age 15 years (or until the end of funding, whichever is earlier). This will allow all children to be followed up to the end of the current funding period in 2016, by which time some will be older than 13 years, as well as adequate time to arrange consent for further extended follow up should additional funding, beyond 2016, become available.
>
> If a child is unable to provide assent because they have not yet reached an adequate level of education and understanding, this will be documented on the assent form. The parent or

guardian's consent will continue to provide for the procedures for such children. Counselling of the children will continue and assent will be sought again at the next routine visit.

Procedures at routine follow up visits: Current approvals provide for the collection of clinical information and examination of stool samples for helminth infections at each routine annual visit to age 10 years. After collection of the stool sample, a single dose of albendazole (400 mg) is provided for all children. A transport refund is provided. Children found to have helminth species that cannot be treated effectively by single-dose albendazole are visited by the field team and provided with the correct treatment. **For this new work** the same procedures will be conducted at annual visits from age 11 to15 years (or to the end of funding, whichever is earlier) and additional investigations will be conducted at age nine years, as set out below.

Nine-year-old assessment: **For this new work** we will conduct a special assessment of asthma, eczema and atopy at the routine visit at age nine years. History and examination pertinent for these conditions will be obtained. A blood sample (15 mls) will be taken for haematological and parasitological examination and immunological studies, including evaluation of total immunoglobulin E (IgE) and allergen-specific immunoglobulin E (asIgE). Skin prick testing will be performed for common allergens, and lung function tests will be performed. Nasal lavage will be performed among children who have evidence of asthma and on a similar number of non-asthmatics for comparison. The non-asthmatics for nasal lavage will be recruited by selecting the next child of the same gender who attends for the nine-year-old assessment, and who is willing to undergo this particular procedure. All the procedures for the nine-year-old assessment are described in detail below. A home visit will be conducted to determine location of residence and to identify environmental risk factors. The location of the home, and of schools attended by study children, will be recording using Geographical Information Systems (GIS).

Stool parasitology: At routine visits aged six years and above stool samples are obtained for parasitology using the Kato Katz method,[14] and stool culture for *Strongyloides*.[15] The stool parasitology results will be used to guide treatment, as discussed above. **For this new work** a portion of stool will also be stored so that, if funds allow, helminth infection status can be investigated, as well, by multiplex real-time polymerase chain reaction. This assay is more sensitive than the Kato Katz method and will also allow us to investigate the presence of intestinal protozoal infections, notably *Giardia* and amoebae. PCR results will not be used to direct treatment, or given to the participants, as the assays are likely to be conducted long after the samples were collected. PCR analyses will be particularly useful for immunological substudies (described below) when smaller subgroups of participants are studied and misclassification of helminth status would be particularly critical.

Haematology and haemoparasitology. **For this new work** a portion of the blood sample (~2 mls) will be used for a full blood count, and will be examined for malaria by thick and thin film, and for *Mansonella perstans* infection using a modified Knott's method. Individuals found to have positive results for malaria will be treated according to government guidelines (currently with artemisinin-based combination therapy as first line), whether or not they are symptomatic. Plasma and cell pellet will be stored for future use, including, if funds allow, PCR assays for malaria which are more sensitive than microscopic examination, and can also allow easy identification of species other than *P. falciparum.* As in the case of stool parasitology, PCR results will not be used to direct treatment, or given to the participants, as the assays are likely to be conducted long after the samples were collected.

Illness visits: Between routine visits, participating children are encouraged to attend the clinic for any illness and free treatment is provided. Details of the illness are documented. If a condition requires investigation or treatment that is not available at the EMaBS clinic, appropriate referrals are made with the support of the clinical team. **For this new work**

doctor-diagnosed asthma and eczema will be particularly important and will continue to be recorded prospectively. Treatment provided to the participants will include provision for these particular conditions and study staff will receive further training in the diagnosis and management of these conditions as noted below.

Aim 1. To describe the phenotypes of asthma and eczema in EMaBS

To describe phenotypes of asthma and eczema within the EMaBS, we will use data collected at illness events, at annual visits, and at the nine-year-old asthma, eczema and atopy assessment.

For asthma and eczema, the most important descriptor of phenotype will be association with atopy. Associations between atopy and asthma or eczema have been reported to be weaker in low- than in high-income countries[16] and risk-factors for atopic and non-atopic phenotypes differ, suggesting differences in causation.[17] If helminths particularly regulate IgE-mediated inflammation, their elimination may result in an increase specifically in atopic phenotypes; if they have a broader effect on allergic inflammation, both phenotypes may increase.

SPT responses will be measured at nine years and allergen-specific IgE will be assessed in stored samples from early childhood (from cord blood up to five years) as well as in samples obtained at nine years.

Other important descriptors will be peripheral blood eosinophilia (and, for asthmatics, nasal lavage eosinophil counts), serum IgE, age of onset, frequency of exacerbations and response to treatment. Data collected during infancy and early childhood will be combined with results obtained at school-age and at the nine-year-old assessment, to provide information on the time-course components of the phenotypes.

For children who miss the nine-year-old assessment, the evaluation will be conducted at their first routine annual visit thereafter and the age of participants will be taken into account in the analyses.

Phenotypes will be defined based on the following investigations.

Reported wheeze and reported eczema will be ascertained by questionnaire based on the International Study on Allergy and Asthma in Children (ISAAC) format, with supplementary questions from the UK diagnostic criteria for atopic eczema.[18-19] Questions based on the ISAAC format have been used at earlier annual visits and this standard format will be maintained for comparability within the study, and with other studies that have been conducted worldwide. Questionnaires will be translated into Luganda after consultation with community members and families affected by asthma and eczema, and with unaffected families, as well as medical colleagues specialising in respiratory disease and skin conditions, to determine which words are best recognised to describe these conditions in our setting. The Luganda version will also be back-translated to verify how it is understood.[20-21] Participants will be interviewed using the English or Luganda version, according to their language preference, and the language of the tool used will be documented. Because experience from our study in primary schools suggests imperfect understanding of wheeze in this community we will also explore use of the ISAAC video questionnaire for asthma, which has been kindly provided by Dr Julian Crane, University of Otago, New Zealand. This will be piloted with the first 100 nine-year-olds to attend for routine visits. If responses are found to differ markedly when compared with the written questionnaire, and use of the video tool is found to be feasible, both methods will be continued for all nine-year-olds and the results will be analysed separately.

Visible flexural dermatitis. Visible flexural dermatitis is the classical physical sign of eczema. In school-age children eczema presents as a recurrent, itchy rash with poorly-defined erythema, and surface changes in the skin such as scaling, vesicles, oozing and crusting, or lichenification (resulting from scratching).[22] It typically occurs in the flexures which, for the standardised sign of visible flexural dermatitis are defined as around the

eyes, around the front or sides of the neck, at the front of the elbows or ankles, and behind the knees. Clinicians and fieldworkers will be trained in diagnosis of this sign[16, 23] using on-line resources (Prof. Hywel Williams, University of Nottingham, UK).[22] For validation, photographs (standard views, taken in baffled light) of cases (and of other rashes for comparison) will be reviewed by Professor Williams.

Doctor-diagnosed asthma and eczema will be established by physicians at either routine or illness visits by detailed history and examination, and exclusion of alternative diagnoses.[24] Because both asthma and eczema are still relatively uncommon in Uganda, and because their management in Uganda often involves the use of relatively out-dated methods (such as oral salbutamol rather than inhaled therapies) lead study physicians will receive additional training in asthma and eczema diagnosis and management with our collaborators at the University of Nottingham, an important centre for asthma and allergy research in the UK.

Atopy will be defined by skin prick testing and by assessment of asIgE as follows.

Skin prick tests (SPT) will be performed by standard methods using allergens which are likely to show a response in this environment,[25] or which have been widely used (for purposes of comparison) such as *Dermatophagoides pteronyssimus*, *Dermatophagoides farinae, Blomia tropicalis,* cat hair, *Alternaria tenuis*, mixed tree and grass pollen and *Periplaneta americana* (cockroach),[26] plus controls (ALK-Abelló). Study staff and participants are already trained in the procedure for SPT, and participants are familiar with it, based on experience in earlier EMaBS sub-studies which have examined responses in subgroups of participants. At age nine years we aim to study responses in all available participants.

Total IgE and antigen-specific IgE (specific to allergens such as *Dermatophagoides* and *Periplaneta americana*) will be measured using methods such as those described in our previous studies.[4] Cord blood and samples obtained at ages one to five, as well as nine, years will be assayed to determine both early and current atopic sensitisation.

Lung function. A spirometer will be used to measure forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1). In children with clinical evidence of asthma or bronchoconstriction, lung function tests will be performed before and after bronchodilation with nebulised salbutamol, in order to determine reversibility.

Exercise induced bronchospasm (EIB) will be assessed by exercise testing, based on free running[27-28] as in our pilot school study, among participants able to undertake this test. Ambient temperature and humidity influence the outcome of this test and will be recorded by thermohygrometer. Exercise induced bronchospasm will be defined based on standard criteria of decline in FEV_1.

Haematology. A full blood count will be performed, with attention to the assessment of eosinophilia.

Nasal lavage for eosinophil and neutrophil counts will be conducted among children with a diagnosis of asthma, and a similar number of non-asthmatics (as described above) in order to further assess proportions of asthmatics with an eosinophilic phenotype.[29] Approximately 10 ml of sterile saline will be instilled, 5 ml in each nostril, with the participant's head tilted backwards by about 30 degrees. The participant will be instructed to hold their breath and not to swallow during a Valsalva manoeuvre, and then to bend the head forwards and breath out to collect the nasal secretions. The fluid will be collected in a sterile container and then transported to the laboratory for processing by centrifugation and cytospin. The cellular fraction will be examined for eosinophils and neutrophils and the supernatant stored for analysis of cytokines and inflammatory mediators.[30]

This work will contribute, together with other studies in our portfolio of work on allergy in Uganda (shortly to be submitted to the committee) to the development of a comprehensive picture of asthma and eczema phenotypes in Uganda, a fundamental basis for understanding allergy in this tropical setting. In particular, assessments within EMaBS will allow us to describe how phenotypes progress from infancy to school-age. If patterns accord with those in Europe, we expect that children with persistent wheeze and atopy, or wheeze, eczema and atopy, will be particularly prone to progress to asthma.[11, 31-32]

Aim 2. To describe risk-factors for asthma, eczema and atopy in EMaBS

To determine risk factors for asthma, eczema and atopy in EMaBS we will conduct observational analyses regarding helminth exposures in utero and childhood, as well as a range of other early-life infections and exposures that may be anticipated to influence these outcomes based on studies in other settings. We hypothesise that prenatal and early-life exposures are critical to subsequent risk of asthma and eczema.

Outcomes:

Outcomes will be allergy events as defined in aim 1, categorised as follows:

Primary outcomes at nine-year-old assessment

reported wheeze in last 12 months
SPT positivity to any allergen
allergen-specific IgE to any allergen

Secondary outcomes

rate of doctor-diagnosed asthma aged five or above
rate of doctor-diagnosed eczema aged five or above
reported eczema in last 12 months at nine years
visible flexural eczema at nine years
FEV_1 at nine years
exercise-induced bronchospasm at nine years

Exposures:

We will use the EMaBS data archive, supplemented by information from serological tests and from questionnaires at age nine (Table 2) to investigate the role of pre-natal and early-life exposures on these asthma, eczema and allergy outcomes at school-age, in multivariate analyses.

Table 2. Entebbe Mother and Baby Study data archive and proposed additional investigations

Exposure	Status	Details
Infections: past and current	Data available for mother in pregnancy, at delivery, child annually	Intestinal helminths; *Mansonella perstans*; Malaria
	Prospective records for all children	Illnesses: malaria, diarrhoea, pneumonia
	Systematic investigation at age five	Mycobacterial infection, isoniazid prophylaxis
	Serology age 1-5, funded; to be done in 2011	Herpes simplex, cytomegalovirus
	Serology age 1-5, proposed	*Oro-faecal infections (Hepatitis A)*
Immunisations	Prospective records, all children	Prospective record of immunisations
Individual factors other than infections	Prospective records, all children	Birth order; birth weight; breast feeding; anthelminthic treatment; growth trajectory; day care & schooling
Maternal factors other than infections	Data available from enrolment during pregnancy	Tribe; *maternal grandparents' tribe*; history of allergy; height; smoking; pregnancy complications; occupation; socio-economic status

Paternal factors other than infections	*To be collected at age nine, if available*	*Tribe; and paternal grandparents' tribe; history of allergy; smoking; occupation; socioeconomic status*
Household environment	Data collected at birth or age five	Building and floor materials; crowding; exposure to domestic animals; cooking fuels; exposure to indoor cooking smoke; socio-economic status
	Data to be collected at age nine	*Exposure to farm animals; cigarette smoke*
Outdoor environment	Available	GIS Location of residence at birth
	Data collection in progress	GIS Location of residence at age five yrs
	To be collected at age nine	*GIS Location of residence and school at age nine yrs*
Atopy	Available for a subgroup of 270 infants	SPT responses age one year
	Available for a subgroup of 303 children	SPT responses age two years
	Available for a subgroup of 569 children	SPT responses age three years
	Preliminary data for subgroup of ~600 aged 1-3; propose to complete	*asIGE in cord blood and at age 3 and five years*

Most data to age five are already available. Those to be added as part of this **new work** are indicated in italics in the table and will be conducted as follows.

Exposure to oro-faecal infections. There is evidence that early exposure to oro-fecal infections may influence susceptibility to asthma, eczema and atopy, and this could confound associations with helminth infection.[33] Hepatitis A is a marker of such exposure. Serological assays on stored samples from our children aged one to five years will be used to assess age of infection with this pathogen. There are no treatment implications for seropositivity to this pathogen so results will not be provided to the participants.

Maternal and paternal factors. Many details of maternal characteristics are available within the EMaBS database. Few details are available regarding paternal factors. Thus additional data will be collected, when possible, on factors which may be pertinent including tribe, and parental history of allergy, parental smoking,[34] occupation and socioeconomic status.

Household and outdoor environment. Many details of household and external environment have been collected for EMaBS at enrolment during pregnancy and at age five years. Additional data will be collected on exposure to farm animals[35] and of location of residence (using Geographical Information Systems) at age nine years.

Atopy. As previously discussed, sensitisation to allergens at an early age is expected to be associated with later eczema and asthma, and assessment of asIgE will be performed in cord blood and at age three and five years to determine its association with later outcomes.

Sample size:

We expect that approximately 1000 children will be seen at age nine years with wheeze reported among 10-15%, SPT positivity and asIgE prevalence approximately 30%. Table 3 estimates power to detect an odds ratio of 1.5 or 2 for these outcomes, according to exposure prevalence.

Table 3. Estimated power to detect effects of exposures on outcomes at p=0.05, according to exposure prevalence and effect size

Outcome	Expected outcome prevalence	odds ratio 1.5 exposure prevalence in cases			odds ratio 2.0 exposure prevalence in cases		
		10%	25%	40%	10%	25%	40%
Wheeze	10%	29%	44%	49%	67%	88%	91%
Positive SPT response	30%	50%	77%	83%	93%	100%	100%
asIgE	30%	50%	77%	83%	93%	100%	100%

Analysis plan:

The main exposures of interest are exposure to helminth infections in utero and in early life. We will develop a conceptual framework describing anticipated relationships between these main exposures and other exposures that we have assessed. Based on these, we will conduct univariable and then multivariable analyses to assess the relationships between the main exposures, the potential confounders, and the primary and secondary outcomes. For helminths that were found to be effectively treated by the trial interventions (hookworm and *Ascaris* for albendazole, *Schistosoma mansoni* for praziquantel)[3] we will stratify this observational analysis by treatment arm. We will explore effect modification to address the hypothesis that risk factors differ between atopic and non-atopic phenotypes of asthma and eczema, although there will be limited power to detect such interaction effects.

These analyses will be conducted using logistic regression for binary outcomes and linear regression for continuous outcomes. For illness incidence rate outcomes we will use Cox regression and will employ methods appropriate for clustered data to allow for the occurrence of multiple illness events within individual children.

This is a unique opportunity to test the hypothesis that early-life helminth exposures protect against later asthma, eczema and atopy in a large, well-documented cohort. It is a study that would be impossible in affluent countries where helminths are no longer prevalent, illustrating the potential global significance of research conducted in Africa.

Added value:

Analysis of stool results from age one to nine years will also allow us to address the reverse hypothesis, that prior atopy is associated with a reduced incidence of helminth infection.

Aim 3. To determine the effect of anthelminthic interventions in utero and in the first five years of life on asthma, eczema and atopy outcomes at school-age.

Given the randomised interventions against helminths in EMaBS, and the effect identified on the incidence of eczema in early childhood,[4] the continued follow up of the cohort provides a unique opportunity to determine whether these prenatal and early life anthelminthic interventions also result in differences in asthma, eczema and atopy at school age.

The outcomes for this trial analysis will be the same as for the observational analysis described above:

Primary outcomes at age nine years

reported wheeze in last 12 months
SPT positivity to any allergen
allergen-specific IgE to any allergen

Secondary outcomes

rate of doctor-diagnosed asthma aged five or above
rate of doctor-diagnosed eczema aged five or above
reported eczema in last 12 months at nine years
visible flexural eczema at nine years
FEV_1 at nine years

Sample size:

We expect a sample size of approximately 1000 children seen at age nine years, so 500 per treatment arm for each randomised intervention. Assuming prevalence of 10%, 30% and 30% for the primary outcomes of wheeze, SPT positivity and asIgE, respectively, we will have 80% power to detect a difference between trial arms of 6% in the proportion of children with wheeze , and 80%

power to detect a difference of 9% in the proportion with positive SPT and in the proportion with positive asIgE, with p=0.05.

Analysis plan:

For each outcome we will first check for two- and three-way interactions between the three interventions. If, as has been the case to date, no interactions are observed, then the effect of each intervention will be analysed separately.

Exposures identified as having important associations with the outcomes will be examined to determine that they are balanced between the randomised treatment arms for each intervention. If this is not the case then factors showing imbalance will be adjusted for in the analysis.

We will first examine the effects of each intervention on each outcome for the whole study population. For the maternal interventions we will then conduct planned subgroup analyses to examine the effects of maternal albendazole among infants of mother who had hookworm (the commonest worm susceptible to albendazole) and to examine the effects of maternal praziquantel among the infants of mothers who had schistosomiasis.

As before, binary outcomes will be examined using logistic regression; continuous outcomes will be examined using linear regression; illness incidence rates will be examined using a method (such as Cox proportional hazards with robust standard errors), that allows for clustering of multiple events within individual children.

Aim 4. To determine the effects of prenatal exposure to helminths and their treatment on the expression of immunological pathways in response to allergens

Using blood samples obtained at the nine-year-old assessment, we aim to identify immunological pathways of response to allergens that are influenced by helminths, programmed in early life, and contribute to susceptibility to allergic disease. Samples will be used fresh, or stored, for use in these assays.

Dendritic cells are critical in responses to allergen.[36] Based on current evidence, we postulate that helminth co-infection influences dendritic cell responses,[37] and hence the relative expression of regulatory and pro-inflammatory pathways in response to allergen stimulation.

Our main aim will be to determine whether maternal helminth infections and their treatment result in persistent differences in immunological pathways at school-age. In addition, we will be able to investigate the effects of current helminth infection by comparing children with and without active helminth infection.

We will investigate gene expression and protein production by whole blood, peripheral blood mononuclear cells (PBMCs) or monocyte-derived dendritic cells (MDDC) with and without stimulation with allergens such as Dermatophagoides (Derp1) and cockroach allergens (Greer Laboratories). Initial assays among a pilot group of participants (and/or anonymous blood packs provided by the Nakasero blood bank) will allow us to optimise assays, which will then be used in selected subjects from among 1000 subjects expected at nine years of age. Optimisation will determine which preparation (whole blood, PBMC or MDDC), which allergen (Derp1, cockroach), and which panels of reagents will be used to investigate factors influencing the *in vitro* response to allergen in terms of gene expression, and protein production. Flow-cytometric studies in small numbers of participants will allow us to identify the cellular source of proteins of interest.

Cell culture: Whole blood will be stimulated, or PBMCs will be isolated and MDDCs prepared by adherence and culture in granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. Whole blood, PBMC or MDDC will be stimulated with allergens or left unstimulated for comparison. Supernatant will be harvested and stored; cells will be harvested and stored in an RNA

preservative. For flow cytometry, cells in additional wells will be exposed to Brefeldin A, then harvested and stored in liquid nitrogen for subsequent staining and analysis.

Gene expression: Molecular methods, such as microarray and reverse transcription Multiplex Ligation-dependent Probe Amplification (RT-MLPA) assay will be used to investigate the effects of helminths on gene expression within immunological pathways. The novel RT-MLPA assay allows simultaneous, quantitative determination of transcription of up to 60 loci. Fluorophore-tagged primer sets are designed such that the product size for each gene of interest is unique. The quantity of each PCR product is determined using a sequencer (Applied Biosystems) and GeneMapper software. The assay has relatively high throughput (96 well plates) and low cost and can be undertaken at the MRC Unit using the available sequencing facilities. Primer sets targeting innate and adaptive immune responses have been developed by our collaborators, Dr Marielle Haks and colleagues, at Leiden University Medical Centre. A scientist from our team will travel to Leiden to learn this technique so that it can be transferred to Uganda. The currently available primer sets will be modified, if necessary, to include genes relevant to allergy.

Luminex technology: Relevant gene products will be measured in supernatants using multiplex Luminex technology. A Luminex instrument is expected to be installed in Uganda within 2012.

Flow cytometry: The cellular source of products found to be of interest will be investigated by multi-parameter flow cytometry.

We anticipate that all assays will be conducted in Uganda. If this is not possible then relevant material transfer agreements will be submitted for approval by the committee.

Analysis plan and sample size:

To determine whether maternal helminth infections and their treatment result in persistent differences in the immunological pathways of response to allergens at school-age, the optimized assay will be used among the samples obtained at the nine-year-old assessment. Using the blood samples obtained from all children, a portion of whole blood will be used fresh, or PBMCs will be separated and used fresh, or stored in liquid nitrogen until required.

To investigate the hypothesis that prenatal exposure to helminths influences long-term programming of the immune response to allergens, and that maternal anthelminthic treatment modifies this effect, the following groups will be identified:

(A) mother had schistosomiasis, received praziquantel-placebo
(B) mother had schistosomiasis, received praziquantel
(C) mother had hookworm, received albendazole-placebo
(D) mother had hookworm, received albendazole

Because albendazole also had an adverse effect on infantile eczema among children of mothers without any helminths (Table 3) we will also include:

(E) mother had no helminths, received albendazole-placebo
(F) mother had no helminths, received albendazole

As far as possible, we will ensure that groups are balanced regarding other important immune modulating exposures (such as gender and malaria infection), for example by selecting samples for inclusion after stratifying for such exposures. Selected assays will be performed for allergen-specific responses with RT-MLPA and bead-array read-outs.

We anticipate that of 1000 nine-year-olds seen, 90 will fall into each of groups (A) and (B), 225 into groups (C) and (D) and 160 into groups (E) and(F). Results from the optimisation studies will be used to refine the design and sample size for experiments among the available participants.

Outcome:

Identifying immunological pathways of response to allergens that show specific and long-term modulation in response to helminth exposure will help to pinpoint targets for therapeutic intervention in children at risk of allergic disease.

ETHICAL CONSIDERATIONS

The intervention component of the EMaBS has been completed and no new intervention is proposed in this study. Further follow up of the children will have the benefit of determining any long term effects of the earlier interventions, and this is ethically important since the principal positive outcome of the trial – the increased incidence of infantile eczema among offspring of mothers who received anthelminthics during pregnancy – was an adverse one.

The participants will suffer the inconvenience and minor discomfort entailed by the study procedures, in particular the blood draws, skin prick testing, exercise testing and (for the small number of asthmatic subjects and the comparison group of non-asthmatic children) nasal lavage. All these procedures are regularly undertaken within epidemiological and clinical studies of asthma, eczema and atopy. The research team are experienced in all these procedures, except nasal lavage, and for this training will be arranged prior to the implementation of the procedure.

The skin prick tests will be carried out by an experienced nurse. They are not painful, but could be viewed as invasive. For that reason, we will obtain a separate consent signature for this procedure. However, our past experience has been that the skin tests are well tolerated and considered a "benefit" by study participants, as many people are keen to find the allergic status of themselves and their families.

At annual visits, participants are provided with a transport refund based on standard MRC Unit rates but tailored to their location of residence (some participants have now moved up-country but continue to attend for routine visits). The nine-year-old assessment will be reasonably time-consuming because it has several components, and physically demanding because of the exercise testing. Participants and their parent or guardian will be provided with a soft drink and an additional allowance of 5000 Ush to recognise the loss of time and possible need to purchase refreshments while waiting.

DATA ENTRY, MANAGEMENT AND QUALITY ASSURANCE

Data collected in the clinic or at field visits will be entered on standard clinical record forms (CRFs). The originals will be collated and batched at the clinic by the Study Clerk, and then sent to the MRC/UVRI statistics section for data entry. Carbon copies will remain in the participants' files at the clinic. Data entry will be overseen by a statistician / data manager at the MRC Unit.

Clinical laboratory results will also be recorded on standard CRFs. Sample identification data will be recorded on these forms at the time of sample collection, and results will be added at the laboratory. These forms will be collated at the MRC/UVRI clinical laboratories and forwarded to the statistics section for data entry.

Data will be double-entered and verified. To assure high quality data sets, the statistician / data manager will review entered data and generate queries at regular intervals which will be addressed by clinic or laboratory staff. Necessary corrections will be made.

Immunological data will be generated in the basic science laboratories and data entry will be conducted there. Some equipment (for example current ELISA reader equipment) can automatically transfer readings into the desired format, promoting data quality by avoiding the need

for numbers to be re-entered. This data will be merged by participant identification number with the clinical data. Again, appropriate queries will be generated for checks and quality assurance.

ANALYSIS PLAN

For each aim, the analysis plan has been set out above, under the relevant section.

POTENTIAL LIMITATIONS; ANTICIPATED PROBLEMS

EMaBS is one of the few birth cohorts that have been established in tropical Africa, and is unique in the randomised interventions against helminth infections that have been studied. It offers a unique opportunity to study the long-term effects these interventions, and of prenatal exposures, on disease outcomes.

In relation to allergy-related conditions, asthma is a long-term outcome of particular interest, which tends to present in children aged five and above. So far 12 EMaBS children have a definite diagnosis of asthma. We do not know how many more children will present with asthma over the forthcoming period, but the number may be small, limiting the power of the study for this outcome.

Some data of interest have not yet been collected – for example some paternal details, such as history of allergy – which may be important. It will only be possible to collect this information from children who are still under follow up (and even for some of those under follow up, information about their fathers may be lacking). This may result in considerable missing data for some variables of interest.

SIGNIFICANCE OF THE PROPOSED WORK

Almost no research has been conducted on asthma, eczema and atopy in Uganda, but it is likely that these conditions will become an increasing health problem over the forthcoming decades, as the country develops and lifestyles change. This work is part of a portfolio of projects on the interactions between infections and asthma, eczema and atopy that we hope to conduct over the forthcoming five years. The work will allow us to inform the Ministry of Health about trends in these conditions in Uganda, and about the likely impact of interventions against infections, particularly helminths.

The proposed immunological studies may help us to understand mechanisms by which helminths modulate susceptibility to asthma, eczema and atopy. The results may have global implications in terms of the design of interventions against these conditions.

Plans for dissemination

Our programme has strong links with the Vector Control Division at the Ministry of Health. For example, the head of the control programme for helminths and two district vector control officers recently attended our programme retreat in Jinja where the proposed new studies were discussed, and gave their input. This close contact enables us to disseminate our results to policy makers in Uganda.

Also, we have a long-standing tradition within the EMaBS of community and participant meetings, and will use these to inform the community of our findings.

The academic community will be informed through local and international scientific meetings and the publication of papers in relevant journals.

Time frame

EMaBS children are continuing under follow up. Participants will start to attain nine years of age in April 2012. The nine-year-old assessments will be undertaken as the children reach this age from 2012 to 2015. Immunological investigations will be conducted during the same period.

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APPENDICES

1. **Parent/ guardian information sheet, English**
2. **Consent form, English**
3. **Parent/ guardian information sheet, Luganda**
4. **Consent form, Luganda**

5. **Child information sheet, English**
6. **Assent form, English**
7. **Child information sheet, Luganda**
8. **Assent form, Luganda**