The impact of helminths on the response to immunisation and on susceptibility to infectious diseases in childhood in Uganda

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Protocol amendments:

1. Effect of praziquantel treatment during pregnancy on anti-schistosome immune responses in pregnant women and their infants. March 2003
2. Introduction of ART. March 2005
3. Extension of follow up beyond five years of age. March 2008.
4. TB infection in five and six year olds. January 2009
5. Schistosomiasis and immune responses in children aged five years who were exposed to schistosomiasis in utero. January 2009.

Hypothesis:

1. That maternal and childhood helminth infection reduce the effectiveness of childhood immunisations.
2. That helminth infection increases susceptibility to viral and bacterial infectious diseases.
3. That treatment of maternal and childhood helminth infection can improve the effectiveness of childhood immunisations and reduce the incidence of infectious diseases in childhood.

Specific Aim:

To examine the impact of helminths on the response to immunisation, and on susceptibility to infectious diseases in childhood, in a cohort of 2500 mothers and their children in Entebbe, Uganda. The study will have three randomised, placebo-controlled comparisons as well as observational components.

1. **To examine the effects of maternal infection with intestinal nematodes, and of the treatment of maternal nematode infection in pregnancy.** Mothers will be randomised to receive treatment with albendazole or placebo during the second to third trimester of pregnancy.

Mothers who are severely anaemic (haemoglobin $\leq$8g/dl) will be excluded from randomisation and treated with albendazole.

2. **To examine the effects of maternal schistosomiasis, and of the treatment of schistosomiasis in pregnancy.** Mothers will be independently randomised to receive treatment with praziquantel or placebo during the second to third trimester of pregnancy.

Mothers who have persistent diarrhoea with blood in the stools will be excluded from randomisation, investigated, and treated for schistosomiasis if present.

Thus the study will comprise four groups of mothers, according to treatment during pregnancy: (1) treated with both albendazole and praziquantel; (2) treated with only albendazole; (3) treated with only praziquantel; (4) no anti-helminthic treatment.

All mothers will have a second stool sample taken at delivery. All mothers will be mass treated with albendazole and praziquantel after delivery.

3. **To examine the effects of acquisition of intestinal nematodes during childhood and of regular mass treatment in childhood.** After one year of age, children will be randomised to receive albendazole or placebo every 3 months. All children will receive annual, selective treatment based on stool analysis.

4. **To examine the effects of childhood schistosomiasis.** This component will be observational. Children will receive annual, selective treatment based on stool analysis.

Co-infections other than helminths may also have immunomodulating effects. As an additional objective we will use stored samples from the cohort to examine the effects of herpes virus infections. Protocol amendment, January 2009. This component will be observational. Stored sera will be used to determine the age of first infection with Herpes simplex and cytomegalovirus. Particular attention will be paid to the relationship between these infections and the immune response following BCG, and the risk of TB infection.

The principal outcomes will be:
(1) immunological responses to childhood immunisations (BCG, tetanus and measles)
(2) immunological responses to HIV in children of HIV-1 infected mothers
(3) the incidence of infectious diseases in childhood (pneumonia, diarrhoea, measles, malaria, tuberculosis disease and tuberculosis infection (protocol amendment, January 2009) and vertical transmission of HIV infection).

Background

Infectious diseases are the most important cause of illness and premature death in Africa today. Pneumonia, diarrhoea, measles and malaria are the leading causes of death in young children.[1] Tuberculosis and HIV infection are the leading causes of death in adults.[2,3] In addition, African populations have a very high prevalence of helminth infection. Over 150 million are thought to be affected by schistosomiasis, and intestinal nematodes are even more widespread.[4,5] Most helminth infections can be treated easily, but they are often ignored, because the effects are unnoticed by the human host. This occurs partly because helminths have a profound effect on the host immune response, which allows parasites to persist for long periods, with relatively little damage to host tissues.[6] However, recent studies, including our own, indicate that the effect of helminths on the immune response extends to other pathogens, and may markedly impair protective responses, especially to intracellular pathogens, such as *Mycobacterium tuberculosis,* and to viral infections, including the Human Immunodeficiency Virus (HIV).[7,8] Similarly, helminth infection may impair the immune response to vaccine antigens. This is a particularly intriguing possibility in relation to Bacille Calmette-Guerin (BCG) immunisation, which has variable efficacy, correlated to distance from the equator and hence with the global distribution of helminth infections.[9,10] Thus helminths may influence susceptibility to other pathogens directly, or through their effect on vaccine-induced immunity, and treatment of helminth infections may resolve these effects.

A role for helminths in susceptibility to other pathogens is suggested by current understanding of the type 1/ type 2 dichotomy of the immune response. In essence, type 1 responses are characterised by production of gamma interferon IFN-γ, promote macrophage and cytotoxic T-cell activation, and contribute to protection against intracellular pathogens. In mice, type 1 responses also stimulate production of the opsonising and complement fixing sub-classes of antibodies required for protection against extracellular microbes, but in humans this function has not been proven. Type 2 responses are characterised by production of IL-4, IL-5, and IL-13, and of antibodies including immunoglobulin (Ig)E, and eosinophilia.[11,12] Mycobacterial and helminth antigens induce highly polarised type 1 and type 2 responses, respectively.[13] Other antigens elicit more balanced, mixed responses, and mixed responses also occur in the early phases of lymphocyte activation and in some subsets of memory T-cells.[14,15] The character of the immune response to a specific antigen may be influenced by the initial, non-specific cytokine response, or the pre-existing cytokine milieu. For example, type 1 responses can be initiated by IL-12 production from macrophages or by interferons released from somatic cells following viral infection.[16] Type 2 responses may be promoted by release of IL-4 from basophils or CD4+NK1+ T-cells.[17] Type 1 and type 2 responses are mutually antagonistic so that an initial type 1 or type 2 bias in the response is likely to be sustained.[18] Thus, by inducing a type 2 bias, helminths may impede development of protective type 1 responses to bacterial and viral pathogens.

In addition, helminths have evolved a broad array of mechanisms to evade host immune defences, several of which may modulate the immune response to other pathogens. Of particular interest in relation to T-cell mediated immune responses are effects which impair antigen presentation or macrophage activation. These functions may be inhibited by molecules, such as phosphocholine and protease inhibitors, secreted by helminths.[6] In addition, cytokines such as IL-10, induced by helminth infection, can inhibit macrophage activation and down-regulate expression of antigen presenting molecules and co-stimulatory molecules for both type 1 and type 2 T-cells.[19-23] Similarly, tissue growth factor (TGF)-β can inhibit macrophage killing of both intracellular and extracellular organisms.[23] Other mechanisms may influence the response to extracellular pathogens. Parasites secrete molecules which can degrade antibodies, interfere with complement activity, inhibit neutrophil chemotaxis and inhibit the oxidative activity of activated leukocytes.[6]

Studies in mice demonstrate that helminth infection can lead to down regulation of type 1 responses, or a switch to a type 2 response, to unrelated antigens. Prior infection with

Schistsoma mansoni reduces type 1 responses to unrelated antigens, and delays clearance of viral infection.[24,25] Prior exposure to filarial antigen induces a switch to a type 2 response to mycobacterial antigens.[26] Prior infection with *Fasciola hepatica* suppresses the type 1 response to *Bordetella pertussis*, and leads to more severe bacterial disease. Furthermore, infection with *Fasciola* after exposure to *B. pertussis* leads to suppression of an established type 1 response.[27] Thus helminth infection can influence both induction and effector components of the type 1 immune response, and increase susceptibility to bacterial and viral pathogens. Interestingly, the potency of the effect of helminth co-infection is further illustrated by studies which suggest that helminth infection ameliorates disease mediated by type 1 responses: co-infection with helminths reduces gastric atrophy induced by *Helicobacter felis*, despite the presence of high numbers of bacteria,[28] and protects against development of diabetes in genetically predisposed mice.[29]

There is evidence that helminth infection modulates responses to unrelated antigens, and influences susceptibility to infectious diseases in humans, also. Reduced responses to unrelated, delayed hypersensitivity, skin test antigens have been demonstrated in individuals with helminth infection.[30-33] Results with respect to cytokine production have been more variable. Some studies have shown a reduction in type 1 responses specific for parasite antigens.[34,35] Others, including our own, have shown suppression of IFN-γ responses to unrelated antigens, but not necessarily a switch to a type 2 profile.[8,36,37] More recently, we demonstrated marked suppression of IFN-γ responses to *M. tuberculosis* antigens following activation of parasite specific responses by treatment for *S. mansoni* in HIV-1 infected subjects, while IL-2 and IL-5 responses to *M. tuberculosis* antigens showed a transient rise (Appendix 1, *Figure 3*). Results may vary between studies for technical reasons, such as use of separated cells rather than whole blood assays, the latter being more likely to retain parasite antigens, or parasite induced lymphokines. Alternatively, in some studies there may have been differences in exposure to unrelated antigens between helminth infected and control subjects, or differences in the sequence of exposure to helminths and unrelated antigens. Studies of the effect of helminths on the outcome of immunisation are helpful in this respect, and several of these demonstrate impairment of cellular responses.[32,33,38-40] These immunological findings have led us to the central question of this proposal, which is whether helminths are also associated with increased susceptibility to bacterial and viral disease. Our data, so far, indicate that this may be so in HIV-1 infected subjects. In one study, eosinophilia, which may represent a type 2 bias or be a marker of helminth infection, was associated with a high risk of progression to active tuberculosis.[8] In another study, activation of the parasite specific response to *S. mansoni* after treatment was associated with a transient increase in HIV load (Appendix 1, *Figure 1*) and persistent helminth infection seemed to be associated with more rapid HIV progression (Appendix 1, *Figure 2*). On the other hand, helminths may also ameliorate disease in humans in some situations. Of particular interest for the proposed study is evidence that helminth infection may be associated with less severe malaria; if so, a detrimental effect of helminths on bacterial or viral infection may be offset by a reduced incidence of severe malaria.[41,42] Helminth infection may also be associated with reduced expression of atopic disease, perhaps because effector mechanisms are suppressed.[43]

Exposures in utero and in early neonatal life may have lifelong effects.[44] This has been illustrated for immunological responses by studies suggesting that exposure to viral and bacterial pathogens early in life leads to a lower risk of atopic disease.[45,46] Similarly, our own findings in HIV-1 infected adults in Uganda, supported by data from school children in Brazil, suggest that BCG immunisation may have a long term influence on susceptibility to helminths.[47,48] Both these observations suggest a potent effect of exposures which induce type 1 responses in infancy. Our hypothesis proposes that helminth infection has an equally important converse effect, suppressing type 1 responses in infancy. Helminth infection is rare in infants, but maternal helminth infection can sensitise neonates by exposure to helminth antigen, or to maternal anti-idiotype antibody, which may mimic antigen.[49-53] Thus it has been shown that children of mothers with microfiladermic onchocerciasis have increased type 2 responses to parasite antigens and reduced type 1 responses to

non-parasite antigens[54] and that children of mothers with schistosomiasis or filariasis have a reduced IFN-γ response following BCG immunisation.[55] These studies emphasise how maternal helminth infection may influence induction of immunity by vaccines given in the first weeks of life.

Together these findings show that helminth infection may have an important effect on susceptibility to infectious disease in the tropics. This possibility needs to be explored further at several levels. First, as discussed, type 1/ type 2 switching is unlikely to be the only mechanism of interaction between helminths and other pathogens. Second, few studies have examined the effects of intestinal nematodes, although these are more widely distributed than schistosomiasis and filarial infections; thus key findings need to be re-examined for intestinal nematodes. Third, the effect of treatment of helminths, and the time course of changes in immune responses to unrelated antigens, need to be explored. Finally, the immunological correlates of protective immunity against many pathogens, including *M. tuberculosis* and HIV, are still uncertain, and immunological studies alone fail to determine the effect of helminths on the actual efficacy of vaccines, and incidence of infectious disease. We now aim to investigate these issues in the studies described in this proposal.

Experimental Design and Methods:

Setting and facilities. The studies will be conducted at the Entebbe Hospitals. The surrounding community has a strong Local Council administration, with over 20 units in the Entebbe Municipality (including the part of Katabi which belongs in the municipality) and 50-60 if the adjacent area of Katabi is added. Laboratory investigations will be done at the Uganda Virus Research Institute (UVRI) where facilities for haematology, serology, bacteriology, parasitology and mycobacterial diagnosis, as well as cell culture, ELISA, PCR and flow cytometry are in place. The studies will build on our collaborations with the Medical Research Council Programme on AIDS, the Uganda National Tuberculosis Control Programme, the Uganda Vector Control Programme, Dr Dunne (Department of Pathology, University of Cambridge), Professor Gotch (Imperial College, London) and the Immunology and Tropical Epidemiology Groups, London School of Hygiene & Tropical Medicine.

Establishing the cohort.

The cohort will be recruited at the Entebbe General Hospital. If necessary, recruitment can be extended to the Private Wing also. Each year, 1200-1500 deliveries take place. About 70% of the mothers who deliver in the General Hospital live in Entebbe Municipality and Katabi, and over 90% of these attend the Hospitals' antenatal clinics before delivery (Appendix 2A). The proposed study profile is summarised in Appendix 3.

Procedures

Mothers: enrolment visit.

Women will be invited to participate in the study at their first antenatal visit if their normal residence is in the proposed study area (Entebbe Municipality; women from the additional, adjacent part of Katabi will be included later if necessary to maintain recruitment rates). Mothers who feel unable to decide to join the study at their first visit to the antenatal clinic, but who wish to do so at later visits, will be accepted provided that they accept all the study procedures, particularly the need for a blood draw on the day of screening.

They will be excluded if they have a history of adverse reactions to anti-helminthic drugs.

They will be excluded if, in the opinion of the doctor or midwife, the current pregnancy is not normal. Normal pregnancies would include twins. Abnormal pregnancies would include, for example, those in which the mother has gestational diabetes, pre-eclampsia or vaginal bleeding.

They will be excluded if they have already participated during an earlier pregnancy within the duration of the study. (Inclusion of more than one pregnancy and child for the same mother would complicate analysis, and the mother's behaviour with regard to antenatal care and use of anti-helminthics for herself and her child might be influenced by earlier participation in the study.)

They will be asked to give informed consent for participation in the study of albendazole in pregnancy, and for follow up of the babies to one year of age.
A questionnaire regarding demographic and socio-economic details, clinical history and examination will be completed.
They will have blood drawn for full blood count, malaria slide, examination for microfilariae, HIV and syphilis serology and studies of the maternal immune response (30 ml).
Urine will be examined for protein and glucose using a dipstick.
They will be asked to provide a stool sample.
They will be provided with haematinics and antimalarial prophylaxis (an initial dose of Fansidar).[57]
Women with positive syphilis serology (RPR and TPHA) will be treated.
Tetanus immunisation will be given.

In keeping with government policy, mothers will be encouraged to receive their HIV result and this study will be integrated with the Ministry's programme for Prevention of mother-to-child HIV transmission. For mothers who agree, pre- and post- HIV test counselling will be given and HIV serology will be performed using a rapid testing algorithm. HIV-positive mothers will be offered nevirapine[56] and advised on infant-feeding practices. Those wishing for specific follow up care will be referred to TASO Entebbe, to the Mildmay Centre, or to appropriate clinics elsewhere, according to their preference.

Protocol amendment, 14th March 2005. In view of the introduction of provision of antiretroviral therapy at Entebbe Hospital, mothers who are HIV-positive will be offered a repeat full blood count and CD4 T cell count to determine whether referral for treatment is indicated. Those who are treated will have full blood count and CD4 T cell count repeated every six months. Those for whom anti-retroviral treatment is not yet indicated will have repeat CD4 counts performed at intervals of six months to one year, to allow referral when necessary. Remaining material from the 5 ml sample will be stored for possible future virological studies.

Mothers: randomisation to albendazole or placebo and to praziquantel or placebo/ no praziquantel.
Women will be requested to return to the clinic with a stool sample.
On receipt of the stool sample (but before analysis of stool results) they will be randomised to receive albendazole (a single dose of 400 mg) or placebo and to praziquantel (a single dose of 40 mg/kg) or placebo/ no praziquantel.
If the first trimester is not yet complete administration of the study drugs will be delayed. The mother will be given an appointment to return during the second trimester to receive the drugs. It is expected that this will only be necessary in a minority of cases.
Women with severe anaemia (haemoglobin $\leq$8g/dl) will be excluded from randomisation and will be treated with albendazole, because of the known benefit of treatment of hookworm, if present. If indicated, additional measures will be taken for management of anaemia. Women with persistent diarrhoea with blood, which might be caused by schistosomiasis, will be excluded from randomisation and will be investigated, and treated for schistosomiasis if present.

Mothers: six weeks after enrolment. *Protocol amendment, March 2003. "Effect of praziquantel treatment during pregnancy on anti-schistosome immune responses in pregnant women and their infants."* All mothers will be requested to provide a second stool sample and a second blood sample (maximum 30 ml) to allow evaluation of the effects of treatment during pregnancy at a

consistent interval after treatment. This is particularly pertinent to the study of the effects of treating schistosomiasis.

Mothers: at delivery.
A second blood (20 ml), urine and stool sample will be obtained at delivery, before the mother leaves hospital. The blood samples will be used to perform a full blood count (for assessment of post-partum anaemia) a malaria slide and studies of the maternal immune response. The urine sample will be used as part of the assessment of pre-eclampsia and eclampsia. The stool sample will be used to determine the presence of persisting helminth infections, and to guide treatment in the case of *Strongyloides*. A placental smear will also be examined for malaria. Mothers found to have malaria will be treated.

After delivery, participants will be taken home if possible, to ensure follow up.

Mothers who receive the study drugs but do not deliver the baby in the hospital will be followed up at home to determine the outcome of the pregnancy.

Mothers: treatment of helminths post delivery.
All mothers will receive mass treatment with albendazole 400 mg and praziquantel 40 mg/kg; both drugs will be given as a single stat dose. Mothers found to have *Strongyloides* will be treated with albendazole 400 mg daily for three days. This treatment will be provided when the mother attends the follow up clinic with her baby six weeks after delivery, by which time both her stool results will be available.

Protocol amendment, March 2003. *"Effect of praziquantel treatment during pregnancy on anti-schistosome immune responses in pregnant women and their infants."* Mothers who have schistosomiasis will be asked to provide an additional blood sample (maximum volume 30 ml) before this post-delivery treatment, and a further follow-up stool and blood sample (maximum volume 30 ml) a further six weeks later. These samples will allow a comparison of both the immunological and the therapeutic effects of treating schistosomiasis in pregnancy, with the effects of treatment after delivery.

Children: at delivery.
For children, cord blood will be obtained at delivery.

Children: follow up visits.
A clinic at which mothers and children can be seen for follow up will be established in the renovated building at the Private Wing. Children will be seen there at scheduled follow up visits, or if they are sick.

Children will be seen routinely at their immunisation visits (6, 10 and 14 weeks) and then every three months up to the age of 18 months. This will allow their immunisations to be documented (at 6, 10 and 14 weeks, and then measles immunisation at 9 months) and their weight to be recorded. At first this will be done at the study clinic. Later it may be possible to devolve some of these visits to community clinics nearer to the mothers' homes. At one year mothers will be asked to give informed consent on behalf of the children for participation in the randomised, placebo controlled trial of three-monthly albendazole treatment. At 15 and 18 months they will receive their first two doses of albendazole or placebo. At 18 months blood samples will be obtained from children of HIV-positive mothers to ascertain the child's HIV status.

At routine annual follow up visits clinical data, blood for full blood count, malaria slide and immunological studies (2-5 ml) and stool samples will be obtained. Children found to have helminths in stool samples collected at annual visits will be treated by the field workers.

Protocol amendment, January 2009. At five years, 10 ml will be obtained to accommodate assessment of TB infection by T-spot.TB® assay and assessment of additional immune responses in children who were exposed to schistosomiasis in utero. At six years, 5 ml will be obtained for a T-spot.TB® assay.

Protocol amendment, January 2009. For more detailed assessment of schistosomiasis infection at the age of five years in children who were exposed to schistosomiasis in utero, three stool samples will be obtained from this subgroup of five year olds.

Children: interim disease events.

To obtain information about disease events, participants will be visited every two weeks by a field worker based in their Local Council area. Each field worker will be equipped with a bicycle, a thermometer and equipment to prepare malaria slides. The child's temperature will be recorded at each visit. Episodes of cough, diarrhoea or fever, and medication, immunisations or clinic visits in the preceding period, will be noted. Two malaria slides will be prepared for any child who is febrile ($\geq$37.5 ^{0}C). One will be brought by the mother to the clinic to be used for clinical purposes. The other will be kept by the field worker for later processing and analysis. Participants will be asked to attend the study clinic for any severe illness. Out of clinic hours they will be asked to attend the General Hospital.

Diagnosis of HIV infection.

- For mothers who wish to know their HIV status, two different rapid HIV tests will be performed in the antenatal clinic. Samples positive on both tests will be considered positive. Samples negative on both tests will be considered negative. Mothers will be informed accordingly. Samples with discrepant results will be sent to UVRI for further tests in Dr Biryahwaho's laboratory. These mothers will be given an appointment in 2 weeks to come for their results.
- For mothers who do not wish to receive their results, the same procedures will be followed but the rapid tests will be performed in our laboratory at UVRI.
- Quality assurance will be provided by the MRC serology laboratory. All of the first 100 samples (positive or negative), and all of the first 100 positive samples, tested in the programme will be tested in parallel. If the results show good agreement we will then send the first 10 samples each month, plus all samples which show discordant results on the two initial rapid tests, for quality assurance. These follow-up quality control samples will be submitted for analysis every quarter.

Diagnosis of syphilis.

- A rapid RPR test will be conducted in the clinic. Samples which are positive on RPR will be further examined using a rapid TPHA test. Mothers will be treated for syphilis if both the RPR and TPHA are positive. These mothers will also be asked to send their partners for treatment.
- Quality assurance for the RPR will be provided by the MRC serology laboratory. All of the first 100 samples tested in the programme will be tested in parallel. If the results show good agreement we will then send the first 10 samples each month for quality assurance. These follow-up quality control samples will be submitted for analysis every quarter.

Parasitology

- Stools will be examined by the Kato-Katz method for helminth ova,[5] and by concentration for larvae.

- Serum will be examined for *Schistosoma* circulating anodic antigen (CAA) in HIV-infected women and their children, in whom stool examination is insensitive.[58,59]
- Urine examination for *S. haematobium* will not be conducted as the prevalence is low in this area. None of 100 samples examined for S. haematobium in a previous study among HIV-positive adults was positive (Dr M. Brown, unpublished data).
- The prevalence of *Mansonella* infection has been found to be about 8% among HIV-positive adults in Entebbe using a modification of Knott's method.[92] However, none of 50 samples tested for *Wuchereria bancrofti* antigen were positive (ICT Filariasis, Amrad ICT, Frenchs Forest NSW, Australia) (Dr M. Brown, unpublished data). The modified Knott's method will therefore be used to examine samples of maternal blood for *Mansonella*.
- Thick blood films and placental smears will be examined for malaria.
- External quality assurance for the Kato-Katz method will be contributed by the Vector Control Programme. The laboratory subscribes to the American College of Pathologists for quality assurance for general parasitology.

Haematology

- Full blood counts and eosinophil counts will be performed in the MRC microbiology laboratory using an automated Coulter counter. When eosinophil counts cannot be performed using the automated method they will be done using a thin film stained with Leishman's stain, and calculated from a differential white cell count.
- CD4+ T cell counts will be performed using a FACScount instrument in the MRC microbiology laboratory.
- The laboratory subscribes to the British National External Quality Assessment Scheme for quality assurance for CD4+ T cell counts.

Special diagnostic procedures in children

Clinical diagnoses will be made for acute lower respiratory infections and diarrhoea, as described below (Outcome (3)); malaria will be defined using clinical criteria and parasite density. Special investigations will be used for measles, tuberculosis and HIV infection.

- *measles.* A serum sample will be obtained 4 weeks after an episode of clinical measles. This will be examined for measles antibody in order to confirm the diagnosis.
- *tuberculosis.* Samples for microscopy and culture for *Mycobacterium tuberculosis* will be obtained as clinically indicated. Where there is sufficient material a portion will be used for microscopy and a portion will be injected into BACTEC bottles and sent for culture at JCRC. Remaining material will be cultured on LJ medium at UVRI, and a portion will be stored for examination by Ligase Chain Reaction (LCR). Two blood samples (approximately 1 ml) will be obtained, one before treatment and the other after 4 weeks of treatment for examination of the whole blood assay response to the *M. tuberculosis* specific antigen, ESAT-6.
- *HIV infection.* Samples will be obtained from children of HIV-positive mothers for examination at 6 weeks (a filter-paper blood spot for DNA-PCR) and at 18 months (a filter-paper blood spot for DNA-PCR, a serum sample for HIV-antibody testing and a plasma sample for measurement of viral RNA.

Assessment of TB infection in 5-6 year olds. Protocol amendment, January 2009.

To determine prevalence and incidence of TB infection among five to six year old children in the cohort (approximately 830 children)

- **Among five and six year olds, questions will be asked regarding history of TB exposure, disease and treatment.**
- **T-spot.TB® assays will be performed for all five and six year olds.** These investigations will allow us to determine the prevalence of T-spot.TB®-positivity at age five and six years and,

hence, to estimate the prevalence of TB infection at age five, and the incidence of TB infection between age five and six years.

- **Tuberculin skin testing (TST) will be performed at age five years plus three weeks** among those who are contacts of a TB patient or T-spot.TB®-positive and among 50 T-spot.TB®-negative controls (expected number, 260 children). TST will be repeated at age six years in this group, excluding those with an initial TST>10mm. This will allow comparison of T-spot.TB® findings with the more conventional TST results.
- **Additional blood samples for repeat T-spot.TB® assays** (each 5 ml) will be requested at 5 years plus 3 weeks for all children with a history of household contact with a TB patient, or with a positive T-spot.TB® result (expected to be a total of about 210 children) and for the 50 T-spot.TB®-negative controls selected to receive a TST; and at 5 years plus 27 weeks for all children with a history of household contact with a TB patient, or with a positive T-spot.TB® result (approximately 175 children). This will allow the stability/ repeatability of the test result to be determined. In this group of children, additional peptide pools will be added to the assay to determine whether responses to antigens expressed by *Mycobacterium tuberculosis* can be detected if, or when, responses to the standard antigens are lost.

Management of children potentially at risk of TB (approximately 210 children) will be as follows (see also flow diagram, Appendix 12). Protocol amendment, January 2009.

- **All children with a history of contact with a TB patient or with a positive T-spot.TB® result will be investigated for active TB:** an X-ray will be performed and the child will be examined by a physician; any further investigations indicated (such as lymph node aspiration, gastric lavage) will be performed.
- **Children with a history of TB disease which has been/ is being treated, or of isoniazid prophylaxis following exposure to a TB patient** (expected number, <10): no additional treatment will be required.
- **Children with untreated active tuberculosis** (expected number, <5). These will be treated.
- **Healthy household contacts of TB patients** (expected number, ~111) who have never received isoniazid prophylaxis (whether T-spot.TB® positive or negative) will be treated with isoniazid prophylaxis for six months, in keeping with the National Tuberculosis Control Programme policy which recommends treatment of household contacts up to the age of five years.
- **Healthy HIV-positive T-spot.TB®-positive children** (expected number, <10). These will receive isoniazid prophylaxis.
- **T-spot.TB®-positive children with no history of exposure to TB patients** (expected number, ~74). This study will be among the first to identify healthy children with no history of exposure to a TB patient, but a positive T-spot.TB® response. The natural history of such a response (whether it is stable/ repeatable, and whether it will resolve spontaneously), the appropriate management, and the effect of isoniazid on such children, is unknown. Therefore children in this group will be randomised to receive isoniazid prophylaxis and monthly monitoring for 24 weeks or monthly monitoring only, and changes in the T-spot.TB® response will be investigated as indicated above. Children in the monitoring only group will be offered isoniazid prophylaxis from the age of six years if their T-spot.TB® response is still positive at that time.
- **Adherence to isoniazid will be monitored by monthly pill counts and urine tests for isoniazid metabolites** in all the treat children. Urine tests for isoniazid metabolites will also be performed in the T-spot.TB®-positive untreated group to detect any children who might be given isoniazid outside the study protocol.
- **All children who are household contacts of a TB patient or T-spot.TB® positive will be seen monthly** from age five to six years by a field worker for a simple health check and any that are sick will be referred to a physician for investigation and management.
- **Children who are household contacts of a TB patient or T-spot.TB®-positive, will have liver function tests performed before isoniazid is commenced.** If the results are abnormal, or

if isoniazid is contraindicated for any other reason (a history of convulsions or psychosis) they will not be treated but will be followed clinically for early detection of TB over the coming year.

To determine the effects of herpesvirus infections during early childhood on the ability of neonatal BCG immunisation to protect against TB infection.

Stored serum obtained annually from age one to five years will be examined using ELISA assays for IgG and IgM to Herpes simplex and cytomegalovirus in order to determine the age of first infection.

Interventions and exposures.

Aims 1 & 2. To examine the effects of maternal infection with intestinal nematodes or schistosomiasis, and of the treatment of maternal nematode infection or schistosomiasis in pregnancy. *Mothers will be randomised to receive albendazole or placebo and praziquantel or placebo during the second to third trimester of pregnancy.* A marked reduction in IFN-γ response after BCG immunisation has been demonstrated for children of mothers with schistosomiasis or filariasis[55] but the effects of intestinal nematodes, and the effects of treatment of helminths in pregnancy, including both intestinal nematodes and *Schistosoma sp*, are not known. We will determine the effect of intestinal nematodes by comparing children of mothers with and without intestinal nematodes during pregnancy (Appendix 4). If intestinal nematodes have an effect similar to schistosomiasis or filariasis, treatment of all or any of these groups of helminths in pregnancy may be beneficial for the response to immunisation in the neonate. This will be determined for individuals known to have had helminths before treatment, and for all participants (Appendix 4). If, however, treatment leads to an increase in type 2 bias, as has been documented in studies for schistosomiasis (Appendix 1, *Figure 3*), suppression of the neonatal type 1 response may be increased. For the same reason, treatment in pregnancy may have benefits, or disadvantages, for the neonate's susceptibility to bacterial or viral infections. For vertical transmission of HIV infection, treatment in pregnancy may have benefits for the immune response, or may enhance vertical transmission through a transient rise in viral load (Appendix 1, *Figure 1*). Thus, for our objectives, a randomised trial of treatment in pregnancy is appropriate. Other considerations are as follows. Treating hookworm in pregnancy can reduce anaemia,[61-63] so women with hookworm and severe anaemia will be excluded from randomisation and treated. Treating helminths may improve the mother's general health, and hence birth outcome (stillbirths, perinatal deaths, birthweight), but this has not been demonstrated in controlled trials; and there is a possible risk of teratogenicity, but this may be confined to the first trimester.[62] Therefore, although these outcomes are not the main issue for this study, they will be recorded and considered in an interim analysis.

Aim 3. To examine the effects of acquisition of intestinal nematodes during childhood and of regular mass treatment in childhood. *After one year of age, children will be randomised to receive albendazole or placebo every 3 months. Both groups will receive annual, selective treatment based on stool analysis.* Prior helminth infection is associated with impaired responses to immunisation,[38-40,55] but it is not certain whether acquisition of helminths leads to loss of an established response, or increased susceptibility to bacterial and viral infections, and whether this can be prevented by regular de-worming. This will be determined by comparing children who receive regular de-worming as well as annual, selective treatment with those who receive annual, selective treatment only (groups C1 vs C2, appendix 4). Prevention of helminth infections may have disadvantages, such as increased risk of severe malaria[41,42] and thus, for our objectives, a randomised trial is appropriate. Other considerations are as follows. Regular de-worming may have benefits for growth and cognitive performance in childhood, but a recent meta-analysis has shown that the evidence, particularly relating to cognitive performance, is still inconclusive.[64] Again, these outcomes are not the main issue for this study, but will be recorded.

Aim 4. To examine the effects of childhood schistosomiasis. This component will be observational because the incidence of schistosomiasis in children under 5 is not expected to be high enough to justify mass treatment. As in the case of intestinal nematodes, we wish to investigate whether acquisition of helminths leads to loss of an established response to immunisations, and influences susceptibility to other infections. For this observational component, important potential nutritional and environmental confounding factors for the effects of helminths are known to exist. These will be recorded and appropriate adjustments will be made.

Aim 5. To examine the effects of herpesvirus infections. Protocol amendment, January 2009. Since the start of our study, there has been new evidence that Herpesvirus infections early in life have a profound effect on the infant immune response. For example, in the Gambia, where 85% of children are infected with cytomegalovirus (CMV) by age one year, early CMV infection has been shown to alter maturation of the infant response, inducing a lymphocyte phenotype which suggests immune dysfunction in the elderly.[99] We will therefore use stored serum from our cohort to investigate the age of infection with herpesviruses (Herpes simplex and CMV) in our community and to determine whether these infections influence the ability of BCG to protect against TB infection

Principal Outcomes.

Outcome (1): immunological responses to childhood immunisations. By studying responses to BCG, tetanus and measles, we will investigate a broad range of immunological responses. BCG evokes a polarised type 1 response, cytotoxic and antibody responses.[65,66] Tetanus evokes an intermediate T-cell and antibody response which may demonstrate type 1 and type 2 switches more readily.[39] For measles, CD8+ T-cell responses can be measured without restimulation *in vitro*.[67]

We will use whole blood assays and antibody assays in all members of the cohort to evaluate the effect of helminths, and their treatment, on the development and maintenance of responses to BCG and tetanus immunisation. *Whole blood assays* can be used in large numbers of subjects and require small amounts of blood.[68,69] They measure responses biased towards CD4+ T-cell, or non-specific responses (monocyte, NK-cell, eosinophil or basophil).[70] Mycobacterial antigens, tetanus toxoid and the mitogen phytohaemagglutinin (PHA) will be used for stimulation. The mycobacterial antigens will be whole cell lysate or culture filtrate proteins of *M. tuberculosis*, which are homologous between *M. tuberculosis* and *M. bovis* BCG, and early secreted antigen of *M. tuberculosis* (ESAT-6), which is specific for *M. tuberculosis*, to distinguish responses to immunisation and *M. tuberculosis* infection.[71,72] We will measure the "type 1" cytokines, IFN-γ and IL-2. Our results suggest that discrepancies between these two responses may be of particular interest: in HIV-1 positive adults, IL-2, but not IFN-γ, responses were associated with increased risk of active tuberculosis;[8] and IL-2 responses increased, while IFN-γ responses declined, after treatment of schistosomiasis (Appendix 1, *Figure 3*). We will now determine whether helminth exposure has a differential effect on these two responses following immunisation. For "type 2" responses, IL-4 is seldom detectable in these assays in response to *M. tuberculosis* antigens, so we will measure IL-5 and IL-13 to examine whether helminths promote type 2 responses following immunisation. In mothers with schistosomiasis and their children, responses to vaccine antigens and to *Schistosoma* antigens will be compared, and we will also measure IL-10 and TGF-β responses, to examine whether helminth-induced immunosuppressive cytokines have a role in helminth associated effects. *Antibody responses* can also be studied in large numbers of subjects. Our existing assays for mycobacterial antibodies and their subclasses will be extended to tetanus. If helminths promote type 2 responses to unrelated antigens we expect a predominance of IgG4 and IgE subclasses following immunisation.

We will use ELIspot assays to evaluate the effect of helminths, and their treatment, on CD8+ T-cell responses following BCG and measles immunisation. Approximately 30% of Ugandans possess

HLA-A2.[73] Mothers and infants with HLA-A2 will be identified by flow cytometry using monoclonal antibodies, according to our current procedures (Appendix 5A), and confirmed by PCR-SSP with specific primers.[73] Selected participants will then be studied, initially at 3-6 weeks after BCG or measles immunisation, since the precursor frequency for vaccine-specific T-cells may decline after this time point.[67] ELIspot assays using selected HLA-A*0201 binding peptides from BCG and measles will be used. We, and others, have detected responses to HLA-A*0201 specific *M. tuberculosis* peptides by ELIspot for patients with tuberculosis (Appendix 5),[75-78] If a response can also be detected following immunisation, ELIspot assays will allow enumeration of IFN-γ and IL-4 producing CD8+ T-cells so that the effects of helminths on type 1/ type 2 responses by CD8+ T-cells can be examined. These assays will first be attempted in small numbers of subjects; the number required to obtain definitive results, and the best assays to use, will be determined by the preliminary results.

Outcome (2): immunological responses to HIV in children of HIV-1 infected mothers. Infants of HIV-infected mothers may be exposed to HIV in utero, at delivery or during breast-feeding.[79,80] The neonatal immune response may have a role in determining vertical transmission. We will estimate the timing of HIV infection by DNA-PCR on blood spots from cord blood, and samples obtained at 6 weeks and 18 months after delivery. We will examine the effect of maternal helminth infections, and their treatment, on immune responses in cord blood and in infants at one year of age, by including HIV p24 antigen in the whole blood assay procedure. More detailed studies will be done on selected participants, using ELIspots or intracellular cytokine staining to identify responses by cell type and cytokine production.[81] Cells will be stimulated with whole viral lysate, or with vaccinia constructs containing HIV genes to ensure class I presentation for CD8+ T cell responses. Potential confounding factors to be considered in these studies include possible associations between maternal helminth infection, or treatment of helminths, and maternal CD4+ T-cell count and viral load (Appendix 1), and the mother's choice of breast feeding practices.[80] The numbers of subjects in the categories of maternal helminth infection and treatment will be small (Appendix 2 B), but will be sufficient to detect effects of a similar magnitude to those in our preliminary studies.

Outcome (3): the incidence of infectious diseases in childhood. *The effect of maternal and childhood helminth infection on disease incidence and severity will be examined in the whole cohort, with helminth species analysed separately and together.* *Pneumonia (Acute lower respiratory tract infection, ALRI)* will be defined by World Health Organisation criteria, based on cough, respiratory rate and chest indrawing.[82] *Malaria* will be defined as fever with a parasite density above a cut-off set using standard methods.[83] *Diarrhoea* (≥3 loose stools per day, or the mother's definition) will be recorded as the principal disease event when there is no evidence of another cause.[84] *Measles* will be diagnosed clinically using standard criteria and confirmed by measurement of specific antibody.[85] Children with suspected *tuberculosis* will be investigated in detail, using gastric aspirates or material from other sites, as clinically indicated.[86] Microscopy will be performed using fluorescence microscopy, and cultures using BACTEC as well as Lowenstein Jensen medium, for greater sensitivity. Proposed algorithms[87] and new diagnostic tests, including the Ligase Chain Reaction (LCR), and whole blood culture with ESAT-6[71,72] will also be used and evaluated. *HIV infection* will be diagnosed by serology and polymerase chain reaction (PCR) in children aged 1-2 years.

Protocol amendment, January 2009. *TB infection* will be added as an outcome. T-spot.TB® assays will be performed for all five and six year olds. These investigations will allow us to determine the prevalence of T-spot.TB®-positivity at age five and six years and, hence, to estimate the prevalence of *TB infection* at age five, and the incidence of TB infection between age five and six years.

The immunological response to schistosomiasis in children exposed to schistosomiasis in utero. Protocol amendment, January 2009. There is increasing evidence that exposure to helminth

infections in utero may modulate the development of immune response to helminths in the offspring and thereby influence susceptibility to both infection and disease induced by the same species. Such effects may be altered by treatment during pregnancy. We will therefore investigate in particular detail the effects of exposure to schistosomiasis, and to praziquantel treatment in utero, on the prevalence of schistosomiasis in children at age five years, and on the regulatory immune response in these children. Furthermore, these effects could "spill over" to influence the development of responses to other antigens such as allergens. This amendment has been developed to examine these possibilities by

1) Determining the prevalence and intensity of S. mansoni among the children at 5 years and effect of the treatment during pregnancy on these parameters.

   Treatment of maternal schistosomiasis during pregnancy could affect the development of responses to schistosomiasis, and hence susceptibility to infection, in several ways. For example, increased *in-utero* sensitisation of the children might increase resistance to schistosomiasis infection and minimise disease morbidity. Reduced sensitisation or no sensitisation of the children might increase susceptibility of the children to infection and predispose to early and severe morbidity. Tolerance on the other hand, if it occurs, might be associated with increased susceptibility to infection as is the case in lymphatic filariasis.[100, 101] These possibilities will be examined by comparing the prevalence as well as intensity of *S. mansoni* infection between the children of women who were treated during pregnancy and those who were not, alongside children from uninfected women.

   The collection of three stool samples at age five years in the nested cohort of *S. mansoni*-exposed children will allow us to fulfil this objective by improving on the sensitivity of parasitological stool analysis for the purpose of identifying any children infected with S. mansoni at 5 years.[96]

2) Determining immune responses including regulatory T cell responses to schistosome antigens and allergen related antigens.

   There may be important differences in the immune profiles between children of women treated during pregnancy and children of women who were not treated during pregnancy. These effects will be examined by determination of schistosome antigen-specific type 1 (IFN_{γ}) and type 2 (IL-4, IL-5, IL-13) cytokine responses and antibody levels. Effects observed in the responses could be influenced by changes in regulatory immune responses. The regulatory immune responses will be examined by determination of schistosome antigen-specific Treg type 1 and Th3 cells as well as naturally occurring Tregs (nTreg) by intracellular cytokine staining (for IL-10, TGF_{β}, and FOXp3 respectively)[103,104] and cell phenotype analysis (for CD3, CD4, and CD25 markers) by flow cytometry.[105]

   In-utero sensitisation to *S. mansoni* antigens may also influence immune responses to allergens since *S. mansoni* infection has been shown to be associated with low reactivity to mite allergens.[106] Treatment of *S. mansoni* during pregnancy may have impact on responses to allergens among the children of the treated women. Indeed, recent data from Dr Harriet Mpairwe's work within this study suggests that treatment of *S. mansoni* during pregnancy is associated with an increase in allergic disease events in infancy. This effect will be examined by determining plasma levels of immunoglobulin (Ig) G and IgE against antigen extracts of dust mites *Blomia tropicalis* and *Dermaphagoides pteronyssinus*.

Sample size estimates

The cohort size of 2500 was proposed, based on clinical outcomes, for 80% power to detect statistically significant differences ($P<0.05$) between the children of mothers treated with albendazole or placebo during pregnancy; or for children randomised at one year of age to

albendazole vs placebo every three months. The change in design to include comparison of treatment with praziquantel or placebo during pregnancy has implications for the sample size required. The incidence of *ALRI* in children under five is expected to be above 10 per 100 person years (pyr);[88] *diarrhoea* over 100 per 100pyr;[89] *clinical malaria,* about 100 per 100 pyr.[90] For these common outcomes, and for effects which are expected to be large, such as the effect on cytokine production in the whole blood assay, the proposed cohort is likely to be large enough to accommodate the change in design.

Our initial objective was to establish a cohort that would be sufficiently large to allow analysis of the effects of helminths and their treatment of the incidence of tuberculosis. For this there are, at present, insufficient data to make accurate calculations regarding the sample size required. The incidence of *tuberculosis* in children under five in Entebbe is not known. In a well-documented community in South Africa the incidence in children aged 0-5 years was 3588/100,000 p.a.; 3.5 times the incidence in adults.[91] The estimated overall incidence in Uganda is 320/100,000 p.a.,[2] so a figure of 500/100,000 p.a. for children under 5 may be reasonable (Appendix 2C)). A study with 1170 children in each arm and median follow up of 3 years would detect a difference in incidence of 250/100,000 to 750/100,000 cases p.a. between two equal groups (e.g. children of mothers treated with albendazole vs children of mothers treated with placebo). This estimate of effect is large but, if helminths are crucial to the reduced effectiveness of BCG in tropical environments, a large effect is expected. On the other hand, the change in design to include an additional intervention means that the required sample size is likely to be even larger. We therefore propose to review the findings when the study has been running for approximately 18 months. Data regarding the impact of interventions in the mothers on incidence of infectious diseases in the first year of life and on immune responses to tuberculosis antigens one year after BCG immunisation will assist in further planning (appendix 9).

Protocol amendment, January 2009. For *TB infection* among five year olds, where the overall prevalence is expected to be about 15%, the study will have over 90% power, with $p<0.05$, to detect a difference of 10% vs 20% between either of the interventions in pregnancy, or between the intervention arms in childhood.

Follow up beyond five years of age. Protocol amendment. March 2008.

It is possible that the interventions described above may have long term effects on children's growth and development, including their susceptibility to worm infections in later life, and their susceptibility to infectious, allergic and metabolic diseases. We will therefore continue observational follow up of our cohort in order to continue to document long-term outcomes. Nothing is known about the long term effects of these interventions, although it is clear that events in pregnancy and early life can have long term effects on health and development. Our initial results suggest possible opposing effects on susceptibility to infections and on susceptibility to allergic disease. These results need to be confirmed and expanded by longer follow up, into the age group where conditions such as asthma become important. The results are likely to have important implications for public health planning.

The additional follow up will be observational. The trial de-worming intervention in children (three-monthly albendazole versus placebo, with annual treatment in both arms for any children found to have worms) is completed at age five years. The aim is to determine the long-term effects of treatment provided during pregnancy and during the first five years of life. During the additional follow up to age 10 years children are expected to receive de-worming at school and in the community, according to national policy and procedures. This will be documented at annual visits, in retrospect, in accordance with current study procedure. Children will continue to have stool samples examined annually and to be treated if worms are found.

Children will continue to be seen at the study clinic for illness events between routine visits.

Until age five years, children have been visited at home twice a month by local council field workers, to document any illnesses that have occurred in the preceding two weeks, in addition to those managed at the study clinic. From age five to 10 years this will be discontinued because children are expected to be ill infrequently, and may often be at school, so unavailable at home. Instead parents/ guardians will be provided with a booklet, an "illness diary", which they will be asked to use if it is necessary for the child to be treated at clinics other than the study clinic. This booklet will consist of carbonised forms such that one copy can be removed at subsequent clinic visits (for data entry) and the other retained by the parent/ guardian.

If funding is not renewed, we will inform participants of the end of the study at that time.

These studies will allow a detailed assessment of the impact of helminths on the response to immunisation and on infectious disease incidence. The results will have important practical applications for health policy in the tropics.

Ethical Considerations

The main ethical consideration for this study is the randomisation of treatment of helminths in pregnancy vs after delivery in mothers, and of mass treatment vs annual, selective treatment in children. Advantages and disadvantages of each intervention are outlined in the proposal. To our knowledge, the only proven benefit of helminth treatment in pregnancy is prevention of hookworm-associated anaemia, so mothers with severe anaemia and hookworm will be excluded from randomisation and treated. Otherwise, risks and benefits of the interventions are not certain and so a randomised design is proposed.

New considerations relating to the proposed change in design, ie to the randomisation of treatment with praziquantel vs placebo during pregnancy followed by selective treatment after delivery are as follows:

- WHO's "Informal Consultation on the use of praziquantel during pregnancy/ lactation, and albendazole/ mebendazole in children under 24 months" recommend that pregnant and breast feeding women should no longer be excluded from treatment with praziquantel, but should be treated in mass treatment programmes or in cases where schistosomiasis is specifically diagnosed.[99] In their report they note that studies of reproductive toxicity in animals showed no harmful effects and that use in breeding animals and in lactating cows is allowed. They also report that several pregnant women have been treated with high doses of praziquantel for cysticercosis without adverse effect and that no reports of adverse outcomes have been made despite the probable, inadvertent treatment of large numbers of pregnant women in mass treatment campaigns.
- However, there is still very little definite information on the use of praziquantel in pregnancy. As far as we know, there is no information on the effect of treating schistosomiasis in pregnancy on the issues addressed in our study, i.e. on the effect on the baby's response to immunisation; on the baby's immune response to other pathogens; or, indeed, on the baby's immune response to schistosomiasis. Many adults with chronic schistosomiasis have minimal symptoms. Treatment might improve the general health of the mother and hence the outcome at birth for both mother and baby, but this has not been shown to be the case. As set out in the study proposal, maternal schistosomiasis has been shown to be associated with a reduced IFN-γ response and increased IL-5 response to mycobacterial antigens in the baby one year after BCG immunisation.[55] It is thought that this profile of immune response is likely to be associated with poor protection against tuberculosis. Treatment of the mother in pregnancy might be beneficial to the baby if it reduces this effect. However, treatment is likely to cause a boost in the maternal bias to a type 2 immune response, as helminth antigens are released, and hence might actually increase the effect (Appendix 1). Almost nothing is known about the effects of

maternal schistosomiasis and its treatment on other immunisations and infections, although another potential disadvantage of treatment of schistosomiasis in pregnancy is a possible increase in viral load in HIV-positive mothers (Appendix 1). This would be particularly undesirable if associated with an increase in intrauterine HIV transmission. Thus we believe that an equipoise exists in the evidence regarding the relative benefits of treatment of schistosomiasis during pregnancy vs after delivery.

- The delay in treating mothers randomised to treatment after delivery would be, at most, just over 7 months, since mothers would not be treated in the first trimester in any case. There appears to be no evidence that progression of disease caused by schistosomiasis is any faster in pregnant women than in other adults. Persistent diarrhoea with blood is considered to be the pathological effect of chronic schistosomiasis most likely to show an immediate response to treatment, so we have planned to exclude women with these symptoms from the study, investigate them, and treat them if schistosomiasis is found. Otherwise, chronic schistosomiasis, including hepatosplenic schistosomiasis, is frequently asymptomatic, or the symptoms are non-specific and often ignored.[94] The severe sequelae of long-term, heavy infection, such as portal hypertension and oesophageal varices, are unlikely to be much affected by a delay in treatment of, at most, just over 6 months. Therefore, as far as we know, the delay in treatment in some mothers is unlikely to have serious adverse consequences for their health.
- The prevalence of schistosomiasis in the pilot study (>20%) would justify a mass treatment approach if treatment in pregnancy were found to be specifically beneficial to the mother or child. In addition, examination of the single stool sample obtained may detect only about 60% of infected mothers.[95,96] Therefore we have proposed to study a mass treatment intervention in pregnancy rather than to use selective treatment.

Advice has been given by colleagues at the Ministry of Health, and Uganda Virus Research Institute (UVRI).

Protocol amendment, January 2009. The following additional ethical considerations relate to the introduction T-spot.TB® testing for TB infection among five and six year olds, and to the use of stored samples for investigation of herpesviruse infections.

- Additional burden of blood draws for cohort children. The increased volume at age five will cause no additional discomfort and 10 ml blood loss can be well tolerated by children of this age. The blood draw at six years is unlikely to cause undue distress as the children are accustomed to annual blood draws. However, additional blood draws at 5 years plus 3 weeks and 5 years plus 27 weeks may be distressing. We therefore plan to introduce the use of anaesthetic cream, as currently used by our colleagues in the ARROW study. Children will not be coerced to provide samples if they are reluctant.
- Tuberculin skin testing causes some discomfort but has been widely used in clinical practice and in population surveys, and is usually tolerated. The required reading after 48-72 hours will cause some inconvenience for the parent or guardian. Transport re-imbursement will be provided and, for those who prefer, or are unable to return on the required day, tests will be read at home.
- This study will provide benefit to the participants through meticulous care of children at risk of developing tuberculosis. Isoniazid prophylaxis is likely to be beneficial among TB contacts but occasionally causes adverse effects, such as hepatitis. This risk will be minimised by initial liver function tests and by monthly clinical monitoring. Among the T-spot.TB®-positive group, where best practice is unknown, close monitoring will ensure that illness is detected early. The data monitoring committee will be asked to consider this component of the work in their review of serious adverse events.
- Testing of stored samples for herpesvirus infections. The original information sheet stated that "blood samples will be used for tests for anaemia, malaria and other infections including HIV"

and consent forms included the statement "I understand that part of the specimen may also be stored for other tests in future." There are no clinical management implications of the results of these tests.

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APPENDIX 1. Preliminary analysis of a study of the impact of helminths on HIV infection.

A study was conducted to examine the hypothesis that helminth infection might be associated with impaired type 1 immune responses and increased rates of viral replication and disease progression in HIV infection. Associations between helminth infection and CD4+ T-cell count and viral load were examined. Type 1 (IFN-γ, IL-2) and type 2 (IL-5) cytokine responses were examined for a helminth antigen from *Schistosoma mansoni*, an unrelated, *Mycobacterium tuberculosis* antigen and a mitogen (phytohaemagglutinin (PHA)).

Methods. Consecutive HIV-1-positive members of the existing adult cohort at the Uganda Virus Research Institute and the AIDS Support Organisation, Entebbe, were enrolled when they attended for routine visits. Stool samples and blood samples were obtained at enrolment and at 5 weeks and 4 months after treatment for helminths, or at a similar interval for those without helminths. Schistosomiasis was treated with two doses of praziquantel, 20mg/kg, 4 hours apart; *Strongyloides* with albendazole 400mg daily for 3 days; other nematodes with mebendazole 100mg b.d. for 3 days. The first dose of each was observed.

Stool samples were examined by the Kato-Katz method for all subjects, and by concentration for subjects with diarrhoea. At the four month visit all samples were examined by both methods to exclude asymptomatic *Strongyloides* infection. For schistosomiasis, serum was also examined for circulating anodic antigen of *S. mansoni* (CAA) using antibodies kindly provided by Dr D. Dunne, Cambridge University, UK.[58] Blood samples were examined for CD4+ T-cell count (FACScount, Becton Dickinson) and viral load (Amplicor HIV-1 monitor version 1.5, Cobas). Cytokine responses were examined using a whole blood assay as previously described.[69] Briefly, unseparated, heparinised blood was diluted to a final concentration of 1 in 4 using RPMI supplemented with penicillin, streptomycin and glutamine, plated in 96-well plates, and stimulated with antigen or mitogen at a final concentration of 10 μg/ml, or left unstimulated. Supernatants were harvested on day one for IL-2 and day 6 for IFN-γ and IL-5 and frozen until analysed. Antigens were crude culture filtrate proteins of *M. tuberculosis* (CFP) and *S. mansoni* adult worm antigen (SWA), kindly provided by Dr J.T. Belisle, Colorado State University, USA and Dr D. Dunne, respectively. The mitogen was phytohaemagglutinin (PHA; Sigma). Cytokine concentrations were measured by ELISA (PharMingen). The sensitivity of the assays was 8 pg/ml. Low level production of cytokine in unstimulated wells was subtracted from the concentration produced in response to stimulation.

Results. Full results for CD4+ T-cell count, viral load and stool samples at enrolment were available for 109 subjects. Of these, 16 had no helminths on the initial examination, but had helminths at later visits, and are excluded for this analysis. Of the remaining 93 subjects, 41 (44%) had helminths; 18 *S. mansoni* only, 10 *S. mansoni* and intestinal nematodes; 12 intestinal nematodes only; 1 *Taenia*. Intestinal nematodes comprised 15 hookworm, 6 *Trichuris*, 2 *Strongyloides, 2 Ascaris*. Several individuals were infected with more than one nematode. Helminths were grouped for this analysis because the numbers of subjects were small.

Before treatment, individuals with helminth infection had higher CD4+ T-cell counts than those with no helminths (Table 1). ***This was somewhat surprising in relation to our hypothesis that helminths might exacerbate HIV disease. The result might be explained if the T-cells were specific for helminths, but two findings suggest that the CD4+ T-cell counts represent a genuinely more intact immune response to unrelated antigens.***

Table 1. CD4+ T-cell counts & viral load before treatment for helminths

	no helminths (n=52)	helminths (n=41)	P value
median CD4+ T-cells/μl	260	467	0.006 [1]
mean $\log_{10}$ viral load	11.3	10.6	0.09 [2]

[1] P value for rank sum test. [2] P value for t test.

First, the viral load was slightly lower in subjects with helminth infection. Second, the proportion of individuals with positive responses to the unrelated antigen, CFP, was higher in the helminth infected group (table 2). Both findings were explained by adjusting for CD4+ T-cell count.

Table 2. Cytokine responses before treatment for helminths

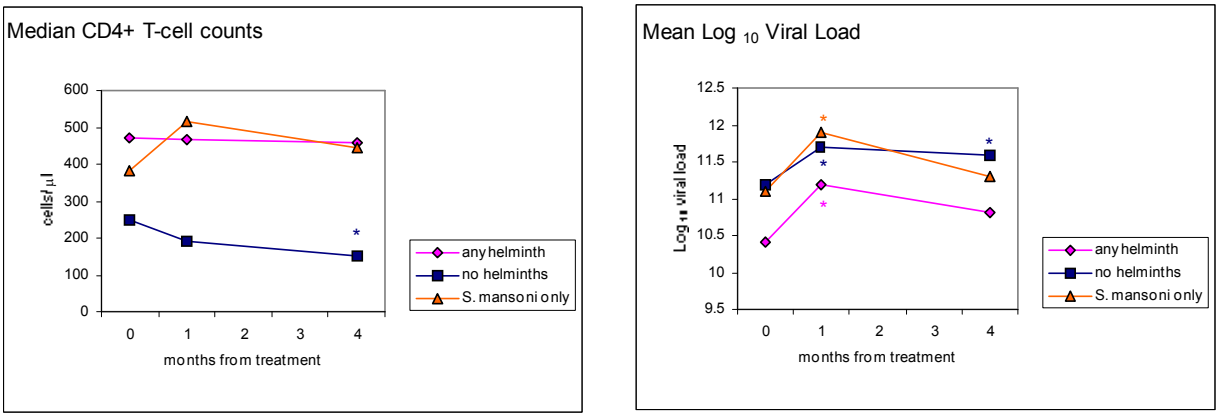
	no helminths (n=52)	helminths (n=41)	P value
PHA: median concentration (pg/ml)			
IFN-γ	1526	1639	0.40
IL-2	62	80	0.80
IL-5	486	1059	0.10
CFP: % with positive response			
IFN-γ	31%	51%	0.05
IL-2	27%	38%	0.28
IL-5	23%	46%	0.02

[1] P value for rank sum test. [2] P value for X^2 test.

Thus it seems that, in this sample, individuals shown to have helminths had a more intact immune response than those without. This might occur if individuals with helminths present earlier in the course of HIV disease than those without, or have a slower disease progression, or die or are otherwise lost from the cohort at higher CD4+ T-cell counts. Alternatively, helminth infection might be less readily detected in individuals with advanced immunosuppression. These possibilities are being explored in further studies.

Following treatment, subjects with helminths showed a transient increase in viral load, but relatively stable CD4+ T-cell counts. Those without helminths showed a decline in CD4+ T-cell counts, and a consistent increase in viral load (Figure 1). Thirty subjects with helminths at enrolment (13 with *S. mansoni* only), and thirty without, had complete data for CD4+ T-cell counts and viral load at all three time points. Of the 30 with helminths at enrolment, 15 still had helminths at the final visit. One of the 13 with *S.mansoni* only still had a positive CAA result at the final visit, and two had acquired intestinal nematodes.

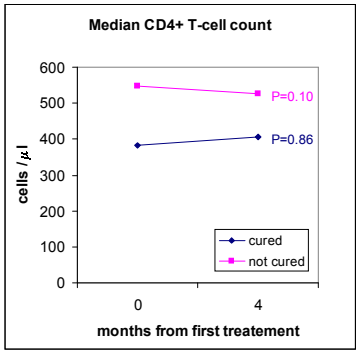
Figure 1. Changes in CD4+ T-cell counts and viral load after treatment for helminths.

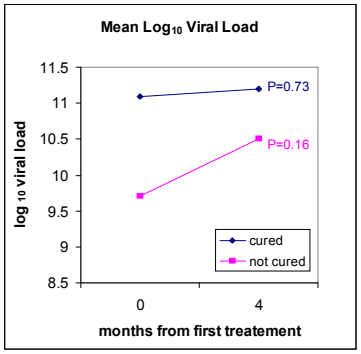


Values which differed significantly form enrolment are indicated * ($P \leq 0.05$)
For subjects with *S. mansoni* only the viral load at 4 months was significantly lower than at 5 weeks.

These results suggest a transient increase in viral replication following treatment, but indicate a satisfactory longer term outcome in individuals treated for helminths. There remains uncertainty as to whether the presence of helminths was detrimental in the first place. To pursue this question further, the outcome was compared between the 15 individuals whose helminth infection cleared, and the 15 with helminths still present at 4 months (Figure 2).

Figure 2. Changes in CD4+ T cell count and viral load for individuals with helminth infection who did, or did not, remain free of infection at 4 months.

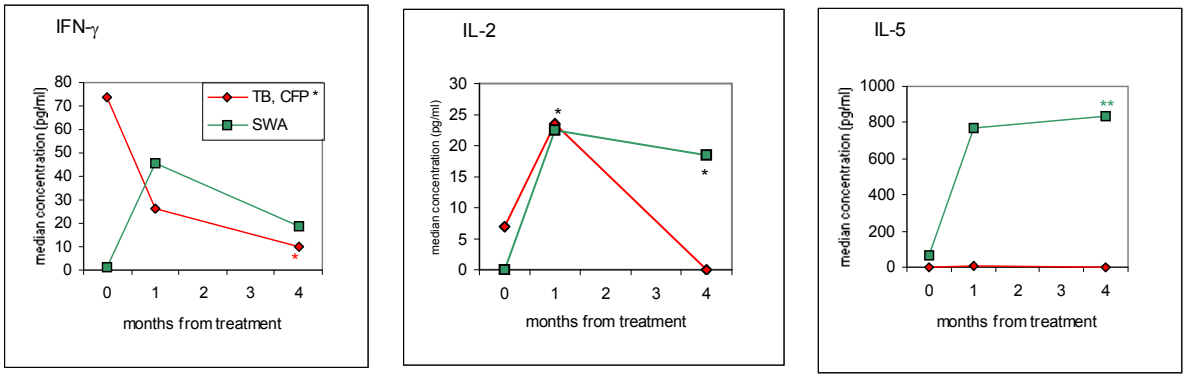




Subjects who were cured maintained their CD4 + T-cell counts, while those with persistent infection had a decline in CD4+ T-cell count and an increase in viral load. However, the differences observed were not statistically significant.

In addition to maintaining relatively high CD4+ T cell counts, individuals treated for helminths showed little change in cytokine production in response to PHA. By contrast, the median response for all cytokines declined significantly in individuals without helminths (P<0.01). Immunological changes following treatment were examined further in the group of individuals with schistosomiasis, for whom a helminth-specific antigen was available. **For subjects with schistosomiasis only at enrolment, an increase in helminth specific responses was observed, while the IFN-γ response to the *M. tuberculosis* antigen declined.**

Figure 3. Changes in cytokine concentration following treatment for schistosomiasis.



* Median cytokine responses to CFP are shown for individuals who had a positive response to CFP for any cytokine at enrolment. Values which differed significantly form enrolment are indicated * * P<0.05; ** P<0.01.

The results show a marked increase in the cytokine response to SWA following treatment, especially for IL-2 and IL-5, which was still present at 4 months. By contrast, the IFN-γ response to CFP declined following treatment, while there was a transient increase in the IL-2 response and a small increase in the IL-5 response at 5 weeks, which coincided with the increase in viral load in these subjects.

<u>Conclusions.</u> Helminth infection showed a strong association with higher CD4+ T-cell counts and this appeared to reflect a more intact immune response to other antigens, rather than immune activation in response to the parasitic infection. The explanation for this association is not clear. Results following treatment are, however, in keeping with the hypothesis that persistent helminth infection may be associated with more rapid HIV progression, and that activation of parasite-specific immune responses may be associated with increased HIV replication and suppression of the IFN-γ response to *M. tuberculosis.*

APPENDIX 2:

A. Mothers delivering at Entebbe Grade B Hospital, January 2001.

Data regarding permanent residence and antenatal attendance were obtained from mothers who delivered their babies during a 4 week period in January 2001. Mothers who delivered their babies in the private wing were not included. The findings were as followed.

- Number of mothers who delivered 106
- Number interviewed 99
- Number (%) with permanent residence in proposed study area 70 (71%)
- Number (%) of those from study area who have attended Entebbe Hospital Antenatal Clinics (EH-ANC) 65 (93%)
- Mean number of EH-ANC visits (range) 5 (1-16)

B. Estimates of helminth infection in mothers.

During enrolment for the study described in Appendix 1, a sample of individuals attending the voluntary HIV testing centre at UVRI, who were found to be HIV-negative, were asked to bring stool samples to identify HIV-negative controls with schistosomiasis. Stools were examined by the Kato-Katz method only, so the results for nematodes are underestimates, and *Strongyloides* was not sought. From the results for these subjects, the minimum prevalence of helminth infections for adult women in Entebbe is expected to be as follows:

	S. mansoni	hookworm	*Trichuris*	*Ascaris*	all nematodes	all worms
prevalence	12%	18%	5%	3%	23%	32%

In a cohort of 2500 women, the minimum expected, numbers by HIV status would be:

	S. mansoni	hookworm	*Trichuris*	*Ascaris*	all nematodes	all worms
HIV- (2250)	270	405	112	68	518	720
HIV+ (250)	30	45	12	8	58	80

C. Estimates for incidence of tuberculosis in childhood.

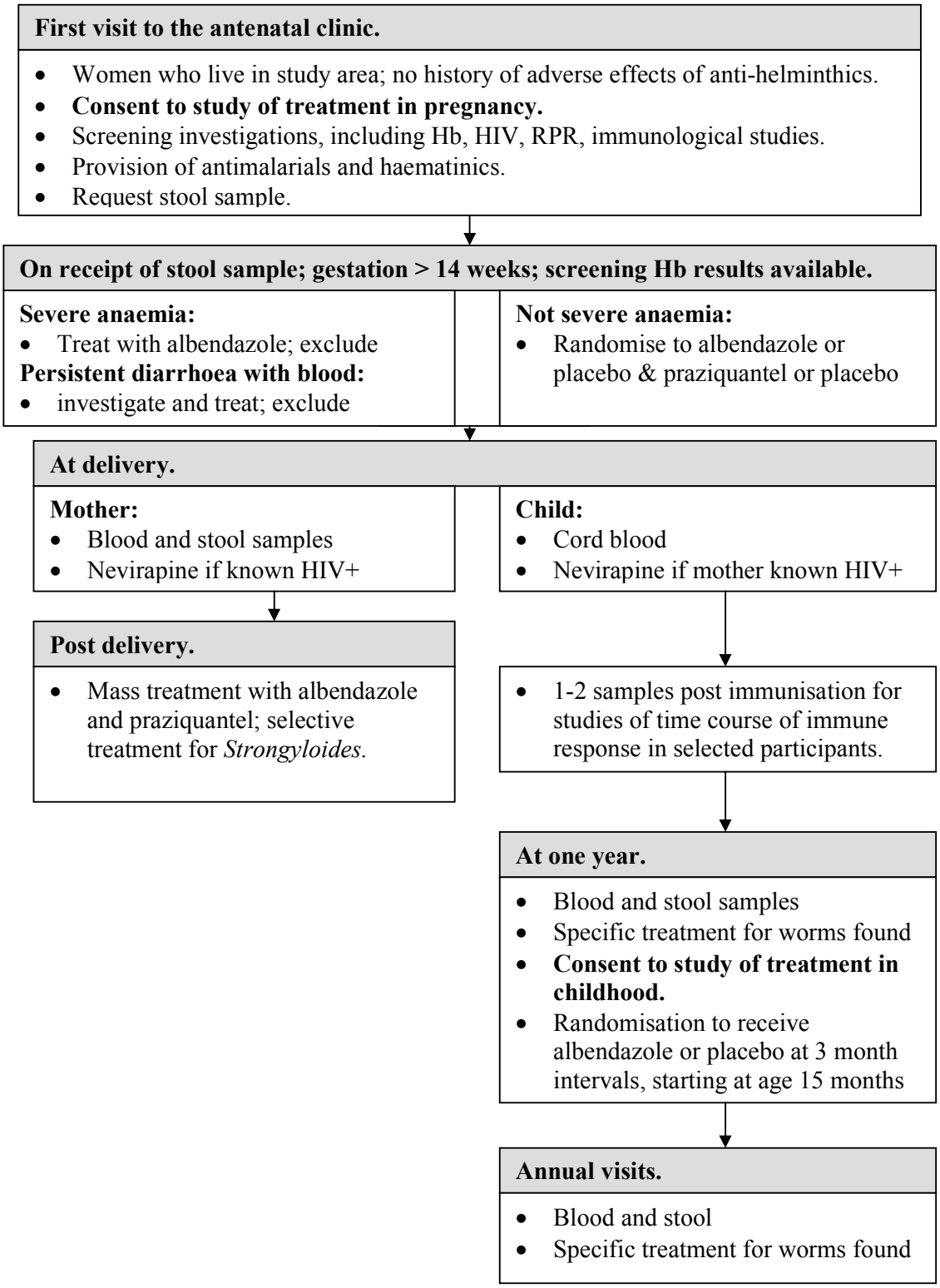
At Mulago Hospital, the main government hospital in Kampala, approximately 1200 cases of tuberculosis in childhood are treated per year, 80% in children under 5.
The population of Kampala is estimated to be about 1,000,000; 18% under 5.
This gives an estimated incidence of 533/100,000 per year in children under 5.

This figure may not be accurate, but more reliable data are not available as childhood tuberculosis is not systematically recorded. The figure will be an overestimate if many cases come from outside Kampala. The figure will be an underestimate if many additional cases from Kampala are diagnosed at the 2 large mission hospitals (Nsambya and Mengo), or if cases of childhood tuberculosis are under-diagnosed or fail to reach hospital.

D. Estimates for incidence of vertical transmission of HIV infection.

In studies at Mulago Hospital, approximately 50% of antenatal women have been willing to receive their HIV results and nevirapine therapy.
Among children of mothers who do not receive anti-viral therapy, vertical transmission of HIV infection at 1 year is likely to be about 25%
Among children of mothers who receive nevirapine, vertical transmission at one year is reported to be about 12%, although it may be possible to reduce this further by avoiding breast-feeding, or by exclusive breast feeding for the first 3 months.
This gives an estimated overall incidence of vertical transmission of, at most, 18% at 1 year.

APPENDIX 3: The impact of helminths on the response to immunisation and on susceptibility to infectious diseases in childhood in Uganda. Proposed Study Profile.



APPENDIX 4: Plan for Analysis.

MOTHERS

At Antenatal Clinic

Mothers will be investigated for helminth infections and randomised to receive albendazole (Alb+) or placebo (Alb-) and praziquantel (Pzq+)or placebo (Pzq-).

Intestinal nematodes +				Intestinal nematodes +				Intestinal nematodes -				Intestinal nematodes -			
Schistosomiasis +				Schistosomiasis -				Schistosomiasis +				Schistosomiasis -			
Alb +		Alb -		Alb +		Alb -		Alb +		Alb -		Alb +		Alb -	
Pzq +	Pzq -	Pzq +	Pzq -	Pzq +	Pzq -	Pzq +	Pzq -	Pzq +	Pzq -	Pzq +	Pzq -	Pzq +	Pzq -	Pzq +	Pzq -
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16

At Delivery	
Helminths present, Treated	Helminths absent

CHILDREN

At One Year Follow-Up

Children will be randomised to receive routine albendazole every 3 months or placebo, starting at 15 months. All will receive annual selective treatment based on stool examination.

Group C1	**Group C2**
Albendazole every 3 months and annual selective treatment.	Placebo every 3 months and annual selective treatment.

Annual Follow-Up Visits to Age 5 Years	
Helminths present, treated	Helminths absent

The analysis for the effect of maternal intestinal nematode infection will compare groups according to the presence or absence of intestinal nematodes at the antenatal visit, and whether or not they were treated; i.e. outcomes will be compared for children of mothers in groups G1-2-5-6 (intestinal nematodes, treated), G3-4-7-8 (intestinal nematodes, not treated), G9-10-13-14 (no intestinal nematodes, treated) G11-12-15-16 (no intestinal nematodes, not treated). The findings will be adjusted for the presence or absence of schistosomiasis, treatment with praziquantel, and the presence or absence of helminths at delivery. The overall effect of albendazole treatment during pregnancy will be determined by comparing G1-2-5-6-9-10-13-14 vs G3-4-7-8-11-12-15-16.

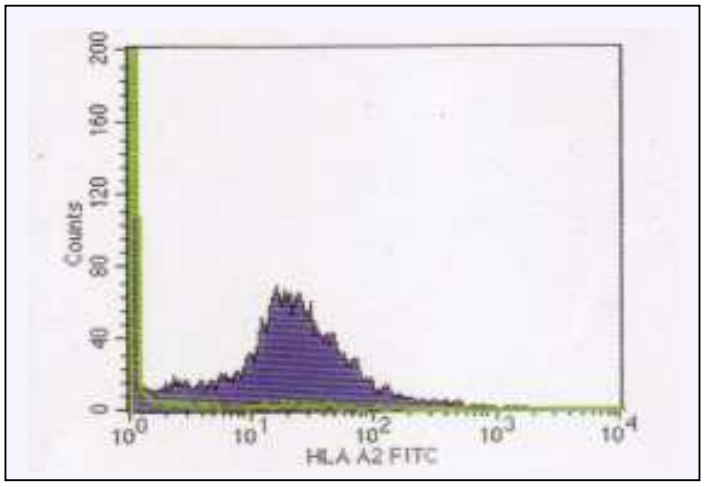
The analysis for the effect of maternal schistosomiasis will compare groups according to the presence or absence of schistosomiasis at the antenatal visit, and whether or not they were treated; i.e. outcomes will be compared for children of mothers in groups G1-3-9-11 (schistosomiasis, treated), G2-4-10-12 (schistosomiasis, not treated), G5-7-13-15 (no schistosomiasis, treated) G6-8-14-16 (no schistosomiasis, not treated). The findings will be adjusted for the presence or absence of intestinal nematodes, treatment with albendazole, and the presence or absence of helminths at delivery. The overall effect of praziquantel treatment during pregnancy will be determined by comparing G1-3-5-7-9-11-13-15 vs G2-4-6-8-10-12-14-16.

The analysis for the effect of intestinal nematodes in children will compare groups according to whether they receive routine albendazole every 3 months or annual selective treatment; i.e. outcomes for children in groups C1 and C2 will be compared. The presence or absence of helminths at annual follow up visits will be taken into account.

APPENDIX 5:

A. Identification of HLA-A2+ individuals by flow cytometry.

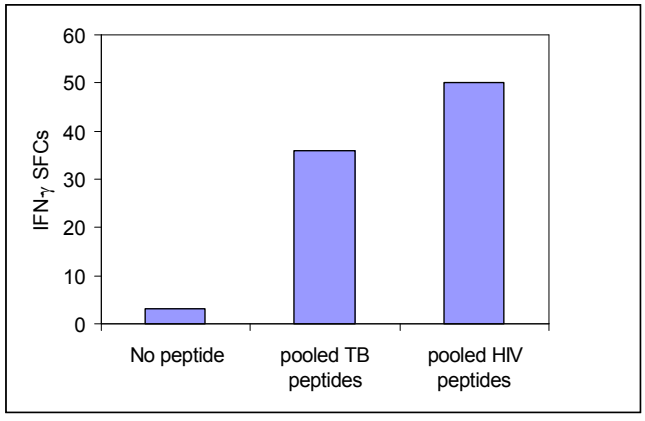
Cells were incubated in cell culture supernatant from the hybridoma B7.2; washed, incubated with FITC goat anti-mouse antibody (DAKO), washed and fixed in 2% paraformaldehyde. Flow cytometry was performed on a FACScan and analysed using CELLQuest software.



HLA-A2 positive individuals show a shift in fluorescence of at least 1 log

B. Enumeration of *M. tuberculosis* and HIV peptide-specific cells in pleural fluid from an HIV-infected patient with pleural tuberculosis; HLA class I haplotype HLA-A2.

Pleural fluid mononuclear cells were separated on Histopaque (Sigma) and stored in liquid nitrogen until analysed. The IFN-γ ELIspot was conducted in a 96 well filtration plate (Millipore Multiscreen) using anti-IFN-γ antibodies supplied by MABTECH. Briefly the plate was wetted with alcohol, coated with capture antibody, washed with RPMI and blocked with culture medium (RPMI containing 10% human serum). Cells were thawed, suspended in culture medium and added to the plate at 500,000 cells per well, in a final volume of 200 μl. Wells were stimulated in duplicate with pooled *M. tuberculosis* peptides, pooled HIV peptides, (each peptide at a final concentration of 10 μg/ml) or left unstimulated, and incubated for 24 hours at 37 ^{0}C with 5% CO_2. The cells were then washed off and the plate was developed with biotinylated second antibody, followed by streptavidin-alkaline phosphatase and then chromogenic substrate (BioRad). Development was stopped with tap water. The plates were dried overnight and spots counted using a dissecting microscope.



SCFs : spot forming cells

Pleural fluid cells from an HIV+, HLA-A2+ Ugandan patient show a positive IFN-γ response for pooled peptides of both *M. tuberculosis* and HIV.

*Peptides selected on predicted HLA-A*0201 binding motif.*

TB peptides: from antigen 85 C.

R**L**RSAATT**L**
A**I**AAMGAV**L**
G**L**PVEYLQ**V**
S**M**SGGSAL**I**
P**M**VQIPRL**V**
R**L**VANNTR**I**
Q**L**VAMKAD**I**
A**M**KADIQH**V**

HIV peptides:

S**L**YNTVAT**L** gag p17, clade D
S**L**FNTVAT**L** gag p17, clade A
I**L**KEPVHG**V** pol, clade D
I**L**KDPVHG**V** pol, clade A

(anchor residues for HLA-A*0201 in bold)

APPENDIX 6: Time-line of the proposed study.

<table>
<tr><th></th><th colspan="3">Year 1</th><th colspan="3">Year 2</th><th colspan="3">Year 3</th><th colspan="3">Year 4</th><th colspan="3">Year 5</th></tr>
<tr><td>Administration & preparations</td><td colspan="2">* Renovations
* Ordering
* Lab set-up
* Staff recruitment
* Sensitise community</td><td colspan="13"></td></tr>
<tr><td>Cohort Study, Original plan.</td><td colspan="2"></td><td colspan="6">Enrolment of participants</td><td colspan="6">Continuing follow up</td><td></td></tr>
<tr><td>Cohort Study, Revised plan.</td><td colspan="4">Pilot enrolment</td><td colspan="8">Enrolment of participants</td><td colspan="4">Continuing follow up</td></tr>
<tr><td>Analysis</td><td colspan="3"></td><td>Pilot: screen -ing data</td><td></td><td>Pilot: screen -ing immuno -logy</td><td>Pilot: maternal & birth outcome & immuno -logy @ delivery</td><td></td><td>Main cohort: Interim analysis for maternal and birth outcome</td><td>Pilot: outcome in babies at 1 year</td><td colspan="2">Main cohort: Interim analysis of effects of maternal helminths on outcomes at 1 year</td><td colspan="3">Plans to be based on earlier analyses</td></tr>
</table>

APPENDIX 7. MATERNAL AND BIRTH OUTCOMES

DEFINITIONS.

Stages of pregnancy:

First trimester:	less than 14 weeks
Second trimester	14 to 27 weeks
Third trimester	28 weeks or more

Major congenital abnormalities:

Structural or functional defects that require surgical or medical intervention; including but not restricted to the following.

Nervous system:

- anencephaly
- Dandy-Walker cysts
- Encephalocele
- Hydrocephalus
- Meningomyelocele
- Microcephaly

Cardiovascular system

- Atrial septal defect
- Fallot's tetralogy
- Patent ductus arteriosus
- Ventricular septal defect

Gastrointestinal tract

- Cleft lip
- Cleft palate
- Imperforate anus
- Pyloric stenosis

Genitourinary tract

- chordae of the penis
- cliteromegaly
- cyst of hydrocele
- hypospadias
- micropenis
- undescended testis
- vaginal polyps

Musculoskeletal system and skin

- arachnodactyly
- congenital dislocation of the hip
- club foot
- deletion of fingers or toes
- micrognathia
- pholcomalia
- neonatal teeth
- polydactyly
- rudimentary external ears
- umbilical hernia

Dysmorphic features

- mongoloid features
- a combination of two or more of -
  - ear tags, low hairline, low-set ears, palmar triradius

Multisystem abnormalities

abnormalities involving 2 or more of the listed systems
prune-belly syndrome
Turner's syndrome

Birth weight: Low birth weight <2500 g
Very low birthweight <1500 g

Perinatal death: Stillbirths and all deaths during the first week from delivery.

Maternal death: Death while pregnant or within 42 days of the termination of the pregnancy.

Severe postpartum maternal anaemia: < 9 g/dl [93]

Pre-eclampsia Hypertension (diastolic pressure ≥95, and/or systolic pressure ≥160 mmHg) and proteinuria (≥2+ on dipstick)

OUTCOMES AT DELIVERY

1. Outcome: birth outcome

Measures:	**Likely incidence:**
major congenital defect	1-2%
stillbirth	1-4%
perinatal deaths	1-7%
low birthweight	18%
very low birthweight	1-2%

Sample size calculations: *risk ratio of 2; P=0.05; 80% power.*
Incidence of 1 vs 2%, e.g. major congenital defects; require 2514 in each group; not feasible
Indicence of 2 vs 4%, e.g. stillbirths + early neonatal deaths, require 1239 in each group; feasible by end of study
Incidence of 10 vs 20%, e.g. low birthweight, require 219 in each group; feasible for interim analysis

Potential confounders / explanatory variables:

Demographic/ socioeconomic
education of mother & father
literacy of mother & father
occupation of mother & father
housing type
property/ items owned
tribe

Clinical
mother's height
mother's weight at first visit
duration of gestation at first visit
mother's gravidity
past obstetric history
alcohol
smoking

mother's general health (nutrition, diabetes etc)
medicines taken during pregnancy
illnesses for which medicines taken
maternal syphilis
maternal HIV

2. Outcome: maternal outcomes

Measures:	**Likely incidence:**
Severe postpartum anaemia	8%
Eclampsia /pre-eclampsia	1-2%
Mortality	<1%

Confounders / explanatory variables in addition to those considered above:

Helminth risks
sanitation
water source
wearing of shoes

Clinical
Malaria in pregnancy -film at enrolment
- mother and placental film at delivery
Malaria risk: environment, bednets, spraying
Use of antimalarials in pregnancy
Use of antihelminthics in pregnancy
Haemoglobinopathies - may be possible to evaluate on stored samples

Sample size calculation: *risk ratio of 2; P=0.05; 80% power.*
Incidence of 1 vs 2%, e.g. pre-eclampsia; require 2514 in each group; not feasible
Incidence of 5 vs 10%, e.g. postpartum anaemia, require 474 in each group; feasible for interim analysis

APPENDIX 8. Procedures for handling study drugs.

1. FOR MOTHERS.

Identical, chewable albendazole tablets and placebo tablets will be supplied in bulk by Glaxo Smith Kline, the manufacturers.

A randomisation code will be prepared in London, and held there, by a member of the Tropical Health Epidemiology Unit of the London School of Hygiene & Tropical Medicine. A copy will be sent to Entebbe and held by a statistician who is not involved in the trial.

Albendazole or placebo and praziquantel or placebo will be packaged in consecutively numbered envelopes using the randomisation codes and these will be sealed. This will be done by two members of the MRC Programme staff who are not involved in the trial.

When mothers bring their antenatal stool samples and are enrolled in the trial they will be given the envelope in numerical order and the number on the envelope will be their study number (designated WS _ _ _ _ on data collection forms).

2. FOR CHILDREN.

Identical bottles of a single dose of albendazole syrup or placebo syrup will be supplied for children aged 15, 18 and 21 months. Identical chewable tablets of albendazole or placebo will be provided for children aged 24 months and above.

A randomisation code will be prepared in London, and held there, by a member of the Tropical Health Epidemiology Unit of the London School of Hygiene & Tropical Medicine. A copy will be sent to the manufacturers who will label the doses of albendazole or placebo accordingly. A full set of doses of syrup and tablets will be provided for each child, bearing the same number.

A copy of the code will be sent to Entebbe and held by a statistician who is not involved in the trial.

When children reach the age of 1 year, and consent is given for this component of the trial, they will be given the next consecutive number and they will receive the doses of medication labelled with this number.

APPENDIX 9. PROPOSED SCHEDULE FOR ANALYSES.

1. PILOT STUDY.

a. Pilot study eligibility and screening data.

Analysis period: August 2002.
Data to be analysed: Eligibility / socioeconomic screening data; clinical laboratory screening data.
Purpose of analysis: To assist in design of main study and improving data collection forms.
Comment: Unblinding not required.

b. Pilot study screening immunological results.

Analysis period: Approximately December 2002-February 2003
(To begin when necessary laboratory data are complete)
Data to be analysed: Immunological findings on samples from pilot study mothers taken at screening.
Purpose of analysis: To assist in identification of immunological parameters of most interest for further study, and in choice of laboratory methods to be used.
To obtain preliminary data on the effects of maternal helminth infections on maternal immune responses.
Comment: Unblinding not required.

c. Pilot study clinical and immunological findings in mothers and baby at delivery.

Analysis period: Approximately March-May 2003.
(When all pilot study babies have been delivered and necessary laboratory data are complete).
Data to be analysed: Clinical and laboratory (haematological, parasitological and immunological) results.
Purpose of analysis: 1. To obtain preliminary data on the effect of albendazole on outcomes at delivery, in particular:

- maternal post-partum haemoglobin*
- maternal and cord blood viral load in HIV-positive mothers*
- maternal parasite burden
- babies' birth weight*
- maternal and cord blood immune responses

2. To examine the effect of albendazole treatment on maternal immune responses by comparing findings at enrolment and delivery in treated and untreated groups.
3. To make preliminary comparisons between maternal and cord blood immune responses.
4. To assist in the design of further nested studies within the cohort.
Comment: Access to unblinded data required. **

d. Pilot study clinical and immunological findings in babies at one year.

Analysis period: Approximately February-April2004.
(When all pilot study babies have reached one year and necessary laboratory data are complete).
Data to be analysed: Clinical and laboratory (haematological, parasitological and immunological) results.
Purpose of analysis: To obtain preliminary data on the effect of maternal helminths and of maternal treatment with albendazole on outcomes in the babies at one year, in particular:

- babies' growth*
- babies' immune responses to selected antigens.

This data will allow us to obtain preliminary information for comparison with earlier studies on the effects of maternal helminth infections on the response to immunisations in the babies, extending the results to new helminth species. We will also obtain preliminary data on the effects of maternal treatment for helminths.

Comment: Access to unblinded data required. **

2. MAIN STUDY.

a. Clinical findings in mothers and baby at delivery.

Analysis period: Approximately October 2003-January 2004.
(When enrolment to the study has completed one year).

Data to be analysed: Clinical and laboratory (haematological and parasitological) results.

Purpose of analysis: To obtain preliminary data on the effect of albendazole and praziquantel on outcome at delivery for mother and baby, in particular:

- maternal post-partum haemoglobin
- maternal and cord blood viral load in HIV-positive mothers
- maternal parasite burden
- eclampsia/ pre-eclampsia*
- maternal deaths*
- babies' birth weight
- congenital abnormalities*
- stillbirths and early neonatal deaths*

Comment: Access to unblinded data required. **

b. Clinical and immunological findings in babies at one year; comparison with maternal and cord blood immunological responses.

Analysis period: Approximately April-September2004.
(When babies of mothers enrolled in the first 6 months have reached one year and necessary laboratory data are complete).

Data to be analysed: Clinical and laboratory (haematological, parasitological and immunological) results.

Purpose of analysis: 1. To obtain preliminary data on the effect of maternal helminths and of maternal treatment with albendazole and praziquantel on outcomes in the babies at one year, in particular:

- babies' growth
- incidence of common disease outcomes in the first year (malaria, diarrhoea, ALRI).
- neonatal and infant mortality*
- babies' immune responses to selected antigens.

This data will provide better information on the effects of maternal helminth infections on the response to immunisations in the babies, and for comparisons between helminth species, as well as on the effects of maternal treatment for helminths.

2. To examine the effect of albendazole and praziquantel treatment on maternal immune responses by comparing findings at enrolment and delivery in treated and untreated groups.

3. To make further comparisons between maternal and cord blood immune responses.
These analyses will make an important contribution to evaluating safety and ethical issues regarding the risks and benefits of anti-helminthic treatment in pregnancy.
These analyses will also allow the TSC to re-evaluate the target sample size for the cohort.

Comment: Access to unblinded data required. **

GENERAL CONSIDERATIONS.

* For some analyses (indicated*) the sample size at the time point proposed is likely to be insufficient to achieve a statistically significant result.

** Unblinding. Where access to unblinded data is required it is proposed that the analysis be conducted by the principal investigator and project statistician(s), who do not have clinical responsibility for participants. Access to the full data set and analysis programmes will be given to the DMEC. Access to the overall results will be given to scientific staff of the project, collaborators and TSC members.

Data from these analyses, especially from the analyses of the main study, may be publishable. This will be reviewed with the TSC.

TIMETABLE.

August 02		**December 02-February 03**	**March 03-April 03**		**October 03-January 04**	**February 04-April 04**	**April 04-September 04**
pilot (a)		**pilot (b)**	**pilot (c)**		**main (a)**	**pilot (d)**	**main (b)**

APPENDIX 10. Serious Adverse Events.

A serious adverse event will be defined as any clinical event considered by the clinician to be severe or life-threatening, or that results in death. This includes events that require unexpected hospitalisation or prolongation of existing hospitalisation or results in persistent or significant disability or incapacity.

For the purposes of this study, severe adverse events include, but are not limited to: miscarriage, intrauterine death, stillbirth, neonatal death, major congenital abnormality; maternal death or death of the infant/ child; anaphylaxis, severe acute bronchospasm, seizures occurring within 24 hours of administration of the study drug.

Maternal deaths will only be reported as "serious adverse events" if they occur during pregnancy and the puerperium (within 42 days of the end of pregnancy) since no further study intervention will be given to the mothers after the treatment of helminth infections found at delivery. However, death of the mother will be documented if it occurs at any time during follow up of the child.

The likelihood of a causal association between administration of the study drug and a serious adverse event will be defined using definitions adapted from WHO terminology, as follows:

1. **Probable:** A clinical event, including laboratory test abnormality, with a reasonable time sequence to administration of the drug, unlikely to be attributed to concurrent disease or other drugs or chemicals.
2. **Possible:** A clinical event, including laboratory test abnormality, with a reasonable time sequence to administration of the drug, but which could also be explained by concurrent disease or other drugs or chemicals.
3. **Unlikely:** A clinical event, including laboratory test abnormality, with a temporal relationship to drug administration that suggests that a causal relationship to administration is unlikely. Other drugs, chemicals or underlying disease provide a plausible explanation.
4. **Not assessable:** A clinical event for which the information received is insufficient to allow reasonable assessment.

A report will be made on any serious adverse event as soon as possible after it occurs, and a copy will be forwarded as soon as possible thereafter to the statistician in charge of the Data Monitoring and Ethical Committee. At his discretion he will consult the DMEC and will inform the chairman of the Trial Steering Committee if there is evidence to suggest that the Steering Committee should consider stopping the study or requesting that an interim analysis should be conducted.

A summary of all reported serious adverse events will be presented at each Trial Steering Committee meeting.

APPENDIX 11. DEFINITIONS FOR OUTCOMES IN CHILDREN

1. ACUTE LOWER RESPIRATORY TRACT INFECTION (PNEUMONIA)[97]

- Cough
- Difficulty in breathing, with at least one of the following:
  Grunting
  Nasal flaring
  Inter-costal recession
  Lower chest wall indrawing
- Fast breathing
  ≥ 60 breaths per minute (< 2 months)
  ≥ 50 breaths per minute (2-12 months)
  ≥ 40 breaths per minute (1-5 years)
- ± Abnormal Chest findings
  Decreased breath sounds
  Bronchial breathing
  Crepitations
  Radiological changes

a. Very Severe Pneumonia

One or more of the above, plus at least one of the following:

- central cyanosis
- inability to breastfeed or drink, or vomiting everything
- convulsions, lethargy or unconsciousness
- severe respiratory distress (e.g. head nodding)

2. TUBERCULOSIS

Using the scoring system[87] below as a screening tool to select children (aged 0-4 yrs) with a high probability for tuberculosis, for further investigations:

Criterion	Score
Close contact with a known case of TB	2
Mantoux skin test positive	2
Persistent cough	2
Low weight for age/weight loss	3
Unexplained/prolonged fever	1
Total score must equal/exceed	**5**

(Positive skin test: Tuberculin skin test, ≥ 10mm reaction is suggestive of TB).

- A total screening score $\geq$ 5.
- Scoring results may be supplemented by a diagnostic chest x-ray such as primary complex or miliary tuberculosis.

Microscopy and culture: Acid-fast bacilli (Ziehl-Nielsen stain) cultured tubercle bacilli, from specimen such as three consecutive early morning gastric aspirates, CSF, pleural fluid, ascites fluid. Owing to low detection rates by these methods in children, a positive culture would confirm tuberculosis but a negative result does not exclude the disease.

3. MALARIA[97]

- Fever (temperature ≥ 37.5 ^{0}C or ≥ 99.5 ^{0}F)

- Positive blood smear (with parasite count above a cut off defined using data from asymptomatic children).[83]

a. Severe Malaria

Both of the above, plus any one of the following;

- Altered consciousness / unrousable coma
- Generalised convulsions
- Severe anaemia (haematocrit <18%; haemoglobin <6g/dl)
- Hypoglycaemia (blood glucose <2.5mmol/litre or <45mg/dl)
- Respiratory distress, acidosis (deep laboured breathing), pulmonary oedema
- Jaundice
- Shock
- Bleeding tendency

4. DIARRHOEA, AND DEHYDRATION[97]

- Frequent loose stool, > 3 times/day

a. Persistent Diarrhoea

Diarrhoea with or without blood, which begins acutely and lasts for 14 days or longer.

b. Dysentery

Loose frequent stools containing blood.

c. Some Dehydration

The child has two or more of the following:

- Restlessness / irritability
- Thirsty and drinks eagerly
- Sunken eyes
- Skin pinch goes back slowly

d. Severe Dehydration

The child has two or more of the following:

- Lethargy or unconsciousness
- Sunken eyes
- Skin pinch goes back very slowly (≥ 2 seconds)
- Not able to drink or drinks poorly

e. No Dehydration

The child does not have two or more of the above signs, which characterise dehydration.

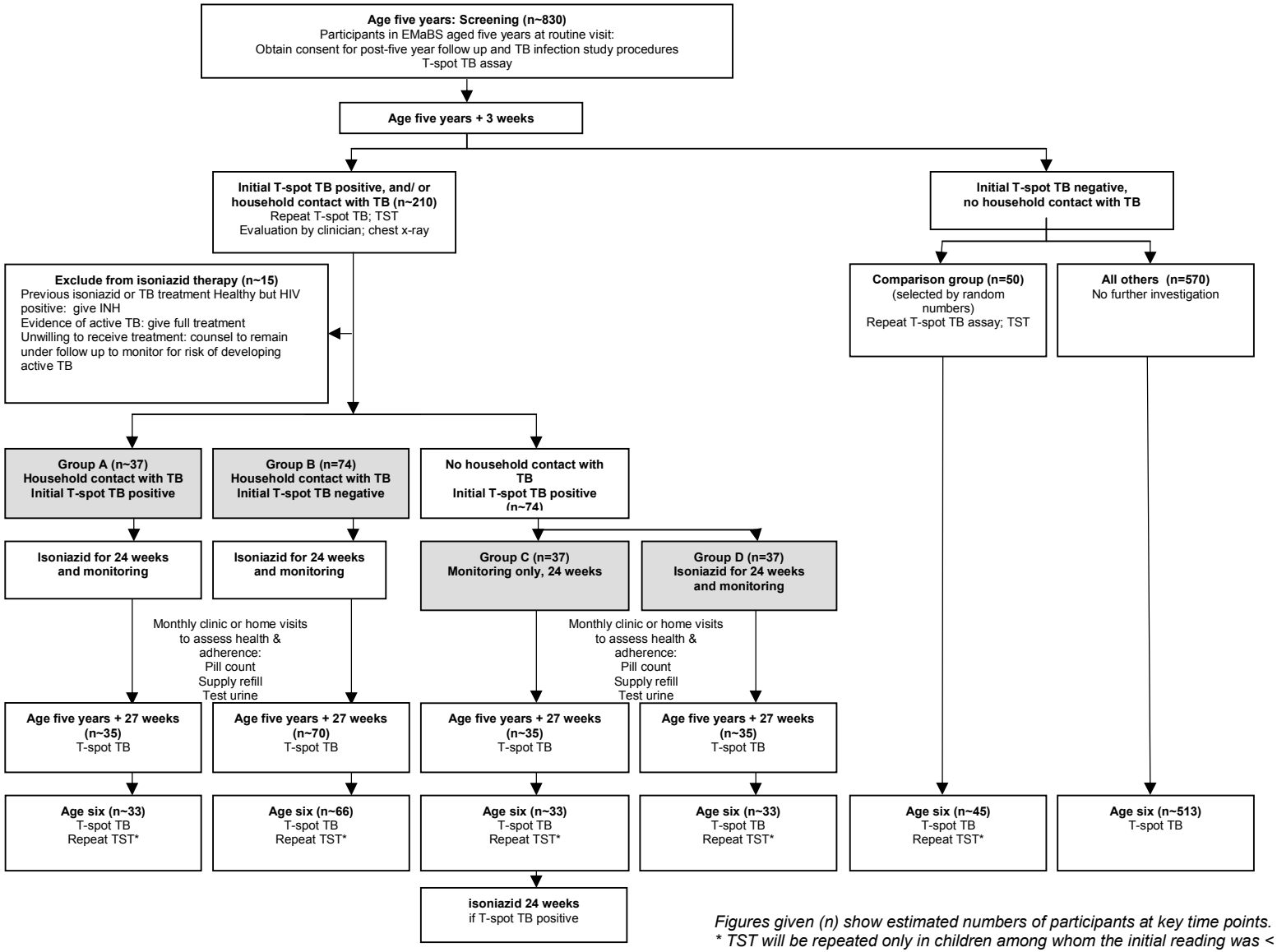
5. MEASLES

- Fever
- Generalised rash (a maculopapular rash that begins behind the ears and along the hair-line, and spreads to become generalised and blotchy)
- One of the following – cough, runny nose, red eyes, Koplik's spots.

- Positive IgM.

a. **Measles with complications**
Any of the following;
- Pneumonia
- Otitis media
- Diarrhoea and dehydration
- Measles croup (laryngo-tracheo-bronchitis)
- Corneal and retinal damage
- Mouth ulcers (cancrum oris)
- Neurological complications (encephalitis)
- Severe malnutrition

APPENDIX 12. Planned profile for TB infection study



Figures given (n) show estimated numbers of participants at key time points.
** TST will be repeated only in children among whom the initial reading was < 10mm*