ASTHMA AND ALLERGIC DISEASE:

THEIR RELATION WITH *NECATOR AMERICANUS*

AND OTHER HELMINTH INFECTIONS

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ABSTRACT

Background

The rate at which the prevalence of allergic disease is increasing in many countries suggests that environmental exposures may be important aetiological factors. Epidemiological evidence indicates that infection with helminth parasites may be one such factor: in particular, in a systematic review and meta-analysis, current hookworm (*Necator americanus*) infection at an intensity of 50 eggs/g faeces was shown to be associated with a halving of risk of asthma. The relation between parasite infection and atopy has not been subjected to the same rigorous and comprehensive review. Based on the results of the studies in asthma, it is possible that hookworm infection may have potential in the treatment of this disease, but to date, no clinical trials have been carried out to test this hypothesis. For ethical and safety reasons, before embarking on a clinical trial in asthma it is necessary to establish the dose of larvae required to produce at least 50 eggs/g faeces, and to determine whether experimental hookworm infection might exacerbate bronchial hyper-responsiveness during larval lung migration.

Aims and objectives

The first aim of this thesis was to establish whether experimental hookworm infection improves asthma by carrying out a series of three intervention studies. The second aim was to determine the association between intestinal parasite infection and atopy (defined as positive allergen skin sensitisation or the presence of specific IgE) and to establish whether the association was species-specific.

This thesis therefore consists of two main components: a series of three clinical trials of experimental hookworm infection; and a systematic review and meta-analysis of the association between intestinal parasite infection and atopy.

Methods and Results

Dose-ranging study of experimental hookworm infection

Aim: To identify the dose of hookworm larvae necessary to achieve 50 eggs/g faeces and to monitor any adverse effects of infection.

Methods: Ten healthy volunteers, without asthma or bronchial responsiveness to inhaled methacholine, received 10, 25, 50, or 100 *Necator americanus* larvae administered double-blind to an area of skin on the arm and were monitored weekly for 12 weeks.

Results: All doses resulted in the production of at least 50 eggs/g faeces in the eight subjects who completed the study. Skin itching at the entry site and gastrointestinal symptoms were common at higher doses.

Study of experimental hookworm infection in allergic rhinoconjunctivitis

Aim: To determine whether hookworm larval migration through the lungs increases bronchial responsiveness in allergic individuals with measurable bronchial responsiveness but not clinical asthma, and to investigate the general tolerability of infection and its effect on allergic symptoms.

Methods: Thirty individuals with allergic rhinoconjunctivitis and measurable bronchial responsiveness to adenosine monophosphate (AMP) but not clinically diagnosed asthma were randomised, double-blind, to cutaneous administration of either ten *Necator americanus* larvae or histamine placebo, and followed for 12 weeks. The primary outcome was the maximum fall from baseline in provocative

dose of inhaled AMP required to reduce one-second forced expiratory volume by 10% (PD_{10}AMP) measured at any time over the four weeks after active or placebo infection. Secondary outcomes included peak flow variability in the four weeks after infection, adverse effect diary scores and rhinoconjunctivitis symptom severity over the 12-week study period, and change in allergen skin sensitisation between baseline and 12 weeks.

Results: Mean maximum change in PD_{10}AMP from baseline was slightly but not significantly greater in the hookworm than the placebo group (-1.67 and -1.16 doubling doses; mean difference -0.51, 95% confidence interval: -1.80 to 0.78; p=0.42). There were no significant differences in peak flow variability, rhinoconjunctivitis symptoms or allergen skin sensitisation between groups. Symptom scores of potential adverse effects were more commonly reported in the hookworm group, but infection was generally well tolerated.

Study of experimental hookworm infection in asthma

Aim: To determine the effects of experimental hookworm infection on asthma.

Methods: Thirty-two individuals with asthma and measurable bronchial hyper-responsiveness to adenosine monophosphate (AMP) were randomised, double-blind, to cutaneous administration of either ten *Necator americanus* larvae or histamine placebo, and followed for 16 weeks. The primary outcome was the change in provocation dose of inhaled AMP required to reduce one-second forced expiratory volume by 20% (PD_{20}AMP) from baseline to week 16. Secondary outcomes included change in several measures of asthma control and allergen skin sensitisation and the occurrence of adverse effects.

Results: Mean PD_{20}AMP improved in both groups, more in the hookworm (1.49 doubling doses (DD)) than the placebo group (0.98 DD), but the difference

between groups was not significant (0.51 DD, 95% confidence interval: -1.79 to 2.80; p=0.65). There were no significant differences between the two groups for other measures of asthma control or allergen skin sensitisation. Infection was generally well tolerated.

Systematic review and meta-analysis of the association between intestinal parasite infection and atopy

Aim: To quantify the association between current intestinal parasite infection and the presence of atopy in a systematic review and meta-analysis of epidemiological studies, and to determine whether, as with asthma, this relation is species-specific.

Methods: MEDLINE, EMBASE, LILIACS and CAB Abstracts (to March 2009); reviews; and reference lists from publications were searched. No language restrictions were applied. Studies that measured current parasite infection using direct faecal microscopy and defined atopy as allergen skin sensitisation or presence of specific IgE were included. Pooled odds ratios (OR) and 95% confidence intervals (95% CI) using data extracted from published papers using random effect models were calculated.

Results: 20 studies met the inclusion criteria. Current parasite infection was associated with a reduced risk of allergen skin sensitisation (OR 0.69, 95% CI: 0.60 to 0.79; p<0.01). When analyses were restricted to current geohelminth infection, the size of effect remained similar (OR 0.68, 95% CI: 0.60 to 0.76; p<0.01). In species-specific analysis, a consistent protective effect was found for infection with *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm. There were insufficient data to pool results for Schistosomiasis or atopy defined by presence of specific IgE.

Conclusions

Experimental infection with ten *Necator americanus* larvae produces at least 50 eggs/g faeces, the intensity of infection seen to protect against asthma in observational studies. This dose is safe, well tolerated, feasible to use in clinical trials and does not cause clinically significant exacerbation of bronchial responsiveness during larval pulmonary migration. In clinical trials, it did not result in significant improvement in symptoms of allergic rhinoconjunctivitis, or in bronchial hyper-responsiveness or other measures of asthma control. However, a non-significant improvement in bronchial hyper-responsiveness was seen, indicating that further studies incorporating revised dosing regimens that more closely mimic natural infection are feasible, and should be undertaken, with the aim of identifying novel treatments for asthma. As with asthma, there appears to be an inverse association between intestinal parasite infection and atopy. Work should continue to identify the mechanisms of this effect and means of harnessing these to reduce the global burden of allergic disease.

PUBLICATIONS DIRECTLY RELATED TO THESIS

2006 **Dose-ranging study for trials of therapeutic infection with *Necator americanus* in man** K Mortimer, A Brown, J Feary, C Jagger, S Lewis, M Antoniak, D Pritchard, J Britton. American Journal of Tropical Medicine and Hygiene 2006. 75(5), 914-920

2009 **Safety of hookworm infection in individuals with measurable airway responsiveness: a randomised placebo-controlled feasibility study** J Feary, A Venn, K Mortimer, A Brown, D Hooi, F Falcone, A Brown, D Pritchard, J Britton. Clinical and Experimental Allergy 2009. 39(7), 1060-8

2010 **Experimental hookworm infection: a randomised placebo controlled trial in asthma** J Feary, A Venn, K Mortimer, A Brown, D Hooi, F Falcone, A Brown, D Pritchard, J Britton. Clinical and Experimental Allergy 2010. 40(2), 299-306

2010 **Atopy and current intestinal parasite infection: a systematic review and meta-analysis** J Feary, J Britton, J Leonardi-Bee. Allergy 2011. 66, 569–578

OTHER RELATED PUBLICATIONS

2009 **Immunological profiles of subjects recruited for a randomised, placebo controlled clinical trial of hookworm infection** D Blount, D Hooi, J Feary, A Venn, G Telford, A Brown, J Britton, D Pritchard. American Journal of Tropical Medicine and Hygiene 2009. 81(5), 911-6

2009 **Antigen-driven basophil activation is indicative of early *Necator americanus* infection in IgE seronegative patients** F Falcone, D Hooi, G Telford, A Brown, R Seabra, J Feary, A Venn, J Britton, D Pritchard. Journal of Allergy and Clinical Immunology 2009. 124(6), 1343-50

CONFERENCE ABSTRACTS ARISING FROM THESIS

2007 **A randomised controlled trial of the effects of hookworm infection on bronchial hyper-reactivity during pulmonary migration phase** JR Feary, AJ Venn, AP Brown, D Hooi, FH Falcone, DI Pritchard, JR Britton. American Journal of Respiratory and Critical Care Medicine, April 2007, 175, Abstracts Issue, A689.

2009 **Experimental hookworm infection: a randomised placebo controlled trial in asthma** J Feary, A Venn, K Mortimer, A Brown, D Hooi, F Falcone, D Pritchard, J Britton. European Respiratory Journal 2007, 34, Supplement 53, P951

(Appendix A)

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STATEMENT OF WORK IN THESIS

Clinical Trials

I was recruited into the projects that gave rise to this thesis as a research fellow in 2005. The original grant proposals and ethics applications had therefore been prepared before I became involved. I was involved in statistical analysis of the data and preparation of the manuscript for the dose-ranging study (Chapter 2), and I am the corresponding author for the resultant paper. For these reasons, and for completeness of the overall work, this study is included in this thesis. I was fully responsible for all aspects of the delivery of both randomised controlled trials, including recruitment of participants, ethics amendments and all the study visits (other than during a 10 day period in the asthma study); and I carried out all clinical measures. The only part of the visit I was not responsible for was the treatment allocation and the unblinding of the participant, due to the need to keep me blinded. I entered and cleaned the data and performed the statistical analyses with assistance from Dr Andrea Venn, the trial statistician. During and after the studies we experienced substantial media interest and I was responsible for writing briefings for the media and being interviewed on local and national radio and television.

Full blood tests and serum albumin tests were performed by the department of pathology at Nottingham City Hospital. Immunology tests were performed by scientists at the University of Nottingham in the School of Pharmacy under the supervision of Professor David Pritchard. Larvae culture and egg counts were performed by Dr Alan Brown who is also based in the School of Pharmacy.

Prior to starting the project, an agreement was made that I would take the lead in reporting clinical aspects of the project and Professor Pritchard's team would be responsible for reporting the laboratory outcomes. This is reflected in the publications that have arisen from the project.

Systematic review

I performed the systematic literature review and extracted potential relevant papers. After checking these papers and, where appropriate, their reference lists, I scored those suitable for inclusion according to recommended guidelines on methodological quality. I entered the data and performed the relevant meta-analyses. This process was then repeated by Dr Jo Leonardi-Bee and our results were cross-checked for discrepancies.

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AMP	adenosine monophosphate
AQLQ	Asthma Quality of Life Questionnaire
AUC	area under curve
CI	confidence interval
DD	doubling dose
ECRHS	European Community Respiratory Health Survey
FEV_1	one-second forced expiratory volume
FVC	forced vital capacity
IFN-γ	interferon-γ
IL	interleukin
ISAAC	International Study of Asthma and Allergy in Childhood
L3	stage 3 larvae
MOOSE	Meta-analysis of Observational Studies in Epidemiology
OR	odds ratio
$PD_{10}AMP$	provocation dose of adenosine monophosphate to reduce one-second forced expiratory volume by 10%
$PD_{20}AMP$	provocation dose of adenosine monophosphate to reduce one-second forced expiratory volume by 20%
PEF	peak expiratory flow
RQLQ	Rhinoconjunctivitis Quality of Life Questionnaire
TGF-β	transforming growth factor-β
T_H0	naïve T-helper cell
T_H1	type 1 T-helper cell
T_H2	type 2 T-helper cell
TNF-α	tumour necrosis factor

1 INTRODUCTION

1.1 Overview of thesis

Asthma, allergic rhinoconjunctivitis and eczema all belong to the spectrum of atopic or allergic disease and are some of the most common causes of chronic morbidity worldwide. Allergic disease affects around one in five children in high income countries and whilst the prevalence has increased over the last 30 years, it appears to have now reached a plateau [1-4]. The prevalence of allergic disease in low and middle income countries is generally lower than that in high income countries, but has increased rapidly over the last 30 years [5]. In the UK alone, asthma affects over 5 million people and causes around 1400 deaths each year [6]. Allergic rhinoconjunctivitis is estimated to affect around 15% of adults in the UK [7] and overall prevalence continues to increase [8]. Eczema affects around 5.8 million people in the UK, with the greatest burden of disease in young children [9-11].

Allergic disease occurs as a result of a complex interaction of genetic and environmental exposures [12], but marked changes in prevalence over recent decades, particularly in economically-rich countries, indicate that environmental factors play a key role. Many environmental factors have been posited to influence the development of allergic disease. One such factor is infection with parasites, particularly the soil-transmitted helminths (or geohelminths), which may be protective, and the association between parasite infection and allergic disease forms the basis of the work in this thesis. The finding of an inverse relation between hookworm infection and asthma [13] has prompted scientific interest in the possibility of a therapeutic role for hookworm infection in the management of

asthma and other allergic disease. This possibility has led to design and execution of a series of three intervention studies of intentional hookworm infection, carried out in the UK and presented in this thesis, to test the hypothesis that hookworm infection improves clinical control of asthma, and also to investigate the feasibility of using hookworm infection as a therapeutic agent for asthma. The first two studies comprised a dose-ranging study, described in Chapter 2, and a safety and feasibility study in people with allergic rhinoconjunctivitis and bronchial responsiveness, described in Chapter 3. These studies were primarily designed to determine the safety and tolerability of intentional hookworm infection in people. A third study was conducted to determine the efficacy of experimental hookworm infection on asthma and is described in Chapter 4. Chapter 5 contains the results of a systematic review of the literature and meta-analysis of the association between parasite infection and atopy. The thesis concludes with a summary and discussion of the clinical implications of the findings of these studies, and identifies areas for further research.

1.2 Definition of asthma

The benchmark definition of asthma was proposed by the 1958 Ciba Guest Symposium as “the condition of subjects with widespread narrowing of the bronchial airways, which changes its severity over short periods of time either spontaneously or under treatment”. This variable bronchoconstriction, and subsequent airflow obstruction, results in the clinical features of asthma - which are abnormal breathlessness (paroxysmal or persistent), wheezing and cough [14] - and is caused by several underlying processes. These include inflammation, due

to inflammatory mediators release during mast cell and basophil degranulation; abnormal contraction of bronchial smooth muscle, in response to a variety of stimuli; thickening of the airway wall, due to oedema, smooth muscle thickening and fibrosis formation; and mucus production in the airways. Asthma is characterised by bronchial hyper-responsiveness to triggers that would not normally cause bronchoconstriction, probably as a result of the underlying airway inflammation. These triggers vary between individuals and range from cold air and exercise, to stimuli such as histamine. The gold standard for measuring bronchial hyper-responsiveness is bronchial challenge testing which can be measured both by "direct" and "indirect" tests depending on the mode of action by the agent on airway smooth muscle contraction [15;16]. A variety of stimuli can be used, the most common in clinical assessment and trials being histamine and methacholine which act directly on smooth muscle contraction, and adenosine monophosphate which acts indirectly. This test involves the sequential inhalation of increasing doses of the stimulant whilst monitoring lung function, with the aim of establishing the provocation dose (or concentration) of stimulant required to reduce one-second forced expiratory volume by 20% [17]. Alternative methods for diagnosing asthma used in clinical practice and epidemiology studies include other objective measures of airflow obstruction such as the occurrence of exercise-induced bronchoconstriction and peak expiratory flow rate variability, and subjective assessment of the presence of symptoms.

1.3 Definition of atopy and allergy

Atopy is the capacity to produce IgE antibodies specific for common environmental allergens, and is a term also used to describe a predisposition to

develop allergic diseases. Allergy is a symptomatic response to normally innocuous environmental antigens. It should be noted that not all atopic responses will be associated with an allergic reaction: around a quarter of atopic individuals will develop clinically relevant allergic disease [18] and it has been suggested that around 40% of asthma is attributable to atopy at the population level [19]. Worldwide studies have suggested the relation between atopy and allergic disease may be linked to the economy of the country [20]. Atopy is diagnosed by testing for allergen skin sensitisation (a form of IgE-mediated immediate hypersensitivity reaction), most commonly using skin prick testing; other methods include intra-dermal and skin patch tests [21]. In skin prick testing, an allergen is introduced just under the surface of the skin where it encounters subcutaneous mast cells. If these are coated with IgE specific to that particular allergen (or “sensitised”), binding between the allergen and IgE will occur. Adjacent IgE molecules directed against the allergen then cross-link on the cell surface and initiate intracellular signalling, with mast cell activation and degranulation releasing vasoactive mediators and triggering *de novo* generation of inflammatory mediators. This results in a visible and measurable "wheal-and-flare" reaction characterised by a central area of superficial skin oedema (wheal) surrounded by erythema (flare).

1.4 The immune system and allergy

A brief description of the normal function of the immune system is necessary to place in context the changes, described later in this thesis, that occur in allergy, and with parasite infection. When potential pathogens are recognised by the immune system (identified by pathogen associated molecular patterns (PAMP) or

pattern recognition receptors such as Toll-like receptors and C-type lectin receptors), a cascade of events is triggered. First, the innate immune system is stimulated with activation of macrophages, neutrophils, Natural Killer cells and dendritic cells resulting in release of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. These cytokines stimulate the phagocytic actions of neutrophils and monocytes-macrophages and activate dendritic cells. In addition to their involvement in the innate immune response, these dendritic cells also have a crucial role as antigen presenting cells in the specific immune response which ensues. Once activated, dendritic cells degrade antigen peptides and present them on their cell surface to naïve T-helper (T_H0) cells; in turn, the T_H0 cells recognise the antigen via specific receptors triggering their transformation into type 1 T-helper (T_H1) cells, type 2 T-helper (T_H2) cells and T-regulatory lymphocytes [22;23]. If exposed to IL-12 from dendritic cells, macrophages and other cells, T_H0 cells develop into T_H1 cells and trigger cell-mediated immunity via production of IFN-γ which activates macrophages, enabling them to destroy intracellular parasites (such as viruses, fungi and Mycobacteria). In addition, Natural Killer cells are activated to destroy viruses and tumour cells and cytotoxic T-cells develop and to provide further immunity specific to the peptide presented by the dendritic cells. If, instead, the T_H0 cells are exposed to IL-4, T_H0 cells transform into T_H2 cells and produce several cytokines (specifically IL-4, -5, -6, -10 and -13), which trigger antibody formation by B-lymphocytes. The antibodies themselves are pro-inflammatory and cause activation of the complement cascade, which results in a further neutrophil-dominated immune response. It should also be noted that some cytokines also have a down-regulatory effect on parts of the immune system (Figure 1.1).

IgE is the principal antibody involved in the allergic response and is formed by the action of IL-4 and IL-13 after an allergen is encountered. If IgE directed against a specific allergen then binds to a mast cell, a type 1 hypersensitivity reaction follows characterised by mast cell degranulation and release of vasoactive mediator release (such as histamine, tryptase and chymase), which are chemotactic predominantly for eosinophils, but also for neutrophils. IgE also provides protection against extracellular parasites such as *Ascaris lumbricoides* and hookworm.

If an immune response is potentially harmful or inappropriate, for example, against self-tissues, food and pollen, it should be blocked or down-regulated through the development of tolerance. This involves formation of antigen-specific T-regulatory cells which produce the immunosuppressive cytokines TGF-β and IL-10 and which then block local immune reactions by other cells. In allergic disease, there is an inappropriate immune response to innocuous antigens driven by the T_H2 cells. In the past, it was thought that this might develop as a result of an immune system imbalance, with insufficient stimulation of the T_H1 arm resulting in an exaggerated T_H2 response. It is now thought that it occurs as a result of a failure in development of fully functioning T-regulatory cells leading to problems with both T_H2 driven reactions (causing allergic disease) and T_H1 driven reactions (resulting in autoimmune disease such as inflammatory bowel disease and multiple sclerosis) [23].

1.5 Prevalence of asthma

Accurate assessment of the prevalence of asthma is hampered by varying definitions of asthma and by different methods of data collection, making comparison of data across studies and globally difficult. Epidemiology studies commonly define disease using question-based criteria, for simplicity, to reduce costs and to increase participation. Where possible, questionnaires are used in combination with objective clinical measures of lung function and bronchial hyper-responsiveness such as peak flow variability, exercise testing and bronchial challenge testing. There are inherent problems with each method used and the biases vary with different approaches. For example, if asthma is diagnosed after clinical assessment, physician criteria will not be standardised and there will be a bias by access to healthcare. Questions pertaining to a history of symptoms will be biased towards more severe cases and influenced by recall bias. Presence of symptoms may not be specific to asthma. For example, wheeze in young children is a poor predictor of asthma [24], is often due to isolated viral respiratory tract infections [25] causing a degree of bronchospasm in narrow immature airways and may lead to an overestimation of asthma prevalence in these age groups. Meanwhile, bronchial challenge testing in the setting of an epidemiology study has a low positive predictive value for asthma [26].

Despite these challenges, two large ongoing international population studies to determine allergic disease prevalence using standardised methods have successfully collected a wealth of data from where much of our knowledge of the epidemiology of these conditions is derived. These are the European Community Respiratory Health Survey (ECRHS), which looked at young adults [27], and the

International Study of Asthma and Allergy in Childhood (ISAAC) [3]. The studies both use well validated standardised questionnaires (based on self-reported asthma symptoms in the preceding 12 months), which have been shown to have good specificity and sensitivity for both bronchial hyper-responsiveness and a diagnosis of asthma [28].

1.5.1 International Study of Asthma and Allergy in Childhood

ISAAC was established with the aims of identifying prevalence and trends in allergic disease in children worldwide, and providing a baseline framework to be used in investigating possible aetiological factors for these diseases. ISAAC Phase One, carried out between 1992 and 1998, involved the participation of 700,000 children from 1565 centres in 56 countries using written, and in some cases video, questionnaires [29]. In ISAAC Phase Two, which began in 1998, detailed questionnaires were carried out and objective measurements of indoor exposure and physiological variables were made in 30 centres in 22 countries [30]. Between 1999 and 2004, ISAAC Phase Three repeated the original cross-sectional survey on around 193,000 6-7 year olds and over 300,000 13-14 year olds to investigate trends in disease prevalence [1;3]. Baseline data were collected on 463,801 13-14 year olds and showed a marked variation between countries in prevalence of self-reported asthma symptoms, with the highest prevalence being about 20 times higher than in the centre with the lowest prevalence (range 1·6–36·8%) and an eight-fold variation seen between the 10th and 90th percentiles (3·9–30·6%). Prevalence of symptoms was greatest in the United Kingdom (UK), Ireland and Australasia (29.4-32.2%) and lowest in Indonesia, Albania and parts of Ethiopia and India (2.1-2.6%). Most countries had similar reported prevalence of symptoms, though large within-country variations were seen in India (1.6-

17.8%), Ethiopia (1.9-10.7%) and Spain (2.7-13.5%) [2;3]. ISAAC Phase III found that, in the (on average) seven year interval between the two studies, the prevalence of asthma had changed by one standard error or more in most of the centres. In 6-7 year olds, 59% of centres showed a change, with two-thirds of these having had an increase in prevalence. In 13-14 year olds, the prevalence changed in 77% of centres, evenly divided between increased and decreased prevalence. Generally asthma prevalence tended to increase in centres where mean asthma prevalence had been low, whereas in centres with already high mean prevalence, decreases were more common for both age groups [1].

1.5.2 European Community Respiratory Health Survey

The ECHRS is a survey of the prevalence, determinants and management of asthma in 20-44 year olds from 48 centres [31], the majority in Western Europe. It was first carried out in 1990 (ECRHS I) with follow-up studies of 11,168 participants in 1998-2002 (ECRHS II). As in ISAAC, significant variation both between countries and within countries was found in ECRHS I and, unsurprisingly, the prevalence of reported wheeze was greater than that of diagnosed asthma. In general, a similar geographical pattern was seen, with a higher prevalence of respiratory symptoms and asthma in centres in English-speaking countries and a lower prevalence in Mediterranean countries [27]. Wheeze prevalence ranged from 25-32% in Australasia, the United States, Ireland and the UK and parts of Spain, compared with prevalence of around 4% in India and Algeria. Diagnosed asthma was reported in 7-11% of those surveyed in the US, the UK and Australasia, and was as low as 2-4% in India, Algeria, Greece, Estonia, Iceland, and parts of Spain, France and Germany [27]. Again, there was evidence of within-country variation: for example, in Belgium, a relatively small

country geographically, prevalences were 21% and 5% for wheeze and asthma respectively in Antwerp city, compared with 13% and 3% in South Antwerp. Similarly, in Germany, asthma prevalence in Hamburg was over twice that in Erfurt (4.4% as against 2.1%). Follow-up studies 5-11 years later in some of these centres in ECRHS II found an overall increase of 0.8% in subjects reporting asthma attacks (95% CI: 0.2 to 1.4) and of 2.1% in the proportion of subjects using medication for asthma (95% CI: 1.6 to 2.6). Interestingly, there was no significant change in reported symptoms [32], possibly reflecting better control of disease. However, using data from ECRHS I and performing a retrospective analysis in 17,613 individuals, Sunyer *et al* demonstrated a progressive increase in yearly incidence of asthma between 1946 and 1971 by birth cohort, in 15 high-income countries, with a relative risk of 2.33 (95% CI: 1.81 to 2.98) in those born in 1966-1971 compared with those born in 1946-1950 [33].

Whilst change in disease classification and increased clinical awareness are probably responsible in part for observed changes in trends of disease, some of the changes will be due to modifications in the risk factors that cause and worsen asthma [34]. Asthma, and indeed other allergic diseases, traditionally affects those living in higher income countries. As described, they have increased in prevalence particularly in the second half of the last century and have probably now reached a peak [1]. The worldwide data from ISAAC, ECRHS and other studies suggest that it is in low and middle income countries where rapid increases in prevalence are now being seen [26;28;35]. In seeking to identify possible aetiological factors, studies have often focussed on countries within which variations in asthma prevalence have been noted. One such example is Ghana where the prevalence of exercise-induced bronchospasm in schoolchildren appeared to double between 1993 and

2003 and was much higher in urban rich schools (8.3%), compared with urban poor (3.0%) and rural (3.9%) schools [36]. Another example is Ethiopia, where prevalence of wheeze was found to be 3.7% in urban areas and 1.2% in rural areas [37]. The finding of such an urban-rural gradient is not unique to these studies and it has been observed that these changes in prevalence seem to occur as communities move from a rural subsistence lifestyle to one which is more urbanised [38;39]. Possible reasons for this are discussed in the next section.

1.6 Aetiology of asthma

1.6.1 Genes and environment

Whilst the aetiology of asthma is not fully established, it is accepted to be multi-factorial and to involve complex interactions between genetic susceptibility and environmental factors. Genetic studies of asthma, which initially looked at areas of broad linkage, have now identified several candidate genes and have revealed complex associations with, for example, differences in the links between ethnic groups [40]. However, evidence for a key role of environmental factors in the aetiology of asthma arises from two main observations. First, the increase over the last 50 years in worldwide prevalence of asthma and allergic disease is too rapid to be due to genetic factors alone. Secondly, as suggested above, significant variation in prevalence of allergic disease exists in genetically similar groups. For example, in a study of nearly 2000 Polynesian children, 11% of those living in Tokelau had a diagnosis of asthma compared with 25.3% of those living in New Zealand [41]. In another study, reversible airflow obstruction (provoked using exercise testing) was assessed in three different areas of Zimbabwe: positive tests were found in 5.8% of children in Northern Harare (high socio-economic

class urban children), 3.1% in southern Harare (low socio-economic class urban children) and 0.1% in Wedza Communal Land (rural children from peasant families) [38]. After the reunification of Germany, 9-11 year olds were studied in West Germany (n=5030) and East Germany (n=2623) and asthma was found to be significantly higher in West compared with East Germany based on a self-administered questionnaire (5.9% vs. 3.9%) and presence of bronchial hyper-responsiveness (8.3% vs. 5.5) [42]. Interestingly, within a few years, the prevalence in the Eastern and Western German populations had become similar [43]. Similarly, studies have demonstrated an increase in risk of atopy, wheeze and eczema in migrants as they move from areas of low allergy prevalence to areas of higher prevalence [44] with this apparent loss of protection from allergic disease occurring in a time-dependent manner [45].

These observations have resulted in a plethora of possible environmental factors being implemented in the aetiology of asthma and allergic disease. Many of these factors have been identified in studies from rural Europe and have the “hygiene hypothesis”, which is discussed below in more detail, as a central theme.

1.6.2 The Hygiene Hypothesis

The development of the immune system is probably influenced both *in utero* and during infancy by early environmental exposure to allergens and infections. In 1989, Strachan put forward the “hygiene hypothesis” to explain the observation that hay fever was associated with number of siblings in the household, with lower prevalences of hay fever in children with higher birth order, possibly as a result of exposure to more infections early in life [46]. Other studies - for example, where an inverse relation between attendance at day care and prevalence of asthma –

have supported this hypothesis [47-49]. Exposure to a farming environment has been shown in several studies to be protective against asthma in children [50;51], with meta-analyses identifying contact with livestock and poultry as specific key factors [52]. In addition, children exposed to stables and/or unpasteurised milk during the first year of life, regardless of where they lived, had substantial protection against the development of asthma and other atopic conditions. Prenatal exposure in this group appeared to have an additional effect [53]. These findings are not entirely consistent, however, with other studies identifying a protective effect of unpasteurised milk consumption on eczema and atopy, but not on asthma symptoms [54]. The role of infections has been looked at in detail. One retrospective study in the United States found positive hepatitis A serology to be protective against hay fever and asthma [55], a finding that was confirmed in other studies [56;57]; but the evidence for this and for other viral infections remains inconclusive [58-61]. Support for the protective effect on allergy of the traditional lifestyle has also come from studies on Steiner school children who follow an anthroposophic lifestyle. This lifestyle involves the minimal use of medications, delayed vaccinations, a lower use of antibiotics and paracetemol, and a diet consisting of organic or fermented vegetables. Children following this lifestyle were shown to have a much lower rate of IgE sensitisation, asthma, hay fever and eczema than children attending non-Steiner schools in the same area [62]. A systematic review and meta-analysis of the literature found that antibiotic use in the first year of life was associated with an increased risk of physician-diagnosed asthma in those aged 1-18 years (OR 2.05, 95% CI: 1.41 to 2.99) and also demonstrated a dose-response relation between numbers of antibiotic courses and risk of asthma or wheeze [63]. This association could of course be attributed to reverse causation. However, *in utero* exposure to antibiotics has also been shown to be associated

with wheeze and asthma in a dose-related response [64]. Endotoxin exposure in these environments may account for some of the reduction in allergic disease, with the suggestion that microbes combine with dendritic cells to cause induction of the T_H1 response via IL-12 [50]. However, subsequent studies specifically designed to test this hypothesis have been inconclusive [65]. The relation between parasite infection and asthma and other allergic disease is also closely linked to the hygiene hypothesis and is explored in detail later in this chapter.

1.6.3 The role of diet and paracetemol

Evidence from Steiner schools and the urban-rural studies led to the hypothesis that changes in diet as populations adopt less subsistence-based lifestyles affects occurrence of allergic disease. Whilst this hypothesis is based on biologically plausible mechanisms, the evidence for a causal relation between diet and asthma is inconsistent: whilst the general trend in observational studies is for the identification of positive associations, intervention studies have not confirmed clinically significant effects [66-68]. ISAAC identified an inverse relationship between the intake of complex carbohydrates and vegetables and prevalence rates of asthma, allergic rhinoconjunctivitis and eczema [69]. It also found a statistically significant positive relation between prevalence of the allergic diseases and intake of trans-fatty acids [70]. But whilst fish-oil supplements were found to reduce exercise-induced bronchoconstriction in asthma [71], further studies showed no evidence of an effect on asthma symptoms or medication usage [72]. Dietary deficiencies of antioxidants may contribute to the presence of asthma [73]: observational studies have shown Vitamin C intake to be associated with a reduced risk of asthma [66;74-77]; but this was not confirmed in longitudinal [78;78] or large intervention studies [79;79]. However, placebo-controlled studies of ozone-

induced bronchoconstriction found a protective effect of Vitamin C and E supplements in adults with asthma [80], and of a combination of Vitamin C, Vitamin E and β-carotene in a small study of Mexican street workers without asthma [81]. Longitudinal data suggested a protective effect of dietary vitamin E [78], but there is less cross-sectional evidence for this relation [66;74;75], and an intervention study of vitamin E supplements found no evidence of clinical benefit in asthma [82]. Low dietary intake of selenium has been identified in some patients with asthma [66;74;75], but this finding is not consistent [83] and two intervention studies of selenium found no objective improvement in disease [84;85]. Several studies of sodium intake have found conflicting results [86;87], but a recent large intervention study of a low-sodium diet (compared with a normal diet) found no effect on asthma [88]. Finally, it may not be individual nutrients which are important, but food groups or diet as a whole; for example, a recent systematic review and meta-analysis of observational studies found that a Mediterranean diet was strongly protective for wheeze [89].

Again, in line with data from the urban-rural studies and Steiner schools, there is increasing evidence that regular paracetemol use carries a dose-dependent risk of developing asthma [90]. Glutathione, an endogenous antioxidant, exists in high concentrations in the airway epithelial lining fluid [91]; local oxidant damage may occur if levels fall [92;93]. It is also involved in paracetemol metabolism [94], and high doses of the drug can cause a reduction in lung tissue levels of glutathione [95;96]. A case-control study of 664 people with asthma and 910 people without asthma in London found the risk of asthma to be related to paracetemol use, with a positive dose response such that there was a more than doubling in risk for daily users compared with never-users [97]. Similar dose-related responses were found using the Third National Health and Nutrition Examination Survey where daily use of

paracetemol was associated with almost a doubling in risk of diagnosis of asthma compared with never use [98]. This association is also seen in low income countries [99] which helps to exclude bias from medical contraindication to use of aspirin/non-steroidal anti-inflammatory drugs in asthma as knowledge of aspirin avoidance with asthma is rare in these countries. It also provides evidence against reverse causation, because use of analgesia in these countries is uncommon. Stronger evidence against reverse causation comes from longitudinal studies where paracetemol use in infancy was associated with wheeze between 1 and 3 years of age [100] and, in adulthood, where it was associated with an increase in new diagnosis of asthma [101]. Furthermore, the relation does not appear to be restricted to adults, and studies have shown that paracetemol use in pregnancy increases the risk of wheezing in the first year months of life [102-104] and persists in children of school age [104-106].

1.6.4 Other environmental factors

Other environmental factors have been identified in relation to the aetiology of asthma and allergic disease and studies continue to try and elucidate which of these factors are the most important [107;108]. Prenatal factors have been extensively researched. Prenatal maternal smoking is consistently associated with early childhood wheezing, and probably occurs (at least in part) because of decreased airway calibre in early life [109]. Indeed, a meta-analysis of studies of parental smoking prenatally and immediately after birth excluded an increased risk of allergic sensitisation in exposed children [110]. Mode of delivery may also be important, with development of atopy more common in children delivered by emergency Caesarean section (although not by elective Caesarean section) [111;112]; the evidence for this, however, is not consistent [113]. The relationship

between breastfeeding and allergy remains controversial, with some studies showing a protective effect [114;115] and others reporting a higher incidence of allergy and asthma amongst breastfed babies [116]. A meta-analysis of prospective studies in 2001 found a protective effect of exclusive breastfeeding during the first 3 months after birth on asthma between the ages of 2-6 years (OR 0.70, 95% CI: 0.60 to 0.81) [117]. The impact of maternal dietary manipulation during lactation on risk of childhood allergy has been explored, but a longitudinal study showed no association with atopic disease at 4 years of age [118].

Finally, exposure to air pollution may be important. Indoor air pollution, which includes smoke emitted from combustion of biomass fuels and coal, and environmental tobacco smoke, is associated with an increased risk of exacerbation of asthma in addition to other adverse health outcomes [119]. Exposure to outdoor air pollution in epidemiological studies is evaluated in different ways including measuring individual pollutants levels, or using surrogate markers for exposure to traffic-related pollution such as proximity of home to main roads and use of different modes of transport. There is reasonable evidence that air pollutants such as particulate matter and ozone trigger exacerbations of asthma [120] but whilst some studies have shown an effect of transport-related pollution on asthma and wheeze, the evidence is inconsistent [26;121]. Much of this evidence on air pollution arises from cross-sectional studies in people with pre-existing asthma. However, there is also evidence from longitudinal studies to suggest an association with new diagnoses of asthma; for example, one study from California found a greater risk of developing asthma in children who spent significant amounts of time exercising in areas of high ozone, compared with children who did not exercise [122]. Similarly, a large birth cohort study in the

Netherlands found an increased incidence of wheeze and physician-diagnosed asthma in children at 2 and 4 years of age exposed to greater levels of traffic-related air pollution [123;124]. There is also longitudinal evidence suggesting a detrimental effect on lung development with one study reporting that adolescents living within 500m of a motorway in the United States had lower increases in lung function over an eight year period compared with those who lived beyond 1500m [125;126].

1.7 Helminth infection

1.7.1 Classification of helminth infection

The evidence for an association between helminth infection (in particular hookworm infection) and allergic disease, and the relative influence of different species, is discussed in detail in sections 1.9 and 1.10. The helminth family includes the nematodes (roundworms), trematodes (flukes) and cesatodes (tapeworms) classes. Most of the evidence is for geohelminths, namely hookworm, *Ascaris lumbricoides*, *Trichuris trichiura* and *Enterobius vermicularis* (Figure 1.2). The two main hookworm species causing significant disease in humans are *Necator americanus* and *Ancylostoma duodenale*. In addition, three zoonotic hookworm species can infect humans but cause less serious pathology: *A. caninum* (dog hookworm), which can cause human eosinophilic enteritis; *A. braziliense*, which may cause cutaneous larva migrans, a self-limiting pruritic eruption (usually on the feet) with burrows up to 5cm in length; and *A. ceylanicum*, which may infect cats, dogs and humans but is generally asymptomatic [127].

Schistosoma species, belonging to the trematode class, have also been found to be associated with allergic disease and are also discussed in this thesis. Those infecting humans include *Schistosoma mansoni* and *S. intercalatum* (causing intestinal Schistosomiasis), *S. japonicum* and *S. mekon* (causing Asian intestinal Schistosomiasis) and *S. haematobium* (causing urinary Schistosomiasis). Their lifecycle is very different from that of the geohelminths in that it includes a snail as an intermediate host.

1.7.2 Epidemiology of helminth infection

Between one and two billion people worldwide, or a quarter of the global population, are estimated to be chronically infected with geohelminth infections [128;129]. Estimates for individual species range from 800 to 1500 million for *A. lumbricoides,* 600 to 1100 million for *T. trichiura* and 575 to 1300 million for hookworm [129;130]. Geohelminths require warm wet climates in which to thrive and be transmitted and this is reflected in their geographical distribution. Infection is associated with poor sanitation, poverty and lack of access to clean water [131]. The areas of highest prevalence of infection are sub-Saharan Africa and Papua New Guinea, followed by China, India, east Asia and the Americas [132]. Of the two main hookworms, *N. americanus* tends to predominate in the countries listed above (other than India), whereas *A. duodenale* predominates in India, North Africa and the Middle East. Schistosoma infection is also very common, with *S. mansoni* and *S. haematobium* affecting 170 million people in sub-Saharan Africa alone and many more being at risk [130]. The intensity of infection for *A. lumbricoides* and *Schistosoma* species peaks during childhood and adolescence. With hookworm, however, there is more variation: in so far as there is a general pattern, it is for a steady increase during childhood, followed by a peak or plateau in adulthood [133].

These trends are reflected in the literature, where the majority of studies have been performed in children.

1.8 Hookworm infection

1.8.1 Lifecycles of helminth infection

N. americanus, the species on which much of this thesis is focused, infects its human host via the cutaneous route by stage 3 (L3) larvae [134]. These larvae, measuring 600 microns in length, burrow through the skin and probably then enter the blood circulation, passing via the right side of the heart to the pulmonary vasculature. Here, usually around ten days after cutaneous infection, larvae rupture into the alveoli, migrate up the bronchial tree into the proximal airways and are expectorated and swallowed. Once in the duodenum, they remain and undergo two molts, maturing into adult male or female hookworm measuring 5-13mm in length at around weeks 6 to 8 [135]. Sexual reproduction occurs, and females lay thousands of eggs every day, which pass out in the host's faeces. In an optimal warm moist environment, the eggs then hatch into stage 1 larvae within 48 hours. These stage 1 larvae molt twice to develop into L3 larvae which can survive on their lipid metabolic reserves for several weeks. The optimal conditions for them to be transmitted to humans, usually through the feet, are in areas with high moisture and silted sand; worldwide rates of infection therefore tend to coincide with countries where walking barefoot, particularly in rural areas, is normal practice [136;137]. Adult hookworms typically live in the host for about five years, although survival has been reported for up to 18 years [138]. The lifecycle of *A. duodenale* is similar to that of *N. americanus*, but infection may occur both cutaneously or after ingestion of eggs.

Humans are also the only major definitive host for the other geohelminth infections *A. lumbricoides* and *T. trichiura*. However, in contrast with *N. americanus*, people become infected with these parasites after ingesting the fully mature eggs [129]. *A. lumbricoides* has a similar lifecycle to *N. americanus*; after ingestion, the larvae penetrate the intestinal mucosa and are carried via the portal and then systemic system into the lungs. Here they mature further before moving through the alveoli walls, ascending the bronchial tree into the proximal airways and are expectorated and swallowed, re-entering the gastrointestinal tract. Approximately ten weeks after egg ingestion, they develop into adult worms and reside throughout the small intestine. Conversely, *T. trichiura* has no pulmonary or systemic phase in its lifecycle and following ingestion, the eggs enter the small intestine where they hatch into larvae develop into adult worms in the large intestine around 12 weeks after egg ingestion.

The lifecycle of *Schistosoma* species are somewhat different from the geohelminth in that they requires a fresh-water source and an intermediate snail host. Eggs are released from the host via faeces or, in the case of *S. haematobium*, in the urine. On contact with water, the egg releases a miracidium which swim and penetrates the snail host [139]. Here the miracidium multiple asexually into sporocysts and then develop into cercarial larvae which are released by the snails into the water. These microscopic cercariae then seek out and penetrate the skin of the definitive human host. Once through the skin, they migrate in the vasculature through the lungs and into the liver where they transform into young worms or schistosomulae. They mature in the portal vein and migrate to the venous system of the gastrointestinal tract (*S. mansoni*) or the

bladder (*S. haematobium*). Here, the females lay eggs which move from the venules into the lumen of intestine (or bladder) and are expelled.

1.8.2 Immune modulation in helminth infection

Helminth infections induce polarised T_H2 responses [140], elevated serum IgE titres and eosinophilic-rich tissue inflammation. Yet helminth infections also appear to be inversely associated with allergic disease where as described in section 1.4, a similar T_H2 response to allergens is seen. Extensive research, much of which has been undertaken using murine models, is slowly elucidating the reasons behind this apparent paradox, and a number of possible mechanisms involved in this immune modulation have been postulated. Helminths have developed defences against host immunity which are necessary for their survival. These defences centre on modulation of the immune system and specifically include suppression of antigen-specific immune responses, modulation of the host immune response from protective to non-protective response and inactivation of immune effector mechanisms [133;138;141]. There is now epidemiological evidence that these effects may be particularly pronounced when exposure to infection occurs in early life [142]. It is thought that a consequence of these effects on the host immune system is a suppressed response to other environmental allergens, conferring a degree of protection in the host against allergic disease. There is also evidence that this decline in host immune responsiveness during infection reverses following parasite eradication [133]. A full discussion of research to date and possible mechanisms explaining the underlying immune modulation is beyond the scope of this thesis; however a brief overview follows, by way of a background to the epidemiology discussed and the intervention studies presented in this thesis.

One early suggestion was the IgE blocking hypothesis [143;144]: this posited that polyclonal total IgE production induced by the helminths saturates the IgE receptor binding sites, stopping binding of allergen-specific IgE and subsequent mast cell or basophil degranulation. There is now little evidence to support this as a key mechanism [145;146]. Over the last 15 years, several hookworm products have been identified, predominantly in animal models, which may contribute to the immune modulation either via a direct effect on the allergic response or through effector mechanisms. These include inactivation of complement-mediated haemolysis and subsequent inflammatory responses by a calcireticulin [147;148]; modulation of T-cell function by potassium channel blockage via a kaliseptine-like molecule [149]; digestion of eotaxin by metalloproteinase secretion controlling tissue eosinophilia [150]; and tissue damage from superoxide dismutase secretion (in response to host phagocytes) and production of hydrogen peroxide. Glutathione S-transferase secretion also protects the hookworm against lipid peroxidation from the host immune response and from the potential effects of its own superoxide dismutase/hydrogen peroxide combination [151;152]. Necator product ES-62 has been shown to inhibit mast cell degranulation [153;154], and other Necator secretory products have been shown to induce T-lymphocytes apoptosis [155].

It is likely, however, that the more important mechanism is induction of a regulatory network by hookworm and other helminths, with T-cell hypo-responsiveness, resulting in suppression of the host immune system. This network involves activity of both T-regulatory and B-regulatory cells [156], and modulation of innate immune cells such as macrophages, dendritic cells and local stromal cells. In turn, this results in an anti-inflammatory environment characterised by high levels of IL-10 (produced by both T and B cells) and TGF-β

[141;157-159]. A key role for IL-10 in this setting has been suggested by the observations of several epidemiology studies [160-162] where, for example, an inverse association between the degree of infection with Schistosomiasis and atopy was associated with the amount of serum IL-10 present [163]. IL-10 and/or TGF-β probably interfere with allergic effector mechanisms by inhibiting mast cell degranulation or T_H2 cell proliferation [141;164]. A role for T-regulatory cells was identified with the observations that forkhead box P3 (FoxP3), a gene expressed on T-regulatory cells and a key regulator in development and function of the T-regulatory cells, protects against allergic disease [165]; and that patients with mutations in the gene show immune-mediated pathologies, including allergy and asthma [166]. Further evidence in support of this hypothesis comes from the observation of proliferation of T-regulatory cells and an increase in IL-10 in successful allergen-specific immunotherapy (resulting in a decrease in allergic symptoms) [167;168]. The changes in T-cell functioning may also be directed towards "bystander" antigens such as vaccine antigens, providing an explanation for the reduced immune responses to Bacillus Calmette-Guérin and cholera vaccines previously observed with chronic helminth infections [169]. Despite the progress made in this area, studies have not always identified changes in IL-10 levels with helminth infection either in healthy individuals [170] or people with atopic disease, indicating that further research is needed [171].

There is also consistent evidence that helminth products fail to induce conventional dendritic cell maturation [172] and that they inhibit PAMP-induced dendritic cell activation, which may impair T_H1 development and bias the immune response toward T_H2 and regulatory responses [159]. Airway epithelial cells and their derived cytokines (in particular, IL-25 and IL-33 and thymic stromal

lymphopoietin), through their interactions with dendritic cells, may also have a role in influencing the immune system in helminth-infected individuals [159]. Induction of alternatively activated macrophages by helminths may also directly suppress T-cell effector functions and thus play a part in suppression of allergic disease [173].

1.8.3 Antibody response to hookworm infection

In natural hookworm infection, there is a strong immune response with elevated levels of all five isotypes of serum antibodies occurring after weeks 2-8 [174]. Specifically, there is marked up-regulation of polyclonal IgE [175;176], raised levels of serum polyclonal IgG and intestinal IgG, IgM and IgE [177;178] and reduced total intestinal IgA [178]. Most types of anti-hookworm antibodies show cross-reactivity with other helminth infection such as *A. lumbricoides* and *S. mansoni* [179-181], but IgG4 or IgE responses are more species-specific [177;182;183]. In primary intentional infections, specific and total IgE responses are sometimes low [184], but with repeated infection they increase progressively and, in such cases, antibody levels reflect cumulative exposure rather than current parasite infection levels [185].

1.8.4 Adverse effects of hookworm infection

In addition to the possible relation between hookworm infection and allergic disease, there are, of course, other symptoms and health consequences of acquiring infection whether naturally, or as a result of iatrogenic intervention. The majority of infections do not result in clinical disease; however, morbidity as a result of infection is closely related to intensity of infection and tends to be most apparent in young children and pregnant women [186]. In response to these effects, the World Health Organisation therefore now advocates anti-helminth treatment for all at-risk school-aged children [187] although re-infection usually occurs within a

few months, necessitating the use of frequent repeated treatments and giving rise to concerns about drug resistance. Consequently, a collaborative initiative, the Human Hookworm Vaccine Initiative (HHVI), has developed an anti-hookworm vaccine (Na-ASP-2) and preparatory work and phase I studies and are currently in progress [188;189].

1.8.4.1 Anaemia

Iron deficiency anaemia is the most important complication of naturally acquired hookworm infection and results in significant morbidity and mortality worldwide [190]. For example, up to 38 million women of reproductive age may be infected with hookworm in sub-Saharan Africa alone [191] and hookworm-related anaemia in pregnancy can cause low birth weight, impaired milk production, and increased risk of maternal and child mortality [192]. Adult hookworm attach to the duodenal mucosa using a cutting plate, contract their muscular oesophagus and use negative pressure to pull a plug of tissue into their buccal mucosa. Capillaries and arterioles rupture both mechanically and by the action of hydrolytic enzymes. The hookworm then releases anti-coagulative agents, allowing ingestion of extra-vasated blood amounting to around 0.3mls/ blood per *N. americanus* worm per day (and probably more in *A. duodenale*) [136;193]. Total blood loss will depend on worm burden, and though probably negligible in well-nourished people who have no other causes of anaemia, is likely to be much more significant in people with poor nutrition, in children and in pregnant women.

1.8.4.2 Rash

A pruritic erythematous maculopapular rash (“ground itch”), often with visible tiny blood speaks representing entry portals, occurs at the time of cutaneous infection

with both *N. americanus* [194] and *A. duodenale* [195]. This appears within minutes and lasts approximately 48 hours before disappearing, but often reappears a few weeks later [184;194]. A skin biopsy taken from the penetration site five days after infection showed a perivascular inflammatory lymphocytic infiltrate with occasional presence of eosinophils, although in the same study a biopsy from another infected individual was unremarkable [195].

1.8.4.3 Gastrointestinal

The peripheral blood eosinophilia which starts to occur around five weeks after infection can cause an eosinophilic gastroenteritis associated with a range of symptoms such as nausea, anorexia and abdominal pain [194;195]. These symptoms are generally self-limiting, abating with the reduction in eosinophilia at around eight weeks after infection [194].

1.8.4.4 Respiratory

There is a theoretical chance of developing respiratory symptoms with parasite infection due to a pulmonary eosinophilic syndrome, or local effects of larval migration. These are rare, however, and tend to be associated with tissue nematodes (particularly *Onchocerca*), *A. lumbricoides* or canine hookworm species [196;197]. One study performed bronchoscopy and bronchoalveolar lavage on four normal volunteers eight to 21 days after being infected with 50 *N. americanus* larvae and found bronchial mucosa erythema in all volunteers [195]. Lavage samples were no different from normal or pre-infection samples in all the volunteers with the exception of one whose lavage contained 2% eosinophils. Wright and Bickle described “mild rawness” in the lower respiratory tract in the third week after intentional hookworm infection; however, they did not perform any

objective clinical assessment of airway functioning, and the infected individual in this study was fully aware of the potential side effects of infection, which may have biased his reporting [170]. There are no other reports in the literature of respiratory symptoms occurring as a result of *N. americanus* infection.

1.8.4.5 Endomyocardial fibrosis

Helminth infection has been implicated as a causal agent in endomyocardial fibrosis, a form of restrictive cardiomyopathy which occurs mainly in populations living in the rainforests of Africa, South India, South America and Thailand [198]. It is thought to occur as the end-stage result of a sustained parasite-induced hyper-eosinophilia [199], though a high eosinophil count may also be a risk factor independent of parasite infection [200]. Other aetiological hypotheses include the effects of poor diet, poverty and geochemical exposures. Although hookworm infection is often listed with other helminths as a potential cause of eosinophilia leading to endomyocardial fibrosis, there are no reports in the literature of it occurring in people infected with hookworm alone, and only two reported cases of it occurring in individuals who were concurrently infected with *A. lumbricoides* [201;202]. In fact, the tissue-based filarial nematodes are the most frequently implicated helminth in inducing eosinophilia causing endomyocardial fibrosis [196;197;203], followed in frequency by *Schistosoma* species. Given that parasite infection is endemic in the tropics, the risk of developing endomyocardial fibrosis with hookworm infection is therefore minimal.

1.9 Observational studies of helminth infection and allergy

There is now a growing body of epidemiological evidence suggesting that intestinal parasite infection is associated with a reduced risk of allergy, and that hookworm infection in particular may protect against wheeze [13], hay fever and atopy [146]. This association is likely to vary according to species, age of acquisition of infection and burden of infection [13]. A likely consequence of one or more of these factors is that the results of studies in the literature are sometimes contradictory. Other explanations for the conflicting nature of the results include the heterogeneity of the study designs and study populations, and the fact that multiple parasite infection is common in areas of endemic infection. Finally, there is always the chance in observational studies that residual or unmeasured confounders may explain any observed associations.

1.9.1 Helminths and asthma

A systematic review and meta-analysis of the relation between parasites and asthma was reported in 2006 [13]. No significant association was found in pooled analyses between infection with *T. trichiura*, *E. vermicularis* or *Strongyloides stercoralis*, and asthma. However, the pooled results of nine studies of hookworm showed a 50% reduction in the risk of asthma with infection (OR 0.50, 95% CI: 0.28 to 0.90; p=0.02) [13]. In contrast, the pooled results of 20 studies of *A. lumbricoides* identified a significant, increased risk of asthma with infection (OR 1.34, 95% CI: 1.05 to 1.71; p=0.02) [13].

Since this review was published, several further studies have been reported. The first, in South Africa, found that isolated *A. lumbricoides* infection was associated

with an increased risk of exercise-induced bronchospasm (adjusted OR 1.87, 95% CI: 1.19 to 2.95), whereas dual infection with *A. lumbricoides* and *T. trichiura* showed no association [204]. The second study was performed in Brazil: here nearly 20% of children had helminth infection, of which the most common were *A. lumbricoides* (12%) and *T. trichiura* (5%). Wheeze and bronchial hyper-responsiveness were significantly associated with *A. lumbricoides* infection at higher loads (>100 eggs/g faeces), but no association was seen for diagnosed asthma or when all helminth infections were considered together [205;206]. The third study, carried out in 1320 children in Cuba, found no relation between current helminth infection (*A. lumbricoides*, *E. vermicularis*, *T. trichiura*, or hookworm) and wheeze, although prevalence was below 5% for the last two of these infections [207]. The fourth study was carried out in Ecuador in over 3500 children, of whom 75% had geohelminth infection. Heavy infection with *T. trichiura* was found to be significantly associated with reduced risk of wheeze in those with positive allergen skin sensitisation; in contrast, no relation was identified between *A. lumbricoides* infection and presence of wheeze [208]. The fifth study was undertaken in 682 school children in Brazil and found that current infection with *T. trichiura*, but not *A. lumbricoides*, was significantly associated with a reported history of wheeze in the last 12 months (adjusted OR 2.60, 95% CI: 1.54 to 4.38) [209]. A sixth study was carried out on over 1000 children in Thailand, and found that hookworm was significantly associated with physician diagnosed wheeze, in contrast, no association was seen for infection with *A. lumbricoides* or *T. trichiura* [210]. The final study was carried out in Uganda and comprised a small case-control study nested within a trial of deworming in pregnancy. The authors found that infection with hookworm was significantly less frequent in women with a history of asthma but no such association was found with Schistosomiasis [211].

1.9.2 Hookworm and asthma

In the meta-analysis described in the previous section, an inverse association between hookworm infection and asthma was identified [13]. The nine studies which looked specifically at this relation are described here in more detail. The first five studies were all case-control studies performed in the 1970s and showed no association between hookworm and asthma in the analyses presented which were all unadjusted for potential confounders. However, except in one study, numbers of either cases or controls were small and the individual studies were therefore unlikely to be adequately powered to show a significant effect (if one did exist). Alshishtawy *et al* studied 68 cases with a clinical diagnosis of asthma and 37 non-asthmatic controls in Egypt. Three cases and just one control had hookworm infection producing an OR of 1.66 (95% CI: 0.17 to 16.56) [212]. Carswell *et al* performed two studies, both with small numbers of cases, in Tanzanian children. The first compared eight children with exercise-induced asthma with 97 non-asthmatic controls. Two of the seven children with asthma from whom stool samples were obtained tested positive for hookworm (29%), compared with 25 of the 97 controls (26%) (OR 1.15, 95% CI: 0.21 to 6.32) [213]. In the second study, 18 children with exercise-induced asthma were compared with 224 normal controls, with no significant difference found in hookworm infection between those with asthma (21%) and normal controls (23%) (OR 0.92, 95% CI: 0.24 to 3.46) [214]. Tullis *et al* reported results from a study in Canada where stool samples from 201 hospital inpatients with asthma were examined. Just two of these patients were found to have hookworm ova, compared with none of 20 non-asthmatic controls (OR 0.51, 95% CI: 0.02 to 11.07) [215]. Cheah and Kan examined stools from 108 cases of asthma and 300 controls of adults and children living in Singapore, and reported 11 cases and 19 controls to have evidence of hookworm infection (OR

1.68, 95% CI: 0.77 to 3.65) [216]. Salako and Sofowora reported the results of a study from Nigeria which was the only study presenting an unadjusted analysis to find a protective effect of hookworm infection on asthma. Here, *N. americanus* was found in 35 out of 250 cases of asthma (14%), compared with 44 out of 100 controls (44%) (OR 0.21, 95% CI: 0.12 to 0.35) [217].

Three larger studies in Ethiopia - two cross-sectional surveys and one case-control study - have been carried out in the last ten years and have reported adjusted analyses. Scrivener looked at 205 cases of self-reported wheeze and 399 adult controls. Hookworm was present in 24% individuals and was associated with a significant reduction in the risk of wheeze (adjusted OR 0.48, 95% CI: 0.24 to 0.93; p=0.03) [58]. Dagoye *et al* studied 7155 children and found the prevalence of hookworm infection to be 10%. Wheezing in the preceding year was reported in 3.4% of children and was negatively associated, though not at a statistically significant level, with hookworm infection (adjusted OR 0.6, 95% CI: 0.2 to 1.8) [218]. Davey *et al* found similar results in 7649 children and adults [219], with a prevalence of wheeze in the preceding year of 10.5% and "asthma ever" in 2% of responders. Hookworm was found in 14.7% of these participants, with no statistically significant relationship between the asthmatics and controls (adjusted OR 0.89, 95% CI: 0.48 to 1.63) or "wheezers" and controls (adjusted OR 0.92, 95% CI: 0.73 to 1.14).

In addition to the studies in the meta-analysis, three further recent studies have also investigated the association between hookworm infection and asthma. The first was carried out in Thailand as part of the Prospective Cohort Study of Thai Children, a birth cohort study of children from four rural areas born in 2001 and

which includes over 1000 children [210]. Data were available from 706 of these children and in contrast to most of the previous studies, the authors found current hookworm infection to be strongly related to physician-diagnosed wheeze in the last six months (adjusted OR 4.20, 95% CI: 1.45 to 12.10). The second, a small nested case-control study carried out in Uganda, found an significant inverse relation between hookworm and asthma. The authors compared 20 women with a history of asthma with 117 non-asthmatic controls; four of the cases and 62 of the controls had had hookworm infection within the past two years (adjusted OR 0.24, 95% CI: 0.07 to 0.81) [211]. Finally, another birth cohort study currently in progress in Butajira, Ethiopia has looked at the association between infection and asthma or wheeze. The first analysis was carried out on 899 children when they were aged one year to look at risk factors for wheeze and eczema. Faecal samples were collected for analysis, but the prevalence of infection with any helminth was less than 4%, with hookworm being the most common, and whilst wheeze was lower in the uninfected than the infected children, the numbers were too low to derive a meaningful estimate of effect [100;220].

1.9.3 Relation between intensity of helminth infection and asthma

In the meta-analysis, five studies (three from Ethiopia [58;218;219] and two from Central/South America [221;222]) presented sufficient data to allow a pooled analysis on effects of burden of infection. A significant dose-related reduction in risk of both asthma and wheeze was seen with increasing hookworm infection, with the risk of asthma in the highest tertile of infection being reduced by approximately two-thirds compared with those with no infection (adjusted OR 0.34, 95% CI: 0.19 to 0.62) [13]. Much of this effect was due to the study by Scrivener *et al* who reported an adjusted OR for the lowest tertile of 0.54 (hookworm eggs 1.00-5.79

per gram of faeces (/g faeces)), 0.60 for the middle tertile (5.80-48.87 eggs/g faeces) and 0.30 for highest tertile (>48.88 eggs/g faeces), all compared to zero eggs [58]. No significant effects on asthma or wheeze were seen for different intensities of infection with *T. trichiura* or *A. lumbricoides* in the studies from the meta-analysis [58;218;219;221;222]. Since then this was published, a study from Brazil reported *A. lumbricoides* infection at higher loads (>100 eggs/g faeces) to be significantly associated with wheeze and bronchial hyper-responsiveness (OR 1.8, 95% CI: 1.0 to 3.0) but not diagnosed asthma [205;206] and in a study from Ecuador heavy infection with *T. trichiura* (>490 eggs/g faeces) was found to be significantly associated with reduced risk of wheeze in participants with positive allergen skin sensitisation (adjusted OR 0.24, 95% CI: 0.09 to 0.63) [208].

1.9.4 Helminths and allergic rhinoconjunctivitis

The first report of the observed association between parasites and allergic rhinoconjunctivitis was by Preston, a naval officer, who in 1970 observed fewer symptoms of hay fever amongst those on board his ship who were infected with parasites [223]. More recently, however, cross-sectional studies of the relation between parasites and allergic rhinoconjunctivitis have been reported which demonstrate little convincing evidence of a significant association. One explanation for this might be that the studies have mainly been performed in areas of endemic *A. lumbricoides* infection and different parasite species are likely to exert different effects on allergic disease. Lynch *et al* found that allergic rhinoconjunctivitis was significantly more common in Venezuelan children living in urban areas than in those in rural areas; however, infection with *A. lumbricoides* was equally prevalent in urban and rural areas, which suggests that other factors were responsible [224]. Two cross-sectional studies of children, one in rural Ecuador

(n=1002) [225] and one in South Africa [226] (n=53), found no significant relation between allergic rhinoconjunctivitis symptoms and current *A. lumbricoides* infection. Similarly, a study of 1320 children in Cuba found no association with *A. lumbricoides*, *E. vermicularis*, hookworm or *T. trichiura* and allergic rhinoconjunctivitis [207]. Finally, in contrast to this last study, Huang *et al* reported a protective effect of *E. vermicularis* against rhinitis in 3107 children in Taiwan (adjusted OR 0.61, 95% CI: 0.45 to 0.84) [227].

1.9.5 Helminths and eczema

Eczema is a complex disease, and a particular problem with large epidemiological studies is the challenge of making a reliable diagnosis when using self-reported questionnaires. Itchy rashes are common in children, and are often incorrectly attributed to eczema, which can cause an overestimation of prevalence. Even when using clinical examination to confirm a diagnosis, the relapsing-remitting nature of the condition may lead to an underestimation of prevalence. This may, in part, explain the mixed findings of the studies that have investigated the relation between parasites and eczema. Some studies have shown an inverse relation between eczema and any helminth infection (predominantly hookworm) [228]; current infection with *A. lumbricoides* [207;229] and a history of infection with *A. lumbricoides* [230]. In contrast, others have found a positive association between eczema and *T. trichiura* infection [231]; a history of infection with *E. vermicularis* [207] and a history of mixed parasite infection (predominantly *A. lumbricoides*) [232]. Moreover, no association between eczema and helminth infection was identified in three other studies where *E. vermicularis* [227], geohelminths (*T. trichiura*, *A. lumbricoides* and hookworm) [221] and “any parasite” (hookworm, Mansonella perstans and *S. mansoni* [211] were the predominant infections.

1.9.6 Helminths and atopy

The association between parasite infection and atopy is much less clear. In almost all epidemiological studies atopy is defined as the presence of positive allergen skin sensitisation and these terms are therefore used interchangeably in this thesis. Where another definition of atopy has been used, such as the presence of specific IgE, it will be explicitly stated. Studies have explored the relation between atopy and parasites generally, and specifically between atopy and helminths and individual species. These studies have found mixed results [163;209;211;221;225;230;232-238]. In view of this, as part of the work forming this thesis, a systematic literature review of the relation between atopy and parasite infection, and meta-analysis of the identified studies, were performed. The results of this review and meta-analysis are presented in Chapter 5.

1.10 Intervention studies of helminths and allergy

Most of the evidence for an association between parasites and allergy arises from observational studies. The majority of these are cross-sectional in design and, as such, can only demonstrate a relation between exposure and outcome, rather than a temporal association or causality. Cohort studies are the only observational study which can show a temporal association, but for several reasons, there are limited numbers of these investigating the relation between parasites and allergy, including the significant length of time the studies take to complete and the large numbers of recruits into the cohort that are usually required. Alternatively, intervention studies may also identify whether a temporal association between parasites and allergic disease exists. These need to look at the effect on allergic symptoms, either after infection of parasite-naïve individuals or after eradication of

parasite infection from areas where it is endemic. Because of the apparently strong inverse relation between hookworm infection and asthma, a series of intervention studies using *N. americanus* was planned and carried out, and forms part of this thesis.

1.10.1 Previous studies of deliberate helminth infection

Several previous intervention studies, with a variety of objectives, of intentional infection with *N. americanus* have been described in the literature. There are two early case reports in the literature describing intentional infection with *N. americanus* and its longevity. In the first, reported in 1941 by Palmer [239], an undetermined number of cultured *N. americanus* larvae were given in one dose to a healthy male and faecal egg counts were measured at regular intervals during the course of infection. A plateau of egg production was attained by the 11th month and remained constant for six years, subsequently declining until egg production ceased at 15 years [240]. In the second case report, reported by Beaver in 1951, a healthy volunteer was given three larvae and faecal egg counts were monitored thereafter. Eggs counts were 1000/g faeces during the first year, 1500/g faeces over the next five years, reducing to less than 200/g faeces over the last three years with egg production ceasing 18 years after infection [138].

Cline *et al* infected 30 healthy volunteers with 45 hookworm larvae to establish efficacy of eradication of infection with a single dose of albendazole. Six days after infection, volunteers were randomised to receive one of the following: albendazole plus a meal, albendazole plus instructions to fast for 24 hours, or placebo. Cutaneous reactions in all volunteers except one, and gastrointestinal symptoms, were reported in those who developed sustained infection. The single

dose of albendazole was unsuccessful in eradicating the infection given six days earlier in 13 of the 21 volunteers (regardless of whether or not they had fasted) who went on to develop established infection, although geometric mean egg counts on day 56 post-exposure were significantly lower in the albendazole groups as against the placebo group (72 vs. 268 eggs/g faeces) [194].

Turton described self-infection with 250 larvae on four occasions over 26 months, (starting in October 1974,) with the two-fold purpose of examining the haematological response to the infection and obtaining a supply of larvae for antigen preparation [241]. These hookworms produced between 3500 and 5000 eggs/g faeces and, interestingly, Turton's pre-existing symptoms of hay fever abated and he remained symptom-free at the time of publication in 1976. This is the only case report in the literature where self-infection caused remission of hay fever symptoms. In 1978, Ogilvie *et al* reported the clinical and antibody responses following Turton's self-infection. Gastrointestinal symptoms were reported from days 25 to 70 after each infection and were most marked following the first infection. A pronounced eosinophilic leucocytosis was also reported with each infection and no significant changes were seen in red cell parameters. Total IgE rose after the second infection and continued to increase after each subsequent infection and over time to more than 500 U/ml. A rise in specific IgE to hookworm antigen occurred after the third and fourth infections. Antibodies to the adult worm secretions and to extract of L3 larvae were detected six weeks after the first infection and rose again after subsequent infections. Antibodies to *Necator* acetylcholinesterase were detected 12 weeks after the second infection and also rose after subsequent infections [184].

In 1987, Maxwell *et al* infected five healthy volunteers with 50 infective larvae of *N. americanus* in order to observe the clinical and immunological responses over 60 days. All volunteers reported gastrointestinal symptoms of flatulence and epigastric/abdominal pain between days 30 and 45, with some also describing nausea, diarrhoea and vomiting. Three described these symptoms as mild to moderate, one as moderate to severe and one had severe symptoms necessitating eradication treatment on day 40. Faecal eggs were identified in three of the volunteers between days 48 and 58 (500-1200 eggs/g faeces); one volunteer temporarily lost to follow-up after day 45 had a count of 200 eggs/g faeces on day 113. The fifth volunteer was treated on day 40 and had never had eggs identified. Eosinophil counts increased after two to three weeks and peaked between days 38 and 64 at between 1.35 and 3.83 $x10^9$/litre. No significant change was seen in other blood leucocytes counts. IgG-specific antibody responses to *N. americanus* antigen, measured by enzyme-linked immunosorbent assay, rose by 1.5-3.0 fold titres, and peaked after around 21 days. A modest increase in total serum IgE was seen by weeks five to eight in four of the five volunteers; specific IgE rose in two volunteers in the period 28 to 38 days post-infection [195].

Finally, in 2005, after the onset of the clinical intervention studies reported in this thesis, Wright and Bickle published a study where a single healthy male was infected with 50 *N. americanus* larvae, and then re-infected 27 months later, with the aim of characterising the immune response. Following the first infection, and in keeping with other reports, a pruritic cutaneous reaction was noted which, in this case, persisted until day 47. Nausea and moderate abdominal pain was experienced between days 26 and 45 post-infection. The infected individual also

reported “mild rawness” in the lower respiratory tract during days 17 to 19 and occasional peripheral oedema between days 27 and 42. Eggs were present from week 7 post-infection, peaked at 1176 eggs/g faeces on day 145 and remained between 600 and 900 thereafter. An eosinophilic leucocytosis was noted with maximum eosinophil counts of 6.4 $x10^9$/litre on day 42 before declining to a persistently elevated level of 1.6 $x10^9$/litre. A similar, though less marked response was seen after the second infection. Antigen-specific IL-5 production increased between days 13 and 27 after infection and then fell to baseline between days 41 and 118. Hookworm specific IgG and IgE increased gradually during the primary infection and peaked during the 20^{th} and 27^{th} month after infection respectively. IL-13 response was noted between days 41 and 118. Little change in levels of IFN-γ, IL-10 or CRP was detected [170].

1.10.2 Eradication studies of helminth infection

Following on from the evidence for a relation between helminth infection and allergy demonstrated in the observational studies, it might be expected that eradication of naturally-occurring infection could result in an increase in allergic symptoms. However, generally, the results from eradication studies of naturally-occurring infection have failed to show an effect on allergic symptoms. Almost all these studies have been carried out in children. Two large eradication studies investigating a variety of different outcomes of allergic disease [242;243] and a number of other studies looking at specific allergic outcomes have been reported.

The first large study was a cluster-randomised controlled trial in schoolchildren based in 68 rural schools in Ecuador [242]. Children were randomly assigned, by school to treatment with albendazole (34 schools and 1164 children) every two

months for 12 months (7 occasions) or to no treatment (34 schools and 1209 children). The primary outcome was atopy to various allergens using skin sensitisation tests at 12 months. Secondary outcomes were presence of allergic symptoms (namely wheeze, rhinitis with itchy eyes, or itchy flexural rash), presence of flexural dermatitis on clinical examination and risk of exercise-induced bronchospasm. In this study, 72% of children were infected with geohelminths with 56% infected with *A. lumbricoides* and 56% infected with *T. trichiura*. They found no evidence of any difference in any of the outcomes in those children receiving albendazole compared with placebo.

The second large study was carried out in Vietnam and 1084 schoolchildren were randomised to receive anti-helminth treatment or placebo at 0, 3, 6 and 12 months [243]. Outcomes were changes in the prevalence of exercise-induced bronchoconstriction, allergen skin sensitisation, flexural eczema on skin examination and reported symptoms of wheeze and rhinitis using a questionnaire between 0 and 12 months. 5% of children were lost to follow-up. The most common helminth infections were hookworm (65%) and *A. lumbricoides* (7%). Anti-helminth therapy was associated with a significantly higher allergen skin sensitisation risk (adjusted OR 1.31, 95% CI: 1.02 to 1.67; p=0.03). This effect was particularly strong for children infected with *A. lumbricoides* at baseline (adjusted OR 4.90, 95% CI: 1.48 to 16.19; p=0.009). There was no effect of mebendazole observed on any of the other outcomes. No other studies have reported the effects of helminth eradication on symptoms of allergic rhinoconjunctivitis or flexural dermatitis.

One smaller study, carried out in Venezuela, looked specifically at the effects of anti-helminth treatment in people with pre-existing asthma [244]. 100 people with asthma were randomised to receive monthly albendazole for 12 months or placebo. At baseline, 41% of participants were infected with helminths, the predominant ones being *T. trichiura* (26%) and *A. lumbricoides* (23%). The primary outcome was asthma control as determined by number of exacerbations, need for inhaled corticosteroids and use of reliever inhaler. Secondary outcomes were allergen skin sensitisation to *Dermatophagoides* species and *A. lumbricoides*. Although this study did report a temporary improvement in clinical asthma control after albendazole therapy, the results are not convincing as they were based on assessments before and after treatment in those in the treatment group, and no statistical analysis comparing the treated and control groups was reported.

Three studies, of varying quality, have looked specifically at the effects of anti-helminth treatment on allergen skin sensitisation and have also found an increase in risk of atopy after treatment. Two were performed in areas where *A. lumbricoides* and *T. trichiura* were most prevalent and involved 375 children in Venezuela [245] and 317 children in Gabon [246]. In the study from Venezuela, 375 schoolchildren were offered anti-parasite treatment and followed for 22 months. 107 children took the treatment and 80 did not for various reasons and were considered as an untreated control group. The authors noted that atopy increased in those treated compared with those untreated at the end of study for a variety of allergens. For example, to positivity to *D. pteronyssinus* changed from 26% to 16% in the untreated group and from 17% to 68% in the treated group. As this study design was not randomised, limited inferences can be made from the

results. In the study in Gabon, schoolchildren were randomised using an open-label protocol to receive praziquantel and mebendazole or placebo every 3 months for the duration of the study. Skin sensitisation tests to *D. pteronyssinus* were checked every 6 months. After 30 months of follow-up, they found a significant increase in the rate of developing skin sensitivity to *D. pteronyssinus* with treatment compared with placebo (adjusted hazard ratio 2.51, 95% CI: 1.85 to 3.41). The third study, also carried out in Ecuador, investigated the impact on allergic disease of a control programme for onchocerciasis where ivermectin, which is also active against helminth infection had been given on an annual or semi-annual basis for the last 15 to 17 years [247]. 3901 children were assessed with similar numbers from treated and untreated areas. The prevalence of helminth infection was greater in those from non-treated areas (86%) than those in the treated areas (67%) with *T. trichiura* being the most prevalent of the infections. Those children living in treated areas had higher odds of atopy after adjusting for confounders (OR 2.10, 95% CI: 1.50 to 2.94). A further analysis was then performed to assess whether this association might be explained by the reduction in infection. They found this to be true for *T. trichiura*, but not for *A. lumbricoides* or hookworm. There was no effect of infection or treatment on prevalence of eczema symptoms. A further randomised controlled trial of the effects of anti-helminth treatment on the prevalence of allergen skin sensitisation and allergic disease is currently in progress in Indonesia and the results are awaited [248].

In addition to these studies described above, a randomised controlled trial of the effects of giving helminth treatment to pregnant women on incidence of eczema in their infants has been carried out in Uganda [228]. Women in the second trimester of

pregnancy were randomised to receive a single dose of albendazole or placebo and the incidence of infantile eczema, was confirmed by clinical examination up until the age of 15 months. 53 participants received albendazole and 50 received placebo; and complete follow-up data were available for 78%. 66% of participants had helminth infection at enrolment into the study. The authors found the incidence of infantile eczema was lower when the mother had helminth infection both during pregnancy and at delivery (adjusted rate ratio 0.26, 95% CI: 0.08 to 0.83). Similarly, there was also an inverse association between presence of helminth infection at delivery alone and the incidence of new eczema (8.6 per 100 person-years vs. 47.5 per 100 person-years; p=0.001) [228]. Whilst incidence of eczema was higher in infants whose mothers had received albendazole compared with placebo, this was not statistically significant in the adjusted analyses. The numbers in the study were small but the inverse association between maternal infection at delivery and infantile eczema may demonstrate the effect of maternal helminth infection priming the infant's immune system *in utero* against development of clinical allergic disease. Following on from the study above, a trial is now in progress in Uganda to investigate the relation between eradication of perinatal and postnatal infection and risk of atopy [249]. 2507 pregnant women were randomised, double-blind to albendazole or placebo, and praziquantel or placebo using a 2x2 factorial design. Primary outcome includes incidence of atopic disease episodes in the children. Worm infection was detected in 68% of women before treatment, of which hookworm infection was most common (45%). Early results from this study have shown that there was an increased risk of physician-diagnosed eczema in infants whose mothers had received albendazole treatment compared with placebo (Hazard Ratio 1.82, 95% CI: 1.26 to 2.64) [249]. This effect was slightly stronger among infants whose

mothers had no albendazole-susceptible worms (compared with infants whose mothers had such worms), although this difference was not statistically significant. Moreover, there was a significant trend of effect with the greatest effect being in those with no worms and smallest effect in those with three or more worm species. Treatment with albendazole was also strongly associated with reported recurrent wheeze in the infants at one year compared with placebo (OR 1.58, 95% CI: 1.13 to 2.22). Similarly, maternal treatment with albendazole (compared with placebo) was weakly associated with reported eczema at one year (OR 1.29, 95% CI: 0.96 to 1.72; p=0.09). In exploratory analyses, when stratified according to maternal hookworm status at enrolment, albendazole showed no effect on reported eczema if mothers had hookworm but a slight increase if mothers had no hookworm (OR 1.46, 95% CI: 0.98 to 2.17; p-value for interaction = 0.10). Reported recurrent wheeze showed a similar trend, with albendazole showing no effect when a mother had hookworm but a significant effect if a mother had no hookworm (OR 1.97, 95% CI: 1.27 to 3.05; p-value for interaction = 0.31).

1.11 Aims and objectives of thesis

Allergic diseases, and specifically asthma, are common and as described, the prevalence of these conditions continues to increase in parts of the world. There is an emerging body of epidemiological evidence supporting an association between parasite infection and allergic disease, some of which is presented in this introduction, but it is not known if this relation is causal. For causality, to be established, a number of different criteria need to be fulfilled [250], one of which is temporality and is best assessed in a cohort study or in a randomised controlled trial. To date, no randomised controlled trials of experimental infection with

parasites have been performed. If the relation is ultimately proven to be causal, there is potential for parasites, or their products, to be used as treatments for asthma and the efficacy of this would also need to be evaluated in an appropriately designed trial.

There are three main advantages in the design of a randomised controlled trial. The process of randomly allocating the exposure, (in this case, infection), should ensure that each intervention group is similar with respect to all factors, including known and unknown potential confounders. This therefore controls for confounding providing the sample size is a sufficiently large. A trial which is placebo-controlled and double-blinded, if successful, removes bias in ascertainment of subjective outcome measures. For example, if study participants are aware of the treatment allocation, this can result in bias in reporting of symptoms experienced; and similarly, if the investigator is aware, there may be bias in recording of outcome measures and interpretation of results. As discussed in section 1.10, one of the problems of cross-sectional studies is that temporality cannot be assessed, and so reverse causation may explain the observed relation between parasite infection and asthma. In a randomised controlled trial temporality is accounted for and the issue of reverse causation is overcome by first giving the exposure (here, the parasite infection) and then following up the effects on outcome.

The need for a randomised control trial prompted the design of a triad of intervention studies with the overall aim to establish whether experimental hookworm infection improves asthma and if so, whether it could potentially be used as a treatment. The first two studies were designed primarily as safety

studies in preparation for the third study in people with asthma. All were carried out in the UK. Hookworm was chosen as the parasite to be used as the size of a protective effect on asthma was the greatest from the meta-analysis [13] and the side effects of a controlled low dose of infection were likely to be limited.

The epidemiological evidence suggests that protection against asthma is related to the intensity of parasite infection, being most evident in the presence of infections generating more than 50 eggs/g faeces [58]. In light of this, experimental studies of therapeutic effects of hookworm infection in asthma should aim to establish infection at this level, while minimising any adverse effects. The first study, reported in Chapter 2, was therefore designed as a dose-ranging study and was carried out in healthy volunteers. The primary aim of this study was to establish the dose of hookworm required to achieve an egg count of 50 eggs/g faeces. Secondary aims were to establish the side effects of different doses of larvae and to explore the effects of infection on the immune response. It also aimed to assess the feasibility of infecting healthy volunteers with hookworm in a clinical trial setting.

The second study, reported in Chapter 3, was carried out in people with allergic rhinoconjunctivitis and again was designed as a safety study. As described in section 1.8.4.4, it was necessary to establish whether there was any increase in risk of bronchial hyper-responsiveness during the phase of larval pulmonary migration with experimental infection. The study was designed to address this question and was carried out in people who had measurable bronchial responsiveness but not clinical asthma. The primary aim of the study was therefore to determine the effects of experimental hookworm infection on

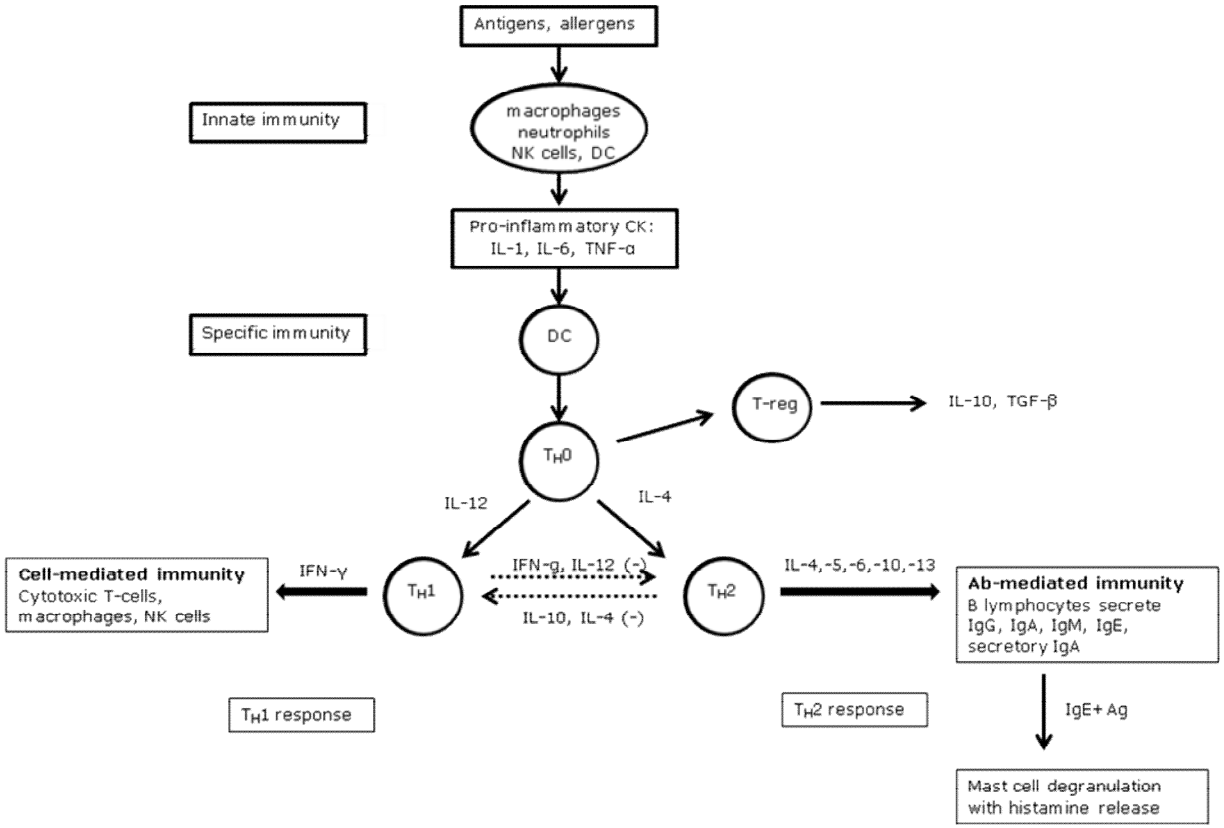
bronchial responsiveness over the first four weeks after infection which corresponds with this period of larval lung migration. Secondary aims were to investigate the effects on symptoms of allergic rhinoconjunctivitis and on allergen skin sensitisation over a 12 week period. The study also aimed to evaluate the immune response and occurrence of adverse effects potentially due to the chosen dose of ten larvae in a greater number of people.

The third and final intervention study was designed to evaluate the therapeutic efficacy of experimental hookworm infection on asthma and is reported in Chapter 4. It was only possible to proceed with this study after favourable results from the first two studies had been achieved. The primary aim of the study was to determine the effects of experimental hookworm infection on bronchial hyper-responsiveness over a 16 week period. Secondary aims were to investigate, over the same time period, the effects on other indicators of asthma control and on allergen skin sensitisation and as before, to monitor the occurrence of adverse effects potentially due to the infection.

The epidemiological evidence for the association between parasite infection and asthma has previously been subject to a systematic review and meta-analysis [13], however, to date, this is not the case for the relation been parasite infection and atopy. This would be particularly useful given the number of studies already carried out with varying results. A meta-analysis of the literature would also indicate whether the inverse association is unique to asthma or applies to allergic disease more generally. Therefore the aims of the systematic review and meta-analysis were to determine the association of infection with any parasite infection

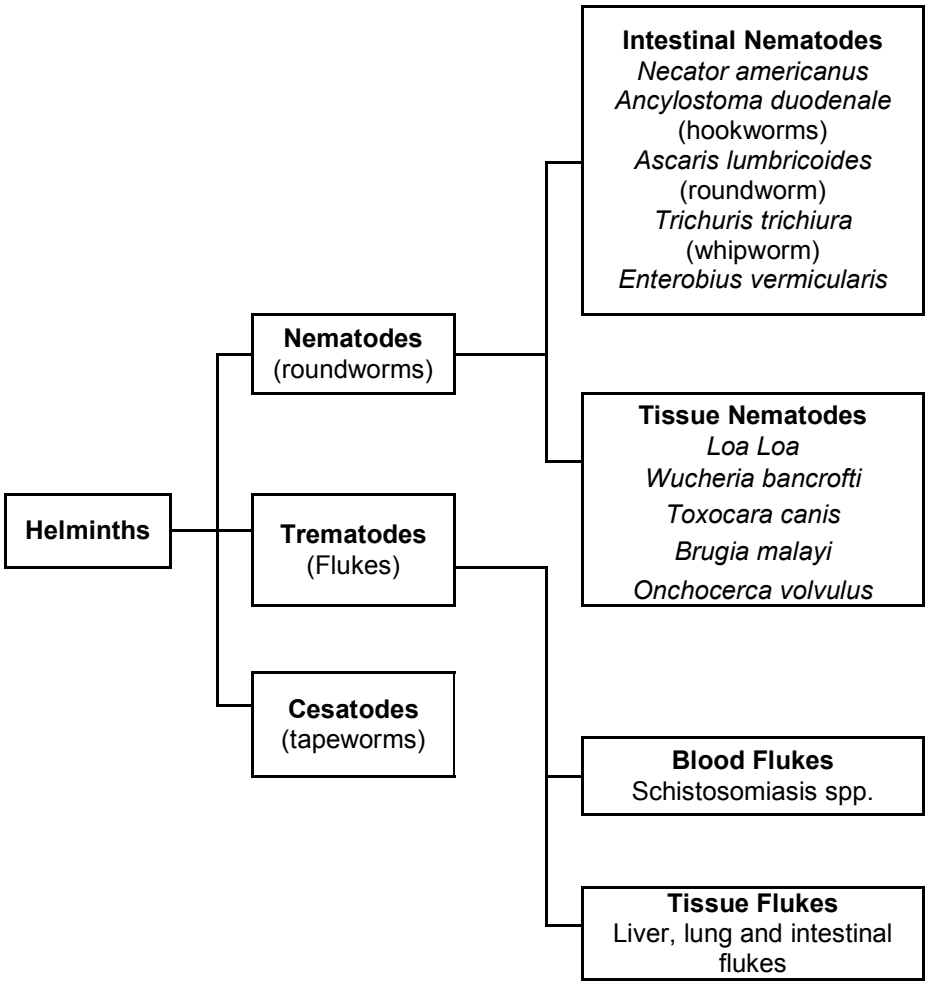
or species-specific infection, and allergen skin sensitisation. The aim was also to determine the relation between parasite infection and allergen-specific IgE.

Figure 1-1: Overview of the immune system adapted from Hanson [251]



Ab: antibody; **CK**: cytokines; **DC**: dendritic cells; **IFN-γ**: interferon- γ; **Ig**: immunoglobin; **IL**: interleukin; **NK**: natural killer; **T_H**: T-helper leucocytes; **TGF-β**: transforming growth factor-β; **TNF-α**: tumour necrosis factor; **T-reg**: regulatory T cell

Figure 1-2: Classification of helminth infection



2 DOSE-RANGING STUDY

2.1 Introduction

Evidence that the effect of hookworm infection on asthma is linked to intensity of infection is described in section 1.9.3. In pooled analyses of the epidemiological studies, a significant dose-related reduction in risk of both asthma, and wheeze, was observed with hookworm infection, with the risk of asthma in the highest tertile of infection being reduced by approximately two-thirds (adjusted OR 0.34, 95% CI: 0.19 to 0.62) [13]. The main study to demonstrate this protective effect of hookworm infection on asthma was a case-control study carried out in Ethiopia, where 205 cases with asthma symptoms were compared with 399 controls [58]. After identifying that hookworm infection appeared to be protective against wheeze the effect of intensity of infection was explored. Hookworm egg counts were analysed in tertiles and the protective effect was found to be most marked where counts were greater than 50 eggs/g faeces. After adjusting for age and sex, the odds ratios for wheeze were 0.54 for the lowest tertile (hookworm eggs <5.79/g faeces), OR 0.60 for the middle tertile (5.80 to 48.87 eggs/g faeces) and OR 0.30 for the highest tertile (>48.88 eggs/g faeces) (Figure 2.1).

Whilst with natural infection female hookworm can produce over one thousand eggs every day, a detailed study of the number of eggs produced with experimental infection has not been previously reported. Before embarking on an intervention study of the effect of experimental hookworm infection in asthma, it was important to establish how many larvae would be needed to generate an effect. A dose-ranging study in healthy volunteers, in preparation for a study in

people with asthma was therefore designed with the aim of determining how many larvae were needed to achieve the apparent protective threshold of at least 50 eggs/g faeces. The other aims of this study were to determine the feasibility of giving experimental hookworm infection in the context of a trial and to establish the side effects resulting from different doses of larvae as the morbidity from natural helminth infection is strongly associated with intensity of infection [186;252]. The study was in part designed as a safety study to assess tolerability of infection and was therefore carried out in healthy volunteers. People with bronchial responsiveness were excluded in case this was exacerbated during the pulmonary phase of larval migration. The primary outcome was the faecal egg count at eight weeks with the different doses of larvae. Secondary outcomes were the side effects reported of different doses of *N. americanus* infection and changes in specific immunoglobulins, differential white cell counting, and interleukin and TGF-β levels over the 12 weeks of the study.

The dose-ranging study was started before I commenced work on this grant and the projects. However, as stated earlier, I was involved with the data analyses and production of the manuscript that reported the findings of the study. As such, for completeness, the study is detailed here.

2.2 Methods

2.2.1 Recruitment, eligibility criteria and screening of participants

Potential study participants were recruited by local advertisement and word of mouth. Initial screening took place to exclude those with a diagnosis of asthma, anaemia or any other significant medical disorder as judged by the study clinician carrying out the visit. In addition, women were also excluded who were pregnant or of child-bearing potential and not willing to use contraception for the duration of the study. Those volunteers who met the initial eligibility criteria were then invited to attend a baseline visit where further criteria were assessed as detailed in section 2.2.2. There were no data available on which to base a sample size calculation as this was the first study of its kind. Therefore, a pragmatic decision was made to randomise five volunteers to each dose of larvae on the basis that this should provide adequate egg count data to fulfil the objective of the study.

2.2.2 Baseline visit

At the baseline visit, the study was explained in detail and written informed consent obtained. Several clinical measures were then taken as detailed below.

2.2.2.1 Lung function and bronchial challenge

Lung function was measured using a Micro Spiro (Micro Medical Limited, Kent, UK) spirometer, taking the one-second forced expiratory volume (FEV_1) as the higher of two values within 100mls, and the higher of two measures of forced vital capacity (FVC) according to international guidelines [17]. Bronchial responsiveness to methacholine was measured by the Yan method [253] to a maximum cumulative dose of 24.5 μM, stopping the test if FEV_1 fell by 20% or more from the post-

saline baseline value. Individuals with a 20% or greater fall in FEV_1 were deemed to have bronchial hyper-responsiveness and were excluded from further participation.

2.2.2.2 Allergen skin sensitisation

Allergen skin sensitisation to grass, cat fur and *D. pteronyssinus* plus positive (histamine) and negative (0.9% saline) controls (Allergopharma, Diagenics Ltd, Milton Keynes, UK) was measured by standard skin prick test methods [254]. A drop of the solution was placed on the ventral surface of the forearm and the skin was broken using a lancet needle. After 15 minutes, the mean of two diameters at right angles to each other (excluding pseudopods), one of which was the largest measurable diameter, was measured for all five solutions.

2.2.2.3 Blood samples

Peripheral blood samples were taken for haemoglobin estimation and differential cell counting (Full Blood Count) which was analysed in the pathology department at Nottingham City Hospital. Total IgE and specific IgG to hookworm levels were estimated using standard enzyme-linked immunosorbent assay techniques and antigen recognition by post–infection sera was analysed by Western blot [183;255]. These immunology tests were performed by scientists in the University of Nottingham School of Pharmacy and the methods used are not described in further detail here.

2.2.3 Randomisation visit

2.2.3.1 Randomisation

Individuals who met all eligibility criteria and who consented to participate in the study were then seen for a second visit where infection was carried out. Participants were randomised to receive a drop of water containing 10, 25, 50 or 100 *N. americanus* larvae, administered under gauze to an area of skin on the ventral aspect of the non-dominant forearm. Randomisation was in blocks of four according to a computer-generated random code, produced by the trial statistician. *N. americanus* larvae were obtained by culture of faecal material [256] from a healthy human source using the method described in section 2.2.3.3, and stored in water until required.

2.2.3.2 Blinding

The trial was carried out double-blind: both the participants and the study clinician responsible for conducting the study visits and carrying out clinical measurements were blind to randomisation code. The randomisation code was shared by the trial statistician with the scientist responsible for larvae culture to allow him to infect participants with the appropriate number of larvae.

2.2.3.3 Necator americanus L3 larval culture

Larval culture was performed by scientists based in the Department of Immunoparasitology in the University of Nottingham School of Pharmacy. Faecal material containing *N. americanus* eggs was obtained from a healthy human source known to be Hepatitis B and C and Human Immunodeficiency Virus

negative, mixed with activated charcoal, 1% (w/v) amphotericin B and water to form a smooth paste and then applied to the upper half of a 5 x 30 cm strip of filter paper. The strips were suspended in 750mls of distilled water in a sealed glass chromatography tank and incubated at 28°C for 7 to 10 days. The water containing the larvae was then transferred to a measuring cylinder and the larvae allowed to settle for 2 hours. After aspirating off the water, the larvae were washed twice to remove any contamination and then re-suspended in approximately 20mls of storage buffer (50mM Na2HPO4, 70mM NaCl, 15mM KH2PO4, pH7.4) [256].

2.2.4 Follow-up visits

After infection, study participants were seen weekly for twelve weeks. On each occasion, FEV_1 and FVC were measured, blood was taken for Full Blood Count and immunoglobulin estimation, and a faecal sample was collected to quantify egg production. Faecal egg counts were carried out by scientists in the University of Nottingham School of Pharmacy by suspending a weighed sample of faeces in one to two mls of saturated salt solution, counting eggs in a MacMaster egg counting chamber under a pre-marked grid, and back-calculating to estimate eggs/g faeces. Between visits, participants completed daily diaries of a variety of adverse effects, scoring severity on an arbitrary scale of 0 (no symptoms) to 10 (maximum possible severity of symptoms) because no validated questionnaire to assess the effects of infection existed. These adverse effects were selected based on previous reports in the literature of past experimental infection and were as follows: local skin reactions at the site of infection, gastrointestinal symptoms (nausea, indigestion, abdominal pain, diarrhoea, wind) and respiratory symptoms (cough, wheeze, breathlessness) [184;194;195]. Participants were also encouraged to

report the presence of any other symptoms (such as tiredness) that they were experiencing and which they felt might be important.

2.2.5 Final visit

At the final visit, 12 weeks after randomisation, in addition to the other clinical measures taken, allergen skin tests were repeated. The study clinician then unblinded the participants and provided them with 100mg mebendazole tablets to be taken twice daily for 3 days to eradicate the infection. A further faecal sample was checked for presence of eggs two weeks later to ensure eradication of the infection in all participants.

2.2.6 Trial monitoring

Data on adverse effects, blood results and faecal egg counts were monitored as the trial progressed, blind to allocation, by the trial statistician.

2.2.7 Ethical approval

The study was approved by the Nottingham NHS and University Ethics Committees.

2.3 Data analysis

2.3.1 Data entry

Data analyses were carried out at the end of the study blind to randomisation code. All data were double entered into Statistical Package for Social Sciences version 13 (SPSS Inc, Illinois, Chicago) and cross-checked for discrepancies.

2.3.2 Primary outcome

2.3.2.1 Faecal egg counts

The primary outcome was the number of faecal eggs produced with different doses of hookworm larvae 12 weeks after being infected. Faecal egg counts for each dose of hookworm larvae were not normally distributed and could not be transformed. The median faecal egg count was therefore calculated for each week and plotted by dose of larvae for each of the 12 weeks of the study and compared descriptively. The lowest maximum egg count for any individual was identified.

2.3.3 Secondary outcomes

The secondary outcomes were the occurrence of adverse symptoms potentially due to hookworm at different doses of larvae and the immune response to infection, determined by changes in leucocyte counts, haemoglobin and various cytokines over the course of the study. Exploratory analyses were also carried out to investigate changes in lung function and allergen skin sensitisation between the start and end of the study. Given the small sample size of the study, the intention was to descriptively assess outcomes and so no statistical tests of significance were therefore performed.

2.3.3.1 Adverse symptoms

The occurrence of adverse symptoms potentially due to the hookworm larvae is presented for each participant separately to demonstrate the range of experiences at each infection dose. Scores for each symptom were plotted over the 12 week study period and then compared between the different doses.

2.3.3.2 Lung function

The change in lung function between the start and end of the study (FEV_1 and FVC) was calculated for each individual. The mean change in FEV_1 and FVC was then calculated for all participants and also according to dose of larvae received. In addition the maximum fall in lung function was determined for each dose and compared between the groups.

2.3.3.3 Allergen skin sensitisation

To remove any effect on the size of the wheal from the saline solution or the act of breaking the skin, a saline-adjusted mean wheal diameter was calculated by subtracting the mean wheal diameter for the negative control from the mean wheal diameter for each allergen. The change in allergen skin sensitisation test (for any participants who had a positive response defined as a saline-adjusted mean wheal diameter equal to or greater than 3mm [254]) was compared for each individual between the start and end of the study.

2.3.3.4 Blood results

Leucocyte (total white cell and eosinophil) counts and immunoglobulin levels were not normally distributed but could be transformed by taking the natural log of each

value. The geometric means were therefore calculated and plotted for each week of the study according to dose of hookworm larvae and compared descriptively. The change in haemoglobin level was calculated for each individual as the difference between week 12 and screening visit values and the maximum fall identified.

2.4 Results

2.4.1 Participant flow

Ten people were randomised to the study (Figure 2.2). Three participants were randomised to each of the lower doses of ten, 25 and 50 *N. americanus* larvae and one to the dose of 100 larvae. Two participants withdrew from the study due to experiencing gastrointestinal symptoms and were subsequently unblinded. The first withdrew on day 35 after experiencing recurrent vomiting and diarrhoea. Unblinding of this participant revealed they had been given 100 larvae; no other volunteers had yet been randomised to this dose and the decision was made by the study investigators not to randomise any further people to this dose in view of the severity of the symptoms. The second, developed diarrhoea and abdominal pain and withdrew on day 28. Unblinding of this participant revealed they had received 50 larvae. Both these participants were treated with mebendazole to eradicate the infection. The remaining eight participants went on to complete the full twelve week study, attending a minimum of ten of the twelve weekly visits. The initial intention had been to randomise five people to each dose of larvae however, recruitment was stopped after three participants had been allocated to each dose of the three lower doses since it became apparent from monitoring the faecal egg count data that the number of larvae required to generate an egg count of more than 50 eggs/g faeces had been established.

2.4.2 Faecal egg counts

Hookworm eggs were not seen in faecal samples at any time in the two participants who withdrew from the study prematurely, but appeared at between four and six weeks after infection in all participants who completed the study. The

highest egg counts occurred in the people who received 50 larvae; median egg counts were similar in participants allocated to the two lower doses. The lowest maximum egg count in an individual was 66 eggs/g faeces (Figure 2.3). Egg counts for individual participants were variable and two participants had one or two weeks in which eggs were not seen, having previously been detected. Two weeks after treatment with mebendazole at the end of the study, faecal eggs were undetectable in all participants.

2.4.3 Adverse symptoms

2.4.3.1 Local skin reactions

Nine of the ten participants reported immediate local skin itching and developed a localised maculopapular rash at the skin entry site that typically lasted for two to five days (maximum score 8/10) but persisted beyond this time in two participants, who had been infected with 50 and 100 larvae (Figure 2.4). In the person who received 100 larvae the eruption was severe and lasted a total of 21 days (maximum score 8/10 on days 4 and 5 after infection). The participant who did not experience skin symptoms received ten larvae. The rash recurred at lower intensity (maximum score 5/10) for a few days at two to three weeks after infection in five participants, of whom two had received 25 larvae, two had received 50 larvae and one had received 100 larvae.

2.4.3.2 Gastrointestinal symptoms

Gastrointestinal symptoms appeared to be dose-related with symptoms generally occurring earlier (within the first two weeks following infection) in those who received 50 or 100 larvae compared with those who received lower doses where symptoms did not occur until after at least three weeks. In addition, severity

scores were tended to be lower in those infected with fewer larvae. Abdominal discomfort was the most commonly experienced symptom and was reported by nine out of ten participants. It tended to be intermittent and in some cases, was related to meals. The maximum score for abdominal discomfort in those who received ten larvae was 4/10; the highest score at any time for abdominal discomfort was 6/10 and was reported by participants who had received 50 and 100 larvae (Figure 2.5).

Most participants also reported occasional episodes of diarrhoea but these were mild, scoring a maximum of 2/10 on the severity scale for all but three participants. Of these three who recorded higher scores, the first had received ten larvae reported diarrhoea on day 56 only (score 6/10), the second who received 25 larvae had two isolated days with diarrhoea on days 2 and 95 (score 6/10). The third of these participants had received 100 larvae and had symptoms which started at day 20 and lasted several days at a time (scores of 5/10 and 6/10). Occasional nausea was reported by five participants, of whom two had received ten larvae (maximum score 2/10), one had received 25 larvae (maximum score 2/10), one had received 50 larvae (maximum score 5/10) and one who had received 100 larvae (maximum score 8/10). Two reported early satiety, and six reported increased flatulence (maximum score 4/10). As described in section 2.4.1, the two participants who withdrew from the study as a result of their gastrointestinal symptoms, had received the two higher doses of larvae.

2.4.3.3 Respiratory symptoms

With the exception of one participant who reported a cough productive of a small amount of phlegm during week seven, no respiratory symptoms were reported by

the three participants who received ten larvae. One participant who received 25 larvae reported mild wheeze (score 2/10) on the second day of infection. The participant who received 50 larvae and withdrew after four weeks reported cough on days 5, 15 and 16 post-infection and mild wheeze (score 3/10) during the third week. The participant who received 100 larvae reported mild breathlessness (score 2/10) on day 11. No respiratory symptoms were reported by any of the other participants.

2.4.3.4 Other reported symptoms

Symptoms of malaise or fatigue were reported by four of the ten participants. Symptoms were mild to moderate (maximum score 5/10) and occurred between weeks six and seven in three participants and at week 12 in one participant. Other symptoms reported were mild neck pain and headache for 2 days by one participant after a period of working at a computer, and pyrexia (38 °C) and coryzal symptoms for 2 days during week seven by another.

2.4.3.5 Response of symptoms to therapy

All symptoms resolved completely after treatment with mebendazole.

2.4.4 Lung function and allergen skin tests

The mean (SD) FEV_1 and FVC at baseline for the eight participants completing the study were 3.60L (0.53) and 4.48L (1.06) and at exit from the study were 3.57L (0.62) and 4.24L (0.98) respectively. The mean change in FEV_1 from the start to the end of the study was a fall by 0.03L (0.18) with a maximum fall in any individual of 0.18L. The mean change in FEV_1 for those who received ten larvae was a fall by 0.01L (0.11) with a maximum fall of 0.13L, for those who received 25

larvae was a fall of 0.00L (0.29) with a maximum fall of 0.18L and for those who received 50 larvae was a fall of 0.13L (0.06) with a maximum fall of 0.17L. The maximum fall of 0.18L was in a participant who had received 25 larvae. Similarly there were no clinical important changes in FVC and no evidence of a dose-related effect.

Only one participant who completed the study had positive allergen skin tests; saline-adjusted wheal diameters in this participant at baseline were 5mm to cat fur and 7mm to grass, reducing to 4mm and 5 mm respectively at the end of the study.

2.4.5 Leucocyte counts and haemoglobin

The mean white cell count increased to a peak at 5 to 9 weeks post-infection (Figure 2.6), due almost entirely to changes in eosinophils (Figure 2.7). All other leucocyte counts remained within the normal range throughout the study. The increase in eosinophil count was lower in the group who received ten larvae compared with the groups who received a dose of 25 or 50 larvae (Figure 2.7). There were no clinically important changes in haemoglobin level during the study. The maximum fall was in a participant who had received ten larvae; their haemoglobin level fell by 1g/dL from 15.5 g/dL to 14.5g/dL.

2.4.6 Immunoglobulin levels

Total IgE levels increased slightly during weeks 2 to 6 in the two higher dose groups but overall there was little difference between the different doses (Figure 2.8). Specific IgG levels increased gradually from time of infection, peaking at week 10 in those participants who received ten larvae and at week 12 in the two

higher larval dose groups (Figure 2.9). Western blots of adult hookworm secretions were probed with sera taken at baseline and from the week 12 blood test prior to treatment. Eight out of ten volunteers demonstrated a typical IgG antigen recognition profile to hookworm infection [255]. The two volunteers who withdrew early from the study failed to show a specific IgG response to hookworm secretions.

2.5 Discussion

2.5.1 Summary of findings

This study explored the dose-related effects of hookworm infection in normal healthy volunteers in preparation for a study in people with asthma. The primary aim was to establish the dose of larvae required to achieve an apparent protective threshold of at least 50 eggs/g faeces. It also aimed to determine the feasibility of giving experimental hookworm infection in the context of a trial and to establish the side effects resulting from different doses of larvae.

The study demonstrates that infection with cutaneous doses of ten or more larvae generated an intensity of infection resulting in faecal egg counts of at least 50 eggs/g, previously suggested to be the approximate threshold of intensity necessary to offer protection against asthma and allergic disease [58]. Localised skin rash and itching occurring in the first few days after infection were reported by almost all the participants and were most marked at the highest dose of larvae. Abdominal discomfort was also experienced by the majority of participants and again appeared to be dose-related. Symptoms were mild-moderate (up to 5/10 on severity score) in all but two participants, who had both received the two higher doses of larvae, and who withdrew from the study as a result. In those who had received ten larvae, gastrointestinal symptoms were generally mild and well tolerated. Respiratory symptoms were unusual, and malaise and fatigue, whilst reported by almost half participants were mild and short lived. There was no clinically significant change in lung function (FEV_1 and FVC) over the course of the study. One participant had positive allergen skin sensitisation to cat fur and grass at the start of the study and although the wheal sizes reduced, their repeat

tests remained positive after 12 weeks of infection. There were no clinically important changes in haemoglobin levels during the study with any dose of larvae. Eosinophil levels rose during the study and peaked as expected at weeks 5 to 9 with higher levels in those receiving greater doses of larvae. Total IgE levels also rose slightly but there was no evidence of a dose effect.

2.5.2 Strengths and weaknesses

2.5.2.1 Measurement error

Non-differential error may have occurred in the reporting of the adverse symptoms. No validated questionnaire was available to use to assess the occurrence of adverse symptoms potentially attributable to the infection, so a set of symptoms based on previous reports of intentional hookworm infection was devised. A visual analogue scale of 0 to 10 was used on which to grade the severity of these symptoms and this is recognised as a sensitive tool for rating subjective experiences [257]. However, there is likely to be a degree of variation between individuals in their definition of symptoms; for example, some individuals may consider three bowel movements a day to be normal, whereas for others, this might be reported as diarrhoea. It is also likely that there will be inter-participant variation in their perception of severity of symptoms and as the questionnaire has not been validated, it is important not to place undue emphasis on absolute scores.

The other main outcomes subject to non-differential measurement error were the faecal egg counts, lung function and allergen skin sensitisation tests. No automated system was used was used to count the faecal eggs, however, a single experienced scientist in the University of Nottingham School of Pharmacy

processed all the samples and followed a strict protocol to reduce the chance of error. Similarly the lung function and allergen skin sensitisation were carried out by a single clinician following a protocol using standard methods. Automated machines in the pathology department at Nottingham City Hospital and University of Nottingham School of Pharmacy were used to analyse the blood samples. These are all used routinely and subject to quality control and so measurement error of these results should have been minimal.

2.5.2.2 Success of blinding and bias

Blinding to the dose of larvae was important to reduce the chance of both reporter and observer bias. Participants were unlikely to have become unblinded as there was no obvious way for them to determine the dose they had been given at any point during the study and it was emphasised at the time of randomisation that the degree of reaction to the infection would be different for each individual, regardless of dose, and so the occurrence of side effects of infection would not be a reliable means of deducing the number of larvae received. The larvae are microscopic and so it would not have been possible to observe the number of larvae given at the time of application on to the skin. There is a theoretical chance that a participant could guess the dose of larvae they had received if they had noticed and counted the number of portals of entry on the skin (assuming one per larva) after the plaster was removed, or alternatively, on the basis of the severity of the rash. These are not likely to be accurate means of determining the dose of larvae, particularly because there was no opportunity for participants to compare their individual rashes and none of the participants had experienced previous infection. If participants had become aware of the dose they had received, reporter bias was most likely to arise in subjective reporting of adverse symptoms.

However, given that symptoms appeared relatively consistent for each dose and in keeping with previous studies, it is unlikely to have been a particular problem. The study clinician carrying out the study visits and measures and who was also responsible for the data analyses, remained successfully blind to dose of larvae given minimising the chance of observer bias. In addition, the majority of the outcomes were measured using objective methods and so less likely to be subject to bias even if the clinician had been aware of the number of larvae given to each participant.

2.5.2.3 Representativeness

One obvious source of bias in this study was the inclusion of four of the investigators as participants. Although not conventional, it was felt on moral and ethical grounds that the investigators should not recruit volunteers to the study without being willing to undergo infection themselves. The doses received by the investigators, as for all participants, were double-blind and allocated at random but at the end of the study proved to be 25 in one instance and 50 in the other three. The lower incidence of adverse effects in the ten larvae group in particular is therefore not attributable to bias due to possible under-reporting of adverse effects by participants who were also investigators.

2.5.2.4 Statistical power

The study was the first to look at the effect of different doses of experimental intentional hookworm infection and there were no data available on which to base a sample size calculation. Therefore, a pragmatic approach was taken to randomise four individuals to each dose of larvae on the basis that this should enable the main aim of the study to be met. Despite the use of randomisation, the

small sample size means that the groups are likely to have been different in terms of participants' baseline characteristics, but this is unlikely to have had an impact on the primary outcomes of the study. However, because of the small number of participants who received each dose of larvae, the study lacked power and it was not appropriate to use statistical tests to determine whether the differences observed between the groups were significant or if they had arisen by chance and so the analyses carried out were all exploratory analyses.

2.5.3 Results in context of other studies

Although previous studies, described in section 1.10.1, have also documented the effect of experimental hookworm infection in humans over the last three decades, none were carried out as a randomised clinical trial and all included only small numbers of individuals [138;184;194;195;240;241]. None of the previous studies have compared the clinical and immunological response (including egg production) to different doses of infection. Those previous studies reporting egg counts following infection with a known number of larvae at a similar dose to the ones used in this study have found comparable results: for example, a mean of 268 eggs/g faeces eight weeks after being infected with 45 larvae [194]. The side effects of infection experienced by participants in this study were also similar to previous reports with an early skin rash and abdominal discomfort between weeks 4 and 8 after infection with around 50 larvae being the most commonly reported [194;195]. Previous studies have also reported a substantial increase in the number of circulating eosinophils following infection with higher doses of larvae [194;195]. Levels of specific IgG increased in all intervention groups throughout the trial period, and Western blots demonstrated a typical antigen recognition profile [258] at all doses of hookworm larvae, but not in the post-infection serum samples of the two

participants (4 and 12) who withdrew early from the study (Figure 2.9). This suggests that infection beyond five weeks is required for this immune response to be detected and may be linked to larval entry into the gastrointestinal tract, which occurs around this time. Total IgE response to all doses were relatively low, and failed to show any significant differences between different doses (Figure 2.8), which is again consistent with other reports [184;185;195;241].

2.5.4 Interpretation of results

All doses of larvae generated a maximal egg count of greater than 50 eggs/g faeces, the apparent protective threshold observed in the studies in asthma. A range of predominantly dose-related adverse effects of infection were reported. The initial adverse effect in most participants was a localised pruritic skin reaction at the site of larval entry, which began within a day or so of infection and typically lasted for up to a week. In about half of participants the rash relapsed or recurred at around two to three weeks after infection for up to 10 days, before settling completely. This biphasic effect is likely to reflect the lag period required for the immune system to mount a response to larval antigens deposited in the skin soon after entry. The most troublesome adverse effects were gastrointestinal symptoms, causing two participants to withdraw from the study. Abdominal discomfort was the most common symptom but the severity appeared to be dose-related and scores were mild and infection well tolerated among those who received ten larvae. Other gastrointestinal symptoms such as diarrhoea occurred sporadically with no obvious association with timing of infection or dose and may have been unrelated to the infection. The other most commonly reported adverse effect was malaise or fatigue, occurring between weeks six and seven after infection. These symptoms occurred when eosinophil counts were at their highest

and may therefore have been indirect effects arising from systemic eosinophilia or an eosinophilic gastroenteritis [259] rather than directly from the parasite.

A major concern in exploring the therapeutic potential of hookworm infection in asthma, and indeed in other conditions, is the theoretical risk of adverse pulmonary effects during the lung migration phase. For this reason people with a history of asthma or with measurable bronchial responsiveness were excluded from participating, and FEV_1 and respiratory symptoms were monitored throughout the study. There was no evidence of a clinically important change in lung function, or of any dose-related effect on lung function. However, symptoms of cough, breathlessness or wheeze were reported by three participants, all of whom had received doses higher than ten larvae, during the first four weeks of infection, the period during which lung migration occurs. These were minimal, with cough troublesome in one participant over the course of one day, while the reported wheeze and breathlessness were not prominent.

The findings on adverse effects demonstrated that of the four doses tested, ten larvae was the dose best tolerated. However, whilst there was a rise in total IgE and eosinophil levels in response to this dose of infection, the peak levels were also less than with the higher doses, so it may be that this greater tolerability is offset by a lesser effect in provoking a potentially beneficial anti-asthma host immune response.

Iron deficiency anaemia resulting from chronic gastrointestinal blood loss is the most important complication of hookworm infection in endemic tropical areas, where infected individuals can carry high loads of worms and are subject to

repeated infections over their lifetime [136]. Anaemia was not apparent in this study, and is unlikely to pose a problem with a low dose of infection given to well-nourished adults with no other risk factors for anaemia such as pregnancy.

2.5.5 Conclusions

In conclusion, infection with a dose of ten *N. americanus larvae* generates at least 50 eggs/g faeces and this dose is well tolerated and elicits a modest host eosinophilic and antibody response. Clinical trials of intentional infection with *N. americanus* are feasible and a dose of ten larvae is for use in preliminary therapeutic trials exploring the effects of *N. americanus* on moderation of the allergic response in atopic individuals.

Figure 2-1: Odds Ratios, adjusted for age and sex, of wheeze in individuals with dust mite sensitisation, according to intensity of total parasite infection (taken from Scrivener *et al* [58])

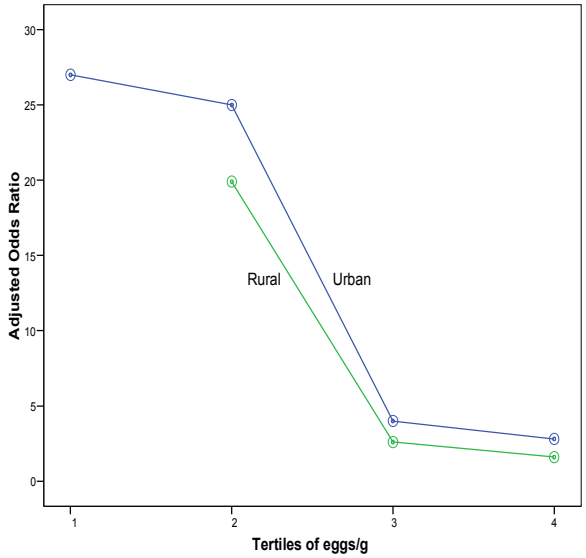


Figure 2-2: Study design and allocation of participants

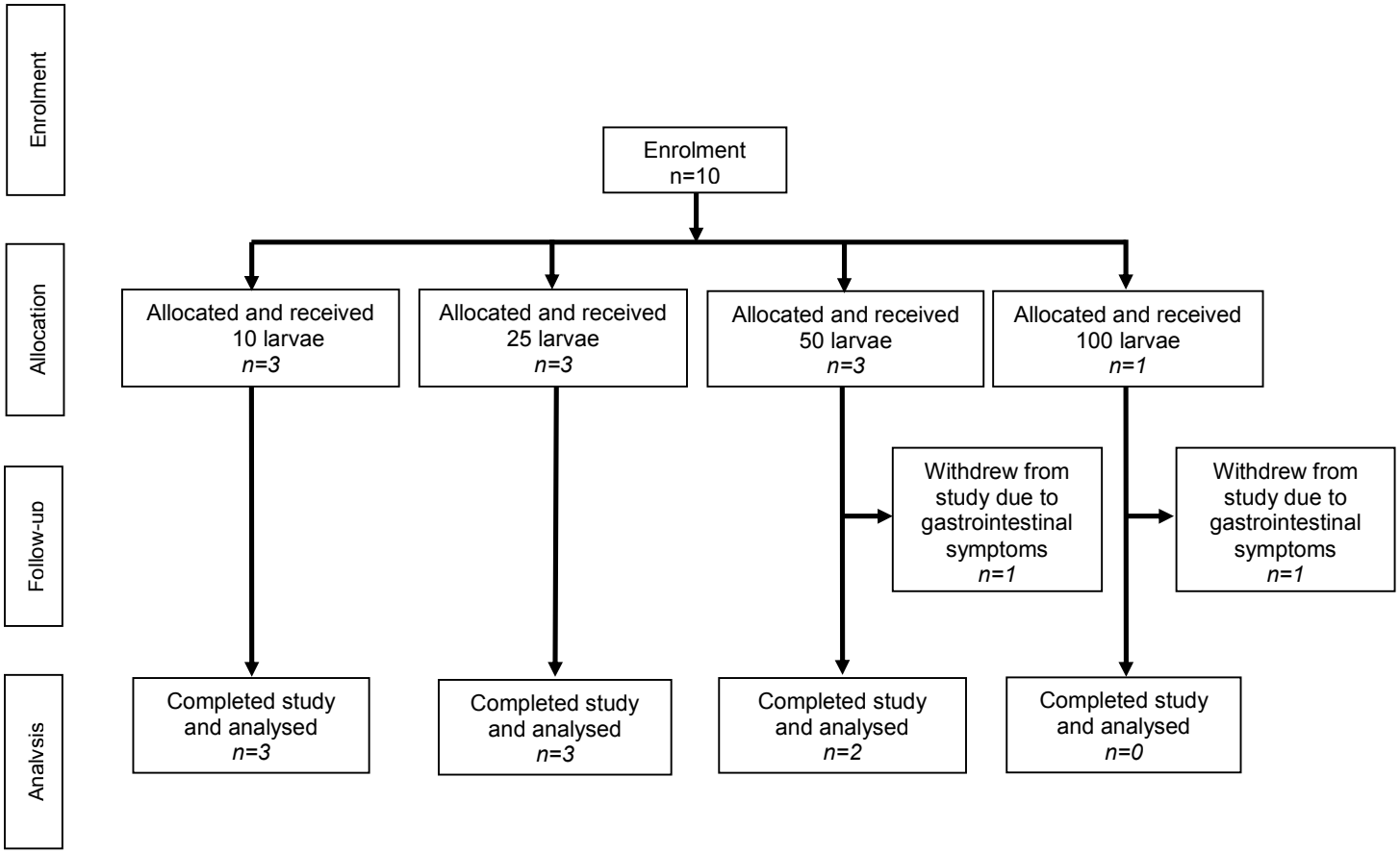


Figure 2-3: Median number of hookworm eggs/g faeces in the eight participants who completed the study by dose of hookworm larvae

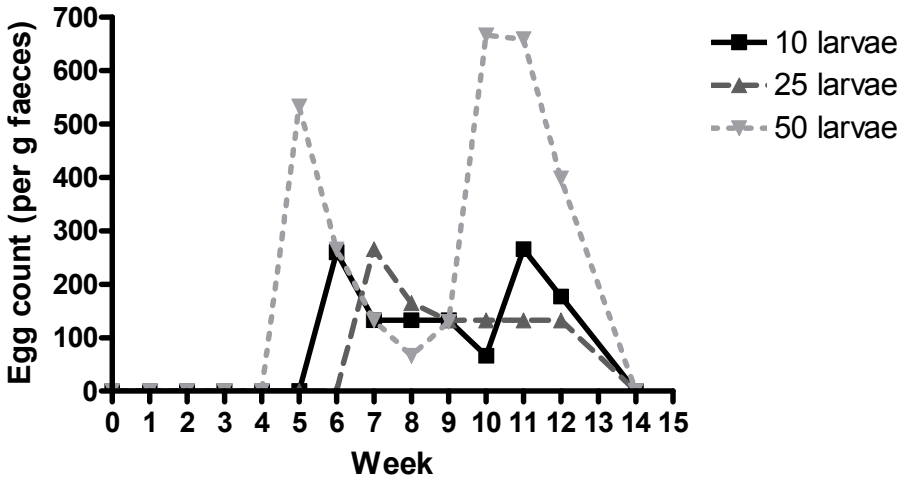


Figure 2-4: Symptom scores for skin rash by dose of hookworm larvae

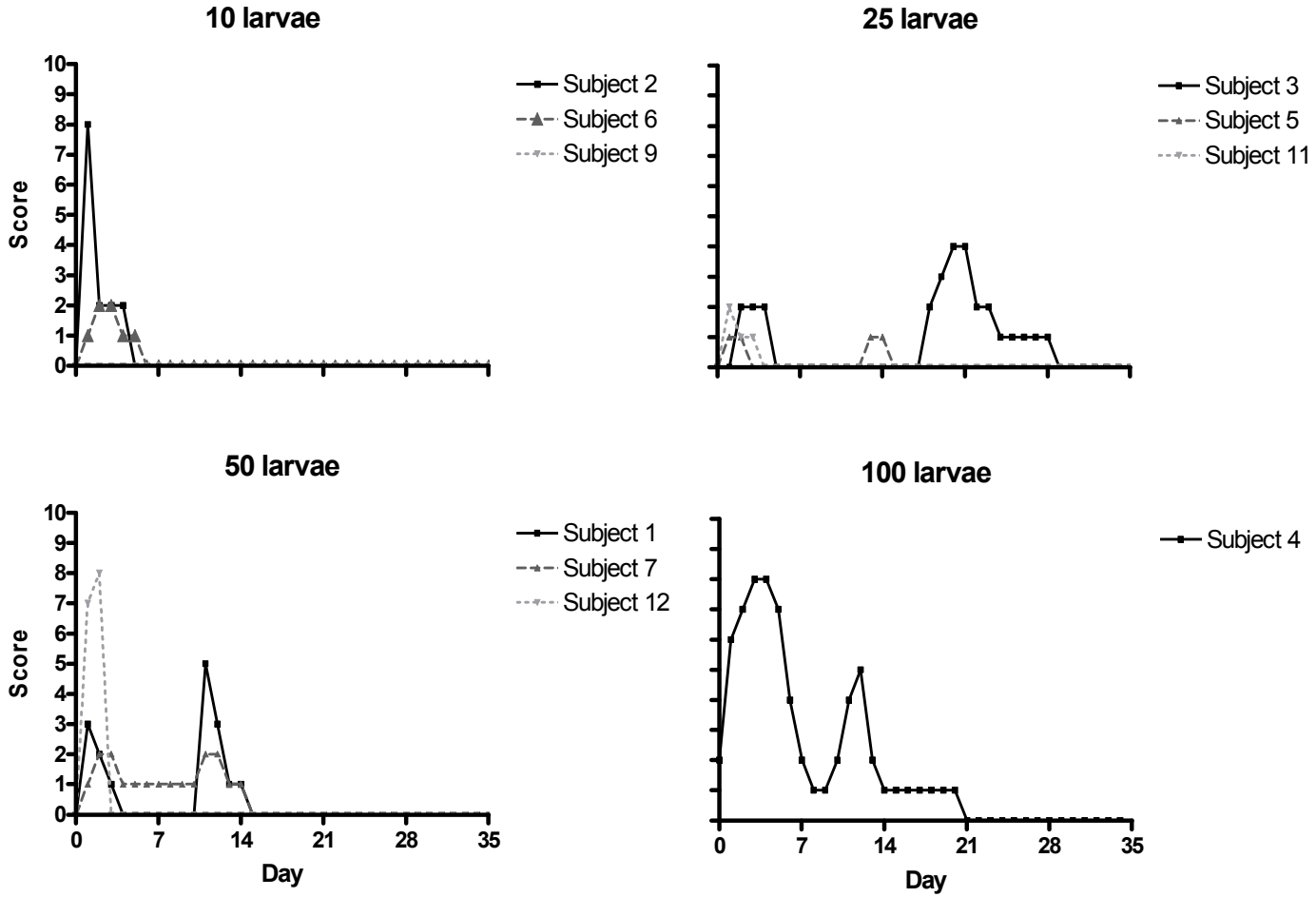


Figure 2-5: Symptom scores for abdominal discomfort by dose of hookworm larvae

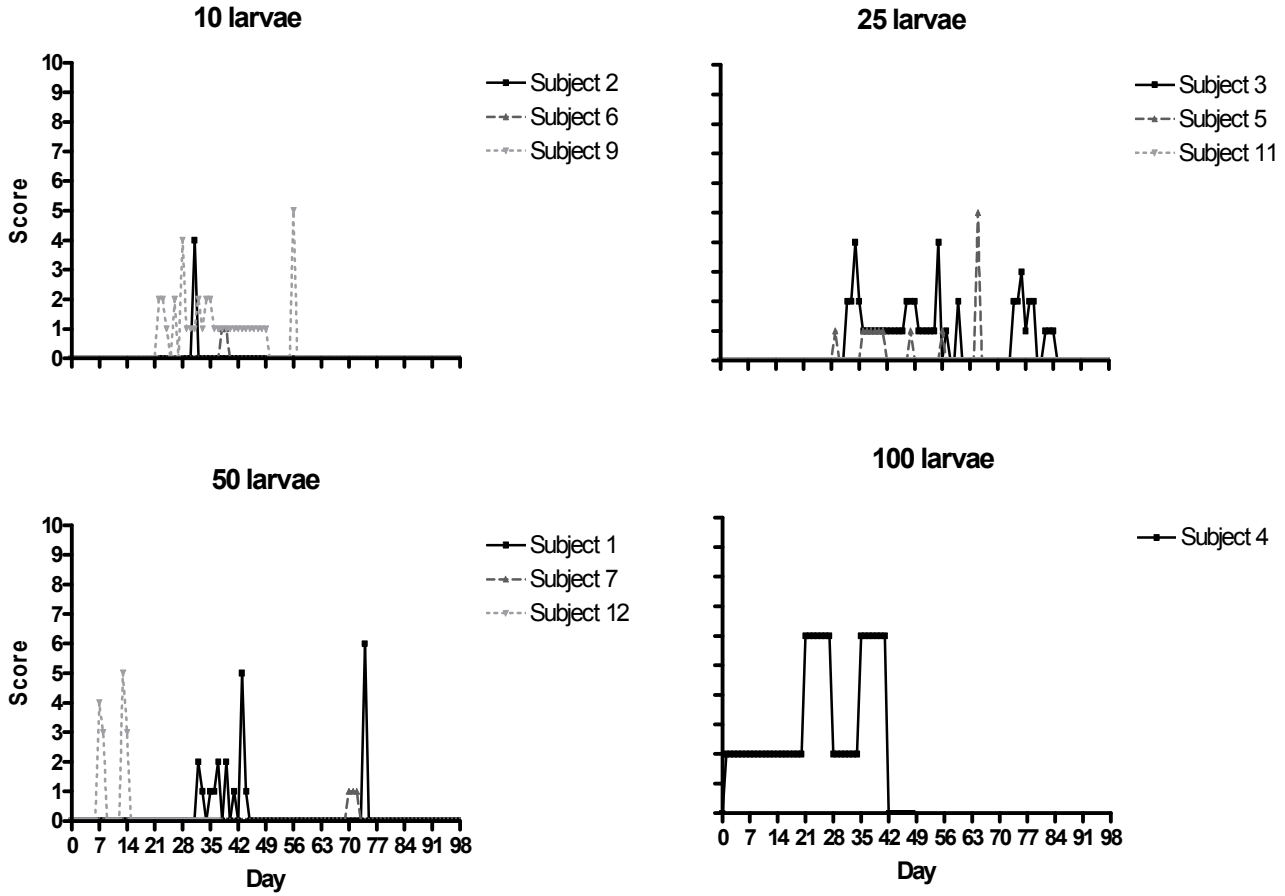


Figure 2-6: Geometric mean total white cell counts in the eight participants who completed the study by dose of hookworm larvae

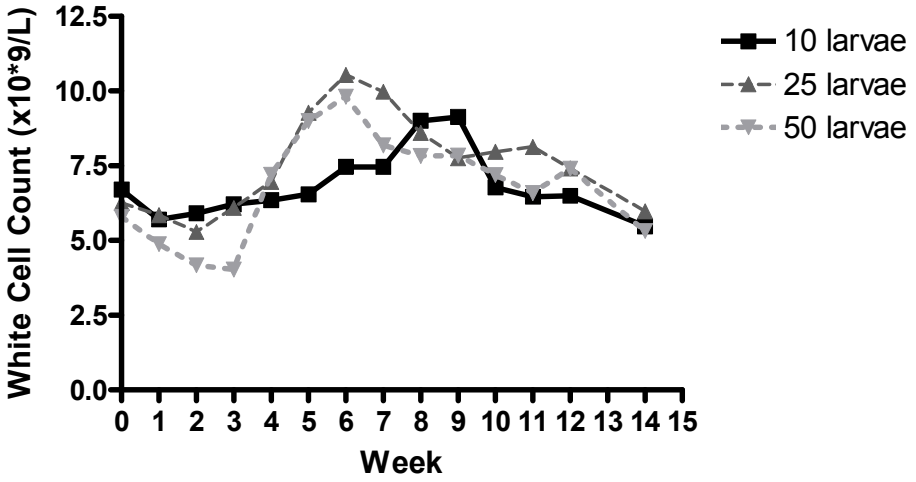


Figure 2-7: Geometric mean eosinophil cell counts in the eight participants who completed the study by dose of hookworm larvae

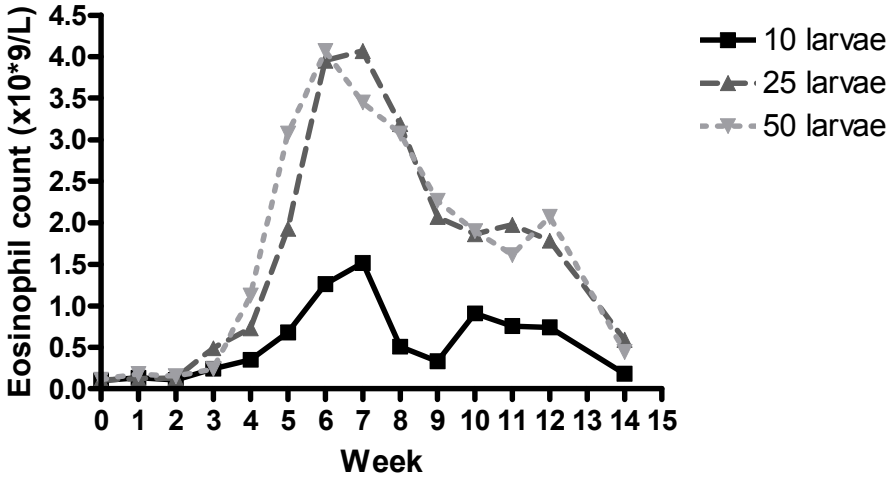


Figure 2-8: Geometric mean total IgE in the eight participants who completed the study by dose of hookworm larvae

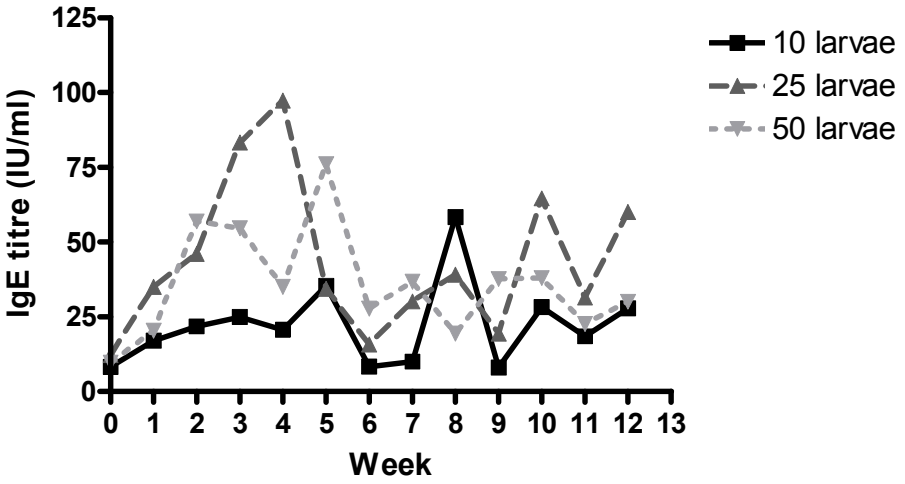
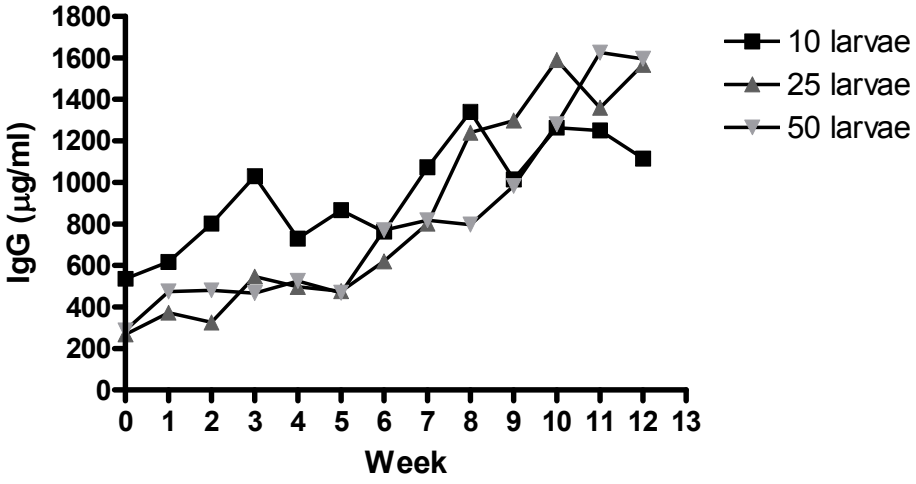


Figure 2-9: Geometric mean specific IgG by dose of hookworm larvae



3 INTERVENTION STUDY OF HOOKWORM IN ALLERGIC RHINOCONJUNCTIVITIS

3.1 Introduction

Whilst there is some evidence that intestinal helminths, such as *A. lumbricoides*, may exacerbate asthma symptoms [260;261] this does not appear to be a problem with hookworm infection, which, as outlined in section 1.9.2, appears to be protective [13]. However, during the design phase of these intervention studies it was considered important to ensure that larval migration through the lung (typically occurring during the first four weeks after infection (described in section 1.8.1)) would be unlikely to exacerbate bronchial hyper-responsiveness before proceeding to a trial of *N. americanus* infection in people with asthma. Safety studies were felt to be required in view of bronchoscopic findings during this time in normal volunteers who had received infection [195] and also because animal studies have demonstrated a pronounced T_H2 phenotype associated with this phase of the lifecycle [262;263], indicating the possibility of exacerbation of clinical disease. The study presented in this chapter was therefore designed as a safety study to determine whether hookworm infection exacerbates bronchial responsiveness during the phase of larval pulmonary migration in preparation for a trial in people with asthma.

The airway inflammation that occurs in asthma may be present in people with asthma but who have no symptoms, and may also occur in atopic individuals who are not asthmatic, suggesting that the inflammation needs to reach a threshold in order to trigger symptoms [264;265]. People with allergic rhinoconjunctivitis and

measurable bronchial responsiveness, but no clinical symptoms of asthma, are an ideal population to study in order to observe whether there is an increase in bronchial responsiveness during larval lung migration. This is because changes in bronchial responsiveness can be measured, but they are unlikely to develop clinically significant airflow restriction when exposed to potential bronchoconstrictors.

The study comprised a randomised, placebo-controlled, double-blind trial of hookworm infection in adults (18 years of age or older) with allergic rhinoconjunctivitis and was the first of its kind to be carried out. Participants were not eligible if they had clinical symptoms of asthma, but needed to have measurable bronchial responsiveness to adenosine monophosphate (AMP) below a threshold considered to be indicative of asthma. The main aim was to compare the maximum change in bronchial responsiveness (using PD_{10}AMP) during the first four weeks after being randomised to receive ten hookworm larvae or placebo. Secondary aims were to establish the side effect profile of infection with ten larvae in a greater number of individuals using a similar questionnaire as in the dose-ranging study. The study also sought to establish effects of ten larvae on symptoms of allergic rhinoconjunctivitis using a Juniper RQLQ, the effect on allergen skin sensitisation and to characterise the immune response to infection over the 12 weeks after randomisation.

3.2 Methods

3.2.1 Recruitment

Potential participants for the study were recruited using several methods. Advertisements were placed in local newspapers and public places around Nottingham, and on websites and notice boards throughout Nottingham City Hospital and the University of Nottingham. Emails advertising the study were also sent to members of staff at the University. The recruitment attracted a large amount of media interest, and the investigators were also approached by volunteers who heard about the study from interviews on local and national radio and television. All people who expressed an interest in the study were contacted by telephone or email and the background to the study, and what participation would entail, were explained. A patient information sheet (Appendix B) was then sent to people who remained interested.

Study visits were held at the University of Nottingham in the Department of Respiratory Medicine. I was responsible for recruiting participants and conducted all the study visits, including the screening visits, and measured all clinical outcomes, thereby eliminating the possibility of inter-observer variability. Recruitment took place during February and August 2006.

3.2.2 Eligibility criteria

3.2.2.1 Inclusion criteria

Participants needed to be aged 18 or over, to have current symptoms of rhinoconjunctivitis at the time of screening and to have a positive allergen skin test to at least one of cat fur, grass or *D. pteronyssinus*. They also needed to have

measurable bronchial responsiveness to AMP at the screening and randomisation visits.

3.2.2.2 Exclusion criteria

Individuals were excluded if they had a history of severe allergic reaction or anaphylaxis, or a diagnosis of asthma, or if they used asthma medication. If they had signs of possible undiagnosed asthma or chronic obstructive pulmonary disease when spirometry was performed (one-second forced expiratory volume (FEV_1) less than 80% predicted (compared with standard reference ranges [266]) or one-second forced expiratory volume/forced vital capacity (FEV_1/FVC) ratio consistent with airflow obstruction (>70%)) they were also excluded. Women who were breastfeeding or pregnant or who were of child-bearing potential and not using contraception were also excluded. Individuals were excluded if they had a positive serum IgG against hookworm signifying previous hookworm infection or positive faecal egg counts signifying current infection at the time of recruitment. Those individuals whose blood tests revealed evidence of anaemia (haemoglobin <13g/dl (male) <11.5g/dl (female), mean corpuscular volume <76fl) were excluded from the study and referred to their General Practitioner for appropriate investigation.

3.2.3 Screening visit

3.2.3.1 Initial consultation and consent

Volunteers who had been sent information sheets and who remained interested in taking part in the study were contacted by telephone or email and a screening visit was arranged once they were experiencing regular (that is, on most days each week) symptoms of allergic rhinoconjunctivitis. At this screening visit, the

study was explained in detail including the background rationale, the study design, what recruitment would involve in terms of tests performed and the required time commitment. The potential side effects of the hookworm infection as listed in the daily diary were described but it was emphasised that the extent to which they occurred, if at all, would vary between individuals. The processes of blinding and randomisation were also described and any questions answered. A brief medical history was taken, and volunteers were excluded at this stage if they had significant medical problems, or if women were pregnant or of child-bearing potential and unwilling to use contraception for the duration of the study. Written informed consent was then taken with two identical consent forms completed: one for the study records and one for the individual to retain for future reference (Appendix C). Permission was gained to inform the individual's General Practitioner of his or her recruitment into the study. A number of measurements were then made to further assess eligibility and to collect data on baseline characteristics and outcomes.

3.2.3.2 Medical history and baseline characteristics

A full medical history was taken including details of allergic symptoms and other atopic disease, past medical history, use of medication and smoking status. Baseline height and weight measurements were made. Urine analysis for β-HCG (QuickVue, Quidel Corporation, San Diego, USA) was performed to confirm that female participants of child-bearing age were not pregnant.

3.2.3.3 Lung function and bronchial challenge

Individuals were instructed on peak expiratory flow measurement (PEF) using a miniWright peak flow meter (European Union scale) (Clement Clarke

International) and the best reading of three adequate attempts was recorded. FEV_1 and FVC were measured according to international guidelines [17] using a Spirometer R model (Vitalograph, Buckingham, UK) and taking the higher of two values within 100mls.

A bronchial challenge was performed to measure bronchial responsiveness to adenosine monophosphate (AMP), which causes bronchoconstriction in people with asthma but also in a proportion of people without asthma but with allergic rhinitis [264;265]. Two grams of AMP (Sigma-Aldrich Company Ltd, Dorest, UK) was dissolved in 5mls of 0.9% sterile saline solution to form an initial solution with a concentration of 400mg/ml and then further diluted to make a number of increasingly less concentrated solutions [267;268] (See Appendix D for dilution scheme).

A breath-activated compressed air dosimeter (MEFAR, Brescia, Italy) was used to deliver each dose of AMP [265;269]. Two mls of each concentration were dispensed using a pipette into a dedicated nebule pot which had been calibrated by delivering 100 breaths of saline with the dosimeter and weighing the pot in grams (to 4 decimal points) to ensure delivery was consistent. For the AMP challenge, an initial dose of 0.9% saline control was inhaled, followed by doubling doses (DD) of AMP (Sigma Chemical Co, UK) dissolved in 0.9% saline from 0.115 to a maximum cumulative dose of 944μM (Appendix E) from a breath-activated dosimeter (MEFAR, Brescia, Italy) set to nebulise for 1 second with a pause of 6 seconds at a pressure of 152kPa. FEV_1 was measured 2 minutes after each dose [267;268] and the inhalation challenge was stopped once FEV_1 had fallen by 10% from the post-saline baseline value, or the maximum dose of AMP had been

inhaled [268]. The provocation dose in adenosine monophosphate (AMP) required to reduce one-second forced expiratory volume (FEV_1) by 10% (PD_{10}AMP) was then calculated by interpolation between the two last doses on the log dose-response scale [17]. The initial intention was to use the PD_{20}AMP measure for bronchial responsiveness but during study recruitment, very few volunteers demonstrated this degree of bronchial responsiveness and therefore the PD_{10} measure was adopted, since this was more easily demonstrable and is a strong predictor of PD_{20} [270].

According to guidelines, individuals were instructed to abstain from the following prior to the bronchial challenge:

- antihistamines for 24 hours [79];
- steroid nasal sprays for 12 hours;
- caffeine-containing food and drink for 12 hours [17];
- strenuous exercise for 12 hours [271].

The bronchial challenge was not performed on anyone who had an FEV_1 of less than 40% predicted or a value of less than 1 litre, or who were pregnant [17].

3.2.3.4 Allergen skin sensitisation

Allergen skin sensitisation to *D. pteronyssinus*, cat fur, grass pollen and positive (histamine) and negative (saline) controls (Diagenics Ltd, Milton Keynes, UK) was measured by standard skin prick test methods as described in section 2.2.2.2.

3.2.3.5 Blood samples

Venesection was performed and samples for Full Blood Count analysis (haemoglobin estimation and differential cell counting) and serum albumin were processed in the Nottingham City Hospital pathology department. Samples were also taken for immunological testing which was carried out in the University of Nottingham School of Pharmacy.

3.2.3.6 Immunological Methods

The immunology tests were all performed by collaborators in the School of Pharmacy at the University of Nottingham. To assess the immune phenotype, cytokine responses to infection were assessed following T-cell stimulation and serological responses to the parasite were assessed by enzyme-linked immunosorbent assay and Western blot; these data are only presented briefly, and for the sake of completeness, in this thesis.

3.2.3.7 Faecal egg count methods

A faecal sample collected within the previous 24 hours was provided by participants to confirm they did not have pre-existing hookworm infection. Faecal samples were analysed within 24 hours of production by a scientist in the Department of Immunoparasitology at the University of Nottingham School of Pharmacy. If it was not possible to provide a fresh specimen, samples were frozen by participants and defrosted prior to analysis to ensure that eggs did not hatch prior to being counted. The methods for determining egg counts are described in section 2.2.4.

3.2.3.8 Juniper Rhinoconjunctivitis Quality of Life Questionnaire

An interviewer-administered Juniper Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ) was completed [272] to ensure that participants had current symptoms and to obtain a baseline symptom score. This questionnaire asks participants to recall their experiences of their symptoms over the previous two weeks and provides a measure of health-related quality of life in clinical trials of rhinoconjunctivitis. It looks at the effects of symptoms on seven different domains (activities, sleep, constitutional disturbance, practical problems, nasal, eye and emotional symptoms) using 28 questions and provides a composite score out of 168 (Appendix F). Higher scores signify a higher impact of symptoms on quality of life. It has been validated to show moderate to strong relations between changes in symptom diary scores and changes in RQLQ scores [273].

3.2.4 Run-in period and daily diary

Individuals who fulfilled the entry criteria at the screening visit then took part in a run-in period lasting approximately two weeks. During this run-in period, and for the duration of the study, participants were asked to complete a daily diary which included a record of the parameters described below (Appendix G).

3.2.4.1 Peak expiratory flow

Participants were asked to record morning and evening PEF, measured as the best of three attempts as another marker of airflow variability.

3.2.4.2 Medication use

Participants were asked to abstain from using antihistamines and steroid nasal sprays for the duration of the study as far as possible. Where occasional usage of antihistamines was unavoidable (for example, due to exceptional circumstances at work), participants were provided with a supply of loratidine 10mg tablets to standardise the antihistamine used, and were instructed to record use in their daily diary; symptoms on that day were subsequently discounted from analysis. A small proportion of participants requested to take antihistamines on a daily basis due to the severity of their symptoms; these participants were asked to substitute their usual medication with loratidine 10mg taken once daily for the duration of the whole study period. Similarly, one participant elected to take a daily steroid nasal spray throughout the entire study. Participants were asked to record use of any medications in their daily diary.

3.2.4.3 Adverse symptoms

Participants were asked to assign a score using a visual analogue scale from 0 (no symptoms) to 10 (maximum possible severity of symptoms) for a range of pre-determined possible adverse effects due to the hookworm, including local skin reactions at the site of infection (redness and itching), gastrointestinal symptoms (nausea, indigestion, abdominal pain, diarrhoea, wind), respiratory symptoms (cough, wheeze, breathlessness) and constitutional symptoms (tiredness). It was not possible to identify an appropriate and previously validated questionnaire for use in the study. These particular symptoms have all been reported to occur in conjunction with hookworm infection and were reported in the dose-ranging study [184;194;195;274]. In addition, a space for free text was included in the diary for the

recording of any other symptoms which the participants felt might be relevant (for example change in eczema severity).

3.2.5 Randomisation visit

3.2.5.1 Clinical measurements

After the two week run-in period, participants were seen for the randomisation visit. The bronchial challenge was repeated, the Juniper RQLQ was completed and blood tests were taken for the same tests as at the screening visit. If any of the eligibility criteria were no longer fulfilled, then the individual was not randomised into the study.

3.2.5.2 Randomisation

Eligible participants then underwent double-blind randomisation to hookworm infection or placebo, allocated in blocks of four according to a computer-generated random code. Larval culture was performed by scientists based in the Department of Immunoparasitology in the University of Nottingham School of Pharmacy, as described in section 2.2.3.3. The solutions were administered by a different member of the research team who was not involved in any of the study measurements. The medical statistician responsible for randomisation was given two sealed eppendorfs: one containing 200µl water into which had been counted out 10 purified L3 *N. americanus* larvae by direct visualisation (active infection); the other containing 200µl histamine dihydrochloride solution (1.7mg/ml) (placebo). Histamine solution was chosen as a placebo solution in the study for the following reasons. First, it is used for allergen skin sensitisation tests and is therefore safe for cutaneous application. Second, it has the same visual appearance as the solution containing the larvae and third, it causes similar

immediate cutaneous effects as the larvae, namely itching and a red rash at the site of application. Depending on the randomisation code, the participant was then either infected with the larvae or, using the same technique, given the placebo. The larvae were pipetted on to a piece of gauze measuring 2cm^2 which was then inverted on the ventral surface of the recruit's non-dominant forearm. A water-tight adhesive dressing ('sleek', BSN medical Ltd, Hull, UK) was applied over the top and pressed over the skin so that it sealed on all edges. Study participants were told to avoid getting the plaster wet and to leave it in place for 24 hours, after which time it could be removed and placed in a universal container containing 70% ethanol (with which they were provided). They were also given one tablet of albendazole 400mg to take home in case they should want to withdraw from the study at any time. Participants were provided with a 24-hour contact telephone number in case of any problems or questions relating to the study. Randomisation codes were placed in a sealed envelope in the department laboratory in case of a medical emergency which might require the participant to withdraw from the study and to be unblinded immediately.

3.2.5.3 Blinding

Various steps were taken in order to try and ensure participants were blind to randomisation code. At the time of consent, they were told that they could expect a degree of cutaneous itching and rash with both infection and placebo but the extent of which would vary between individuals. They were also told to keep the plaster on their arm for 24 hours after randomisation so that when it was removed the most intense phase of the rash had subsided. In addition, the variation in the extent to which individuals experienced side effects of infection (as listed in the daily diaries) was emphasised to reduce the importance the individuals placed on

the presence or absence of these symptoms. Steps were also taken to ensure that I, the clinician conducting study visits (and therefore responsible for making study protocol measurements and performing data analysis), remained blind to the treatment allocation. At randomisation, the solutions were administered by an independent member of the research team not involved in any of the study protocol measurements. If participants had questions or concerns during the study about any symptoms they were told to discuss these only with an independent member of the research team (and were provided with 24-hour contact details for this person). They were also asked to cover their forearms at the site of application of the treatment solution for the first six weeks of the study during visits, in case of the presence or absence of a visible rash. During the study, Full Blood Count results were seen only by the trial medical statistician and were checked and entered into a spreadsheet. At the end of the study, the trial medical statistician or another independent researcher unblinded the participants and, where appropriate, gave them the mebendazole tablets. Any visits conducted after the participant had been unblinded (for checking of eosinophil counts or collecting faecal samples) were arranged by another clinician who was not otherwise involved with the study and were carried out without my knowledge.

3.2.6 Follow-up visits

After randomisation, participants attended study visits every week for four weeks to cover the phase of lung migration (which typically occurs at between days 8 and 21 [195]), then every two weeks for a further eight weeks, until they had been in the study for a total of 12 weeks post-randomisation (Figure 3.1).

At each study visit, the bronchial challenge to measure PD_{10}AMP was performed, the Juniper RQLQ was completed and venous bloods were taken for the same tests as at the screening visit. Participants were asked to provide a faecal sample less than 24 hours old to confirm establishment and survival of the adult hookworms in the gastrointestinal tract of those in the treatment arm of the study and to quantify egg burden [274]. Finally, their daily diaries and symptom cards were collected and participants were issued with new diaries and cards to last until their next study visit.

3.2.7 Final visit

At the week 12 visit, in addition to the usual tests and measurements outlined above, the allergen skin sensitisation testing was repeated, participants were weighed and the female participants had a further urinary pregnancy (β-HCG) test.

Participants were then asked by an independent member of the research team whether they thought they had received placebo or hookworm and to justify their response, before being unblinded. If they had received placebo, no further follow-up was arranged. Those in the hookworm group were provided with a course of mebendazole tablets 100mg to be taken twice a day for three days in order to eradicate the infection. This dose of mebendazole was found to be efficacious in the dose-ranging study [274]. Those participants who chose to take the tablets were followed-up fortnightly and faecal egg counts and blood eosinophil counts were checked until egg counts were zero and eosinophils had returned to within 0.2 $x10^9$/litre of their screening value, or to within normal reference ranges, on two successive occasions.

Those with hookworm infection but who chose not to take the tablets were given written information outlining the potential risks (albeit minimal) of long-term hookworm infection, namely anaemia and endomyocardial fibrosis. They were also told to inform the blood transfusion service before donating blood in the future and were advised that mebendazole was contraindicated in pregnancy and breastfeeding (Appendix H). These participants were asked to sign two copies of a document to show that they understood the risks of infection and that they declined at that time to take the mebendazole tablets; one copy was retained by the participant, the other was filed in their records. No further follow-up was arranged for these participants.

3.2.8 Trial monitoring committee

All members of the trial monitoring committee were based at the University of Nottingham and so were easily contactable at any time. The committee comprised:

- Reader in Primary Care (chair);
- Professor of Medical Statistics in the Division of Epidemiology and Public Health;
- Professor of Respiratory Medicine at Nottingham City Hospital;
- Senior Lecturer/Consultant in Respiratory Medicine at Nottingham City Hospital.

Data on the adverse effects and haemoglobin, eosinophil and albumin levels were monitored every week as the study progressed by the trial statistician, who was aware of the randomisation codes. After discussions between the study investigators and monitoring committee, the following parameters were set as *a*

priori indicators of when the committee was to be informed immediately of abnormal results:

- fall in haemoglobin by 2g/dl from baseline or to less than 10g/dl;
- fall in albumin by more than 5g from baseline;
- fall in baseline FEV_1 by 20% from baseline;
- change in PD_{10}AMP by greater than 2 doubling doses;
- any other serious adverse events or symptoms of concern to either the participant or investigators on review of diary cards.

A decision would then be made by the committee, independent of the study investigators, as to whether the participant in question needed to be withdrawn from the study.

3.2.9 Ethical approval

The original study protocol and subsequent amendments were approved by the Nottingham Research Ethics Committee and by the Research and Development department at the Nottingham University Hospitals NHS Trust. The Medicines and Health Regulatory Association was also informed of the studies and did not require additional specific documentation to be completed, as hookworm was not considered to be a medicine or drug at that time. The study was registered with the Clinical Trials register (http://clinicaltrials.gov/ (trial reference NCT00232518)).

3.3 Data Analysis

3.3.1 Data entry and checking

All the results were entered on to a spreadsheet (Microsoft Excel (2007)), cross-checked for any discrepancies and then imported into SPSS version 14.0 (SPSS Inc., Chicago, IL). SPSS was used for all the data analyses (unless otherwise stated). I carried out all the data analyses (including entry of data and data manipulation) blind to randomisation code.

3.3.2 Primary outcome

3.3.2.1 Computation of primary outcome variable

The primary outcome was maximum change in $PD_{10}AMP$ over the first four weeks following randomisation. After entry into the study, participants who did not demonstrate a fall in FEV_1 of 10% with the maximum dose of AMP (944μm) were assigned values of 944μm for analysis.

The equation for calculating PC_{20} (provocation concentration required to reduce FEV_1 by 20%) [17] was extrapolated to calculate the $PD_{10}AMP$:

$$PD_{10} = \text{anti log } [LN (C1) + ((LN (C2) – LN (C1)) \times (10-R1)/(R2-R1)]$$

where

C1 = penultimate AMP dose

C2 = final AMP dose

R1 = % fall in FEV_1 after C1 from saline baseline

R2 = % fall in FEV_1 after C2 from saline baseline

PD_{10}AMP were calculated on two separate occasions for each study visit, including the screening visit, and the two values obtained were cross-checked to ensure they were identical. Where there was a discrepancy, the values were recalculated by an independent researcher. On each occasion, the independent researcher's calculation correlated with one of the first values obtained and this estimate of PD_{10}AMP was therefore assigned for the visits.

To determine the primary outcome, first the lowest value obtained over the first four weeks after randomisation was identified. PD_{10}AMP values were not normally distributed but could be transformed by taking the natural logarithm of each value. The maximum change in PD_{10}AMP over the first four weeks after randomisation was then calculated for each participant as the difference between the natural logarithm of the lowest PD_{10}AMP value in the first four weeks and the natural logarithm of the PD_{10}AMP from the randomisation (week 0) visit. The result was transformed into a doubling dose by dividing by the natural logarithm of 2 to obtain the final outcome variable which does follow a normal distribution.

$$\text{Change in } PD_{10} \text{ in doubling doses} = \frac{\ln(\text{lowest } PD_{10}\text{AMP in first 4 weeks}) - \ln(\text{baseline } PD_{10}\text{AMP})}{\ln 2}$$

A positive value represented an increase in PD_{10}AMP (that is, reduced bronchial responsiveness, so an "improvement") and a negative value represented a fall in PD_{10}AMP (that is, increased bronchial responsiveness, a "deterioration").

The original intention had been to define the baseline measurement as the mean of the two pre-infection values (screening and week 0). However, due to the learning process observed in performing the bronchial challenge, there were

concerns that the values obtained at the screening visit were not reliable and therefore the week 0 values were used as the baseline for the primary analysis. The results using the mean of screening and week 0 values as baseline were also computed for comparison.

3.3.2.2 Statistical analysis of primary outcome variable

The final change in $PD_{10}AMP$ variable was normally distributed and compared between the two intervention groups by computing means and the mean difference (with 95% CI), and an independent samples t-test was performed to assess statistical significance. Multiple linear regression was used to adjust, where necessary, for any baseline differences in demographics. Using SPSS version 14.0 (SPSS Inc., Chicago, IL), Levene's test was used to check for equality of variances and appropriate adjusted results were used if there was evidence of unequal variances.

3.3.3 Secondary outcomes

A variety of secondary outcomes were measured. In addition to the primary outcome $PD_{10}AMP$, PEF variability was used to assess safety during the larval phase of lung phase over the first four weeks after randomisation. Change in allergen skin sensitisation and the Juniper RQLQ were used to assess the impact on allergic disease severity over the whole 12 week study period. The tolerability of infection was assessed both over the whole period and different high risk periods by measuring a range of symptoms potentially due to the infection. Finally immunological parameters were measured over the 12 week study period to identify the extent to which the infection had an impact on the immune system.

3.3.3.1 PEF variability

PEF variability during the first four weeks after infection was computed as the two-lowest percentage mean (mean of the two lowest PEF values during this period as a percentage of the period mean), which has been shown in a comparison paper to be the best performing PEF variability index [275]. The final variable for analysis was the two-lowest percentage mean for the first four weeks of the study; with the values close to 100% signifying less variability and therefore less airway lability.

$$\text{Two-lowest \% mean} = \frac{\text{mean of lowest two readings in period}}{\text{period mean}} \times 100\ \%$$

3.3.3.2 Quality of Life Scores

The Juniper Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ) questionnaire [272] was completed using a Microsoft Access 2007 database and the results were imported into SPSS version 14.0 (SPSS Inc., Chicago, IL). An allergic symptom score out of 168 was recorded for each visit by summing individual symptoms recorded on the Juniper RQLQ. The scores were logged to achieve a normal distribution and then summarised over the 12 week study period using area under the curve (AUC) (GraphPad prism 4, GraphPad Software Inc., San Diego, CA) to obtain the final variable for analysis.

3.3.3.3 Allergen skin sensitisation

Change in allergen skin sensitisation was computed for each allergen using the same method as described in section 2.3.3.3.

3.3.3.4 Adverse Symptoms

For each adverse effect potentially attributable to the hookworm infection, the mean daily score was computed for both the whole 12 weeks and also a pre-determined 'high-risk' period chosen as the period during which time the symptoms were predicted to be most likely to occur. This was to ensure that the magnitude of effect of important adverse symptoms was not diluted by averaging over the full 12 weeks of the study. These periods were days 1 to 21 (for localised skin reactions as observed in the previous study) [274], days 1 to 28 (for respiratory symptoms coinciding with the period of larval lung migration in the hookworm lifecycle) [127], and days 29 to 70 (for gastrointestinal symptoms and tiredness correlating with the period of elevated eosinophil counts which can result in an eosinophilic gastroenteritis) [259].

3.3.3.5 Statistical analysis of secondary outcome variables

The PEF variability, Juniper RQLQ scores and allergen skin sensitisation variables were normally distributed and the mean values for the two intervention groups were therefore compared using the independent samples t-test with multiple linear regression to adjust where necessary for any baseline differences in demographics. The equality of variances was checked using Levene's test in SPSS version 14.0 (SPSS Inc., Chicago, IL) and appropriate adjusted results were used if there was evidence of unequal variances. The adverse symptom score variables were not normally distributed and could not be transformed and so medians were used as the summarised averages and the non-parametric Mann-Whitney U test was used to compare the two intervention groups.

3.3.4 Sensitivity analysis

Two sensitivity analyses were performed.

the PEF analysis was repeated excluding any participants who had provided less than 75% of potential readings during the first four weeks;

the Juniper RQLQ analysis was repeated excluding participants who had used antihistamines more than three times per week (unless used daily).

3.3.5 Other outcomes

3.3.5.1 Haemoglobin and albumin

Haemoglobin and albumin levels were monitored as the study progressed by the trial statistician to ensure participants did not become anaemic or show signs of becoming malnourished. At the end of the study, change in haemoglobin and albumin levels were computed as the difference between week 12 and baseline values. In addition, the net change in weight of each participant was calculated as the difference between the weights at the week 12 visit and screening visit.

3.3.5.2 Markers of hookworm infection

Eosinophil counts and faecal egg counts were monitored at regular intervals during the study as described and used to confirm presence of infection in those who received hookworm infection.

3.3.5.3 Immunological parameters

The immunology tests were performed by collaborators in the University of Nottingham School of Pharmacy and the results are included here for completeness. Levels of total IgE, parasite-specific IgG, interleukins IL-10 and IL-

13 and cytokines TNF-α and IFN-γ and numbers of T-lymphocytes and T-regulatory cells were measured at each study visit. Each time point was plotted and the AUC computed as a summary measure for each individual. If the AUC values were normally distributed, the mean value for AUC was then calculated for the hookworm and placebo groups and the groups compared using the independent samples t-test; otherwise the groups were compared using the non-parametric Mann-Whitney U test.

3.3.6 Sample size and power calculation

The primary objective was to detect an increase in bronchial responsiveness, which was defined *a priori* to be one doubling dose or more in magnitude, in the active group, relative to the placebo group, over the first four weeks of the study. This time period correlates with the lung phase of migration of the stage L3 larvae. With a sample size of 30 (15 in each group) the study would have approximately 80% power to detect a difference of this magnitude in the maximum fall in $PD_{10}AMP$ between active and placebo groups, assuming a similar repeatability to that reported for $PD_{20}AMP$ [267;268].

3.4 Results

3.4.1 Participant flow

Thirty people were randomised to the study: 15 were randomised to active hookworm infection and 15 to placebo (Figure 3.2). Three participants withdrew from the study and were subsequently unblinded: one from the placebo group on day 6, due to an inter-current viral illness; one from the hookworm group on day 12, after becoming pregnant despite a negative pregnancy test at entry into the study and use of contraception; and one from the hookworm group on day 40, due to abdominal pain and diarrhoea, who was treated with mebendazole to eradicate the infection. The participant who withdrew because of pregnancy kept the infection and completed an otherwise uneventful and successful pregnancy. Four participants were unable to attend the week 12 visit and were seen at the earliest possible occasion thereafter which for three of the four was during week 13, and for the fourth was day 112 (during week 16).

3.4.2 Baseline characteristics of participants

There were more current smokers in the placebo group, but otherwise the demographic characteristics of the two groups were similar (Table 3.1). 60% of participants in each group were male, and all were Caucasian with the exception of two participants in the hookworm group. Participants were relatively young, with mean ages of 30.3 (SD 8.97) (hookworm group) and 33.2 (SD 8.82) (placebo group). Exclusion of data from the participants who withdrew early from the study did not appreciably change the overall demographic characteristics of the two groups.

3.4.3 Primary outcome

Data on bronchial responsiveness over the first for 4 weeks following randomisation were available for 28 participants. The mean of the maximum fall in $PD_{10}AMP$ during this period was slightly greater in the hookworm group than the placebo group (-1.67 and -1.16 DD respectively), but the difference between the groups was small (-0.51 DD, 95% CI: -1.80 to 0.78 DD) and not statistically significant either before or after adjusting for the baseline difference in smoking status (p=0.42 and p=0.34 respectively, Table 3.2). The individual greatest falls, or if no fall, then the smallest improvement, ranged from -5.55 to 0.81 DD in the hookworm group and -4.26 to 1.47 DD in the placebo group.

3.4.4 Secondary outcomes

3.4.4.1 Peak expiratory flow variability

Peak flow variability over the first four weeks was slightly less (i.e. closer to 100% which means no variability) in the hookworm group (mean 92.31% (SD 3.73%)) compared with placebo (mean 89.30% (SD 6.70%)), but not significantly so (adjusted mean difference 3.62%, 95% CI: -0.66 to 7.90%; p=0.09) (Table 3.2). Two participants did not complete 75% of their PEF recordings; excluding them from the analysis had little impact on the results (adjusted mean difference 3.70%, 95% CI: -0.99 to 8.39%; p=0.12).

3.4.4.2 Allergen skin sensitisation

The size of wheal increased on average in both hookworm and placebo groups for grass (mean change over study period = 0.73mm and 0.11mm respectively) and *D. pteronyssinus* (1.27mm and 0.54mm respectively) allergens and reduced in both groups for cat fur (-0.27mm and -0.75mm respectively). There was no

significant difference between the two intervention groups in change in the magnitude of the wheal response to any of the individual allergens tested (table 3.3).

3.4.4.3 Allergic rhinoconjunctivitis symptoms

The hookworm group reported higher scores for the Juniper RQLQ over the 12 week study period than the placebo group (mean log AUC 6.01 (SD 0.82) vs. 5.68 (SD 0.85)), but again this difference was small and not statistically significant either before or after adjustment for smoking and baseline score (adjusted mean difference 0.26, 95% CI: -0.45 to 0.97; p=0.46 (table 3.3)). There was also no difference between the two groups after excluding the seven participants who had used antihistamines on more than three occasions in a one week period (unless taken daily; adjusted mean difference 0.42, 95% CI: -0.49 to 1.32; p=0.34).

3.4.4.4 Adverse effects

Table 3.4 shows the reported symptom scores for the potential adverse effects attributable to hookworm. Both localised skin itching and redness were significantly higher in the hookworm compared with the placebo group, and differences were even greater during the high risk period (difference in medians for mean daily score for itching 1.12 (p=0.001) and redness 1.02 (p<0.001)) (Figure 3.3). These symptoms peaked in the hookworm group in the first week with some participants experiencing a second, less marked peak in week 2. For the non-skin symptoms, there was more of a range of different participant experiences, with many reporting mean daily scores of zero (i.e. no symptoms). Scores tended to be higher in the hookworm than the placebo group, but the magnitude of the differences was fairly small. A significant difference was seen for

indigestion (difference in medians 0.1, p=0.03) during the high risk period, and a borderline significant effect was seen for abdominal pain (difference in medians 0.48, p=0.06). Breathlessness was also higher in the hookworm group but not statistically so, and during the high risk period this difference was even less marked. Notably, there was no difference between the two groups for the other respiratory symptoms (Table 3.4).

3.4.5 Other outcomes

3.4.5.1 Effect on eosinophil levels

Twelve of the 13 participants in the hookworm group who completed the study had a rise in eosinophil counts, which typically started between 21 and 28 days after infection and reached a peak at weeks 6 to 8 (days 42 to 56), with maximum counts ranging from 1.53 $x10^9$/litre to 9.70 $x10^9$/litre (Figure 3.4). All eosinophil counts decreased after this time but had not returned to baseline values by the end of the study at week 12 (day 84). The change in eosinophil counts was reflected by a rise in total white cell counts, which also peaked during this time with a maximum eosinophilia (eosinophil count as a percentage of total white cell count) ranging between 21% and 60% (normal range 1% to 5%). No change was seen in numbers of any other leucocyte type. There was no rise in the eosinophil counts in any of participants in the placebo group.

3.4.5.2 Other clinical parameters

All participants had haemoglobin and albumin levels within the normal ranges at entry into the study. No clinically important falls in haemoglobin were seen in either group, with the maximum fall in haemoglobin from baseline being 1.4g/dL in the hookworm group and 0.8g/dL in the placebo group (Figure 3.5). Similarly,

there was no suggestion of any significant changes in serum albumin levels, with the greatest fall from baseline being 7g/dL in both groups, to a minimum of 36g/dL and 31g/dL in the hookworm and placebo groups respectively. There were no clinically important changes in participants' weight during the study.

3.4.5.3 Immunology results

The immunology tests were performed by collaborators in the University of Nottingham School of Pharmacy and the results are included here for completeness. In those who received hookworm, a significant increase was seen in parasite-specific IgG from week 8 onwards (Figure 3.6); this was confirmed using Western blot. There was a non-significant fall in production of the pro-inflammatory cytokines TNF-α and IFN-γ at week 6, which coincides with detection of appearance of eggs and may suggest a degree of regulation by mature hookworm in the gut (Figures 3.7, 3.8). Otherwise, there was no significant change in total IgE levels, number of T-lymphocytes (absolute numbers or relative percentage of T-regulatory cells) or IL-10 or IL-13 production (Figures 3.9-3.13) [276].

3.4.5.4 Confirmation of hookworm infection

Eggs were found in faecal samples of nine participants out of the 13 completing the study in the hookworm group, appearing at either week 6 (six people) or 8 (three people) after infection. The presence of these eggs was confirmed by culturing faecal material obtained at week 12 to detect presence of infective larvae. Three of the four participants in the hookworm group with negative faecal samples had a rise in their eosinophil count (peaks of 3.3-9.6 $x10^9$/litre), implying that the hookworm larvae had reached the bowel and successfully developed into

adult worms. In all nine participants with positive samples, more than 50 eggs/g faeces were found on each occasion. Eggs were not seen in any participants in the placebo group. Repeating the primary outcome analysis after excluding the four participants without eggs in their faeces yielded similar results (mean change in $PD_{10}AMP$ for hookworm group -0.62 DD: adjusted mean difference -0.81 DD, 95% CI: -2.12 to 0.51 DD). In addition, a rise in eosinophil count (section 3.4.5.1) was also used to confirm the presence of establishment of adult worms in the duodenum.

3.4.6 Assessment of participant blinding

Before being unblinded at their final study visit, participants were asked if they thought they knew whether they had received hookworm or placebo infection. Of the 14 individuals in the placebo group who completed the study, three correctly thought they had received placebo, five thought they had received hookworm, and six did not know. Of the 13 with hookworm infection who completed the study, eight correctly thought that they had received hookworm, two thought they had received placebo, and three did not know.

3.4.7 Post study follow-up

All participants who received hookworm were provided with mebendazole at the end of the study to eradicate the infection. Eleven of the 13 participants who completed the study chose not to take the treatment, citing either a perceived improvement in hay fever symptoms, or that they wished to see if they had an improvement in symptoms the following year. In accordance with the protocol, participants in the placebo group were offered hookworm infection at the end of the trial, of whom 11 chose to be infected. These individuals were provided with

an information sheet outlining the adverse symptoms reported in the dose-ranging study (Appendix I) and were asked to sign three copies of a consent form, one for the study records, one for their General Practitioner and one for them to retain. They were provided with a contact telephone number to use in case of a question or problem relating to the study, but were otherwise asked to see their General Practitioner if they had concerns regarding the infection in the future.

3.5 Discussion

3.5.1 Summary of findings

This is the first reported randomised placebo-controlled double-blind study of hookworm infection in people with allergic disease. The study was designed primarily as a safety study to determine whether hookworm infection exacerbates bronchial responsiveness during the phase of larval pulmonary migration in preparation for a trial in people with asthma. A dose of larvae was used which was shown in the dose-ranging study (Chapter 2) to be well tolerated and to result in an infection intensity of over 50 eggs/g faeces, the level associated with a reduced risk of wheeze in a previous observational study [58]. Participants with allergic rhinoconjunctivitis and measurable bronchial responsiveness, but without clinical asthma, were chosen [277] to enable evaluation of an increase in bronchial responsiveness in the active treatment group while minimising the risk of serious clinical exacerbation of asthma.

The study found no evidence of a clinically important increase in bronchial responsiveness during the first four weeks following infection, which corresponds to the period of larval lung migration. The mean of the maximum fall in PD_{10}AMP during this period was slightly greater in the hookworm group than the placebo group (-1.67 and -1.16 DD respectively), but the difference between the groups was small and not statistically significant after adjusting for the baseline difference in smoking status (-0.51 DD, 95% CI: -1.80 to 0.78 DD; p=0.34). Similarly, there was no clinically important difference between the two groups in change in PEF variability. In addition, though only designed and powered to detect effects on bronchial responsiveness, the study also provided an opportunity to explore the

effect of infection on rhinoconjunctivitis symptom severity and allergen skin sensitivity. No clinically important differences between the two groups were found for either allergen skin sensitisation test or Juniper RQLQ scores over the course of the study.

Participants were also monitored for any symptoms potentially attributable to the hookworm infection. The infection was generally well tolerated, with only one participant withdrawing from the study as a result of adverse symptoms. The majority of people who received hookworm reported a pruritic erythematous rash at the site of infection. Gastrointestinal symptoms were also commonly reported, occurring around six weeks after infection.

3.5.2 Strengths and weaknesses

3.5.2.1 Measurement error

During the screening visits it was observed that certain participants took some time to learn how to use the dosimeter properly, resulting in unreliable screening measurements of their bronchial responsiveness. This would not have been affected by the group to which they had been randomised, however, to try and minimise the impact of this measurement error, week 0 results were chosen as the baseline measurement for the primary analysis but the results are also presented using the mean of the screening and week 0 values as baseline, as per protocol (Table 3.2). In the hookworm group, the mean value of the primary outcome variable, maximum fall in PD_{10}AMP, was similar using the two different definitions for baseline, but this was less so in the placebo group where the mean value was lower when the definition included the screening data (-0.62 DD) than when based on week 0 data alone (-0.16 DD; Table 3.2). Further exploration

revealed that this discrepancy was primarily due to one individual who responded rapidly to AMP at screening and thus had a very low value, but who had a much higher value at week 0. Exclusion of this participant from the analysis resulted in a similar estimated mean difference between hookworm and placebo groups regardless of baseline definition (-0.51 DD using week 0 as baseline and -0.55 using mean of screening and week 0).

Allergic rhinoconjunctivitis is a particularly difficult condition to study in view of the variability of symptoms from week to week and year to year. This study was not designed to look at the effect of infection on allergic rhinoconjunctivitis, but given that the participants had symptoms at the time of enrolment, the opportunity was taken to see if there were any changes in symptoms over the course of the study. Symptoms were measured using the Juniper RQLQ which is well validated to show moderate to strong relations between changes in symptom diary scores and changes in RQLQ scores [273] but no other measures of allergic rhinoconjunctivitis disease activity were included in the study design. It is therefore possible that infection did have an effect on participants' symptoms of allergic rhinoconjunctivitis but because of the limited methods used, it was not detected.

There is also the possibility of measurement error when evaluating the occurrence of adverse symptoms of infection. No validated questionnaire was available to record the presence of adverse symptoms of infection and so a diary, based on the symptoms reported by other reports of experimental hookworm infection and symptoms reported in the dose ranging study was instead used. However, as described in section 2.5.2.1 there is likely to be variation between participants in their definition of symptoms and in their perception of severity of symptoms.

Blood samples were all measured using automated mechanisms used regularly in the laboratories and are subject to regular quality control; as such the measurement error in these results should have been minimal.

3.5.2.2 Success of blinding and bias

Blinding in a clinical trial is important to try and reduce reporter and observer bias, especially for outcomes which are subjectively measured and so particularly susceptible to differential bias. Objective measures were used for the majority of outcomes, including the primary outcome, in this trial and therefore theoretically should not have been subject to bias even if the participants or clinician carrying out the study visits had correctly deduced the treatment allocation. The procedures adopted to ensure that participants and clinical investigators remained blind to treatment allocation were successful for the investigator, and predominantly so for participants. Most of those participants who did correctly guess their treatment allocation were in the active treatment group, and usually based their judgment on seeing entry portals on their arm when the plasters were removed 24 hours after infection, or the presence of gastrointestinal disturbance at a later stage. The histamine solution used as a placebo did cause local redness and itching, as reflected in the adverse symptoms scores, but these symptoms lasted for a shorter period than those attributable to hookworm. Despite the fact that some participants were aware of the treatment to which they had been randomised, it is unlikely that they would have been aware of how to influence the primary outcome results. In theory, observer bias may have been slightly harder to overcome as, for example; the bronchial challenge tests are not truly objective in that a degree of judgement is involved in carrying out the test and recording the

results. However, the investigator carrying out study visits remained successfully blind to treatment allocation and thus observer bias should have been minimal.

If participants had correctly guessed the intervention to which they had been allocated, it could have resulted in reporter bias, particularly for the two trial outcomes which were subjective. First, participants' may have under-reported the impact of their allergic rhinoconjunctivitis symptoms in completing the Juniper RQLQ if they thought they had received infection. However, several participants in the placebo group also thought they had infection and may also have under-reported symptoms. Second, adverse symptoms potentially attributable to infection were also measured subjectively and were therefore subject to reporter bias if participants correctly guessed the intervention group to which they had been allocated. This is most likely to have resulted in over-reporting of symptoms in people who thought they had received hookworm and possible under-reporting of symptoms in those who believed they had received placebo. Despite this, the reported adverse effects were broadly similar to those previously reported, again suggesting little effect, if any on the results. For example, the cutaneous rash peaked in the hookworm group in the first week with some participants experiencing a second, less marked peak in week 2 [274].

3.5.2.3 Success of infection

Hookworm infection was confirmed by the presence of eggs in faeces for nine of the infected group; of the other six participants randomised to hookworm, two withdrew before eggs were expected to be seen in faeces, and three demonstrated increases in eosinophil counts similar to those with proven infection, suggesting that they were actively infected but with same-sex organisms

or non-fecund females. It therefore appears that the infection process failed in only one participant in the hookworm group, in whom there was no evidence of active infection. Excluding this individual or all participants with negative egg counts from the analyses had no material impact on the findings of the study.

3.5.2.4 Representativeness and loss to follow-up

The entry criteria for this study were fairly strict, requiring people to have regular symptoms of hay fever and measureable bronchial responsiveness and being willing to commit to regular study visits over the course of four months. People volunteering to take part in a study of experimental hookworm infection are as such unlikely to be representative of the population in general; however, there is no reason to suspect that any such differences would interact with the clinical effect of hookworm infection and impact on the trial results. It is therefore reasonable to assume that the results are likely to be generalisable.

Three participants withdrew from the study, two for reasons unrelated to the study, and one as a result of developing gastrointestinal symptoms. There is no evidence that these individuals were experiencing different effects from the other participants on the primary outcome (such as greater changes in $PD_{10}AMP$) and other than the one individual with gastrointestinal symptoms, on the secondary outcomes. It is therefore unlikely that the results of the study would have been significantly different had these participants remained in the trial.

3.5.2.5 Statistical power

The sample size of the study was small (n=30) and the power calculation was based on the primary outcome. Three participants withdrew from the study, and

so the final analysis was performed on 27 individuals which resulted in a slightly smaller sample size than planned and reduced power. The effect on power is likely to be minimal as the number of participants withdrawing was small, but there is still a possibility that an adverse effect on PD_{10}AMP was missed as a result. Regardless, the confidence intervals suggest that there is unlikely to have been a true large effect of infection. In addition to the effects on primary outcome, it is also possible, that a difference between the two groups may have existed for some of the secondary outcomes, such as symptoms of allergic rhinoconjunctivitis, but the study was not powered to show this difference.

3.5.2.6 Confounding and success of randomisation

The process of randomisation was generally successful in that the baseline characteristics of two groups were similar with the exception of smoking status. Data were collected on several different potential confounders at baseline and were accounted for in the data analyses as appropriate. However, given the small sample size, there may still be differences between the two groups in unmeasured confounders which could have had an impact on the results.

3.5.3 Results in context of other studies

Although there is some observational evidence suggesting that allergic rhinoconjunctivitis is less common in individuals infected with intestinal parasites [223], and specifically *E. vermicularis* [227], the majority of studies have shown little convincing evidence of a significant association [207;224-226]. One possible explanation for this is that different species may exert different effects and the studies have mainly been carried out in areas of endemic *A. lumbricoides* infection. Previous intervention studies with hookworm in allergic

rhinoconjunctivitis until recently were limited to an early anecdotal report, which described an improvement in hay fever symptoms after self-infection with hookworm [241]; and an eradication study in Vietnam in an area where hookworm was endemic, which found no effect of anti-helminth treatment on symptoms of allergic rhinoconjunctivitis [243]. Since the studies reported in this thesis were carried out, a Danish group has published the first randomised controlled trial specifically designed to evaluate parasite infection as a potential treatment for allergic rhinoconjunctivitis. In this study 2500 *Trichuris suis* (pig whipworm) ova, or placebo, were administered orally at 21-day intervals on eight occasions during 2008 [278]. Measured outcomes were daily symptom scores for allergic rhinitis [279], use of rescue medication, exhaled nitric oxide, haemoglobin levels, leucocyte counts, total histamine levels (as a proxy for the basophil count), serum antibody titres and symptoms of asthma, diarrhoea, flatulence and *pruritus ani*. Data from 96 individuals were analysed. Significantly more gastrointestinal disturbance was reported by those who received infection compared with those who received placebo. Predictably, a significantly greater increase in eosinophil counts during the study was seen in the treatment group compared with placebo. *T. suis*-specific antibody titres were also significantly higher in the treatment group. There was no difference in symptom scores; percentage "well" days; medication scores; nasal spray and eye drop usage; percentage of individuals assessing an improvement in their pollen allergy symptoms compared with preceding year; specific IgE to grass titres; total histamine levels; or exhaled nitric oxide or change in allergen skin sensitisation. Tablet usage was less in the treatment group compared with placebo ($p=0.04$). In this context, it is perhaps not surprising that in the study reported in this thesis, no evidence of a significant improvement in

allergic rhinoconjunctivitis symptoms in the infected group relative to the controls was detected; particularly as it was not powered or specifically designed to do so.

3.5.4 Interpretation of results

Since it is not possible to predict when lung migration will take place in any individual participant, the largest observed increase in bronchial responsiveness at any time during the first four weeks after infection was used as the primary outcome. A small increase in bronchial responsiveness was observed in the hookworm group relative to placebo, estimated to be less than one doubling dose of AMP in magnitude, but this effect was not statistically significant. Furthermore, this size of effect is unlikely to be clinically significant, considering the magnitude of normal repeatability for bronchial responsiveness to bronchial challenge testing is large (95% confidence limits of approximately ±1.5 doubling doses for two-week repeated challenge to methacholine) [17]. The magnitude of effect observed is also less than that seen to be associated with established therapies for asthma [280;281]. Bronchial challenge is the gold standard method of measuring bronchial responsiveness but PEF was also used to increase the chance of seeing an effect if one did exist.

Infection was generally well tolerated with only one participant withdrawing from the study as a result of adverse effects of infection. The most common adverse symptoms to be reported were localised skin reactions and gastrointestinal symptoms, which is consistent with previous reports of intentional hookworm infection and the dose ranging study in Chapter 2 [194;195]. All participants in the hookworm group experienced skin reactions (both itching and redness) which occurred in the first few days following infection and, in some, the redness

reappeared in the second week as has been previously reported. The gastrointestinal effects occurred in conjunction with a rise in eosinophil counts and are therefore likely to be due to an eosinophilic gastroenteritis [259] although it should be noted that not all participants reported gastrointestinal symptoms with several reporting scores of zero. Whist the highest scores were around 6/10, the questionnaire is not validated and undue emphasis should not be placed on absolute scores. Some of the symptoms experienced were spurious, occurring on individual days and are likely to be due to coincidence rather than directly related to the infection.

There were no clinically important changes in allergen skin sensitisation in any of the participants nor was there any effect of infection on symptoms of allergic rhinoconjunctivitis. Whilst the study was neither designed nor powered to detect changes in these allergic disease outcomes, there are other possible reasons why if there is a true effect, it was not seen. First, it may be that the hypothesis is incorrect, and that hookworm has no effect on allergic disease outcomes. If this is so, then previously observed associations between infection and allergy may be due to the reverse causation and unmeasured confounders. Second, it may be that the design of this intervention study was wrong and that the duration of the study was too short, or the dose of larvae too small. This is likely to be particularly important given the lack of immune modulation demonstrated by the immunology results. In observational studies of natural infection where an inverse association between infection and allergy has been identified, infection is generally acquired in childhood, with repeated re-infection thereafter. It may be therefore that the timing of infection is paramount and the protective effect of hookworm infection arises from infection in early childhood, rather than from current infection in adults

who have an established allergic phenotype. In this study a single dose of larvae were given to people already sensitised to allergens and it may be infection needs to be given much earlier to have an effect.

In addition to the reasons given above, a true effect of infection on allergic rhinoconjunctivitis may not have been seen because of the limited assessment of the condition that was carried out in this trial. As discussed in section 3.5.2.1, allergic rhinoconjunctivitis is a particularly challenging condition to study because of the intermittent nature of symptoms and its variability from year to year. This trial was designed as a safety study; however, the opportunity was also taken to investigate the effects of infection on allergic rhinoconjunctivitis symptoms using the Juniper RQLQ. The clinical effectiveness of hookworm infection in allergic rhinoconjunctivitis needs to be tested in larger specifically designed studies and should include a number of specific outcome measures; these are discussed more fully in section 6.2.1.

The immunology results showed a fall in the levels of TNF-α and IFN-γ at week 6, which coincides with detection of appearance of eggs and may suggest a degree of regulation by mature hookworm in the gut. However these changes were not significant, and neither were there significant changes in levels of T-lymphocytes IL-10 or IL-13 production which would be expected if a notable effect on the immune system had taken place. There was no significant rise in total IgE levels but this has been previously noted in another report of intentional infection and levels in this study rose when infection was repeated [184]. However, overall this suggests that the single dose of ten larvae had not been adequate to exert the

desired immunoregulatory effect; possible strategies for overcoming this in future trials are discussed in section 6.2.1.

3.5.5 Conclusions

This is the first randomised double-blind placebo-controlled trial of parasite infection to be carried out in people with allergic disease. The trial found no evidence to suggest that hookworm infection was likely to cause clinically relevant increases in bronchial responsiveness, thereby indicating that infection with ten larvae is unlikely to exacerbate asthma. The adverse effects reported by participants who were infected with hookworm were principally skin itching and gastrointestinal disturbance, and in the majority of cases were mild, with only one person choosing to withdraw from the study as a consequence. This study thus indicates that this level of infection is likely to be tolerated by the majority of participants in trials, and that exacerbation of asthma is unlikely. On the basis of these results, further definitive trials are feasible and it is safe to proceed with studies to determine whether infection is effective in the management of asthma.

Table 3-1: Baseline characteristics of study participants

	Hookworm (n=15)	Placebo (n=15)
Males (%)	9 (60)	9 (60)
Mean age (years) at entry (SD)	30.3 (8.97)	33.2 (8.82)
Current smoker (%)	3 (20)	6 (40)
Caucasian (%)	13 (87)	15 (100)
Median $PD_{10}AMP$ at screening (IQR)	13.3 (4.3, 48.3)*	24.8 (9.2, 68.8)*
Median $PD_{10}AMP$ at week 0 (IQR)	19.8 (6.4, 52.1)**	42.1 (23.6, 102.2)*

*n=13; ** n=14

IQR: inter-quartile range; **$PD_{10}AMP$**: provocation dose of adenosine monophosphate to reduce one-second forced expiratory volume by 10%; **SD**: standard deviation

Table 3-2: Respiratory outcomes measured over the first four weeks following randomisation

	Hookworm mean (SD) (n=14)	Placebo mean (SD) (n=14)	Mean difference (95% CI)	P value	Adjusted* mean difference (95% CI)	P value
Change in bronchial reactivity using week 0 as baseline (DD $PD_{10}AMP$)	-1.67 (1.72)	-1.16 (1.60)	-0.51 (-1.80, 0.78)	0.42	-0.63 (-1.97, 0.70)	0.34
Change in bronchial reactivity using mean of screening and week 0 values as baseline (DD $PD_{10}AMP$)	-1.52 (1.55)	-0.62 (1.92)	-0.89 (-2.25, 0.46)	0.19	-0.99 (-2.40, 0.43)	0.16
Peak flow variability (Two-lowest%mean)	92.31 (3.73)	89.30 (6.70)	3.01 (-1.21, 7.22)	0.15	3.62 (-0.66, 7.90)	0.09

*adjusted for smoking status

95% CI: 95% confidence interval; **DD**: doubling dose; **$PD_{10}AMP$**: provocation dose to reduce one-second forced expiratory volume by 10%; **SD**: standard deviation

Table 3-3: Allergic outcomes measured over the 12 week study period

	Hookworm mean (SD) (n=13)	Placebo mean (SD) (n=14)	Mean difference (95% CI)	P value	Adjusted* mean difference (95% CI)	P value
Juniper RQLQ score (log AUC)	6.01(0.82)	5.68 (0.85)	0.33 (-0.33, 1.00)	0.31	0.26 (-0.45, 0.97)	0.46
Change in grass SPT reaction (mm)	0.73(2.65)	0.11 (2.65)	0.62 (-1.48, 2.73)	0.55	1.18 (-0.94, 3.30)	0.26
Change in cat fur SPT reaction (mm)	-0.27 (2.09)	-0.75 (1.83)	0.48 (-1.07, 2.03)	0.53	0.64 (-1.01, 2.29)	0.43
Change in *D.P.* SPT reaction (mm)	1.27 (2.19)	0.54 (2.59)	0.74 (-1.18, 2.64)	0.44	0.85 (-1.19, 2.90)	0.40

*all adjusted for smoking status; Juniper RQLQ additionally adjusted for baseline score

95% CI: 95% confidence interval; **AUC**: area under curve; ***D.P.****: Dermatophagoides pteronyssinus*; **RQLQ**: rhinoconjunctivitis quality of life questionnaire; **SD**: standard deviation; **SPT**: skin prick test

Table 3-4: Adverse effects reported in participants with and without hookworm infection

Symptoms	Mean daily score (scale 0-10) over total 12 week period				Mean daily score (scale 0-10) over high risk period†			
	Hookworm group median (range)	Placebo group median (range)	Difference in medians	P value	Hookworm group median (range)	Placebo group median (range)	Difference in medians	P value
Localised skin itching	0.30 (0.05 – 1.10)	0.00 (0 – 1.32)	0.30	0.003*	1.12 (0.19 – 3.11)	0.00 (0 – 1.76)	1.12	0.001*
Localised skin redness	0.22 (0.02 – 1.04)	0.00 (0 – 0.48)	0.22	0.001*	1.02 (0.10 – 5.00)	0.00 (0 – 1.90)	1.02	<0.001*
Wheeze	0.30 (0 – 1.11)	0.07 (0 – 0.98)	0.23	0.14	0.36 (0 – 2.07)	0.14 (0 – 2.00)	0.22	0.29
Cough	0.22 (0 – 1.02)	0.10 (0 – 1.54)	0.12	0.56	0.43 (0 – 1.35)	0.11 (0 – 2.79)	0.32	0.30
Breathlessness	0.14 (0 – 6.04)	0.00 (0 – 0.98)	0.14	0.07	0.05 (0 – 5.14)	0.00 (0 – 1.86)	0.05	0.34
Nausea	0.17 (0 – 2.45)	0.00 (0 – 1.85)	0.17	0.10	0 (0 – 4.72)	0.00 (0 – 2.07)	0.00	0.13
Diarrhoea	0.12 (0 – 3.37)	0.11 (0 – 3.88)	0.01	0.59	0.06 (0 – 5.64)	0.06 (0 – 3.95)	0.00	0.84
Abdominal pain	0.24 (0 – 3.81)	0.02 (0 – 3.00)	0.22	0.06	0.48 (0 – 5.92)	0.00 (0 – 3.64)	0.48	0.06
Flatulence	0.28 (0 – 1.76)	0.13 (0 – 2.62)	0.15	0.36	0.31 (0 – 1.98)	0.05 (0 – 3.05)	0.26	0.16
Indigestion	0.11 (0 – 2.39)	0.00 (0 – 0.87)	0.11	0.02*	0.10 (0 – 3.92)	0.00 (0 – 1.19)	0.10	0.03*
Loss of appetite	0.14 (0 – 2.25)	0.03 (0 – 2.21)	0.11	0.67	0.24 (0 – 4.28)	0.00 (0 – 2.60)	0.24	0.30
Tiredness	0.86 (0 – 6.34)	0.14 (0 – 2.99)	0.72	0.25	0.41 (0 – 6.55)	0.15 (0 – 2.93)	0.26	0.51

* $p<0.05$ (p value for Mann-Whitney U test)
†High risk periods: localised skin symptoms (days 1-21), respiratory symptoms (days 1-28), gastrointestinal symptoms and tiredness (days 29-70); Range = minimum-maximum

Figure 3-1: Allergic rhinoconjunctivitis study visit timeline

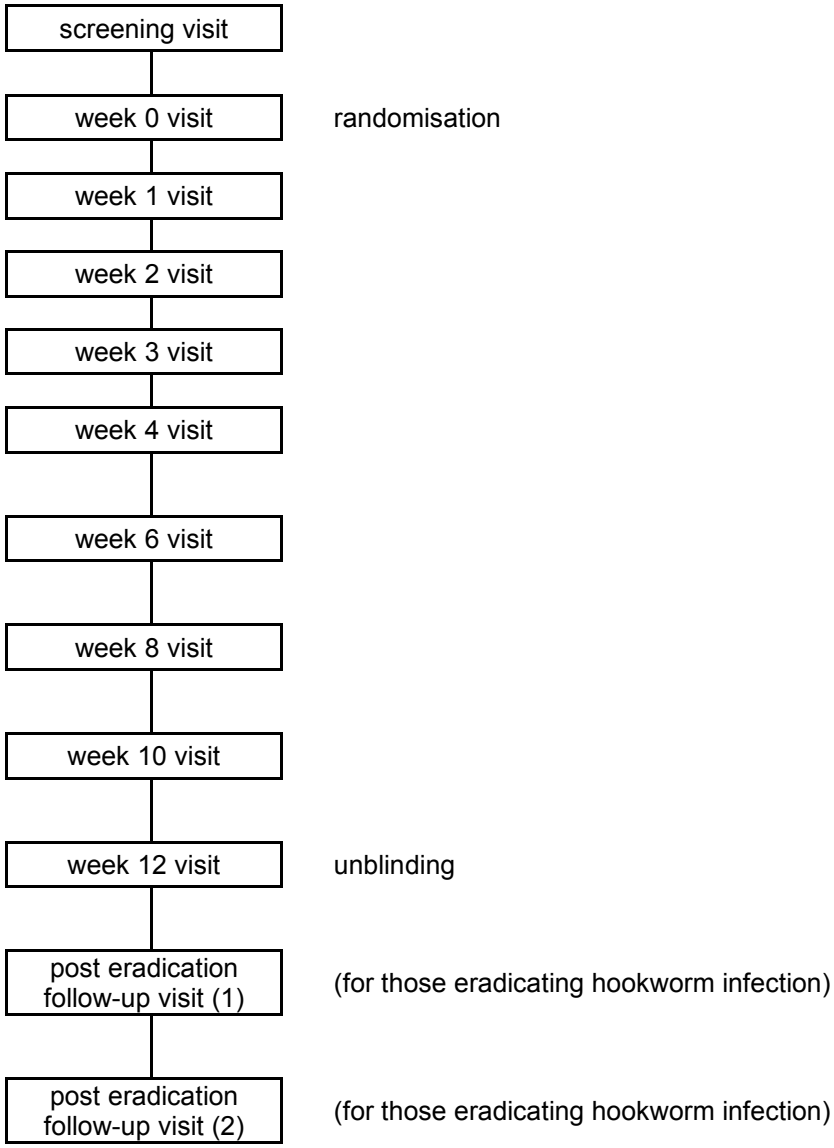


Figure 3-2: Flow chart of study participants

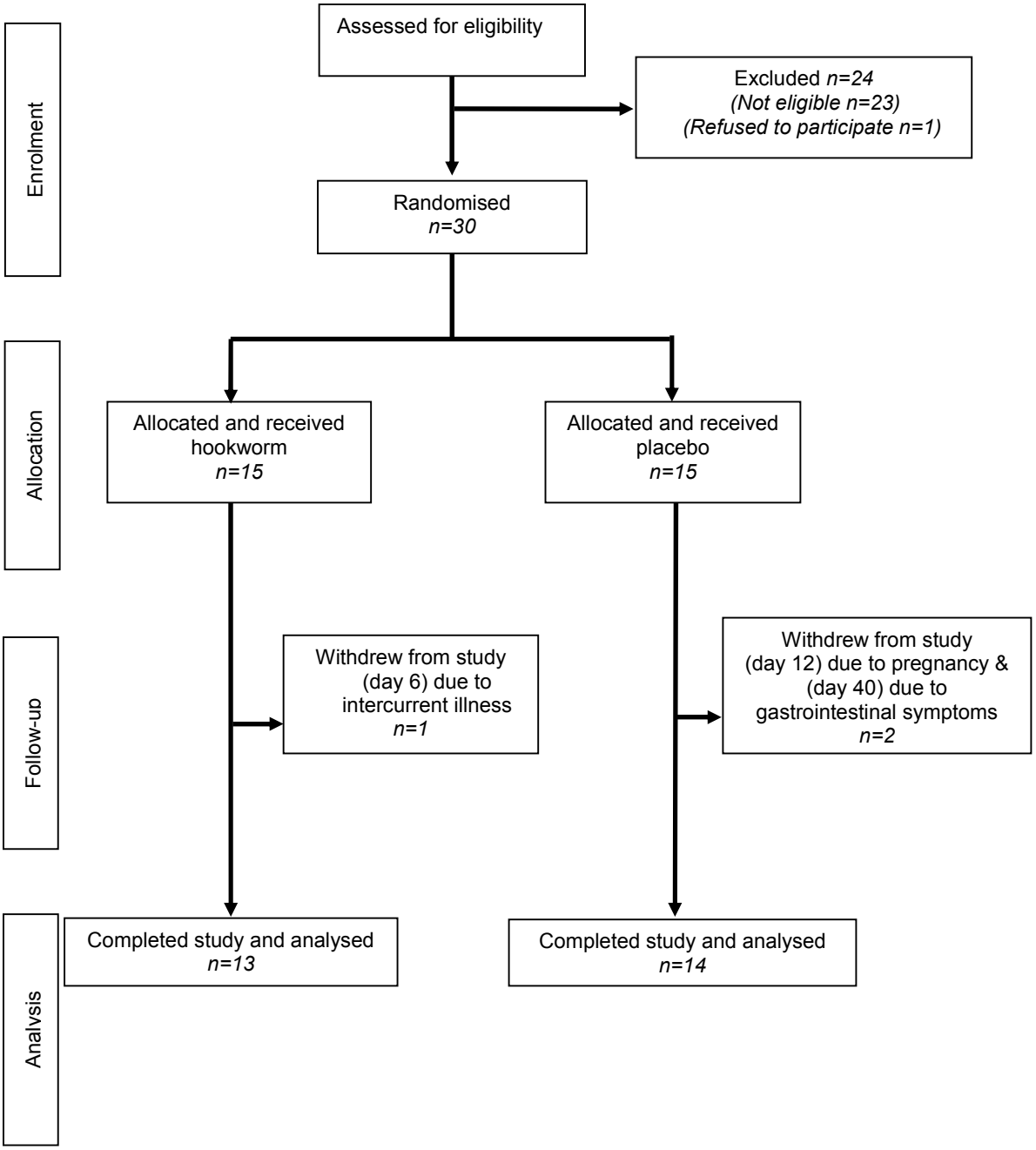
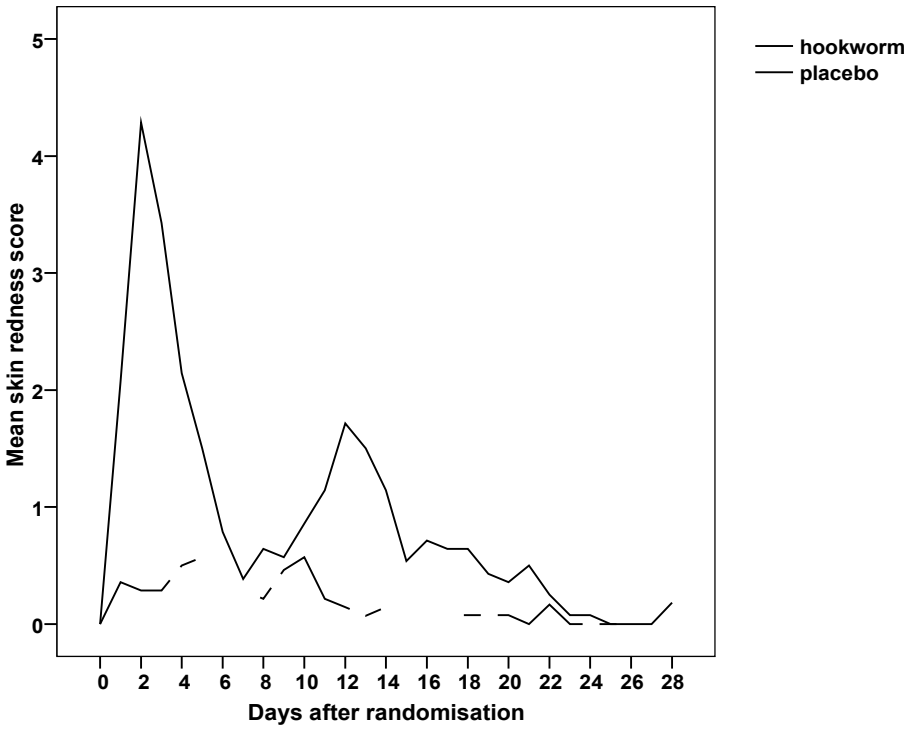


Figure 3-3: Skin symptoms measured on a visual analogue scale (0-10) over the first four weeks after randomisation

Skin redness



Skin itching

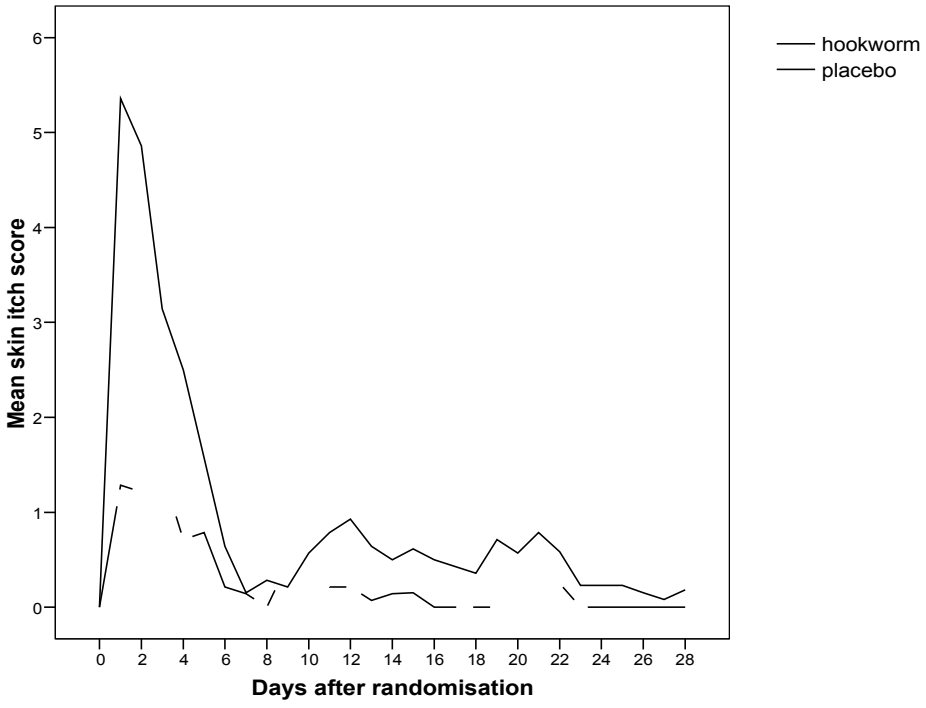
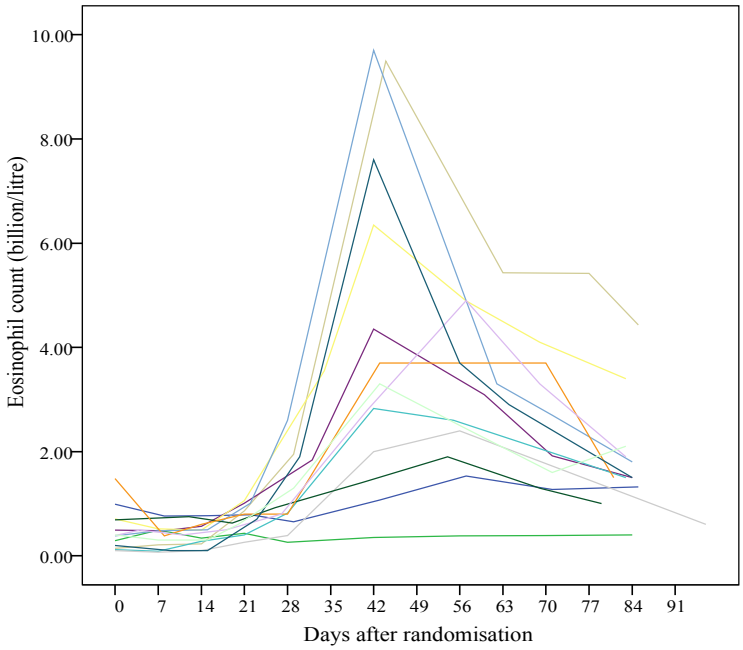


Figure 3-4: Individuals' peripheral blood eosinophil counts over the 12 week study period

Hookworm group



Placebo group

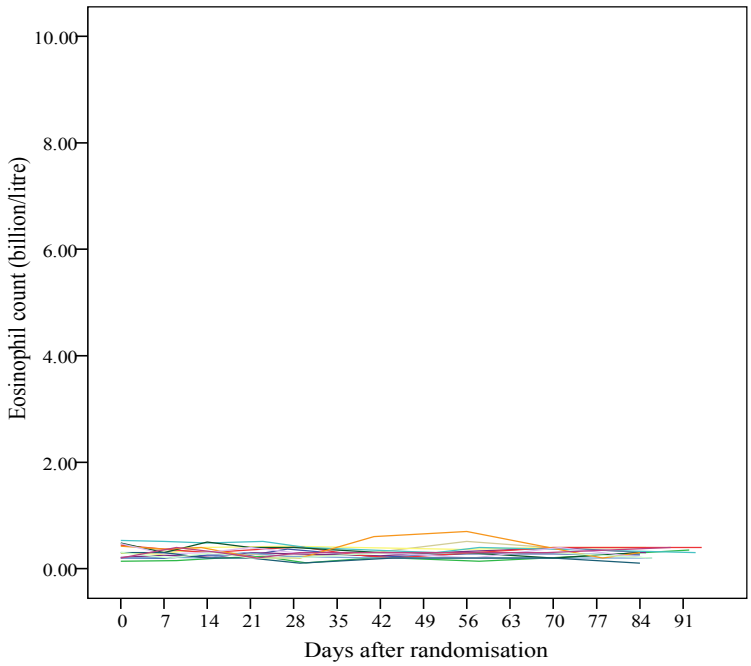
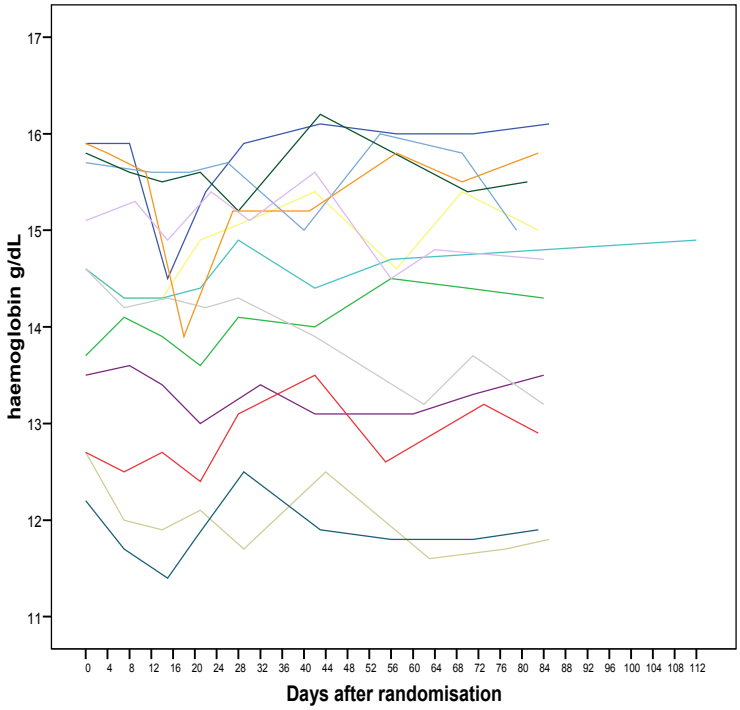


Figure 3-5: Individuals' haemoglobin levels over the 12 week study period

Hookworm group



Placebo group

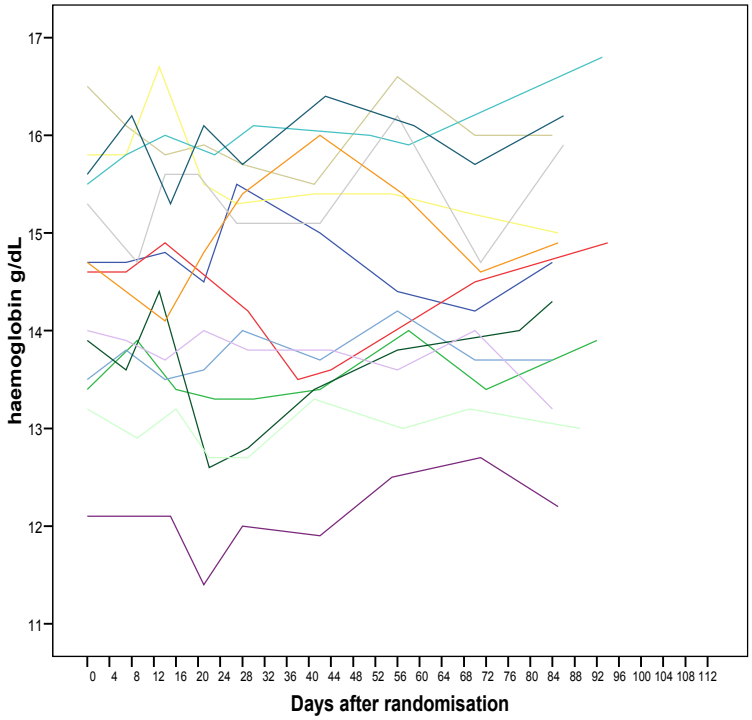


Figure 3-6: Mean parasite specific IgG in those with hookworm and placebo

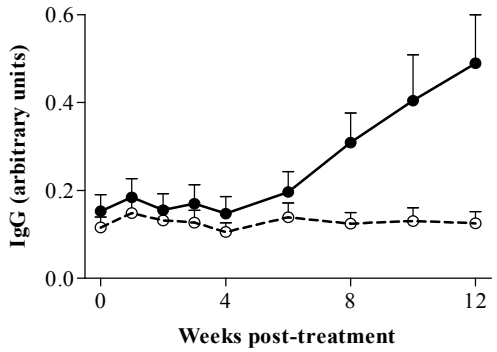


Figure 3-7: Mean TNF-α in those with hookworm and placebo

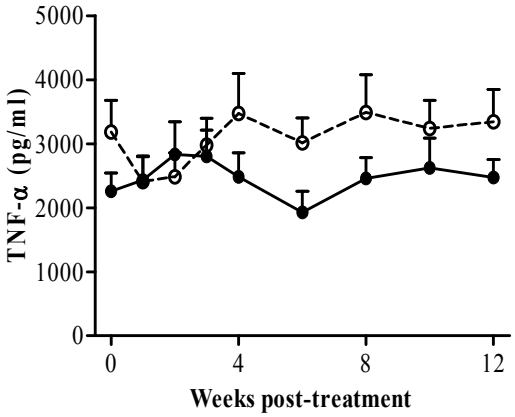


Figure 3-8: Mean IFN-γ in those with hookworm and placebo

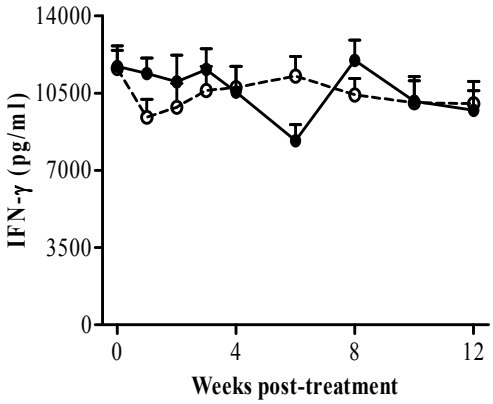


Figure 3-9: Total IgE counts in those with hookworm and placebo

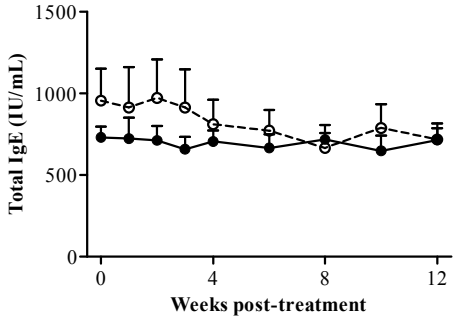


Figure 3-10: Mean T-cell counts in those with hookworm and placebo

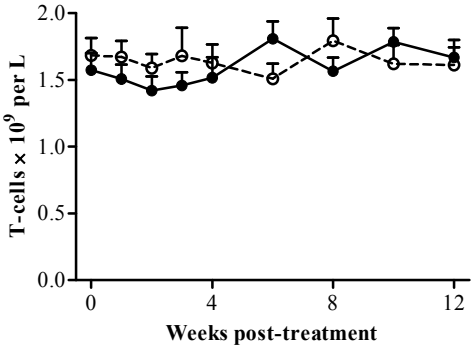


Figure 3-11: Mean T-regulatory cell counts in those with hookworm and placebo

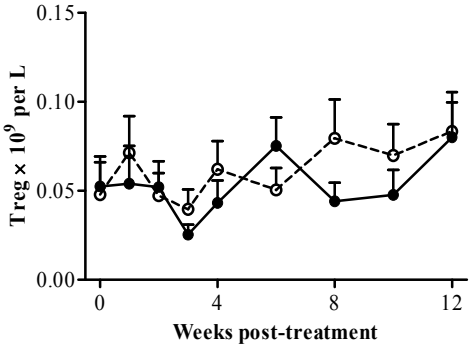


Figure 3-12: Mean IL-10 in those with hookworm and placebo

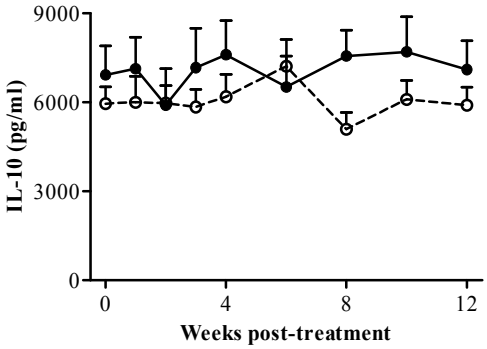
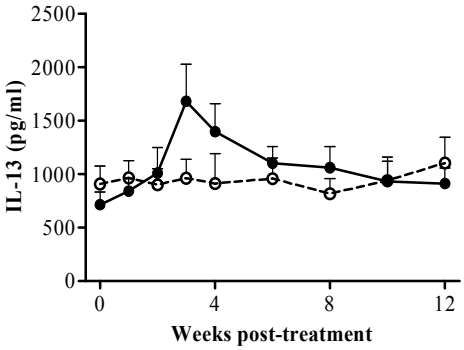


Figure 3-13: Mean IL-13 in those with hookworm and placebo



4 INTERVENTION STUDY OF HOOKWORM IN ASTHMA

4.1 Introduction

The dose-ranging study in normal volunteers (described in Chapter 2) found that infection with ten *N. americanus* larvae established a level of intensity of infection identified in previous epidemiological work to be strongly associated with protection against asthma symptoms [58;274]. The safety study in people with allergic rhinoconjunctivitis and measurable bronchial responsiveness but not clinically diagnosed asthma (presented in Chapter 3) established that the lung migration phase of intentional hookworm infection did not significantly exacerbate bronchial responsiveness and that infection at a dose of ten larvae was generally well tolerated [282]. The results of these preliminary studies allowed us to conduct the randomised controlled trial now described, in people with asthma, to test the hypothesis that infection improves people's asthma.

The use of data from epidemiological studies on the risk of having asthma to inform a study investigating the potential effect of intervention on asthma in people with established disease is not ideal as the risk factors for asthma development and those for disease severity and exacerbations are not likely to be the same. However, it was felt important to determine any effect on those with established disease - and thus whether it could be used in treatment for affected individuals - before embarking on studies of the possible preventative effects of hookworm infection on development of disease.

The primary aim of this study was to determine the effects of experimental hookworm infection on bronchial hyper-responsiveness by comparing change

in PD_{20}AMP over a 16 week period after being randomised to receive ten hookworm larvae or placebo. Secondary aims were to investigate the effects on other indicators of asthma control over the same 16 week time period using records of PEF variability, asthma symptom scores, reliever inhaler usage and quality of life scores (using a Juniper AQLQ). Other secondary aims were to investigate the effect of infection on allergen skin sensitisation and to monitor the occurrence of adverse effects (using the same questionnaire as the study reported in Chapter 3) due to the infection over the 16 weeks after randomisation.

4.2 Methods

4.2.1 Recruitment

Potential participants for the study were identified by local advertisement using posters, articles in local newspapers and the University of Nottingham and Nottingham City Hospital websites. All those who expressed an interest in the study were contacted by telephone or email and the background to the study and what participation would entail was explained. A patient information sheet (Appendix J) was then sent to people who remained interested.

Study visits were held at the University of Nottingham in the Department of Respiratory Medicine. I conducted all the study visits (other than those between May 13th and 23rd 2007), including the screening visits, and measured all clinical outcomes, thereby reducing the possibility of inter-observer variability. Recruitment took place during January and June 2007.

4.2.2 Eligibility criteria

4.2.2.1 Inclusion criteria

Participants needed to be aged 18 or over and to have a diagnosis of asthma treated with regular inhaled corticosteroids at a dose of up to 1000mcg beclomethasone (or equivalent) per day as reported by the participant and which was checked at the screening visit. They needed to have measurable bronchial hyper-responsiveness to AMP with a fall in FEV_1 by 20% at baseline and positive skin sensitisation test to at least one of *D. pteronyssinus*, cat fur and grass pollen.

4.2.2.2 Exclusion criteria

Women who were breastfeeding or pregnant, or who were sexually active and of child-bearing potential but unwilling to use contraception for the duration of study, were excluded. Individuals were also excluded if they had a history of severe allergic reaction or anaphylaxis or if they had any significant medical problems (other than asthma) in my judgement as the study clinical researcher, such as inflammatory bowel disease. Individuals were excluded if they had a positive serum IgG against hookworm signifying previous hookworm infection or any evidence of current parasite infection on faecal analysis at the time of recruitment. Those individuals whose blood tests revealed evidence of anaemia (haemoglobin <13g/dl (male) <11.5g/dl (female), mean corpuscular volume <76fl) were excluded from the study and referred to their General Practitioner for appropriate investigation.

4.2.3 Screening visit

4.2.3.1 Initial consultation and consent

Volunteers who had been sent information sheets were contacted by telephone and a screening visit was arranged for those who remained interested in taking part in the study. At this visit, the study was explained in detail including the background rationale, the study design, what recruitment would involve in terms of tests performed and the required time commitment. The potential side effects of the hookworm infection and the processes of blinding and randomisation were described and any questions answered. A brief medical history was also taken from the volunteer. After excluding those with significant medical problems (other than asthma) and women who were pregnant or of child-bearing potential and unwilling to use contraception for the duration of the study, written informed consent was obtained and two identical

consent forms completed: one for the study records and one for the potential participant to retain for future reference (Appendix C). Permission was obtained to inform the potential participant's General Practitioners of his or her recruitment into the trial. A number of measurements were then made to further assess eligibility and to collect data on baseline characteristics and clinical outcomes.

4.2.3.2 Medical history and baseline characteristics

A full medical history was then taken and the diagnosis of asthma confirmed by the study clinician. This included taking details of the severity of the asthma, presence of any other allergic disease, past medical history, use of medication and smoking status. Baseline height and weight measurements were made. Urine analysis for β-HCG (QuickVue, Quidel Corporation, San Diego, USA) was performed to confirm that female participants of child-bearing age were not pregnant. Participants were asked to bring their inhalers to the visit so that the drug and dose and expiry date could be checked and inhaler technique was assessed and optimised in all individuals. For the duration of the study, participants were asked to continue with their usual asthma medication at a static dose of inhaled corticosteroids and to use inhaled short-acting β2-agonists as normal for relief of asthma symptoms.

4.2.3.3 Lung function and bronchial challenge

Individuals were provided with a miniWright peak flow meter (European Union scale). Instruction on peak expiratory flow (PEF) measurement was given and the best of three attempts recorded. One-second forced expiratory volume (FEV_1) was measured according to international guidelines [17] using a Spirometer R model (Vitalograph, Buckingham, UK) and taking the higher of

two values within 100mls. Bronchial hyper-responsiveness was then measured by adenosine monophosphate (AMP) challenge. This was performed using an identical method to that described in section 3.2.3.3 with sequential inhalations given in doubling increments from 0.115 to 944μM at two-minute intervals [282]. The challenge was continued until FEV_1 fell by at least 20% from post-saline baseline or until the maximum dose of AMP had been inhaled. The provocation dose of AMP required to reduce FEV_1 by 20% (PD_{20}AMP) was estimated by interpolation between the two last doses on the log dose-response plot [17].

According to guidelines, potential participants were instructed to abstain from the following prior to the bronchial challenge:

- antihistamines for 24 hours [79];
- steroid nasal sprays for 24 hours;
- caffeine-containing food and drink for 12 hours [17];
- strenuous exercise for 12 hours [271];
- inhalers containing long-acting β2-agonists (e.g. Salmeterol, Seretide, Symbicort) for 12 hours;
- inhalers containing short-acting β2-agonists for 4 hours (e.g. Terbutaline, Salbutamol).

The bronchial challenge was not performed on individuals who had an FEV_1 of less 40% predicted or a value of less than 1 litre, or who were pregnant [17].

4.2.3.4 Allergen skin sensitisation

Allergen skin sensitisation to *D. pteronyssinus*, cat fur, grass pollen and positive (histamine) and negative (saline) controls (Diagenics Ltd, Milton

Keynes, UK) was measured using standard skin prick test methods as described in section 2.2.2.2.

4.2.3.5 Blood samples

Venesection was performed, and samples for Full Blood Count analysis (haemoglobin estimation and differential cell counting) and serum albumin were processed in the Nottingham City Hospital pathology department.

4.2.3.6 Faecal egg count methods

Individuals were asked to provide a faecal sample collected within the previous 24 hours. This was analysed for parasite eggs using the methods described in sections 2.2.4 and 3.2.3.7.

4.2.3.7 Juniper Asthma Quality of Life Questionnaire

An interviewer-administered Juniper Asthma Quality of Life Questionnaire (AQLQ) based on individuals' recall of their experiences over the preceding fortnight was completed [283]. This questionnaire has been validated to provide a quantified impact of asthma on symptoms, physical activities, emotional function and exposure to environment stimuli. It contains 32 questions and provides a composite score out of a total of 224, with lower scores indicating a greater impact of asthma on quality of life. (Appendix K)

4.2.4 Run-in period and daily diary

Individuals who fulfilled the entry criteria at the screening visit then took part in a run-in period lasting approximately two weeks. During this run-in period, and for the duration of the study, participants were asked to complete a daily diary which included a record of the parameters detailed below. (Appendix L)

4.2.4.1 Peak expiratory flow

Individuals were asked to record twice-daily (morning and evening) PEF, measured as the best of three attempts.

4.2.4.2 Asthma symptom scores

Individuals were asked to record twice-daily scores (morning and night) of how severe they perceived their asthma symptoms to be in the preceding 12 hours using a scale of 0 (no symptoms) to 5 (maximal symptoms).

4.2.4.3 Use of reliever medication

Individuals were asked to record on a twice-daily basis (morning and night) the number of puffs of their asthma reliever inhaler they had used in the preceding 12 hours.

4.2.4.4 Adverse symptoms

The same method of recording adverse symptoms were used for this study as for the study in people with allergic rhinoconjunctivitis, as an appropriate validated questionnaire did not exist. Individuals were asked to assign a score using a visual analogue scale from 0 (no symptoms) to 10 (maximum possible severity of symptoms) for a range of pre-determined possible adverse effects due to the hookworm, including local skin reactions at the site of infection (redness and itching), gastrointestinal symptoms (nausea, indigestion, abdominal pain, diarrhoea, wind) and constitutional symptoms (tiredness). In addition, a space for free text was included in the diary for the recording of any other symptoms which the potential participants felt might be relevant (for example, change in eczema severity).

4.2.5 Randomisation visit

4.2.5.1 Clinical measurements

After the two week run-in period, potential participants were seen for the randomisation visit. The bronchial challenge, Juniper AQLQ and blood tests were repeated. If any of the eligibility criteria were no longer fulfilled, then the individual was not enrolled into the study.

4.2.5.2 Randomisation

Eligible participants then underwent concealed randomisation to active or placebo infection, allocated in blocks of four according to a computer-generated random code. Larvae were obtained by a culture of faecal material as described in section 2.2.3.3. To ensure that I, the clinical researcher carrying out the protocol measures, remained blind to the treatment allocation, solutions were administered by an independent member of the research team who was not involved in any of the study measurements, using the methods described in section 3.2.5.2.

As in the previous study, participants were told to avoid getting the plaster wet for 24 hours and after that time to remove the plaster and place it in a universal container containing 70% ethanol with which they were provided. They were also given one tablet of albendazole 400mg to take home in case they should want to withdraw from the study at any time. Participants were provided with a 24-hour contact telephone number in case of any problems or questions relating to the study. Randomisation codes were placed in a sealed envelope in the department laboratory in case of a medical emergency which might require the participant to withdraw from the study and to be unblinded immediately.

4.2.5.3 Blinding

The same steps were taken as described in section 3.2.5.3 to ensure that both trial participants and I remained blind to the treatment allocation.

4.2.6 Follow-up visits

After randomisation, participants attended study visits every fortnight for eight weeks, and then at 12 and 16 weeks. The original protocol specified a total of 12 weeks of follow-up, but because the eosinophilia observed in the study in allergic rhinoconjunctivitis was still decreasing at 12 weeks [282] it was decided, before commencing the present study, to increase follow-up to 16 weeks. In addition, participants had a blood sample taken for Full Blood Count analysis three weeks after randomisation to coincide with the expected time of onset of hookworm-associated eosinophilia observed in the dose-ranging study and study in allergic rhinoconjunctivitis [274;282].

At each study visit PD_{20}AMP was measured, the Juniper AQLQ was completed, venous blood taken for the same tests as at the screening visit and a faecal sample, collected within the preceding 24 hours, provided to confirm establishment and survival of the adult hookworms in the gastrointestinal tract of those in the treatment arm of the study and to quantify egg burden [274]. Participants' daily diaries and symptom cards from the preceding fortnight were also collected and participants were issued with new diaries and cards to last until their next study visit. .

4.2.7 Final visit

At the final visit at week 16, in addition to the test and measurements performed at the follow-up visits, allergen skin tests were repeated, participants were weighed and female participants had a repeat urinary

pregnancy test. The appearance of the eggs in faecal samples is relatively non-specific and thus can be mistakenly identified, for example, as pollen grains. For this reason, at the final visit, all faecal samples were cultured and infection was confirmed by the presence of visible larvae.

Participants were then seen by an independent member of the research team (to ensure that I remained blind to allocation) and asked whether they thought they had received placebo or hookworm and to justify their response, before being unblinded. If they had received placebo, no further follow-up was arranged. Those in the hookworm group were supplied with a course of mebendazole 100mg to be taken twice a day for three days in order to eradicate the infection. Those participants choosing to take the tablets were followed-up fortnightly and faecal egg counts and blood eosinophil counts were checked until egg counts were zero and eosinophils had returned to within 0.2 $x10^9$/litre of their screening value or were within normal reference ranges on two successive occasions (Figure 4.1).

Those participants in the hookworm group who chose not to eradicate the infection were given written information outlining the potential risks (albeit minimal) of long-term hookworm infection, namely anaemia and endomyocardial fibrosis. They were also told to inform the blood transfusion service before donating blood and were advised that mebendazole was contraindicated in pregnancy and during breastfeeding. (Appendix H) They were asked to sign two copies of a document to show that they understood the risks of infection and that they declined at that time to take the mebendazole tablets; one copy was retained by the participant, the other was filed in their records. These participants had no further follow-up arranged.

4.2.8 Trial monitoring committee

The same trial monitoring committee was used for this study as for the study in allergic rhinoconjunctivitis, and identical parameters were set to indicate when the committee was to be informed of abnormal results and circumstances where a decision regarding possible withdrawal from the study needed to be made (see section 3.2.8).

4.2.9 Ethical Approval

Data on adverse effects and haemoglobin, eosinophil and serum albumin levels were monitored regularly by the trial statistician and a data monitoring committee. The study was approved by the Nottingham Research Ethics Committee and by the Research and Development department at Nottingham University Hospitals NHS Trust and was registered with the Clinical Trials register (http://clinicaltrials.gov/ (trial reference NCT00469989)). The Medicines and Health Regulatory Association was also informed of the studies and did not require additional specific documentation to be completed.

4.3 Data analysis

4.3.1 Data entry and checking

All the results were entered on to a spreadsheet (Microsoft Excel (2007)) and cross-checked for any discrepancies; they were then imported into Stata version 10 (STATA Corp 2007, College Station, Texas, USA). Stata was used to perform data manipulation, computation of variables and all analyses unless otherwise stated. I carried out all the data analyses, including entry of data and manipulation of data, blind to randomisation code.

4.3.2 Primary outcome

4.3.2.1 Computation of primary outcome variable

The primary outcome was change in PD_{20}AMP from baseline to the week 16 (final visit) value, expressed in doubling doses. If after entry into the study participants no longer responded with a 20% reduction in FEV_1 at below the maximum administered dose of AMP, a censored value of one doubling dose higher than the maximum dose of AMP administered (1888mcg) was assigned [284;285]. The equation for calculating PC_{20} (provocation concentration required to reduce FEV_1 by 20%) [17] used to calculate the PD_{20}AMP:

$$PD_{20} = \text{anti log } [LN (C1) + ((LN (C2) – LN (C1)) \times (20-R1)/(R2-R1)]$$

where

C1 = penultimate AMP dose

C2 = final AMP dose

R1 = percent fall in FEV_1 after C1 from saline baseline

R2 = percent fall in FEV_1 after C2 from saline baseline

PD_{20}AMP were calculated on two separate occasions for each study visit, including the screening visit, and the two values obtained were cross-checked

to ensure that they were identical. Where there was a discrepancy, they were recalculated by an independent researcher. On each occasion, the independent researcher's calculation correlated with one of the first values obtained, and this estimate of PD_{20}AMP was therefore assigned for the visits.

PD_{20}AMP values were not normally distributed but could be transformed by taking the natural logarithm of each value. So, for each participant, the difference between the natural logarithm of the PD_{20}AMP of the final visit and the natural logarithm of the baseline PD_{20}AMP was computed and then divided by the natural logarithm of 2 to obtain the change in doubling doses which was the final variable for analysis and which does follow a normal distribution.

$$\text{Change in } PD_{20} \text{ in doubling doses} = \frac{\ln(PD_{20}\text{AMP at final visit}) - \ln(\text{mean of baseline } PD_{20}\text{AMP})}{\ln 2}$$

A positive value represented an increase in PD_{20}AMP (that is, reduced bronchial hyper-responsiveness, so an "improvement"), and a negative value represented a fall in PD_{20}AMP (that is, increased bronchial hyper-responsiveness, a "deterioration").

Baseline PD_{20}AMP was defined as the mean of the screening and randomisation visit values. However, if participants were found to have breached the protocol for bronchial challenge testing following measurement of PD_{20}AMP at either the screening or randomisation visit (for example, due to caffeine ingestion or use of β_2-agonist medication on the morning of the visit), the value for that particular visit was discounted. In such cases, the single valid value for either screening or the randomisation weeks was used for the baseline PD_{20}AMP instead of the mean.

4.3.2.2 Statistical analysis of primary outcome variable

The final change in PD_{20}AMP variable was compared between the two groups by computing means and mean difference (with 95% CI) and an independent samples t-test was performed to assess statistical significance. Analyses were repeated, first excluding any participants without both a screening and randomisation value, and second, with baseline defined as the randomisation week only since for some participants there appeared to be a learning effect in using the dosimeter properly and performing the bronchial challenge test, possibly resulting in less reliable measurements of PD_{20}AMP at their screening visits.

4.3.3 Secondary outcomes

Secondary outcomes were changes in the following variables over the course of the study:

- peak expiratory flow variability;
- asthma symptoms;
- reliever inhaler usage;
- Juniper AQLQ scores [283];
- allergen skin sensitisation (mm);

Other secondary outcomes were the occurrence of adverse symptoms potentially attributable to hookworm larvae during the study.

4.3.3.1 PEF variability

PEF variability was computed as the two-lowest percentage mean for each two-week period of the study i.e. mean of the two lowest PEF values during each fortnight, as a percentage of the fortnight mean. This measure of PEF

variability has been shown in a comparison paper to be the best performing PEF variability index [275].

$$\text{Two-lowest \% mean} = \frac{\text{mean of lowest two readings in period}}{\text{period mean}} \times 100\ \%$$

The final variable for analysis was computed as the two-lowest percentage mean for the final two weeks of the study (weeks 15 and 16) minus the two-lowest percentage mean for the run-in period (baseline).

4.3.3.2 Asthma symptom scores

Using the twice-daily asthma control symptoms scores recorded in the daily diary, the percentage of symptom-free days was determined for each two-week period of the study using the equation below.

$$\text{\% symptom-free days} = \frac{\text{Number of days with daytime symptom score of zero in two-week period}}{\text{Number of days in two-week period score completed}} \times 100\ \%$$

The final variable for analysis was then computed as the percentage symptom-free days for the final two weeks of the study (weeks 15 and 16) minus the percentage symptom-free days for the run-in period (baseline). This computation was then repeated substituting symptom-free nights (that is, a night symptom score of 0) for symptom-free days to generate a second variable for the final analysis.

4.3.3.3 Reliever inhaler use

Using the records from the daily diary, the percentage of reliever inhaler-free days was determined for each two-week period of the study using the equation below. The final variable for analysis was then computed as the

difference between the percentage reliever inhaler-free days for the final two weeks of the study (weeks 15 and 16) and the percentage reliever inhaler-free days for the run-in period. This computation was then repeated substituting reliever inhaler-free nights for reliever inhaler-free days to generate a second variable for the final analysis.

$$\text{\% reliever inhaler-free days} = \frac{\text{Number of days where reliever inhaler not used in two-week period}}{\text{Number of days in two-week period record completed}} \times 100\ \%$$

4.3.3.4 Quality of Life scores

The Juniper AQLQ questionnaire was completed using a Microsoft Access 2007 database and the results were then imported into Stata version 10 for analysis as described in section 4.3.1. A score out of 224 was recorded at each visit which reflected participants' experiences over the preceding fortnight (computed by summing individual symptoms recorded on the AQLQ). The final variable for analysis was calculated as the score for the final two weeks of the study (weeks 15 and 16) minus the score at the week 0 visit (reflecting experiences in the fortnight preceding randomisation).

4.3.3.5 Allergen skin sensitisation

Change in allergen skin sensitisation was computed for each allergen using the same method as described in section 2.3.3.3.

4.3.3.6 Adverse effects

A mean daily symptom score variable for each adverse effect potentially attributable to the hookworm infection was computed by taking the mean of the daily score over the whole 16 weeks and also for a pre-determined 'high-

risk' period, chosen as the period during which the symptoms were most likely to occur, as observed in previous studies of deliberate hookworm infection [127;259], the dose ranging study and the study in people with allergic rhinoconjunctivitis (see section 3.3.3.4).

4.3.3.7 Statistical analysis of secondary outcome variables

The variables for change from baseline to the end of the study (4.3.3.1 – 4.3.3.5) were compared between the intervention groups using the independent samples t-test and a mean difference and 95% CI computed. The adverse symptom score variables were not normally distributed and could not be transformed and so the medians were used as the summarised average in each intervention group and statistical significance was assessed using the non-parametric Mann-Whitney U test.

4.3.4 Area under the curve analyses

To ensure that an effect was not missed if the results from the final two week period of the study had been spurious, time trends were also explored by plotting two week period variables for PD_{20}AMP, PEF variability, Juniper AQLQ scores, asthma symptom-free day and nights and reliever inhaler-free days and nights, to check if there were any obvious trends. The area under the curve (AUC) (GraphPad Prism 5, GraphPad Software Inc., San Diego, CA) was then computed for each diary outcome using the period from weeks 5 to 16 (for PEF and symptoms and reliever inhaler usage) and weeks 6 to 16 for those variables measured at study visits (all other outcomes) as an alternative summary variable to ensure that any effect of larval migration through the airways in the first four weeks after randomisation did not affect the results. For the asthma symptom-free day and nights and reliever inhaler-free days and nights the period from week 5 to 16 was adjusted for by subtracting the

AUC value for the run-in period. For those AUC variables which were normally distributed, the means were compared and independent samples t-test used to assess for a significant difference between the two intervention groups. For those AUC variables which were not normally distributed, the medians were used as summarised averages for each intervention group and statistical significance between the two groups was assessed using Mann-Whitney U tests.

4.3.5 Sensitivity analyses

Three sensitivity analyses for each outcome were also performed, whereby analyses were repeated excluding:

i) participants who breached the protocol (such as changing dose of inhaled steroid during the study);

II) participants who had less than 75% of potentially available data complete for the outcome variable;

III) participants who were infected with hookworm but did not have a positive faecal culture for larvae at week 16.

4.3.6 Other clinical parameters

Haemoglobin and albumin levels were monitored as the study progressed by the trial statistician to ensure participant did not become anaemic or show signs of becoming malnourished. At the end of the study, change in haemoglobin and albumin levels were computed as the difference between week 16 values and baseline. In addition, the net change in weight of each participant was calculated as the difference between the weights at the week 16 visit and screening visit.

4.3.7 Markers of hookworm infection

Eosinophil counts and faecal egg counts were monitored at regular intervals during the study as described and used to confirm presence of infection in those who received hookworm infection.

4.3.8 Sample size and power calculation

With a sample size of 30 (15 in each group), the study had an estimated 80% power to detect one doubling dose difference in the primary outcome, change in $PD_{20}AMP$, between hookworm and placebo groups assuming a standard deviation of approximately one doubling dose.

4.4 Results

4.4.1 Participant flow

Thirty-four participants were recruited between December 2006 and June 2007, with 17 randomised to each intervention arm. Two participants withdrew before the primary endpoint could be measured, and indeed before parasite maturation was complete (one from the placebo group on day 6, due to psychological problems, and one from the hookworm group on day 34, due to abdominal pain). These participants were excluded from further analysis (Figure 4.2).

4.4.2 Baseline characteristics of participants

Baseline characteristics of the two study groups were similar with respect to gender, age, body mass index, smoking history, social deprivation (Townsend score [286]), use of asthma medication (Table 4.1); baseline clinical measures, namely bronchial hyper-responsiveness (PD_{20}AMP), lung function (percentage predicted FEV_1 and PEF variability), Juniper AQLQ score and symptom-free and reliever inhaler-free days and nights, were also similar (Table 4.2). All participants were Caucasian.

4.4.3 Primary outcome

PD_{20}AMP improved relative to baseline (positive value of variable) in 11 of the 16 participants finishing the study who received hookworm (69%), and in 8 of the 16 who received placebo (50%). The mean change in PD_{20}AMP was slightly higher in the hookworm group at 1.49 (SD 2.00) DD, than in the placebo group at 0.98 (SD 4.02) DD, but the difference in means between the two intervention groups was not statistically significant (0.51 DD, 95% CI: -1.79 to 2.80; p=0.65, Table 4.3).

When the analysis was repeated excluding the four participants whose baseline PD_{20}AMP was based on only the screening or randomisation visit values rather than the mean, this made little difference to the results: the mean change in PD_{20}AMP was higher in the hookworm group (n=15) at 1.62 (SD 2.00) DD than in the placebo group (n=13) at 1.06 (SD 3.99) DD (difference in means = 0.56 DD, 95% CI: -1.84 to 2.96; p=0.64). Using the randomisation week values as the baseline instead of the mean also made minimal difference to the results: the mean change in PD_{20}AMP was higher in the hookworm group (n=15) at 1.20 (SD 1.83) DD than in the placebo group (n=16) at 0.71 (SD 4.05) DD (difference in means = 0.49 DD, 95% CI: -1.85 to 2.83; p=0.67).

4.4.4 Secondary outcomes

4.4.4.1 PEF Variability

PEF variability changed on average between run-in and weeks 15-16 by just over 1% in both groups, with an improvement (mean increase) of 1.03% in the hookworm group (SD 6.17) and a deterioration (mean decrease) of 1.21% (SD 7.17) in the placebo group. However, the difference between these was neither clinically important nor statistically significant (difference in means = 2.24%, 95% CI: -2.60 to 7.06; p=0.35, Table 4.3).

4.4.4.2 Asthma symptoms

Self-reported asthma symptom-free days and nights scores improved in both groups, more in the placebo group than the hookworm group (mean change in % symptom-free days 12.72% vs. 9.05%; mean change in % symptom-free

nights 12.59% vs. 8.43%), but the differences between the two intervention groups for each outcome were not significant (Table 4.3).

4.4.4.3 Reliever inhaler usage

Percentage of reliever inhaler-free days improved in both groups during the study, more so for the placebo group than the hookworm group (mean change in reliever-free days 1.39% vs. 0.37%). Percentage of reliever inhaler-free nights improved in the placebo group but fell in the hookworm group by a minimal amount (mean change in reliever-free nights 3.59% vs. -0.01%). However, there were no significant differences between the two intervention groups for either of these outcomes (Table 4.3).

4.4.4.4 Quality of life scores

Juniper AQLQ scores improved in both groups during the study indicating an improvement in asthma related quality of life. The mean change in score from baseline to the end of the study was greater in the placebo group (15.34) than in the hookworm group (10.66), but the difference between the two intervention groups was not statistically significant (Table 4.3).

4.4.4.5 Allergen skin sensitisation

On average, size of wheal increased between baseline and week 16 in both the hookworm and placebo groups for cat fur allergen (mean change of 0.19mm and 0.03mm respectively) and reduced in both the hookworm and placebo groups for grass (mean change of -0.59mm and -0.19mm respectively) and *D. pteronyssinus* allergen (mean change of -0.66mm and -0.19mm respectively), but there was no statistically significant difference

between the intervention groups for any of the individual allergens tested (Table 4.3).

4.4.4.6 Adverse effects

Higher scores for localised skin reactions at the site of infection were reported in the hookworm group compared with the placebo group, particularly between days 1 to 21 after infection (median daily score for itching 1.40 vs. 0.00, difference in medians = 1.40; $p<0.001$, and median daily score for redness 2.18 vs. 0.00, difference in medians = 2.18; $p<0.001$). Daily scores of gastrointestinal symptoms were generally low but tended to be slightly higher in the hookworm group than the placebo group, significantly so for abdominal pain (median = 0.23 vs. 0.03, difference in medians = 0.20; $p=0.02$), and additionally for loss of appetite (median = 0.08 vs. 0.00; difference in medians = 0.08; $p=0.04$) and nausea (median = 0.05 vs. 0.00; difference in medians = 0.05; $p=0.04$) during the high-risk period (days 29 to 112). Reported respiratory symptoms did not suggest a worsening of asthma during the first four weeks of the study (Table 4.4).

4.4.5 Area under the curve analyses

The time trends showed no evidence of spurious results in the last two week period of the study for any outcomes. There was no statistically significant differences between the two intervention groups when outcomes were assessed using AUC (weeks 5/6–16) instead of change from the beginning to the end of the study (Table 4.5).

4.4.6 Sensitivity analyses

At the final visit, three participants were deemed to have breached the protocol during the study. The first had a change of dose of inhaled

corticosteroid in week 15, the second started using a volumatic device with their inhaler in the first week of the study and the third changed their dose of inhaled corticosteroid throughout the study according to their symptoms.

The majority of participants had more than 75% of all data available for each variable during the study, the exceptions being three participants for adverse effects and three for PEF variability (one had minimal data from the run-in period and the other two had data missing in the study after randomisation).

Seven participants were infected with hookworm but did not have a positive faecal culture for larvae at week 16.

When the sensitivity analyses were carried out, the measures of effect did not change materially for the majority of the outcomes (Table 4.6 for those who breached the protocol, Tables 4.7 and 4.8 for those with missing data and Table 4.9 for those in the hookworm group without positive cultures) and with the exception of some of the adverse effects, those outcomes not statistically significant originally did not become significant in the sensitivity analyses.

When those participants who had breached the protocol were excluded the effects on PD_{20}AMP, PEF variability, Juniper AQLQ scores and allergen skin sensitisation tests were minimal (Table 4.6). There was, however, a greater difference between the two groups for symptom-free days and nights, largely due to better control in the placebo group. For example, the difference between the two groups in % symptom-free days was 8.49% compared with 3.66% in the original analysis (Table 4.6). A similar change was also observed for the reliever inhaler-free days with a greater % of reliever inhaler-free days in both groups, but more so for the placebo group resulting in a larger overall difference between the groups (1.03% in the original analysis vs. 4.34% in the sensitivity analysis).

Excluding those participants with missing data on adverse symptoms resulted in a change for nausea and loss of appetite, which were originally significant but no longer in the sensitivity analysis (Table 4.7). However, the size of difference between the groups was the same and therefore the loss of significance was as purely due to the smaller sample size. There was also a change in the symptom-free days and nights and reliever inhaler-free nights when these participants were excluded but the differences between the hookworm and placebo groups did not become statistically significant (Table 4.8). Values did not change for those in the hookworm group, but excluding the two participants with incomplete data who were both in the placebo group resulted in a greater difference between the two groups. For example, the % of symptom-free days in the original analysis was 3.66% and 9.56% in the sensitivity analysis.

Excluding the seven people who received hookworm but who did not have positive cultures resulted in a smaller difference between the two groups for the primary outcome but the improvement was still greater in the hookworm group. There was a change in direction of effect with a fewer symptom-free days and nights for those in the hookworm group, and therefore a greater difference between the groups (3.66% symptom-free days in the original analysis and 14.05% in the sensitivity analysis; 4.16% symptom-free nights in the original analysis and 14.46% in the sensitivity analysis). However the numbers of participants were much smaller and confidence intervals wide and the difference was not significant. A similar change was seen for reliever free days, but to a lesser extent (Table 4.9).

4.4.7 Other clinical parameters

All participants had haemoglobin and albumin levels within the normal ranges at entry into the study. No clinically important change was observed during the study in either of the intervention groups in haemoglobin or serum albumin levels. The maximum fall from baseline in haemoglobin in the hookworm group was 0.9g/dL and in the placebo group was 0.4g/dL. There were no significant differences in change in weights seen between the two intervention groups. The mean change in weight for the placebo group was an increase in weight of 0.48kg (SD 2.96; range -6.4kg to 5.4kg) and for the hookworm group was an increase in weight of 0.11kg (SD 2.21; range -4.1kg to 3.6kg).

4.4.8 Markers of hookworm infection

All participants who received hookworm infection had a rise in eosinophil counts, which began at around day 21 and peaked at between 1.4 and 8.5 $x10^9$/litre between days 42 and 84. The eosinophil counts in the hookworm group were all higher at the end of the study than at the time of infection (Figure 4.3). Nine of the 16 participants who received hookworm and completed the study had eggs detected in their faecal samples, appearing by week 6 in three participants and by week 8 in six participants. At the final visit, faecal cultures were positive for nine participants, eight of whom had had detectable eggs in their faecal samples previously. All positive egg counts at this final visit were between 95 and 213 eggs/g faeces.

4.4.9 Assessment of participant blinding

At the end of the study, 11 participants in the hookworm group correctly thought that they had received infection (due to visible portals of entry on the skin and gastrointestinal disturbance) and four did not know. Of those who

received placebo, eight correctly thought they had been allocated placebo, two incorrectly thought they had received hookworm and six did not know.

4.4.10 Post study follow-up

Thirteen out of 16 participants who received hookworm and completed the study elected to keep their infection. Follow-up for these individuals, and for the three who chose to eradicate the infection, was the same as described in section 3.2.7.

4.5 Discussion

4.5.1 Summary of findings

This is the first reported intervention study of experimental hookworm infection in people with asthma. It was carried out following the two safety studies reported in Chapters 2 and 3 in this thesis and comprised a double-blind placebo-controlled trial.

The primary aim was to determine the effects of experimental hookworm infection on bronchial hyper-responsiveness by comparing change in PD_{20}AMP over a 16 week period after being randomised to receive ten hookworm larvae or placebo. Secondary aims were to investigate the effects on other indicators of asthma control over the same 16 week time period using records of PEF variability, asthma symptom scores, reliever inhaler usage and quality of life scores. The study found that bronchial hyper-responsiveness improved in individuals with hookworm infection on average by half a doubling dose more than in those who received placebo but this was not statistically significant (difference in means = 0.51 DD, 95% CI: -1.79 to 2.80; p=0.65). There was no significant difference between the two groups for any of the other markers of asthma control.

Other secondary aims were to investigate the effect of infection on allergen skin sensitisation and to monitor the occurrence of adverse effects potentially due to the infection. There was no significant difference between the two groups in change in allergen skin sensitisation testing. As in the previous study, infection with a dose of ten larvae was generally well tolerated with only one participant withdrawing from the study as a result of symptoms due to the infection. The most commonly occurring side effects were of a localised rash

at the site of infection occurring in the first few days after infection and gastrointestinal disturbance coinciding with the increase in eosinophil counts. The study also proves that trials of experimental hookworm infection are feasible and since the majority of participants chose to retain their infection at the end of the trial, provides evidence that sustained infection is acceptable to patients.

4.5.2 Strengths and weakness of the study

4.5.2.1 Measurement error

A number of different markers of asthma control were used as study outcomes to try and increase the chance of finding an effect of infection, if one existed. It was also important to use a variety of outcome measure as there can be poor correlation between different outcome measures of asthma such as PEF variability and symptoms [287] or lung function and quality of life [288]. Bronchial challenge testing is often considered as a gold standard method of evaluating bronchial hyper-responsiveness; similarly, PEF variability provides a well validated objective measure of day to day airflow obstruction [275;287]. Guidelines published since the completion of this trial have recommended that clinical trials of asthma include the use of daily diaries which detail presence of symptoms, night-time waking, and reliever inhaler usage as was done here [287]. It also suggests that symptom free days are a useful discerning variable provided the population is not experiencing either very frequent or very infrequent symptoms. It is important to assess the impact of any chronic disease on quality of life and some treatment effects will only be identified by the patient. The Juniper AQLQ used in this study has been validated to quantify the impact of asthma on various aspects of patients daily life [283] and has been widely used in clinical trials [288;289]. Biomarkers of airway inflammation are now increasingly being used in clinical trials of asthma and include

measurement of the fraction of exhaled nitric oxide and analysis of induced sputum to phenotype patients. These were not used in this trial, but it is unlikely that there would have been a significant effect of hookworm on these biomarkers given that no effect was seen on any other asthma outcomes.

There is a possibility of random error in the measurement of PD_{20}AMP values, and to reduce the impact if any spurious results had occurred at the randomisation visit, the mean of the randomisation and screening visits values were used to define baseline PD_{20}AMP, in accordance with the protocol. It was observed that there was a tendency in both groups for PD_{20}AMP values to be higher at the randomisation visit than at the screening visit, most likely due to a combination of improvement in inhaler technique and compliance with asthma treatment following education at the screening visit. However, a similar estimated difference in change in PD_{20}AMP of 0.5 DD was observed between the groups regardless of whether the value from the screening visit, from the randomisation visit or the mean of the two was used as the baseline value.

As discussed in section 3.5.2.1, there is a chance of measurement error in the reporting of adverse symptoms because the questionnaire used has not been validated, although the experiences of participants who received ten hookworm larvae in the trials in allergic rhinoconjunctivitis and in asthma were broadly similar suggesting this wasn't a major problem. As before, measurement error in blood sample measurements should be minimal due to the use of automated equipment and use of quality controls.

4.5.2.2 Success of blinding and bias

Blinding of treatment allocation was successful for those who received placebo and for the clinical researcher who conducted the study visits and

analysed the data, but less so for the participants who received hookworm. This is unlikely to have influenced the primary outcome results or PEF recordings as they were both measured objectively. Other secondary outcomes were measured subjectively and therefore could have resulted in differential bias for those participants who were aware of the intervention to which they had been allocated. For example, those participants who correctly guessed that they had been given hookworm may have perceived there to be an improvement in their asthma with under-reporting of symptoms (possibly reduced frequency of reliever inhaler use) and higher Juniper AQLQ scores. This group may also have reported higher scores for adverse symptoms potentially due to infection. This bias would have led to a greater difference in secondary outcomes between the two groups and an apparent increase in effect of infection on improving asthma. Given that there was minimal difference between the two groups for most outcomes, it would suggest that reporting bias of this nature was minimal. Of course, if they thought the hookworm was not likely to help their asthma, it may have had the reverse effect on their perception of their asthma with over-reporting of the presence of asthma symptoms, and the impact on their quality of life (with lower Juniper AQLQ scores) and under-reported adverse symptoms in their daily diary.

4.5.2.3 Success of infection

Although the two previous studies described in Chapers 2 and 3 showed ten larvae to be effective in establishing infection to an intensity producing more than 50 eggs/g faeces, in the present study eggs were not observed at any time in faeces from several participants. However, all active group participants exhibited marked elevation of peripheral blood eosinophilia, indicating that infection had occurred and therefore that those with no eggs in faeces had perhaps been infected with non-fecund or same-sex organisms. Previous

research in Papua New Guinea has also shown that total IgE levels are inversely related to hookworm fecundity, so it is possible that the presence of high levels in these participants (due to allergic disease) may have reduced hookworm fecundity, though IgE was not tested in this study so no evidence is available either to confirm or refute this theory [290].

4.5.2.4 Representativeness and loss to follow-up

Whilst people volunteering to take part in a study of experimental hookworm infection (or indeed any clinical trial) may not be representative of the asthma population in general, there is no reason to suspect that any such differences would interact with the clinical effect of hookworm infection and impact on the trial results. The study in allergic rhinoconjunctivitis found no effect of infection on people with very mild bronchial hyper-responsiveness and the participants in the study all had moderate asthma (steps 2 or 3 of treatment ladder) [291]. Only around 5% of people with asthma are classed as having severe asthma and will be receiving higher doses of medication (on steps 4 or 5) [292] and therefore the participants in this study are representative of the majority of the population with asthma.

Only two participants withdrew from the study; one from the placebo group for psychological reasons, and one as a result of abdominal discomfort. There is no evidence to suggest that they were any different from the other participants with respect to their asthma symptoms and it is therefore unlikely that the results of the trial would have varied significantly had they remained in the study.

4.5.2.5 Statistical power

The observed level of variability (SD) in the primary outcome, change in PD_{20}AMP, was anticipated to be one doubling dose (DD), but in the study it was far greater, being standard deviations of 2.00 and 4.02 DD in the hookworm and placebo groups respectively. As a result, the study had insufficient power to detect a true effect and this may, as such, have resulted in a false negative result for the primary outcome.

4.5.2.6 Confounding and success of randomisation

Randomisation appeared to be successful in ensuring baseline characteristics of each group were broadly similar. This included data on several different potential confounders (ethnic origin, gender, age, body mass index, smoking history, social deprivation, use of asthma medication) and baseline clinical measures, namely bronchial hyper-responsiveness (PD_{20}AMP), lung function (percentage predicted FEV_1 and PEF variability), Juniper AQLQ score and symptom-free and reliever inhaler-free days and nights. However, there is always the possibility of there being other unmeasured differences in confounding factors between the two groups given the small sample size.

4.5.3 Results in the context of other studies

The hypothesis tested in this study arises from multiple epidemiological studies which demonstrate an inverse relation between helminth infection, especially hookworm, and asthma. Whilst some of the observational data are inconsistent, the larger, more recent and better designed studies were in agreement, which suggests that the effect could be real and that the magnitude of effect might even be greater than that found in the meta-analysis [13]. It should be remembered, however, that these observational studies have

looked at whole populations and have looked at the prevalence of asthma; these associations therefore, may reflect protection from developing asthma. In contrast, in this study the intention was to improve symptoms in people already with a diagnosis of asthma. Previous intervention studies of parasites and asthma are limited to eradication studies and have produced largely negative results. These are described more comprehensively in Chapter 1. The studies were mostly carried out in areas where *T. trichiura* and *A. lumbricoides* were the predominant endemic infection, so one possible reason for their findings might be that there is a species-specific effect with hookworm being the only parasite to exert a protective effect.

4.5.4 Interpretation of results

If the difference between the two groups in PD_{20}AMP is true, it is very small and unlikely to have clinical significance as it lies within the limits of intra-observer repeatability [17]. However, the sample size in the study was small and the confidence interval wide and if the true size of effect is towards the upper limit of the confidence interval (2.80 DD) then this would be equivalent to the effect seen in drug trials and would be clinically important [280;281]. For this reason, it is important to not to exclude the possibility of there being an effect on asthma and to ensure that modified, larger trials are carried out in the future.

A number of factors may explain why this trial did not demonstrate a statistically significant effect on asthma. First it may be that hookworm infection does have a true effect on asthma but it was not observed in this study as a result of the study design. Whilst no immunological parameters were measured in this study, it may be that the dose of ten larvae failed to generate an adequate host immune response resulting in the finding of a

small, non-significant effect. The dose of larvae may have been too small, or repeated infections more closely mimicking natural infection may be needed to induce a full immune response. It may also be that sustained infection and a longer period of follow-up are required in order to observe an effect. The age of infection may be crucial, with parasite exposure needing to occur in childhood whilst the immune system is still developing; this may be particularly relevant, as many of the observational studies which have identified an association between infection and allergy have been performed in children. Moreover, as described above, it may be that the effect on asthma only relates to initial development of disease (in which case infection would need to occur in childhood), rather than having a modifying effect in people who already have established disease.

Another explanation for the findings of the study may be that the underlying hypothesis on which these studies were based is incorrect and that there is no true effect of hookworm infection on asthma. The cross-sectional observations may be due to reverse causation, whereby immunological bias in atopic individuals renders them less likely to establish hookworm infection, but the high level of infection in the intervention studies in people with allergic rhinoconjunctivitis does not support this [282]. Alternatively, other bias or unmeasured confounders, that is, factors independently associated with both hookworm infection and asthma, might explain the observations from previous observational studies. This seems unlikely, given the consistency of the evidence from the more recent, larger studies that support the hypothesis [13]. Polyparasitism is common in the areas where natural infection occurs, and this may be a pre-requisite for the observation of a protective effect, though the results of the meta-analysis imply that single infection should be sufficient [13]. Host genetic factors have been demonstrated to be important in the immune

interaction between individual and parasite and it may be that the participants in these intervention studies had genetic profiles which were unfavourable [293-295], although the high level of infection in the previous study, carried out in individuals with allergic rhinoconjunctivitis, argues against this.

It may also be that the anticipated immune modulation by hookworm could have been suppressed by the use of inhaled corticosteroids, though this is only likely if triggered during larval pulmonary migration, as there is little evidence of systemic immunosuppression at the doses of inhaled corticosteroids taken by these participants [296]. Although it was not possible to measure immunological parameters in this study, it is reasonable to assume that the immunological response would be the same infection as in the study in allergic rhinoconjunctivitis (Chapter 3), where ten larvae had no significant effect on specific or total IgE, T-regulatory cells or IL-10 [276]. The dose-ranging study (Chapter 2) produced higher peak levels of IgE with doses of 25 and 50 larvae, indicating that higher doses are necessary to generate such a response, though this work was carried out in non-allergic participants who may mount different responses to the infection compared with atopic individuals [274].

As with the study in people with allergic rhinoconjunctivitis, there were no clinically important changes in the size of wheal in allergen skin sensitisation testing in any of the participants. This study in asthma was different in that sample size was slightly greater and the infection was given for longer. In addition, it could be argued that the participants in this study had more severe allergic disease in that they had asthma treated with inhaled corticosteroids rather than allergic rhinoconjunctivitis. Regardless, it provides stronger

evidence for a lack of an effect on atopy with a dose of ten larvae and possible explanations for why this might be are discussed in section 3.5.4.

The side effects of infection experienced in this study were broadly similar to those previously reported in the two previous intervention studies and in other reports of intentional hookworm infection [194;195]. This is particularly important as the consistent results suggest that there is unlikely to be any serious adverse effects of infection with ten *N. americanus* larvae which have not been detected because of the small sample sizes of the individual studies. This thus provides further evidence that, at this dose, infection is safe.

4.5.5 Conclusions

This is the first randomised double-blind placebo-controlled trial of the effects of parasite infection on asthma. It found no evidence to suggest that hookworm infection had a positive effect on a number of clinical outcomes of asthma in people with mild to moderate disease. There are a number of possible explanations for this which have been discussed above, and further intervention studies, more closely mimicking naturally acquired infection, should proceed and are discussed further in Chapter 6.

Table 4-1: Baseline characteristics of participants completing the study

		Hookworm (n=16)	Placebo (n=16)
Demographics			
Gender	Male	8 (50%)	9 (56%)
	Female	8 (50%)	7 (44%)
Age	Mean (SD)	40.9 (10.67)	39.8 (15.18)
Body mass index	Median (IQR)	25.6 (23.52, 26.77)	26.9 (23.81, 29.63)
Townsend score†[286]	Median (IQR)	-0.27 (-3.49, 2.48)	-0.11 (-2.75, 1.89)*
Smoking status	Never	10 (63%)	11 (69%)
	Ever	6 (38%)	5 (31%)
Asthma Medication			
Daily inhaled corticosteroid dose	<500 mcg/d	9 (56%)	10 (63%)
	≥500 mcg/d	7 (44%)	6 (38%)
Past oral steroid usage	Never	8 (50%)	4 (25%)
	None in last 2 years	5 (31%)	8 (50%)
	Some <2 years	3 (19%)	4 (25%)
Daily long-acting β2-agonist usage	None	11 (69%)	9 (56%)
	Some	5 (31%)	7 (44%)

*n=12 (Townsend scores not available for participants living in houses built after 2004)
†higher Townsend scores donate more deprived and disadvantaged areas

IQR: Inter-quartile range; **SD**: standard deviation

Table 4-2: Baseline clinical measures

	Hookworm (n=16)	Placebo (n=16)
Lung function (mean (SD))		
% predicted FEV_1 at screening visit	83.38 (15.77)	87.06 (23.13)
% predicted FEV_1 at randomisation	80.25 (17.98)	85.93 (18.37)
Bronchial responsiveness (median (IQR))		
PD_{20}AMP (DD) at screening visit	2.34 (0.78, 7.30)	3.67 (1.49, 7.52)*
PD_{20}AMP (DD) at randomisation	4.10 (1.53, 11.26)**	6.98 (1.93, 17.22)
Skin sensitisation to allergen at screening visit (wheal size in mm) (mean (SD))		
Grass	5.13 (3.00)	5.31 (5.23)
Cat fur	4.25 (2.58)	3.91 (3.72)
Dermatophagoides pteronyssinus	6.53 (3.29)	6.38 (4.36)
Juniper AQLQ symptom scores† (median (IQR))		
Juniper score at screening visit for run-in period	193.00 (186.00, 212.50)	175.00 (154.00, 203.00)
Juniper score at randomisation for run-in period	200.00 (186.50, 212.00)	186.50 (174.00, 209.00)
PEF variability†† (median (IQR))		
PEF variability (%) during run-in period	91.99 (87.96, 93.10)	91.68 (85.62, 94.25)
% Symptom-free days/nights (median (IQR))		
% symptom-free days during run-in period	66.07 (21.43, 88.31)	46.43 (8.12, 96.43)
% symptom-free nights during run-in period	66.76 (40.66, 95.83)	85.71 (57.69, 92.58)
% Reliever inhaler-free days/nights (median (IQR))		
% reliever-free days during run-in period	84.52 (7.42, 92.26)	60.71 (3.57, 92.86)
% reliever-free nights during run-in period	75.65 (44.78, 100.00)	92.86 (76.92, 100.00)

*n=13; **n=15
†Maximum score of 224 where the higher scores indicate better quality of life
††% PEF variability: a value of 100 indicates no variability, i.e. perfect control

% predicted FEV_1: percentage predicted one-second forced expiratory volume; **AQLQ:** Asthma Quality of Life Questionnaire; **IQR:** interquartile range; **PD_{20}AMP (DD):** provocation dose of adenosine monophosphate to reduce one-second forced expiratory volume by 20% in doubling doses; **PEF**: peak expiratory flow; **SD**: standard deviation

Table 4-3: Change in outcomes from baseline/run-in period to week 16 for asthma and allergic outcomes

	Hookworm (n=16) mean (SD)	Placebo (n=16) mean (SD)	Difference in means	95% CI	P value‡
Bronchial responsiveness (DD)					
Change in PD_{20}AMP (DD)(final visit - baseline)	1.49 (2.00)	0.98 (4.02)	0.51	-1.79, 2.80	0.65
Skin sensitivity in mm					
Change in skin sensitisation to grass (final visit – screening visit)	-0.59 (2.42)	-0.19 (2.37)	-0.41	-2.14, 1.32	0.63
Change in skin sensitisation to cat fur (final visit – screening visit)	0.19 (1.74)	0.03 (1.63)	0.16	-1.06, 1.37	0.79
Change in skin sensitisation to *D. pteronyssinus* (final visit – screening visit)	-0.66 (1.46)	-0.19 (2.45)	-0.47	-1.94, 1.00	0.52
Juniper AQLQ symptom scores†					
Change in Juniper score (final visit - mean of screening and randomisation visits)	10.66 (14.08)	15.34 (22.41)	-4.69	-18.20, 8.82	0.48
PEF variability††					
Change in PEF variability (%) (final 2 weeks – run-in period)	1.03 (6.17)	-1.21 (7.17)	2.24	-2.60, 7.06	0.35
% Symptom free days/nights					
Change in % symptom free days (final 2 weeks - run-in period)	9.05 (32.50)	12.72 (48.98)	-3.66	-33.68, 26.35	0.80
Change in % symptom free nights (final 2 weeks - run-in period)	8.43 (39.67)	12.59 (46.39)	-4.16	-35.32, 27.01	0.79
% Reliever free days/nights					
Change in % reliever free days (final 2 weeks - run-in period)	0.37 (24.87)	1.39 (34.46)	-1.03	-22.72, 20.67	0.92
Change in % reliever free nights (final 2 weeks - run-in period)	-0.01 (21.49)	3.59 (27.17)	-3.60	-21.28, 14.09	0.68

‡ P value for independent samples t-test; **†** Higher scores indicate best quality of life **††** % PEF variability a value of 100 indicates no variability, i.e. perfect control
95% CI: 95% confidence interval; **AQLQ:** Asthma Quality of Life Questionnaire; **DD:** doubling dose; **PD_{20}AMP**: provocation dose of adenosine monophosphate to reduce one-second forced expiratory volume by 20%; **PEF**: Peak Expiratory Flow; **SD**: standard deviation

Table 4-4: Symptoms potentially attributable to hookworm infection experienced during the 16 week study period and high-risk period for participants with and without infection

Symptoms	Mean daily score (scale 0-10) over total 16 week period				Mean daily score (scale 0-10) over high-risk period†			
	Hookworm median (range) (n=16)	Placebo median (range) (n=16)	Difference in medians	P value‡	Hookworm median (range) (n=16)	Placebo median (range) (n=16)	Difference in medians	P Value‡
Localised skin itching	0.28 (0.04, 2.17)	0.00 (0, 0.16)	0.28	<0.001*	1.40 (0.24, 4.43)	0.00 (0, 0.67)	1.40	<0.001*
Localised skin redness	0.45 (0.06, 2.13)	0.00 (0, 0.23)	0.45	<0.001*	2.18 (0.33, 4.19)	0.00 (0, 1.19)	2.18	<0.001*
Nausea	0.08 (0, 1.51)	0.01 (0, 0.46)	0.07	0.17	0.05 (0, 1.84)	0.00 (0, 0.61)	0.05	0.04*
Diarrhoea	0.06 (0, 2.53)	0.06 (0, 0.52)	0.00	0.69	0.04 (0, 1.90)	0.05 (0, 0.29)	-0.01	0.85
Abdominal pain	0.23 (0, 2.05)	0.03 (0, 1.04)	0.20	0.02*	0.30 (0, 2.59)	0.01 (0, 0.92)	0.29	0.02*
Flatulence	0.24 (0, 5.43)	0.12 (0, 1.65)	0.12	0.25	0.20 (0, 6.07)	0.05 (0, 1.77)	0.15	0.23
Indigestion	0.00 (0, 0.67)	0.01 (0, 0.43)	-0.01	0.24	0.07 (0, 3.03)	0.00 (0, 0.67)	0.07	0.24
Loss of appetite	0.08 (0, 0.42)	0.00 (0, 1.18)	0.08	0.08	0.08 (0, 0.38)	0.00 (0, 0.83)	0.08	0.04*
Tiredness	0.18 (0, 4.47)	0.67 (0, 5.00)	-0.49	0.51	0.20 (0, 5.10)	0.36 (0, 5.52)	-0.16	0.52

*P value <0.001
‡P value for Mann-Whitney U test
†High risk periods: localised skin symptoms (days 1-21), gastrointestinal symptoms and tiredness (days 29-112)

Table 4-5: Comparison of asthma and allergic outcomes for the period weeks 5 to 16 between treatment groups

	Hookworm		Placebo		Difference in means	95% CI	P Value
	n	mean	n	mean			
Log PD_{20}AMP (AUC weeks 6 to 16) (mean (SD))	14*	4.38(2.19)	15*	4.56 (2.35)	-0.18	-1.91, 1.56	0.84†
PEF variability (AUC weeks 5 to 16) (median (IQR))	16	456 (428, 473)	15	457 (432, 464)			0.83††
Juniper AQLQ (AUC weeks 6 to 16) (median (IQR))	16	1975 (1861, 2170)	15	2037 (1881, 2178)			1.00††
Change in % symptom free days/nights (mean (SD))							
% of symptom free days from weeks 5 to 16 – run-in period	16	4.44 (30.72)	16	20.30 (33.24)	-15.86	-38.97, 7.25	0.17†
% of symptom free nights from weeks 5 to 16 – run-in period	16	4.65 (32.52)	16	15.34 (30.64)	-10.68	-33.49, 12.13	0.35†
Change in % reliever inhaler free days/nights (mean (SD))							
% of reliever free days from weeks 5 to 16 – run-in period	16	-2.51 (27.89)	16	7.33 (23.16)	-9.84	-28.35, 8.67	0.29†
% of reliever free nights from weeks 5 to 16 – run-in period	16	-3.99 (17.49)	16	6.66 (20.35)	-10.65	-24.35, 3.05	0.12†

*no PD_{20}AMP for week 6 so unable to calculate AUC
†independent samples t-test; ††Mann-Whitney U test

95% CI: 95% confidence interval; **AQLQ**: Asthma Quality of Life Questionnaire; **AUC**: Area under Curve; **IQR**: Interquartile range; **PEF**: Peak Expiratory Flow; **PD_{20}AMP**: provocation dose of adenosine monophosphate to reduce one-second forced expiratory volume by 20%; **SD**: standard deviation

Table 4-6: Change in outcomes from baseline/run-in period to week 16 for asthma and allergic outcomes excluding those who breached the protocol

	Hookworm		Placebo		Difference in means	95% CI	P value‡
	n	mean (SD)	n	mean (SD)			
Bronchial responsiveness (DD)							
Change in PD_{20}AMP (DD)(final visit - baseline)	14	1.15 (1.45)	15	0.72 (4.02)	0.44	-1.90, 2.77	0.70
Skin sensitivity in mm							
Change in skin sensitisation to grass (final visit – screening visit)	14	-0.29 (2.44)	15	-0.10 (2.42)	-0.19	-2.04, 1.67	0.84
Change in skin sensitisation to cat fur (final visit – screening visit)	14	0.18 (1.87)	15	0.03 (1.68)	0.15	-1.21, 1.50	0.83
Change in skin sensitisation to *D. pteronyssinus* (final visit – screening visit)	14	-0.68 (1.51)	15	-0.20 (2.53)	-0.48	-1.13, 2.08	0.55
Juniper AQLQ symptom scores†							
Change in Juniper score (final visit - mean of screening and randomisation visits)	14	12.14 (14.47)	15	16.27 22.88)	-4.12	-18.84, 10.59	0.57
PEF variability††							
Change in PEF variability (%) (final 2 weeks – run-in period)	14	1.17 (6.60)	15	-1.64 (7.20)	2.81	-2.46, 8.09	0.28
% Symptom free days/nights							
Change in % symptom free days (final 2 weeks - run-in period)	14	9.83 (34.58)	15	18.33 45.06)	-8.49	-39.26, 22.28	0.58
Change in % symptom free nights (final 2 weeks - run-in period)	14	9.64 (42.47)	15	16.66 44.96)	-7.03	-40.41, 26.35	0.67
% Reliever free days/nights							
Change in % reliever free days (final 2 weeks - run-in period)	14	1.95 (26.27)	15	4.34 (33.51)	-2.39	-25.45, 20.67	0.83
Change in % reliever free nights (final 2 weeks - run-in period)	14	-0.01 (23.08)	15	3.83 (28.11)	-3.84	-23.52, 15.84	0.69

‡P value for independent samples t-test; **†**Higher scores indicate better quality of life; **††**% PEF variability a value of 100 indicates no variability, i.e. perfect control.
95% CI: 95% confidence interval; **AQLQ:** Asthma Quality of Life Questionnaire; **DD:** doubling dose; **PD_{20}AMP**: provocation dose of adenosine monophosphate to reduce one-second forced expiratory volume by 20%; **PEF:** Peak Expiratory Flow; **SD**: standard deviation

Table 4-7: Symptoms potentially attributable to hookworm infection experienced during the 16 week study period and high-risk period for participants with and without infection excluding those with missing data for more than 25% readings

Symptoms	Daily score (scale 0-10) over total 16 week period						Daily score (scale 0-10) over high-risk period †					
	Hookworm		Placebo		Difference in medians	P value ‡	Hookworm		Placebo		Difference in medians	P Value ‡
	n	median (range)	n	median (range)			n	median (range)	n	median (range)		
Localised skin itching	15	0.27 (0.04, 2.17)	14	0 (0, 0.13)	0.27	<0.001*	16	1.40 (0.24, 4.43)	15	0 (0, 0.67)	1.40	<0.001*
Localised skin redness	15	0.46 (0.06, 2.13)	14	0 (0, 0.23)	0.46	<0.001*	16	2.18 (0.33, 4.19)	15	0 (0, 1.19)	2.18	<0.001*
Nausea	15	0.08 (0, 1.51)	14	0.03 (0, 0.46)	0.05	0.35	16	0.05 (0, 1.84)	14	0 (0, 0.61)	0.05	0.08
Diarrhoea	15	0.05 (0, 2.03)	14	0.07 (0, 0.52)	-0.02	0.95	16	0.04 (0, 1.90)	14	0.07 (0, 0.29)	-0.03	0.87
Abdominal pain	15	0.25 (0, 2.05)	14	0.05 (0, 1.04)	0.20	0.04*	16	0.30 (0, 2.59)	14	0.04 (0, 0.92)	0.26	0.04*
Flatulence	15	0.22 (0, 5.43)	14	0.12 (0, 1.65)	0.10	0.26	16	0.20 (0, 6.07)	14	0.05 (0, 1.77)	0.15	0.21
Indigestion	15	0.13 (0, 2.56)	14	0.01 (0, 0.43)	0.12	0.19	16	0.07 (0, 3.03)	14	0 (0, 0.45)	0.07	0.20
Loss of appetite	15	0.07 (0, 0.42)	14	0.01 (0, 1.18)	0.06	0.22	16	0.08 (0, 0.38)	14	0 (0, 0.83)	0.08	0.10
Tiredness	15	0.15 (0, 4.47)	14	0.67 (0, 5.00)	-0.52	0.65	16	0.20 (0, 5.10)	14	0.47 (0, 5.52)	-0.27	0.53

***** P value <0.001; **‡** P value for Mann-Whitney U test; **†** High risk periods: localised skin symptoms (days 1-21), gastrointestinal symptoms and tiredness (days 29-112)

Table 4-8: Change in outcomes from baseline/run-in period to week 16 for asthma and allergic outcomes excluding those with missing data for more than 25% readings

	Hookworm		Placebo		Difference in means	95% CI	P value‡
	n	mean (SD)	n	mean (SD)			
PEF variability ††							
Change in PEF variability (%) (final 2 weeks – run-in period)	14	1.02 (6.39)	15	-1.51 (7.35)	2.52	-2.71, 7.76	0.33
% Symptom free days/nights							
Change in % symptom free days (final 2 weeks - run-in period)	16	9.05 (32.50)	14	18.61 (48.40)	-9.56	-40.04, 20.92	0.53
Change in % symptom free nights (final 2 weeks - run-in period)	16	8.43 (39.67)	14	17.79 (47.01)	-9.37	-41.77, 23.05	0.56
% Reliever free days/nights							
Change in % reliever free days (final 2 weeks - run-in period)	16	0.37 (24.87)	16	1.39 (34.46)	-1.03	-22.72, 20.67	0.92
Change in % reliever free nights (final 2 weeks - run-in period)	16	-0.01 (21.49)	14	8.49 (22.35)	-8.50	-24.91, 7.91	0.30

‡P value for independent samples t-test; **††**% PEF variability a value of 100 indicates no variability, i.e. perfect control.

95% CI: 95% confidence interval; **PEF:** Peak Expiratory Flow; **SD**: standard deviation

Table 4-9: Change in outcomes from baseline/run-in period to week 16 for asthma and allergic outcomes excluding those in hookworm group without positive cultures

	Hookworm mean (SD) (n=9)	Placebo mean (SD) (n=16)	Difference in means	95% CI	P value‡
Bronchial responsiveness (DD)					
Change in PD_{20}AMP (DD)(final visit - baseline)	1.15 (1.77)	0.98 (4.02)	0.17	-2.77, 3.11	0.91
Skin sensitivity in mm					
Change in skin sensitisation to grass (final visit – screening visit)	-0.56 (2.63)	-0.19 (2.37)	-0.37	-2.49, 1.75	0.72
Change in skin sensitisation to cat fur (final visit – screening visit)	0.11 (1.95)	0.03 (1.63)	0.08	-1.43, 1.58	0.91
Change in skin sensitisation to *D. pteronyssinus* (final visit – screening visit)	-0.56 (1.55)	-0.19 (2.45)	-0.37	-2.25, 1.51	0.69
Juniper AQLQ symptom scores†					
Change in Juniper score (final visit - mean of screening and randomisation visits)	12.05 (12.27)	15.34 (22.41)	-3.29	-20.09, 13.52	0.69
PEF variability ††					
Change in PEF variability (%) (final 2 weeks – run-in period)	2.81 (5.58)	-1.21 (7.17)	4.02	-1.72, 9.76	0.16
% Symptom free days/nights					
Change in % symptom free days (final 2 weeks - run-in period)	-1.34 (25.60)	12.72 (48.98)	-14.05	-50.54, 22.44	0.43
Change in % symptom free nights (final 2 weeks - run-in period)	-1.87 (31.20)	12.59 (46.39)	-14.46	-50.43, 21.52	0.41
% Reliever free days/nights					
Change in % reliever free days (final 2 weeks - run-in period)	-4.24 (21.78)	1.39 (34.46)	-5.63	-32.05, 20.78	0.66
Change in % reliever free nights (final 2 weeks - run-in period)	-1.37 (17.67)	3.59 (27.17)	-4.95	-25.89, 15.99	0.63

‡P value for independent samples t-test; †Higher scores indicate better quality of life; ††% PEF variability a value of 100 indicates no variability, i.e. perfect control
95% CI: 95% confidence interval; **AQLQ:** Asthma Quality of Life Questionnaire; **DD:** doubling dose; **PEF:** Peak Expiratory Flow; **PD_{20}AMP**: provocation dose of adenosine monophosphate to reduce one-second forced expiratory volume by 20%; **SD**: standard deviation

Figure 4-1: Asthma study timetable of visits

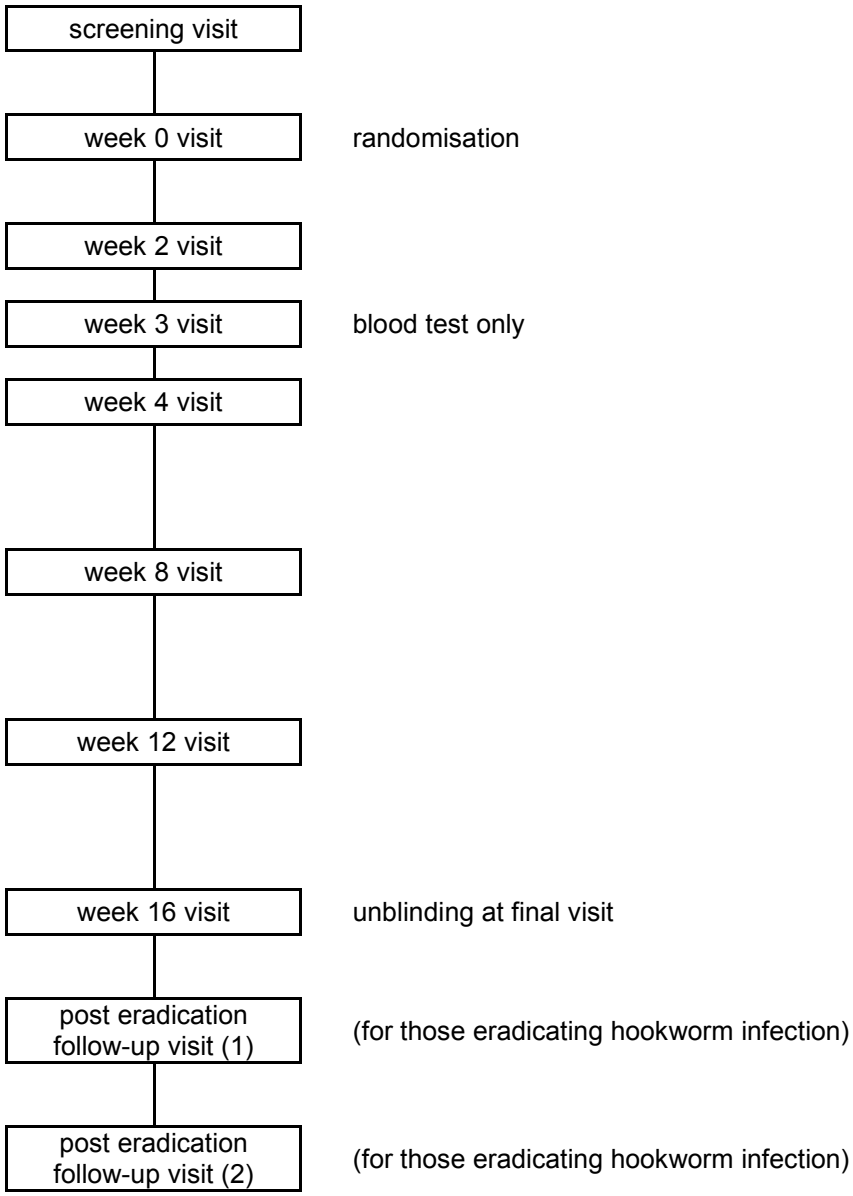


Figure 4-2: Flow chart of asthma study participants

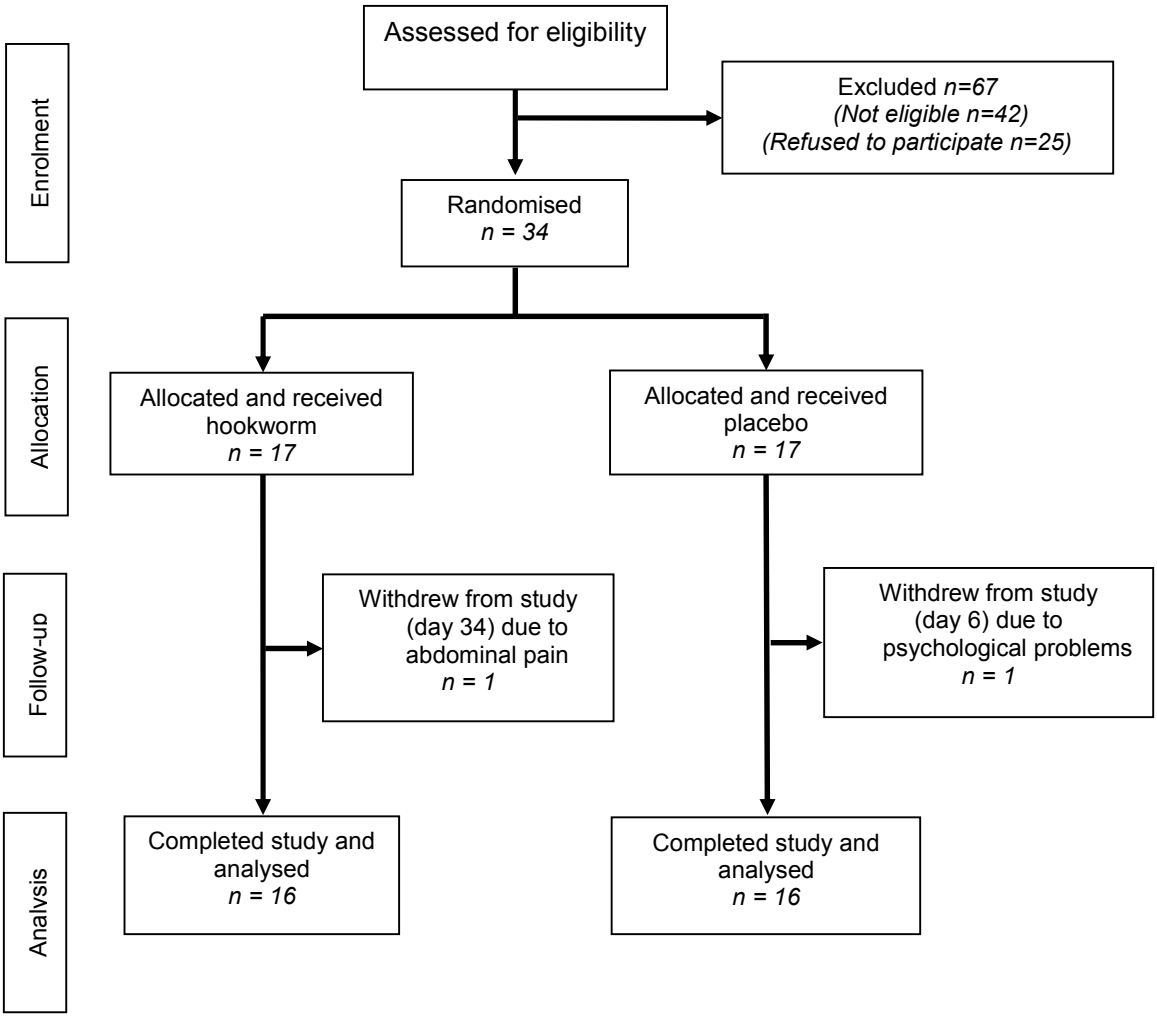
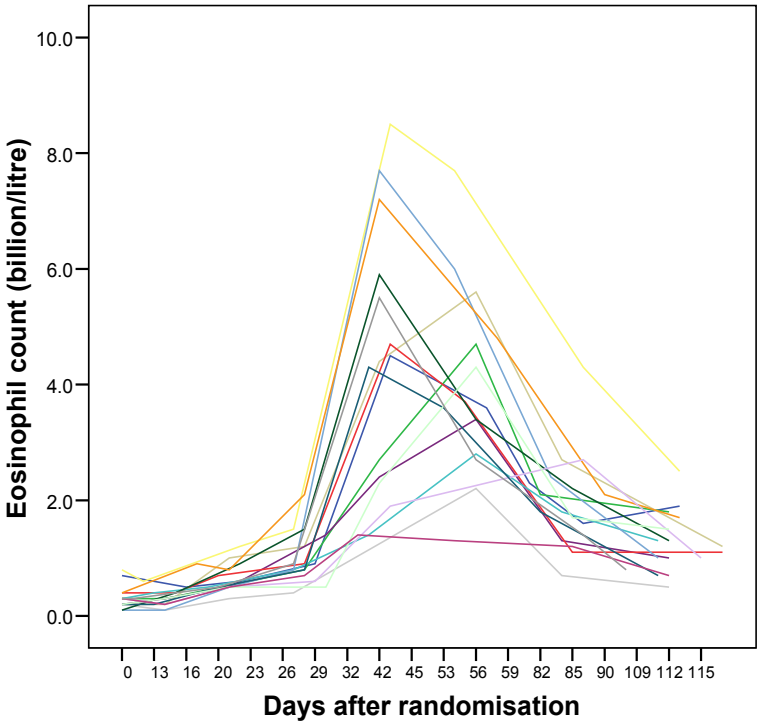
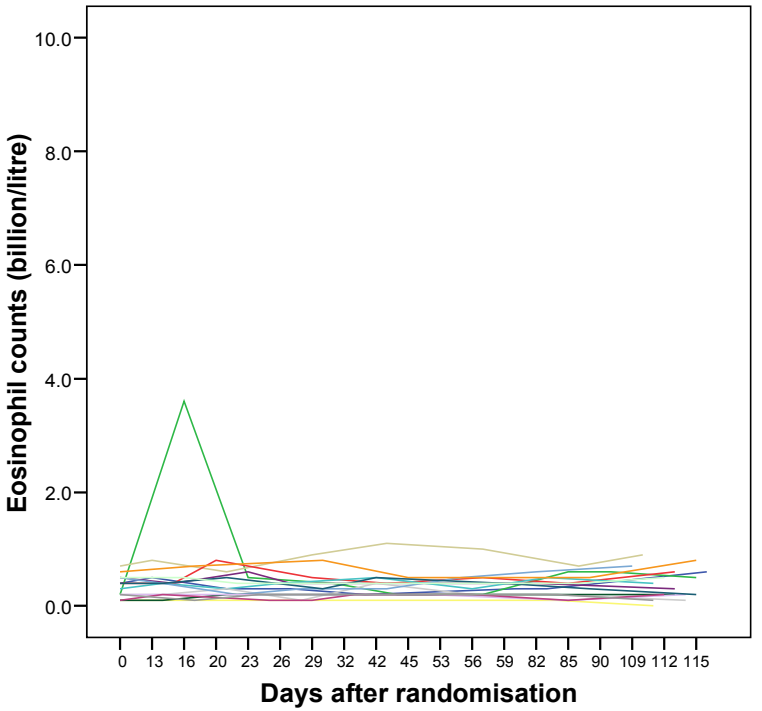


Figure 4-3: Individuals' peripheral blood eosinophil counts in over 16 week study period

Hookworm group



Placebo group



5 SYSTEMATIC REVIEW AND META-ANALYSIS OF THE ASSOCIATION BETWEEN PARASITE INFECTION AND ATOPY

5.1 Introduction

Current hookworm infection was shown to halve the risk of asthma in a systematic review and meta-analysis [13]. Similarly, several studies of the association between atopy and parasite infection have also suggested a possible link, although this relation has not been subjected to a rigorous and comprehensive review [146]. A systematic review and meta-analysis of the epidemiological literature was therefore performed to determine the relation between intestinal parasite infection and atopy and to establish whether, as with asthma, the relation was species-specific [13]. The specific aims of the study were therefore as follows: first, to determine the relation between current infection with any intestinal parasite and allergen skin sensitisation. Second, to determine the relation between current intestinal parasite infection and the presence of allergen-specific IgE. Finally, to establish if any observed associations were species-specific.

5.2 Methods

5.2.1 Systematic review methods

A comprehensive literature search in MEDLINE/PUBMED (1966 to March 2009), EMBASE (1980 to March 2009), LILACS (1982 to March 2009) and CAB Abstracts (January 2000 to March 2009) was performed according to standard guidelines [297] to identify all epidemiological studies, with no restrictions on language, using the search strategy detailed in section 5.2.2. Studies were included if they met the following criteria: [1] the design was a comparative epidemiological study (cross-sectional, cohort, case-control) or presented baseline data from a randomised controlled trial; [2] atopy was described using current allergen skin sensitisation or presence of specific IgE to allergens; [3] direct faecal microscopy was used to measure current parasite infection. Studies were initially selected on the basis of their titles and the abstracts, and full texts were then obtained for those potentially fulfilling the inclusion criteria. To identify any additional papers, the reference lists of published reviews and papers for which the full text was obtained were checked. Assessment of eligibility of papers and data extraction were independently performed by two researchers (myself and a statistician) and cross-checked, with discrepancies decided by consensus opinion. Studies by the same research groups were checked for replicated data to ensure they were not included more than once. Included studies were scored for methodological quality using the Newcastle-Ottawa quality assessment scale [298] with a score of 7 or more (from a maximum of 7 for cross-sectional studies (unable to score for selection or definition of controls) and 9 for case-control studies) chosen *a priori* to indicate a high standard for comparative observational studies (see Appendix M). Cochrane Collaboration's tool was used to assess risk of bias for randomised controlled trials [299]. The systematic

review was carried out in accordance with the MOOSE (Meta-analysis of Observational Studies in Epidemiology) guidelines [300].

5.2.2 Search strategy

The following search strategy, using appropriate Medical Subject Headings prefixes and suffixes [301], was used to identify potential papers.

Incidence or exp mortality or follow-up studies.mp or prognos.tw or prognosis.tw or predict.tw or exp cohort studies or exp risk or (odds and ratio).tw or (relative and risk).tw or (case and control) or (Systematic adj review.tw) or (data adj synthesis).tw or *(published adj studies).ab or meta analysis/ or meta analysis.ti or comment.pt or letter.pt or editorial.pt or (data adj extraction).ab

AND

(Pteronyssinus.mp or skin test.mp or RAST.mp or house dust.mp or feather.mp or cotton flock.mp or skin reaction.mp or antigen.mp or dermatophagoides,.mp or mold.mp or skin prick.mp or mite.mp or skin test.mp)

AND

(Parasite infection.mp or helminth infection.mp or parasites or helminths or *Ascaris*.mp or *Trichuris*.mp or enterobius.mp or hookworm.mp or *Necator*.mp)

NOT

(HIV.mp or AIDS.mp or wounds.mp or TB.mp or leprosy.mp or transplant.mp or cancer.mp or virus.mp or arthritis.mp or coeliac.mp or hepatitis,mp)

LIMIT TO HUMAN

Key to Medical Subject Headings suffixes and prefixes:

exp = exploded search; tw = text word; mp= key word; adj = adjacent words; ab= in abstract; ti= in title; pt = publication type

5.2.3 Data extraction

Data were analysed to yield effect estimates using unadjusted odds ratios (OR) from extracted data from the papers, or preferably, if available, using adjusted ORs using a data extraction form designed for the study. This form was used to record the study design, the method used to measure parasite infection, the parasite species present, the method of measuring atopy, the allergens to which skin sensitisation or IgE were tested and the country in which the study was carried out. It also recorded inclusion and exclusion criteria for the study, the number of participants studied and lost to follow-up and their ages. Finally, it recorded the methodological quality using the Newcastle-Ottawa scale [298] and the study results (Appendix M). Where exposure was expressed based on burden of infection, the highest exposure category was compared with those without infection.

5.2.4 Statistical analysis

Data were analysed using Review Manager Version 5.0 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2008). Where possible, the individual effect estimates from the studies were combined using a random study effects model [302] to estimate the pooled OR with 95% confidence intervals (95% CI) to allow for heterogeneity between the estimates of effect. Publication bias was assessed where adequate numbers of studies were included in the meta-analyses, using a funnel plot where the size of effect (OR) was plotted against the standard error [303]. Heterogeneity (I^2) between studies was anticipated due to inherent biases in the studies and so heterogeneity between study estimates was assessed using established methods [304] and any differences explored using subgroup analyses.

For the primary analysis of current infection with any intestinal parasite and skin sensitisation to at least one allergen, subgroup analyses were carried out according to the study methodological quality, study design, size of wheal used to define atopy, study population (children, adults or both) and geographical area (by continent) where the studies were undertaken.

Subgroup analyses were also performed to look at current infection with any geohelminths (which included *T. trichiura*, *A. lumbricoides*, hookworm, *E. vermicularis*, *Toxocara canis* and *T. cati* and *Strongyloides stercoralis*) and the individual parasite species *T. trichiura*, *A. lumbricoides*, hookworm and intestinal *Schistosoma* species. In addition, the possibility of estimating pooled effects according to burden of infection was explored by identifying any studies which had reported analyses where presence of parasite infection had been stratified according to number or quantile of parasite eggs detected. Whilst the main measure of outcome was skin sensitisation to at least one allergen, several studies reported separate results for specific skin sensitisation to cockroach and mite and therefore these effects are reported individually as secondary outcomes. The results of the studies where atopy was defined as presence of specific IgE were analysed separately.

5.3 Results: skin sensitisation used to define atopy

5.3.1 Overview of studies

The search strategy initially identified 1273 studies published between 1966 and March 2009. The full texts of 225 papers were obtained and, of these, 20 met the inclusion criteria using allergen skin sensitisation to define atopy, including one paper which contained unpublished data but which was identified to us by the authors [305] (Figure 5.1). The most common reasons for excluding papers were that appropriate data were not presented, or that a different definition of atopy had been used, such as history of allergic disease.

Table 5.1 presents the details of each of the 20 studies which were included. Fifteen of the 20 included studies used a cross-sectional design [142;160;205;207;219;221;225;226;233-235;237;305-307]; four used case-control designs [58;218;260;308]; and the remaining study was a randomised controlled trial from which the baseline data were used [242]. No studies using a cohort design were found.

Twelve of the studies were undertaken in children aged 5 to 19 years with one in infants aged 1 to 4 years [218]. Five were performed in both adults and children [160;219;233;307;308] and two in adults alone [58;237]. The total number of individuals included in all 20 included studies was 28,305. Eleven studies presented data using “any intestinal parasite infection” as an exposure [142;205;207;219;221;225;234;237;242;305;306] and these were used for the primary analysis; the remaining 9 studies had results only for species-specific infection [58;160;218;226;233;235;260;307;308]. A positive skin sensitisation test was defined in one study as a saline-adjusted wheal of ≥2mm [218] and in another as ≥1mm [260]; all other included studies used adjusted wheal sizes of ≥3mm

[58;142;205;207;219;221;225;226;233-235;237;242;305-308] or ≥4mm [160]. Six studies presented results for sensitisation to cockroach and mite separately [58;218;219;235;305;307] with the remaining 14 studies presenting results for sensitisation to "any allergen". The details of the allergens tested for in each study are presented in Table 5.2. The majority tested for mite and cockroach and then of the 20 studies, 12 tested for mould or fungi, 11 for animal extracts and 10 for grass or tree extracts. One study did not give details of which allergen were tested for [308]. All allergens were common aeroallergens in the region in which the study was conducted; one study additionally tested for sensitisation to peanuts [305]. Finally, one study analysed data for rural and urban populations independently [58].

5.3.2 Methodological quality and publication bias of studies

16 of the 20 included studies were of higher quality and four were judged to be of lower quality due to an inadequate definition of their control population, a failure to adjust for age or other factors, or omission of non-response rate. In total, 16 of the included studies presented their data after adjusting their analyses for confounders. The median overall score was 7 (range 3 to 9) (Table 5.1) confirming that the quality was generally high using this particular scoring system [298]. The randomised controlled trial also scored a higher level of methodological quality by stating adequate methods of randomisation, allocation concealment and blinding [242].

5.3.3 Effects of infection with any intestinal parasite

A funnel plot of the studies related to the primary outcome of any current intestinal parasite infection and positive skin sensitisation to at least one allergen was generated and showed no clear deviation from symmetry;

however, due to the small number of included studies it is difficult to determine the presence of asymmetry (Figure 5.2). The pooled analysis of estimates from the eleven studies of current infection with any intestinal parasite demonstrated a statistically significant reduction of around 30% in the risk of atopy in individuals with infection (OR 0.69, 95% CI: 0.60 to 0.79; p<0.01) [142;205;207;219;221;225;234;237;242;305;306]. Moderate levels of heterogeneity were observed across these studies (I^2=45%). Whilst there was a significant effect on sensitisation to any allergen, no significant effect was seen for specific sensitisation to mite or cockroach although this was based on only two studies (Figure 5.4).

Subgroup analysis indicated that the findings did not differ according to the methodological quality of the study (higher quality studies (Newcastle-Ottawa scale score ≥7); OR 0.67, 95% CI: 0.59 to 0.75; p<0.01), study design (cross-sectional studies; OR 0.67, 95% CI: 0.57 to 0.78; p<0.01) or the definition of wheal response used (all of these studies used a wheal size of ≥3 mm to define a positive skin sensitisation test) (Table 5.2). Limiting studies to the eight studies of just children made little difference to the results (OR 0.70, 95% CI: 0.61 to 0.80; p<0.01). Restriction of the analyses by geographical area to the seven studies conducted in Central or South America found similar effects (OR 0.68, 95% CI: 0.60 to 0.75; p<0.01). When the analysis was restricted to those nine studies of "any current geohelminth infection" the effect was similar (OR 0.68, 95% CI: 0.60 to 0.76; p<0.01) [142;207;219;221;225;234;237;242;305].

A number of papers provided information on egg counts but pooled effects of burden of infection could not be determined due to the format of the data presented. Some of these studies did find a stronger effect with higher burden of infection for the relation between *A. lumbricoides* [205;225;234] and *T. trichiura*

[225;234] and sensitisation to any allergen; and also with hookworm [235] and *T. trichiura* [58] and sensitisation to mite. In contrast, other analyses found no effect of intensity of infection was in the relation between *A. lumbricoides* and sensitisation to any allergen [307]; *A. lumbricoides* or hookworm and sensitisation to mite [58] or *T. trichiura* and sensitisation to cockroach [218].

5.3.4 Effects of infection with individual intestinal parasite species on atopy

Fifteen of the 20 studies provided species-specific data on current intestinal parasite infection, all of which were helminths. The individual effects of four helminth species that were present in at least 1% of the study populations were analysed.

5.3.4.1 Ascaris lumbricoides

Fifteen studies described the relation between current *A. lumbricoides* infection and the risk of atopy [58;142;205;207;218;219;221;225;226;234;235;260;305;307;308]. A pooled analysis of nine studies demonstrated a statistically significant reduction in the odds of atopy to at least one allergen in individuals with current *A. lumbricoides* infection (OR 0.69, 95% CI: 0.59 to 0.80; p<0.01), with little heterogeneity between the studies (I^2=30%) [142;205;207;221;225;226;234;260;308]. In contrast to this general effect on allergen sensitisation, there were no statistically significant effects in four studies on specific sensitisation to cockroach (OR 0.90, 95% CI: 0.75 to 1.08; p=0.25 (I^2=0%)) [218;219;235;305] or in six studies to mite (OR 1.03, 95% CI: 0.73 to 1.46; p=0.86 (I^2=47%)) [58;218;219;235;305;307] (Figure 5.5).

5.3.4.2 Hookworm

Nine studies reported the association between hookworm infection and the risk of current atopy [58;142;207;218;219;221;234;235;305]. Pooled data from four homogeneous studies (I^2=14%) indicated that current infection with hookworm was associated with a borderline significant reduction in skin sensitisation to at least one allergen (OR 0.68, 95% CI: 0.46 to 1.01; p=0.06) [142;207;221;234]. The pooled effects of hookworm infection on sensitisation to cockroach in four of these studies were not significant (OR 0.81, 95% CI: 0.62 to 1.08; p=0.15 (I^2=52%)) [218;219;235;305]; nor did hookworm have a significant effect in the five studies of sensitisation to mite (OR 0.94, 95% CI: 0.65 to 1.36; p=0.73 (I^2=62%))[58;218;219;235;305] (Figure 5.6).

5.3.4.3 Trichuris trichiura

Eight studies looked at the effects of *T. trichiura* infection on the risk of current atopy [58;142;207;218;221;225;234;305]. Pooled effects of five homogenous studies reporting current *T. trichiura* infection showed a significant reduction in atopy to at least one allergen (OR 0.75, 95% CI: 0.65 to 0.86; p<0.01 (I^2=7%)) [142;207;221;225;234]. In contrast, two studies of sensitisation to cockroach found that infection was associated with a significant increase in current atopy (OR 1.86, 95% CI: 1.24 to 2.80; p=0.003 (I^2=13%)) [218;305]; and three studies of sensitisation to mite found that infection was non-significantly associated with an increase in atopy (OR 1.72, 95% CI: 0.90 to 3.29; p=0.10 (I^2=47%)) [58;218;305] (Figure 5.6).

5.3.4.4 Other individual intestinal parasite species

Three studies reported the effects of *S. mansoni* infection on atopy [160;233]. Studies were only included if the infection had been detected in the faecal

samples and studies reporting the association with *S. haematobium* which is detected in the urine were not included in this analysis [305]. Two studies, both judged to be of lower methodological quality, reported the association with sensitisation to any allergen and found infection to be associated with a reduction in atopy (OR 0.14, 95% CI: 0.03 to 0.63) [233] and (OR 0.01, 95% CI: 0.00 to 0.15) [160]. The third study found no significant effect of infection on sensitisation to mite in an urban population (OR 1.78, 95% CI: 0.45 to 6.98) [58]. Pooled analyses were not carried out due to the small numbers of studies involved. No effect on the risk of current atopy was seen with *E. vermicularis* [207;306], *Giardia intestinalis* [306] or *Blastocystis hominis* [306].

5.4 Results: specific IgE used to define atopy

Six studies were identified which defined atopy as a positive specific IgE to mite or other aeroallergens and looked at its association with intestinal parasite infection [160;233;260;307;309;310]. Extraction of data was not possible in three studies due to the format of the data [307], because no uninfected individuals had specific IgE tests performed [160], or because no data were presented [260], although the authors of this last paper did comment that they had found no significant association between *A. lumbricoides* infection and specific IgE.

The three remaining studies reported inconsistent findings in the relation between infection and presence of specific IgE. One study found the combination of *A. lumbricoides* and *T. trichiura* infection to be associated with a statistically significant increase in the likelihood of having raised specific IgE (>1.0 IU/ml) to mite (OR 1.94, 95% CI: 1.20 to 3.15; $p<0.01$) [310]. In contrast, another study reported no significant association in multivariate analyses between specific IgE to mite and infection with either *A. lumbricoides* (OR

1.74, 95% CI: 0.55 to 5.51) or *T. trichiura* (OR 2.70, 95% CI: 0.95 to 7.67) [309]. Finally, a study of *S. mansoni* found current infection to be associated with a significant reduction in presence of aeroallergen-specific IgE (OR 0.24, 95% CI: 0.09 to 0.60; $p<0.01$) [233].

5.5 Discussion

5.5.1 Main findings

This systematic review and meta-analysis of the epidemiological literature provides strong evidence that current intestinal parasite infection, in particular geohelminth infection, is associated with a reduced prevalence of allergic skin sensitisation to at least one allergen (OR 0.68, 95% CI: 0.60 to 0.76; p<0.01) (although not specifically to cockroach or mite which were analysed separately). A similar pattern was seen for the individual geohelminth species with the strongest effect for hookworm although this was based on only four studies and therefore lacked power and the association had only borderline statistical significance. There was no evidence that current intestinal parasite infection was associated with an increase in risk of skin sensitisation to cockroach or mite, with the exception of *T. trichiura*, which was associated with a significant increase in risk of specific skin sensitisation to cockroach. There were too few studies reporting the association between parasite infection and specific IgE to combine data.

5.5.2 Strengths and limitations

The literature search was comprehensive and, so far as it is possible to ascertain, identified all potentially suitable studies in this area. In addition, the funnel plot did not suggest that the results would have been significantly affected by publication bias. There was a surprisingly low degree of heterogeneity between some of the studies, which may be attributable to the majority of studies being published by a small number of research groups. The methodological quality of the studies included in this review were, with few exceptions, very good. The MOOSE guidelines recommend assessing the impact of key components of study design (such as case-control, cohort and

cross-sectional) using subgroup or sensitivity analysis [300]; however, it was not possible to do this as most of the studies were of the same design, namely cross-sectional. The impact of quality on the findings was therefore explored using subgroup analyses (as recommended by the MOOSE guidelines); removing the one study judged lower quality made little difference to the results (Tables 5.2 and 5.3). The majority of studies were published within the past 10 years. Since 18 of the 20 included studies were carried out in Central or South America or Africa, (the remaining two studies were from Vietnam [235] and Turkey [306]), the findings are particularly applicable to those regions of the world, although there are no grounds to suggest that the findings will not be generalisable elsewhere where similar environmental conditions exist.

Whilst the primary analysis was for infection with any intestinal parasite, of the 20 included studies, only two contained data on infection with species other than helminths, and the majority of parasites were, in fact, geohelminths. It may therefore be that other parasite infections will demonstrate a different relationship with atopy but the studies have not yet been carried out and further studies with these infections are needed before any conclusions can be drawn. To maximise the validity of the results a definition of current infection ascertained by direct faecal microscopy was used, rather than reported history of infection or serological testing, neither of which differentiates between past and current infection. Outcomes were defined using skin sensitisation, a relatively simple and cheap test and unlikely to be subjected to measurement bias (compared with a subjective history of atopic disease). Validity was also increased by using sensitisation to different allergens. The main limitation of the meta-analysis was that it was not possible to account for the effects of age, gender, socio-economic status and other confounders in a number of studies which presented unadjusted estimates of effect. However, less than

half of the studies included in the meta-analysis did not adjust for confounding factors (and were therefore assigned a lower score for methodological quality), and low levels of heterogeneity were generally detected between the studies, indicating that the findings were similar. Age could be of particular importance as different intestinal parasites are acquired at different ages throughout childhood, most commonly after weaning or as infants become mobile. The subgroup analysis of studies including only children did not suggest that the size of effect varied significantly between adults and children. However, because skin sensitisation to allergens increases with age there is a chance that the study of infants aged less than five years may be misleading.

The paucity of studies describing the association between current intestinal parasite infection and specific IgE to allergens makes it difficult to draw definitive conclusions. The preference of researchers for using allergen skin sensitisation over serological testing to define atopy is unsurprising; it is logistically easier and cheaper to perform skin prick testing, which is particularly important given that the sample size of most epidemiology studies is large. In addition, results are obtained faster with skin prick testing, and this method is also likely to be more acceptable to participants compared with venesection.

5.5.3 Possible explanations for observed results

The variation in effect estimates observed between species is likely to reflect in part, the relatively small number of available species-specific studies, and also genuine differences of effect on allergy between species. Previous research has suggested that the protective effect of intestinal parasites on asthma may arise from a host systemic phase in the parasite life cycle [58] but there is no biological reason for this to be required to effect atopy and *T.*

trichiura has no such systemic phase. It is of note that none of the parasites was associated with an increase in the odds of atopy to at least one allergen, though *T. trichiura* infection was associated with an increased risk of specific sensitisation to cockroach. Several authors presented results separately for sensitisation to cockroach and mite. Interestingly, Flohr *et al* were the only authors to find both *A. lumbricoides* and hookworm infection to be significantly associated with a reduction in atopy to mite and cockroach [235], which may reflect geographical variation as this was the only study performed in Asia. With these few exceptions, no association was found between mite and cockroach and skin sensitisation, suggesting that other allergens, namely grasses, fungi or animal extract are responsible for the reduced risk of atopy.

It has been previously reported that, in the relation between current intestinal parasite infection and asthma, hookworm was associated with a 50% reduction in wheeze, in contrast to *A. lumbricoides* which was associated with an increase in wheeze [58]. This association between *A. lumbricoides* infection and asthma is surprising in the context of the results from this meta-analysis of atopy and raises questions about the relation between intestinal parasite infection and different allergic conditions, and indeed about the relation between allergic sensitisation and clinical symptoms. Whilst atopy is a strong risk factor for allergic disease in higher income countries, studies have shown that it appears to be less closely related, or not at all, in low and middle income settings [221;311].

Finally, whilst this meta-analysis suggested a clear inverse association between hookworm and atopy measured using skin sensitisation, there were no consistent changes in allergen skin sensitisation tests over the course of the three intervention studies reported in this thesis. This however, is maybe

unsurprising as it was not the primary aim of the trials and the trials were neither powered nor designed to show an effect. In addition, the relatively short duration of the study may have been a factor.

5.5.4 Conclusions

This study thus provides evidence, from a synthesis of observational studies, that intestinal parasite infections, particularly geohelminth infections appear to protect against atopy. A clearer understanding of the aetiology of allergic disease and the relation between environment and host is important for many reasons. Allergy is already a common cause of chronic disease in childhood in economically developed countries. The prevalence of allergic disease is increasing, particularly in low and middle income countries, placing a huge burden on already stretched health services. An increase in comprehension of the mechanisms underlying development of allergic disease may help to direct future therapeutic advancements, which are at present limited to treatments with undesirable side effects. Parasite infection is endemic in large parts of the world, and whilst eradication is acknowledged to be an important aspect of ensuring public health, there is a chance that this may in fact lead to an increase in allergic disease. It is for this reason that characterisation of the relation between the two is especially important.

Table 5-1: Studies included in meta-analysis of skin sensitisation

Author, year	Study Design	Number in study	Age in years mean (SD) or range	Parasite	Country	Adjusted data	Methodologic quality score‡
Araujo 2000 [233]	XS	175	U: 20.2 (11.9); I: 18.0 (9.7)†	*Schistosoma mansoni*	Brazil	Yes	5
Araujo 2004 [160]	XS	43	6-40	*S. mansoni*	Brazil	No	4
Bahceciler 2007 [306]	XS	997	4-12	Any parasite[1]	Turkey	No	6
Calvert 2005 [260]	CC	743	8-13	*Ascaris lumbricoides*	South Africa	Yes	8
Cooper 2003a [234]	XS	2865	5-18	Any geohelminth *A. lumbricoides;* hookworm; *Trichuris trichiura*	Ecuador	Yes	8
Cooper 2003b [221]	XS	3681	5-19	Any geohelminth *A. lumbricoides;* hookworm; *T. trichiura*	Ecuador	Yes	8
Cooper 2004 [225]	XS	987	7-17	Any geohelminth *A. lumbricoides; T. trichiura*	Ecuador	Yes	8
Cooper 2006 [242]	RCT	2331	C: 9.8 (2.1); V: 9.6 (2.0)†	Any geohelminth	Ecuador	Yes	A*
Dagoye 2003 [218]	CC	563	1-4	*A. lumbricoides;* hookworm; *T. trichiura*	Ethiopia	Yes	9
Davey 2005 [219]	XS	7508	5-95	Any geohelminth *A. lumbricoides;* hookworm	Ethiopia	Yes	8
Flohr 2006 [235]	XS	1601	6-18	*A. lumbricoides;* hookworm	Vietnam	Yes	8
Joubert 1979 [308]	CC	109	12-44	*A. lumbricoides*	South Africa	Yes	4
Nyan 2001 [237]	XS	429	≥15	Any geohelminth	Gambia	Yes	8
Obeng (unpublished data)[305]	XS	2019	5-16	Any geohelminth *A. lumbricoides;* hookworm; *T. trichiura* *Schistosoma haematobium*	Ghana	Yes	7
Obihara 2006 [226]	XS	359	6-14	*A. lumbricoides*	South Africa	Yes	8
Peireira 2007[205]	XS	1011	9-13	Any helminth *A. lumbricoides*	Brazil	No	8
Ponte 2006 [307]	XS	113	12-30	*A. lumbricoides*	Brazil	Yes	7
Rodrigues 2008 [142]	XS	1055	4-12	Any geohelminth *A. lumbricoides;* hookworm; *T. trichiura*	Brazil	No	7
Scrivener 2001 [58]	CC	403	≥16	*A. lumbricoides* hookworm; *T. trichiura; S. mansoni*	Ethiopia	Yes	8
Wördemann 2008 [207]	XS	1313	4-14	Any geohelminth *A. lumbricoides;* hookworm; *T. trichiura* *Enterobius vermicularis*	Cuba	Yes	8

*****A denotes a higher methodological quality for the randomised controlled trial; **†** means (standard deviation) presented separately for uninfected (**U**), infected (**I**), control (**C**) and intervention (**V**) groups **‡**Based on Newcastle Ottawa quality assessment scale from a maximum score of 8 for cross-sectional studies (**XS**)/randomised controlled trials (**RCT**) and 9 for case-control (**CC**) studies;
1 *E.vermicularis* 23.3% (using perianal tape), *Blastocystis hominis* 22.4%, *Giardia intestinalis* 13%, *Entamoeba coli* 2.2%, *Entamoeba histolytica* 1.7% (other parasites contribute less than 1%)

Table 5-2 Allergens to which skin sensitisation tests were performed in studies included in the meta-analysis

Author, year	Mites	Cockroaches	Grasses	Mould and Fungus	Animal extract	Other
Araujo 2000 [233]	*Dermatophagoides. pteronyssinus* *Blomia tropicalis*	*Blattella germanica* *Periplaneta americana*		mixture of fungi	cat epithelium, dog epithelium	
Araujo 2004 [160]	*D.pteronyssinus*, *D.farinae*, *B. tropicalis*,	*P. Americana, B. germanica*				
Bahceciler 2007 [306]	*D.pteronyssinus, D. farinae*		mixture of 12 grasses, Betulaceae, mixture of 4 cereals, Salicaceae,	*Alternaria*, *Aspergillus* mix, *Cladosporium*, *Penecillium* mix, *Candida albicans*	cat hair, dog hair, feather mixture	composites
Calvert 2005 [260]	*D.pteronyssinus*, *D.farinae*, *B. tropicalis*	cockroach	Timothy grass, Bermuda grass	*Aspergillus*, *Cladoporium*, *Alternaria*	cat, dog	
Cooper 2003a [234]	*D.pteronyssinus, D.farinae*	American cockroach	grass pollen mix (9 Southern grass mix), tree pollen mix (11 North American tree mix)	*Alternaria tenuis*	cat	
Cooper 2003b [221]	*D.pteronyssinus, D.farinae*	cockroach	grass pollen, tree pollen	*Alternaria tenuis*	cat fur	
Cooper 2004 [225]	*D.pteronyssinus, D.farinae*	American cockroach	grass pollen mix (9 Southern grass mix), tree pollen mix (11 North American tree mix)	*Alternaria tenuis*	cat	
Cooper 2006 [242]	*D.pteronyssinus, D.farinae*	American cockroach	grass pollen mix, tree pollen	*Alternaria tenuis*	cat	
Dagoye 2003 [218]	*D.pteronyssinus*,	*B. germanica*				
Davey 2005 [219]	*D.pteronyssinus*	cockroach				
Flohr 2006 [235]	*D.pteronyssinus*, *D.farinae*	*P. americana*				
Joubert 1979 [308]						15 common aeroallergens
Nyan 2001 [237]	*D.pteronyssinus,*	cockroach mix (American and German)	grass mix	mould mix (including *Aspergillus, Alternaria, Penicillium*)		
Obeng [305] (unpublished data)	*D.pteronyssinus*, *D.farinae*, mixed mite,	cockroach				peanut
Obihara 2006 [226]	house dust mite		Bermuda grass, Rye grass	*Aspergillus*, *Cladoporium herbarium*, *Alternaria alternata*	cat dander, dog dander	
Peireira 2007[205]	*D.pteronyssinus, D.farinae*		grass mix, tree mix	*Alternaria alternata*	cat dander	
Ponte 2006 [307]	*D.pteronyssinus*, *D.farinae*, *B. tropicalis*					

Rodrigues 2008 [142]	*D.pteronyssinus*, *B. tropicalis*	*P. americana*, *B. germanica,*		fungi *(Aspergillus amstelodami, A.fumigatus, A.niger, A.terrus, Penicillium brevicompactum, P. expansum, P.notatum, P. roquefoti, Cladosporium fulvum. C. herbarum*)	cat epithelium, dog epithelium
Scrivener 2001 [58]	*D.pteronyssinus*	cockroach	grass mix, mixed cereals	mould mix, aspergillus	cat fur, dog dander
Wördemann 2008 [207]	*D.pteronyssinus, D.farinae*	cockroach	mixed grass, mixed tree	*Alternaria alternata*	cat dander

Table 5-3: Subgroup analyses for association between any parasite and sensitisation to at least one allergen

Subgroup analyses	Total trials, n	Odds Ratio (95% CI)	I^2, %	P Value for Heterogeneity	P Value for association
Methodological quality score					
≥7	8	0.67 (0.59, 0.75)	29%	p=0.20	p<0.01
<7	1	1.10 (0.71, 1.70)	n/a	n/a	p=0.67
Skin wheal size					
≥3mm	9	0.69 (0.60, 0.79)	45%	p=0.07	p<0.01
<3mm	0				
Study population					
Children only	8	0.70 (0.61, 0.80)	41%	p=0.11	p<0.01
Adults only	1	0.30 (0.11, 0.80)	n/a	n/a	p=0.02
Adults and children	0				
Geographical location					
South/central America	7	0.68 (0.60, 0.75)	17%	p=0.30	p<0.01
Africa	1	0.30 (0.11, 0.80)	n/a	n/a	p=0.02
Europe/Asia	1	1.10 (0.71, 1.70)	n/a	n/a	p=0.67
Study design					
Cross-sectional study	8	0.67 (0.57, 0.78)	44%	p=0.09	p<0.01
Randomised controlled trial	1	0.78 (0.64, 0.95)	n/a	n/a	P=0.01
Case-control study	0	n/a	n/a	n/a	n/a

Table 5-4 Subgroup analyses for association between individual parasite species and allergen sensitisation to at least one allergen, cockroach and mite

Parasite	Allergen skin sensitisation	Total trials, n	Odds Ratio (95% CI)	I^2, %	P Value for Heterogeneity	P Value for association
A. lumbricoides	≥1 allergen	9	0.69 (0.59, 0.80)	30	p<0.01	p<0.01
	cockroach	4	0.90 (0.75, 1.08)	0	p=0.25	p=0.25
	mite	6	1.03 (0.73, 1.46)	47	P=0.86	P=0.86
hookworm	≥1 allergen	4	0.68 (0.46, 1.01)	14	p=0.06	p=0.06
	cockroach	4	0.81 (0.62, 1.08)	52	P=0.15	P=0.15
	mite	5	0.94 (0.65, 1.36)	62	p=0.73	p=0.73
T. trichiura	≥1 allergen	5	0.75 (0.65, 0.86)	7	p<0.01	p<0.01
	cockroach	2	1.86 (1.24, 2.80)	13	p=0.003	p=0.003
	mite	3	1.72 (0.90, 3.29)	47	p=0.10	p=0.10

Figure 5-1: Flow diagram of included and excluded studies for skin sensitisation

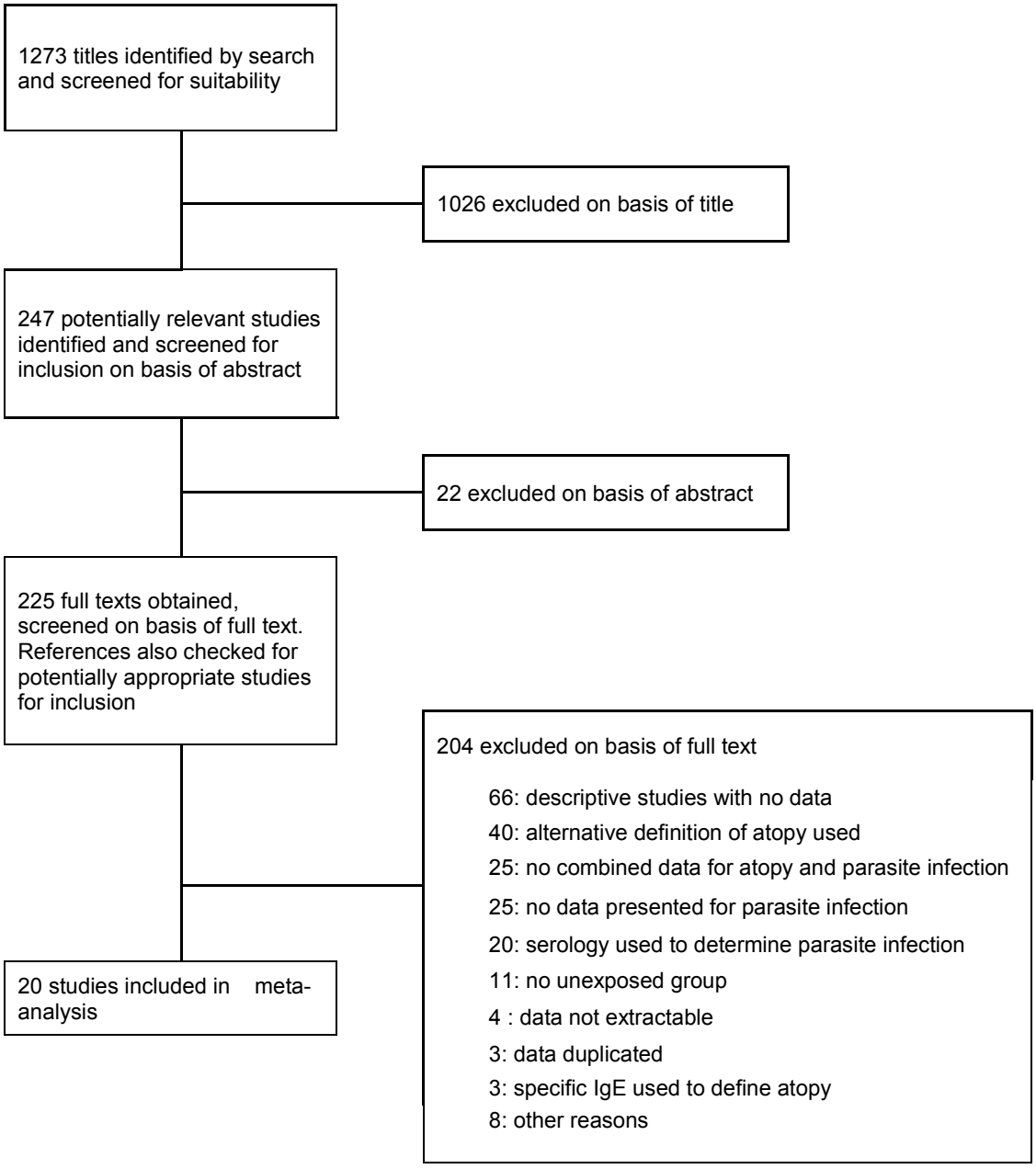


Figure 5-2: Funnel plot for studies of the association between any current parasite infection and skin sensitisation to at least one allergen

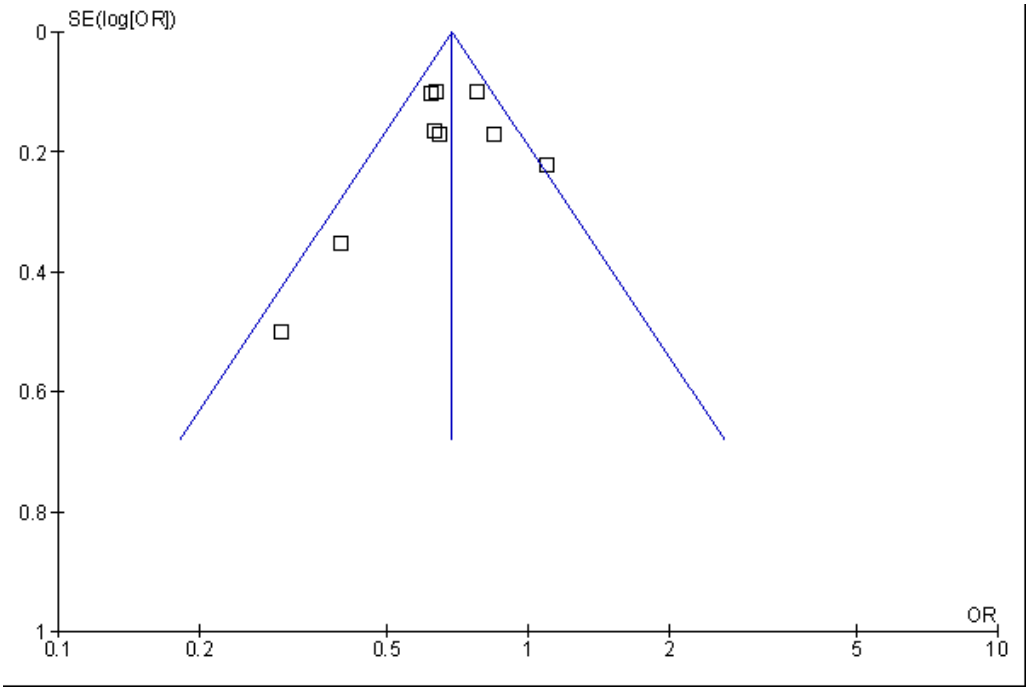


Figure 5-3: Forest plot of studies of the association between infection with any parasite and atopy

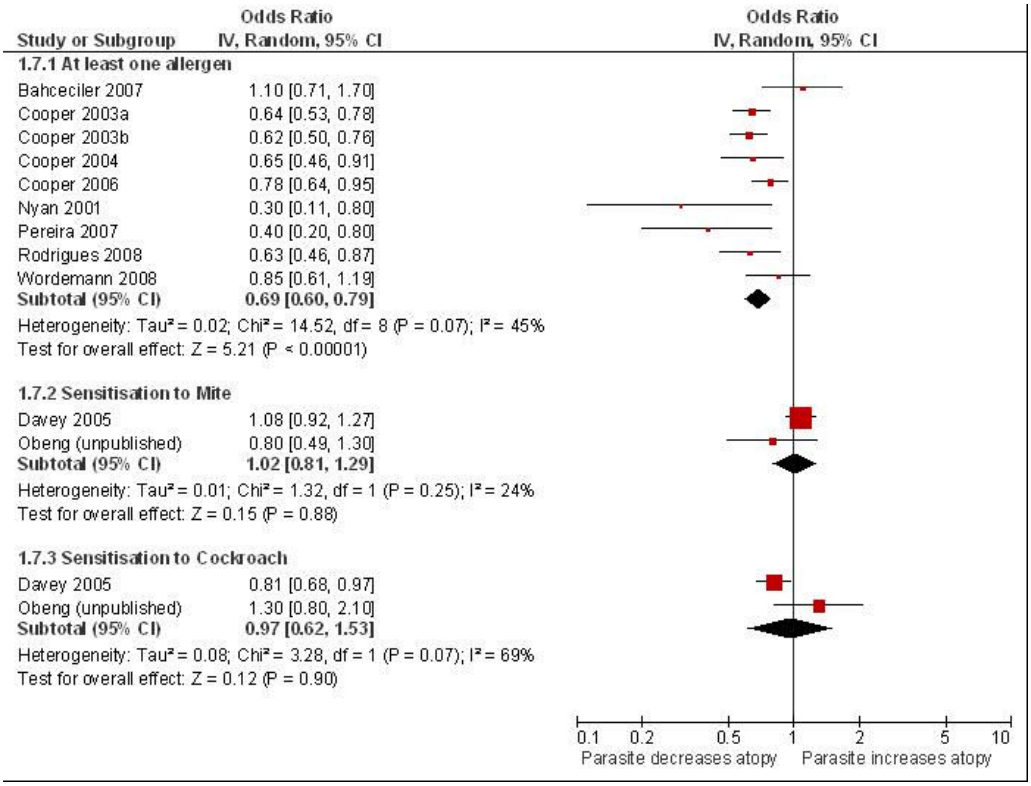


Figure 5-4: Forest plot of studies of the association between infection with *Ascaris lumbricoides* and atopy

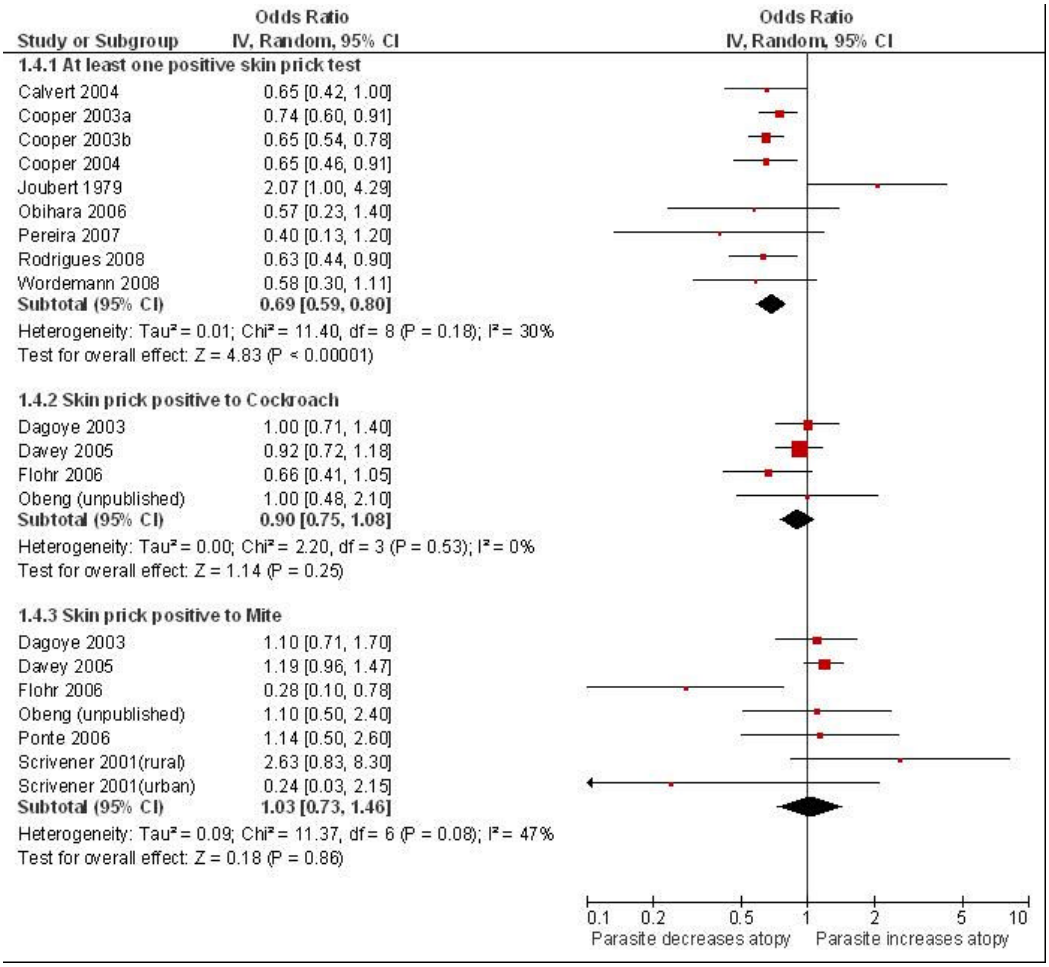


Figure 5-5: Forest plot of studies of the association between infection with hookworm species and atopy

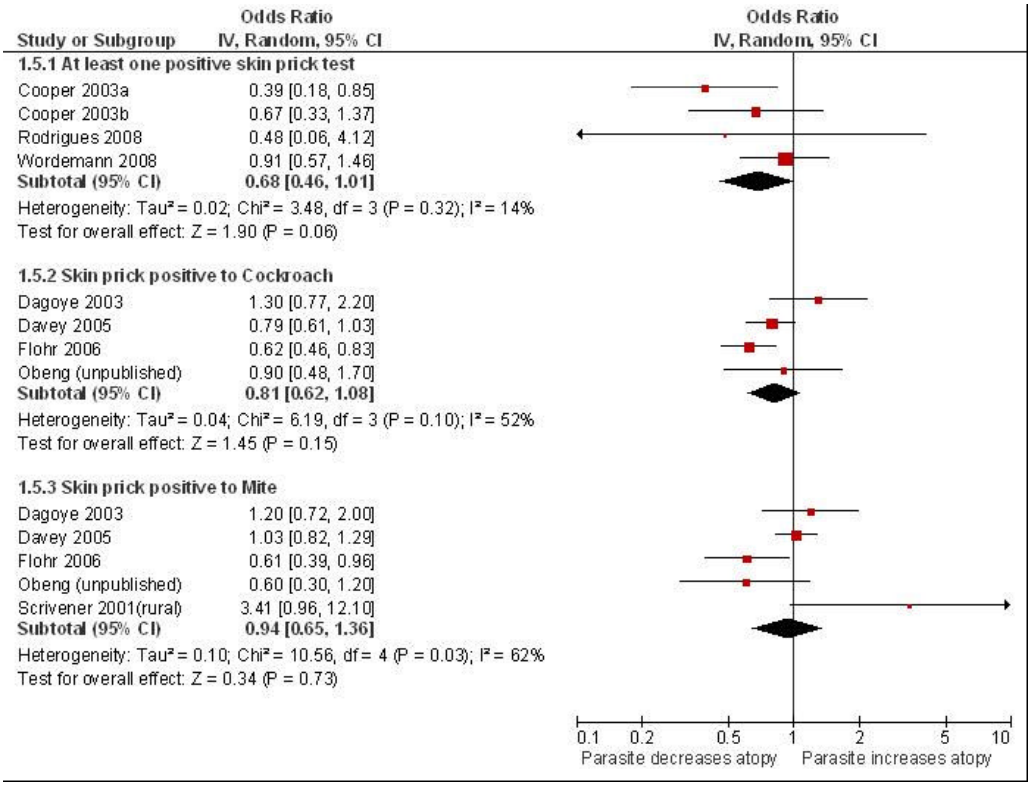
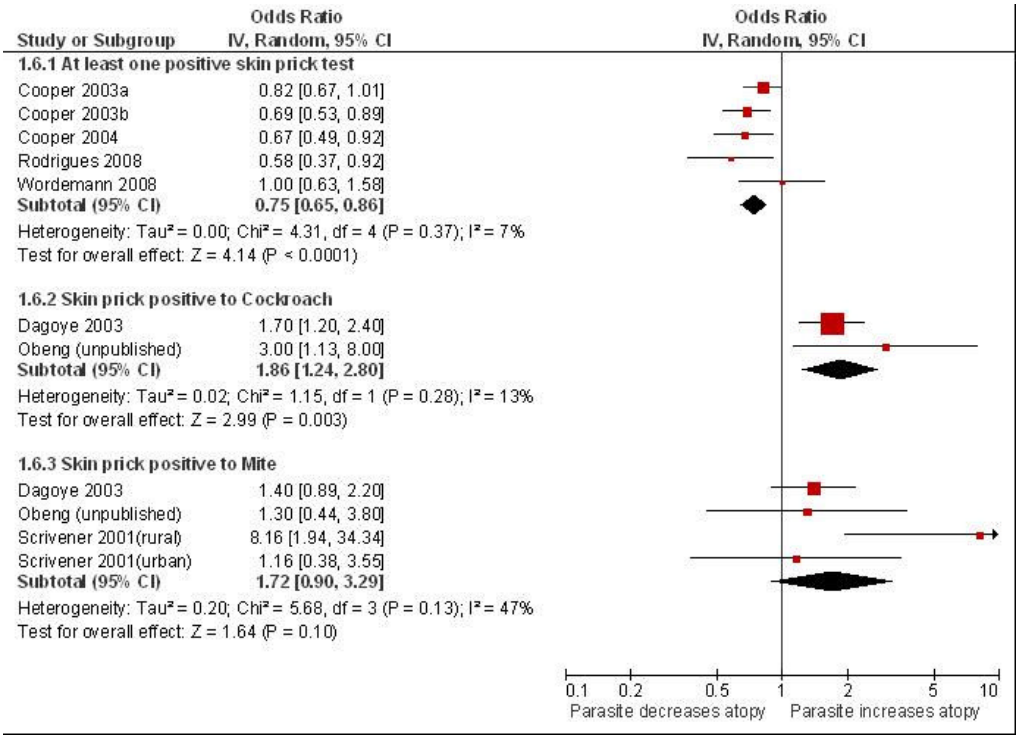


Figure 5-6: Forest plot of studies of the association between infection with *Trichuris trichiura* and atopy



6 GENERAL DISCUSSION AND CONCLUSIONS

6.1 Main findings of thesis

This thesis has looked at several different aspects of the association between parasite infection and asthma and allergic disease. A growing body of epidemiological evidence, mainly from observational studies carried out in areas of the world where parasite infection is endemic, has supported an inverse relation between helminth infection and allergy, but no placebo-controlled randomised controlled trials had been conducted, and meta-analysis of observational studies was only available for asthma. In this thesis, a systematic review and meta-analysis of observational studies has confirmed that helminth infection appears to protect against atopic sensitisation, thus supporting the previous review showing a protective association between infection and asthma [13]. The clinical trials in this thesis have shown that intervention trials of experimental hookworm infection are feasible to pursue in a clinical setting, that infection with ten larvae is generally well tolerated, but the findings did not provide evidence that a single infection at this dose leads to a significant improvement in asthma. The primary reason why it was important to carry out these studies is that despite substantial investment and research, there are still a (currently unquantified) proportion of patients who appear to have refractory disease and remain symptomatic despite using standard available treatments [312] so new therapeutic approaches are still needed. Infection with parasites, or with therapeutically active products derived from them, could prove to be a novel effective treatment for asthma. In addition, the studies contribute data that further characterise the relation between infection and allergy observed in countries where helminth infection is endemic.

6.1.1 Association between parasite infection and atopy

The meta-analysis confirmed that there is a clear inverse association, in observational studies, between helminth infection and atopy measured using skin sensitisation. This relation appears to vary only little according to species, with a similar size of effect for the geohelminths hookworm, *A. lumbricoides* and *T. trichiura. S. mansoni* may have had a greater effect but there were too few studies to pool the results and the neither of the studies included in the meta-analysis were judged to be of high methodological quality. As in asthma, the association seems to be weakest for infection with *A. lumbricoides*. Whilst this meta-analysis was performed after the clinical trials, it provides more robust evidence using pooled analyses that an association between these infections and allergy does exist and that the clinical trials were justified.

Despite the evidence from the observational studies, in the three intervention trials, there were no consistent changes in allergen skin sensitisation tests although this was not their primary aim and none were appropriately powered to show an effect if one did exist. No previous intervention studies of intentional infection with hookworm have reported the effects on atopy. However, given the results of eradication studies where anti-helminth treatment appeared to increase atopy, these results are perhaps surprising. There are several possible explanations for why no change was observed in atopy in the clinical trials which are explored in more detail in section 3.5.4. In brief, one explanation is that the studies were not adequately powered to explore this as an outcome. Other possible explanations relating to the study design include that the duration of the study was too short and too few larvae were given. Importantly, the age at which study participants were infected when compared with naturally acquired infection may be relevant. Regardless, research should continue in order to ascertain the association between atopy

and allergic disease, with particular attention to any variations according to different economic settings [313].

6.1.2 Effects of hookworm infection on asthma and allergic rhinoconjunctivitis

The first clinical study reported in this thesis established that a dose of ten hookworm larvae was sufficient to produce an intensity of infection generating at least 50 eggs/g faeces, the level found to be protective in an observational study in Ethiopia [58]. However, using a single dose of ten larvae was not enough to induce an effect on immune modulation. Nor did this dose demonstrate a role in the intervention studies for infection in treatment of allergic rhinoconjunctivitis or asthma, although in the case of allergic rhinoconjunctivitis the study was not designed or powered to do so. The only other study to intentionally infect people with allergic rhinoconjunctivitis with parasites, in this case using 2500 *T. suis* ova, also found no effect on symptoms [278]. There are several possible explanations for why no effect was seen on asthma which are explored more fully in Chapter 4. In brief, one explanation is that there is a true effect but it was not seen due to problems with the design of the intervention study, the most important of which are likely to be that the study was not adequately powered to show an effect if one did exist and that the dosing regimen was wrong. This is likely to be particularly important given the lack of immune modulation observed in the study in allergic rhinoconjunctivitis. It may also be that there is a true effect but this is only on the development of asthma and not on improving control of pre-existing asthma and the timing of infection needs to be earlier. Finally, it may be that the hypothesis is wrong and the observed relation between parasites and allergic disease is due to other unmeasured factors or reverse causation. The results of the intervention studies neither prove nor refute the hypothesis

that hookworm infection may confer protection against asthma, and further studies - probably using different dosing regimens - are required in order to provide a more conclusive answer.

Despite the apparent lack of effect of infection on allergic disease, the clinical trials presented here are unique, and contribute important data to an area where there are still many unanswered questions. They are the first to report the effects of intentional hookworm infection in the setting of a randomised controlled clinical trial. More specifically, they are the first to report the egg counts and side effects of different dosing regimens and the first to measure the effects of infection on allergic disease. Whilst the studies were limited in size, the results are useful in informing future trials in terms of dosing regimens, length of study and safety data including the occurrence and range of adverse effects infection notably the effects on bronchial responsiveness. In addition, whilst there was no significant effect of infection on asthma, the sample size was small and the effect on the primary outcome, bronchial hyper-responsiveness, was in the right direction suggesting that further larger trials are worth pursuing.

6.1.3 Adverse effects of hookworm infection

The three clinical trials reported the adverse effects of hookworm infection which were broadly consistent with the findings of other published studies and which appeared to be related to the dose of larvae administered. The most common of these was a localised pruritic erythematous rash in the first five days following infection, with a recurrence in many participants during the second week. Gastrointestinal symptoms (particularly nausea, abdominal pain and indigestion) were also reported in several cases and, as they coincided with a rise in eosinophil counts, probably related to an eosinophilic

gastroenteritis [259]. Whilst the severity of the gastrointestinal symptoms in a few of the participants led to them withdrawing from the studies, the average scores were not high and the range of experiences was varied, with a number of people reporting scores of zero. These are the most comprehensive studies to date to describe the adverse effects of intentional hookworm infection in a clinical trial setting and can be used in informing future trials of infection, not just in allergic disease but also in other conditions.

6.2 Recommendations for further areas for research

Several different research strategies could be employed in order to advance our understanding of the association between parasite infection and allergic disease, and which of these are used will be determined by the primary aim of the recommended intervention study, as detailed below.

6.2.1 Future intervention studies

Research should continue to focus on the use of parasites as novel therapeutic agents in the treatment of asthma and allergic disease. As the studies presented here have established that infection is safe, further intervention studies can now proceed, but these should be designed to mimic natural infection more closely and, in doing so, to aim to induce more of an effect on the immune system. This is likely to involve administration of either repeated low-dose infection or a higher single dose of infection, though the former may be more appropriate as this would be likely to reduce the undesirable adverse effects experienced with the higher doses and to imitate naturally acquired infection more closely. One to five larvae could be administered on a weekly, fortnightly or monthly basis for between three and six months (depending on the tolerance of any adverse effects). In such a

study, allergic symptoms should be monitored for a longer period of time (for example, three years) than in the studies presented in this thesis. This could be important as the immune modulatory effect of chronic infection may differ from that of acute infection [314] and thus a beneficial effect, if one exists, may only be seen after a longer period of time than has been measured previously. In addition, allowing a longer period between infection with hookworm and assessment of allergic symptoms is more likely to represent the circumstances observed in cross-sectional studies, where acquisition of infection may have occurred several years before the study took place.

The choice of population in which these further intervention studies should be carried out needs careful consideration. It would seem sensible to conduct such studies in countries where helminth infection is uncommon and participants are parasite-naïve, and then to focus on different populations according to disease severity. The caveat to this approach is the possibility that the effects of parasite infection on the immune system - specifically, with regard to allergy - may be different in helminth-endemic populations compared with populations where infection is uncommon. Logistically, however, it would be challenging to undertake an intervention study of experimental hookworm infection in a country where parasite infection is endemic, because of the likelihood of naturally acquired infection. One option therefore would be to undertake a large study in a country such as the UK, of people with mild to moderate asthma, using a different dosing regime from the intervention studies reported in this thesis (which have already shown no effect of a single dose of ten larvae in such a population). Alternatively, a study could be performed in a cohort of people with poorly controlled or severe asthma in whom conventional treatment is often ineffective. There are two main reasons why current available treatments fail in up to 10% of asthmatics. The first

reason is that in a small proportion of asthmatics, the disease is driven by non-eosinophilic inflammation which does not respond to usual asthma treatments. Asthma research is increasingly using phenotyping of disease to characterise the underlying pathology occurring at a cellular level in patients with difficult disease. A study of patients with difficult disease would require detailed assessment of asthma phenotype (using techniques such as sputum sample cell typing); and the heterogeneous nature of their disease would require a very large sample size to observe a treatment effect, if one existed. The second reason for failure of current treatment lies in psychosocial reasons such as non-compliance and problems associated with social deprivation. An intervention study in this particular group of patients might, however, prove particularly difficult at a logistical level. So whilst a trial of the efficacy of intentional hookworm infection in people with severe asthma is possible, it is likely to be challenging and may not be worthwhile.

If a trial was to be carried out to determine the efficacy of hookworm in treatment of allergic rhinoconjunctivitis, the design should include a number of measures, in line with guidelines, that were not carried out in the safety trial in people with allergic rhinoconjunctivitis reported in this thesis [315]. Specifically, the outcome measures should include a composite daily symptom score based on nasal symptoms, possibly ocular symptoms, and use of anti-allergen medication. More objective measures of disease activity such as nasal nitric oxide or rhinometry could also be used [316]. In addition, when designing a trial to evaluate the efficacy of hookworm as an immune modulatory agent, it should be considered comparable to a trial of immunotherapy. For example, trials of immunotherapy usually start several months prior to the onset of symptoms and last a minimum of six to 12 months. In contrast, trials of pharmacological agents usually enrol participants when allergen exposure is

sufficient to cause significant symptoms and are relatively short as was the case in the study presented here [315].

In addition to using hookworm infection in people with established disease, studies should also be designed to investigate the effects of hookworm infection on development of allergic disease. Such a study should be carried out in a population of young children whose immune systems are still developing in order to maximise the possible immunomodulatory effect of the infection. A significant proportion of young children will go on to develop allergic disease (as described in chapter 1) and therefore conducting a study in a population such as this will provide the greatest chance of seeing an effect, if one does exist, using a reasonable sample size. In contrast, a prevention study carried out in adults who do not have allergic disease is not likely to be feasible, first because their immune system is already mature and second, because relatively few of these individuals will go on to develop asthma, and so the sample size would need to be extremely large for an effect to be seen. Clearly the ethical considerations of giving intentional hookworm infection to children under the age of five are huge and without stronger evidence of advantageous effect, the potential risks of infection are likely to outweigh the benefits at this point in time.

6.2.2 Studies of the immunomodulatory effects of infection

Future intervention studies should seek to identify the likely key helminth molecules involved in immune modulation, which appears to involve T-regulatory cells. This may be particularly useful in increasing our understanding of how the immune system can be manipulated so as to develop future new treatments, or preventative strategies for allergic disease. The information from this could be used in two ways: either helminth products

could be manufactured and administered in controlled conditions to modify the immune system, which is likely to be more acceptable than infecting people with larvae; or novel pharmaceutical products mimicking these effects could be designed. In doing this research, it would also be important to elucidate whether the immune modulation that occurs varies between different helminth infections and/or between different populations. Any findings to that effect may explain some of the variation in observational study results.

6.2.3 Future observational studies

Epidemiological studies should continue with the aim of establishing the relative importance of parasite infection in the aetiology of allergic disease and, in turn, the magnitude of its role in relation to changes observed in the prevalence of allergic disease worldwide. Further observational studies to address this would be of most use if they were large birth cohort studies, carried out in areas where both parasite infection and allergic disease are common. They should be designed to include collection of data on both symptoms of allergic disease (using well validated questionnaires and objective measures of disease) and immune function. Such longitudinal studies would also help to address the issue of reverse causation, that is, the question of whether infection reduces the chance of developing allergy or whether allergic individuals are protected against parasite infection. A longitudinal study, designed to investigate the aetiology of allergic disease (and in particular the role of geohelminth infection and acetaminophen use), is currently in progress in Ethiopia [100]. In 2005/6 a population-based cohort was established in the rural town of Butajira comprising 1065 pregnant women, to whom 1006 live singleton babies were born. At ages one and three, data were collected using questionnaires on wheeze, eczema, child's use of acetaminophen and various potential confounders, along with a stool sample

for geohelminth analysis. Data on those children without wheeze (n=756) or eczema (n=780) at age one were analysed to determine the independent effects of geohelminth infection and acetaminophen use in the first year of life on the incidence of wheeze and eczema by age three. The results showed that the incidence of wheeze and eczema between the ages of one and three was reported in 7.7% (58/756) and 7.3% (57/780) of children respectively, but the prevalence of geohelminth infection was insufficient (<4%) to compute estimates of effect on allergic symptoms [100]. As the cohort matures, and the prevalence of geohelminth infection increases, this study should provide additional useful data on the role of infection on allergic disease.

6.2.4 Studies of the effects of parasite eradication programmes

Studies should also be performed to try to predict the effect of parasite eradication campaigns and hookworm vaccination programmes on prevalence of allergic disease. To date, no eradication studies have had a convincing effect on asthma symptoms, allergic rhinoconjunctivitis or eczema. Conversely, some studies have identified a [243;245-7] increase in atopy after anti-helminth therapy, but the clinical relevance of this may be less important. Current evidence thus suggests a minimal impact of such parasite eradication programmes on prevalence of allergic disease. However, hookworm eradication programmes are now in place in some areas of the world and provide the ideal setting in which to conduct studies to ascertain whether the prevalence of allergy and atopy has increased in these areas and whether these effects have been sustained. Such studies should be of high priority for public health departments: if a potential side effect of eradication programmes is a surge in allergy prevalence, then such programmes may need to be reconsidered.

6.3 Intervention studies of parasite infection in other immune-mediated disease

The intervention studies presented in this thesis were the first intervention studies of hookworm infection in people with allergic disease. The results provide safety data and information about adverse effects of infection and can be used to inform trials of other diseases. In addition to the interest in the use of parasite infection in treatment of allergic diseases, parallel studies are in progress to investigate the use of helminths to treat other immune-mediated diseases and a small number of trials have been carried out in inflammatory bowel disease with a number more in progress. At the time of submission of this thesis, three other intervention studies of parasite infection have been published. The first two are trials of the effects of *Trichuris suis* (pig whipworm) on inflammatory bowel disease [317;318]. In the first, a feasibility study, 29 patients with active Crohn's disease were given 2500 *T. suis* ova orally on eight occasions over 24 weeks. Multiple doses were required to ensure ongoing infection, as humans are not a fully permissive host. Over two-thirds of patients reported an improvement in symptoms of Crohn's disease at the end of the study period, but results should be interpreted with caution as there was no control group for comparison [317]. In the second study, 54 patients with active ulcerative colitis were randomised, double-blind, to receive 2500 *T. suis* ova or placebo every two weeks for 12 weeks. At the end of the study, 43% of those with ova reported clinical improvement, compared with 17% of those with placebo (p=0.04). Perhaps surprisingly, both studies stated that there were no adverse or side effects of infection. The third study, published recently by a Danish group, is a randomised double-blind placebo controlled trial of the effects of *T. suis* on allergic rhinitis [278] and is described in more detail in section 3.5.3.

Reports from observational studies of the effect of parasites on other autoimmune diseases such multiple sclerosis are also emerging in the literature [319]. Following on from this, further trials by other research groups in rheumatoid arthritis [320], multiple sclerosis and Coeliac disease have been proposed, or are registered on the International Clinical Trials Register, but results are not yet available.

6.4 Potential hazards of using infection to treat allergy

There are, of course, potential problems and obstacles associated with the use of helminth infections in the treatment of asthma and other allergic disease. The first relates to the psychological and social acceptability of infecting individuals with whole parasites: the intervention studies reported here suggest this may not be a major issue, although the volunteers for these studies may not be representative of the general population. One obvious way of overcoming this objection in the future is the possibility of using specific parasite products identified to be important, rather than whole organisms. The second problem is that, as with all forms of immunosuppression, there is in theory an increased risk of opportunistic infections which can cause significant morbidity. Third, whilst we are aware of the effects of chronic infection acquired naturally, there are no long-term data of side effects of intentional infection which, depending on doses and frequency of infection used, may differ. As a result of immune modulation, there may be negative effects on other parts of the immune system, such as a reduction in T_H1 responses (with a resultant increase in autoimmune disease) or reduced response to vaccines, the impact of which should not be underestimated. Fourth, there is a theoretical risk of anaphylaxis and an increase in allergic reactions, if given in high doses to already atopic individuals. Fifth, experimental hookworm

infection causes gastrointestinal effects as demonstrated in the intervention studies. The long-term impact on development of bowel pathology from chronic infection is again unknown: whilst there is no evidence of an increase in bowel disease from countries where infection is endemic, there are large differences in population demographics and environmental exposures, which will be important contributory factors. There is also the fact that life expectancy is often shorter in these countries, and so the risks of long-term infections - particularly in the eighth and ninth decades of life - may not be realised.

Irrespective of the evidence of its efficacy, it is important to exercise caution before embarking on the use of helminths (or their products) as a treatment modality in allergic disease, bearing in mind the importance in medical ethics of the principle of non-maleficence. Focusing on the use of antigen-specific products as immune regulators may enable avoidance of potentially harmful adverse effects.

6.5 Conclusions

The findings reported in this thesis have direct relevance to the improvement of human health. Whilst further elucidation is required of the environmental factors which are responsible for the rapid change in prevalence of asthma and allergic disease that occurs as subsistence populations become more urbanised and affluent, parasite infection is likely to provide a partial explanation of the observations. In addition to the existence of an inverse association between hookworm infection and asthma, there is now confirmation of a similar relation between parasite infection and atopy.

Hookworm infection as a treatment modality has now been established as feasible, acceptable and well tolerated. The lack of positive effect on asthma in these studies could be attributable to a number of factors already discussed, but further larger studies of asthma and other allergic disease, incorporating revised dosing regimens, are required. At this stage, it is too early to exclude the possibility that cultured hookworm or hookworm products may have a role to play in the management of asthma, allergic rhinoconjunctivitis and other allergic diseases. The adverse effects of chronic hookworm infection are, however, unquestionable. Moreover, whilst the results of eradication studies do not, to date, suggest that an increase in allergic disease is inevitable; clinicians must be mindful of the potential impact of an international hookworm eradication programme on allergic disease prevalence wherever such a programme is introduced.

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APPENDICES

Appendix A:

Abstracts of work and publications arising from thesis presented at conferences

Appendix B:

Patient Information Sheet (Allergic Rhinoconjunctivitis study)

Patient Information Sheet

Placebo-controlled study of hookworm infection in non-asthmatic people with allergies

Investigators: Dr J Feary, Professor D Pritchard & Professor J Britton

You are invited to take part in a research study. Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with a friend or relatives if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. You can keep this information sheet. If you take part in the study, you will be given a copy of the signed consent form to keep.

Thank you for taking time to read this.

Why are you doing this study?
Asthma and hayfever have become increasingly common in more affluent societies over the last few years but the reason for this increase is uncertain. One possible explanation is that as we live in an increasingly clean environment, and are therefore exposed to fewer infections, our immune systems start to respond to normally harmless things like pollen. It is this inappropriate immune response that causes hayfever.

One infection that is now rare in more affluent societies is hookworm. This is a small worm which has evolved alongside humans and lives in the bowel. Millions of people in the developing world have hookworm and studies in Ethiopia suggest that infection with hookworm is associated with a lower risk of asthma and allergic disease. This raises the intriguing possibility that deliberate hookworm infection may be of benefit in the treatment of asthma and allergy. To investigate this properly we need to do a clinical study in a similar way to how a new drug would be tested. This study will look at the possible benefits and side-effects of hookworm infection in people with hayfever.

We recently completed a pilot study of hookworm infection in people without hayfever or asthma. Ten people (including the investigators for this study) received 10, 25, 50 or 100 hookworm larvae to work out which was the lowest dose that stimulated an immune response but caused a minimum of side effects. Both the 10 and 25 doses were well tolerated but we have chosen 10 larvae because this dose stimulated a good immune response and we want to use the lowest possible dose.

Why have I been chosen?
You have allergies but not asthma and have expressed an interest in assisting with research in Nottingham.

Do I have to take part?
No, you don't. It is up to you whether or not to take part. If you do decide to take part, you will be given a copy of this information sheet to keep and asked to sign a consent form. Also, if you decide to take part, you will be free to withdraw at any time, without giving a reason if you don't want to. This will not affect the standard of any medical care you may be receiving.

What will happen to me if I take part?
The study will involve visits to the Division of Respiratory Medicine at City Hospital over 16 weeks. These will be weekly at first and then fortnightly towards the end of the study. The first visit will take around 2 hours, however each subsequent visit last about an hour. We realise this is a considerable time commitment but we would like to keep a close eye on you.

At the first visit, we will take about 20ml of blood to make sure you are not anaemic and have no evidence of having had hookworm infection in the past. Female volunteers will also be asked to give a blood sample for a pregnancy test. We will also do skin tests for allergy to cat, dust and grass extract: this involves a small scratch on the skin and a drop of each extract to see if this causes an itchy reaction. If you do have some itchiness from this test it should settle in about 20 minutes. We will then measure your sensitivity to an inhaled aerosol called adenosine monophosphate. This involves inhaling adenosine monophosphate given by us through an inhaler device and then doing a simple lung function test. We will then repeat the inhalation at stronger doses until the lung function test shows that you are responding. This is the end of the test and you can take a reliever inhaler (salbutamol, Ventolin) if you wish. At all times during the tests, you can stop whenever you wish.

At the second visit, we will put a gauze pad on your arm under a plaster. This will either contain a placebo solution containing histamine or a solution containing 10 hookworm larvae. In both cases, there may be some skin itching and redness where the solution is applied. The larvae then find their way through the skin, via the lungs to the intestine, where they stay until eradicated. You should keep the plaster on for 24 hours after which time you can remove it and put it in a special container which we will provide.

At each of the visits over the next 12 weeks, we will ask you to complete a questionnaire about your allergic symptoms, we will take about 30ml of blood to measure your blood count, inflammatory and allergic responses and repeat the lung function tests with adenosine monophosphate. We will also need a stool sample to look for hookworm eggs. Between visits we will ask you to complete a diary of your allergic symptoms, any side effects you experience and a record of your peak flow (we will show you how to do this).

After 12 weeks, or if you choose to withdraw from the study, we will treat the hookworm infection with mebendazole tablets (100mg twice each day for three days). These are very effective at treating hookworm infection but are only effective once hookworm is in the bowel (about 4 weeks from the start). If you decide to withdraw from the study before this point we will give you tablets with clear instructions about when to start them to ensure they work. Female volunteers will have another blood test to ensure they are not pregnant before taking the tablets.

We would like to see you again two and four weeks after treatment to make sure you are well and the infection has cleared. This will involve a final blood test and stool sample, skin prick tests and lung function tests with adenosine monophosphate. All information gained during the study will be confidential. We will reimburse reasonable travel expenses incurred during the study.

What is the drug or procedure being tested?
We are assessing the effects of hookworm infection in people with allergies**.**

What are the possible disadvantages and risks of taking part?
There is likely to be some itching and redness of the skin where we give the histamine placebo or hookworm larvae. This should settle within a week but may return 7-14 days later before settling completely. The larvae pass through the lungs on their way to the bowel and so in theory this could cause breathlessness, cough or wheezing. When the hookworm arrives in the bowel, some 'stomach upset' may occur but we expect this to be mild if it occurs. The side effects which are possible are abdominal cramps, nausea, diarrhoea and flatulence. If you experience these symptoms and find them unacceptable, you are free to finish the study at any point. Hookworm infection is very common in many parts of the world and other than in people who are badly nourished, or in very young children, in whom it can cause anaemia or weight loss, no other major common adverse effects are known to occur. One extremely rare complication of some long-term worm infection is called endomyocardial fibrosis. Here, inflammation of the heart muscle occurs and can cause scarring which can, in turn, lead to heart failure. However this condition usually occurs with worms which are very different from the one that we are using and only in people who have very long term infections. We are eradicating the worm infection after 3 months and therefore we do not expect it to be a problem in this study.

If there is a chance you may be pregnant (or become pregnant in the next four months), you should not take part in the study. If you are a blood donor, you should not donate during the study. Before donating again after the study you should inform the blood transfusion service that you have taken part in this study so they can give you up to date advice about when you can start to donate again. Hookworm infection is not contagious in developed countries with normal standards of hygiene and sanitation. Therefore, if you wash your hands carefully after going to the toilet, you will not be contagious to others.

Mebendazole can cause abdominal pain, diarrhoea or rashes and should not be taken in pregnancy. Its effects can also be increased by an anti-acid drug, cimetidine: we will ask you to change this if you are on it.

We suggest that you inform your private health insurance company (if you have one) that you are taking part in this study.

What are the possible benefits of taking part?
The information we get from the studies may help to improve treatment of allergies and asthma in the future.

What will happen to the results of the research study?
We will send you a summary of the final results.

Who is funding the research?
The Wellcome Trust are funding the research

For more information contact:
Dr Johanna Feary
Clinical Research Fellow
University of Nottingham
Clinical Sciences Building
City Hospital
Nottingham NG5 1PB
Telephone 0115 8231936

Appendix C:

Consent Form for Allergic Rhinoconjunctivitis and Asthma studies

CONSENT FORM

Feasibility studies of hookworm infection in the treatment of allergies and asthma

Division of Respiratory Medicine, City Hospital, Nottingham

Investigators: Dr J Feary, Professor D Pritchard & Professor J Britton

The patient should complete the whole of this sheet himself/herself.

Please cross out as necessary

Have you read & understood the patient information sheet	YES/NO
Have you had opportunity to ask questions & discuss the study	YES/NO
Have all the questions been answered satisfactorily	YES/NO
Have you received enough information about the study	YES/NO

Who have you spoken to Dr ……..

Do you understand that you are free to withdraw from the study:

at any time	YES/NO
without having to give a reason	YES/NO
without affecting your future medical care	YES/NO

Do you agree to take part in the study YES/NO

Signature (Patient) Date

Name (In block capitals)

I have explained the study to the above patient and he/she has indicated his/her willingness to take part.

Signature (Doctor) Date

Name (In block capitals)

Appendix D:

Dilution Schemes for the AMP dosing schedules

Volume of AMP solution	Add 0.9% saline	**Obtain final dilution (mg/ml)**
2 mls of 400 mg/ml	2 ml	**200 mg/ml**
1 mls of 200 mg/ml	3 ml	**50 mg/ml**
1 ml of 50 mg/ml	3 ml	**12.5 mg/ml**
1ml of 12.5 mg/ml	3 ml	**3.125 mg/ml**

Appendix E:

Protocol for AMP Challenge with Mefar dosimeter

AMP CHALLENGE WITH MEFAR

Initals/ID …….. Visit ……

Date ……..... Time ……

Last caffeine ……

Last cigarette …… No. per day …….

Last bronchodilator …… Last ICS/LABA ……

Last antihistamine ……..

FEV1 …… Predicted …… % Predicted ……..

FVC …… Predicted …… % Predicted ……..

AMP CHALLENGE *

DOSE	DOSE IN PUFFS	AMP IN µmol	FEV1	% fall in FEV1
0	3 x saline			
1	1 x 3.12 mg/ml	0.115		
2	1 x 3.12 mg/ml	0.230		
3	2 x 3.12 mg/ml	0.460		
4	4 x 3.12 mg/ml	0.92		
5	2 x 12.5 mg/ml	1.84		
6	4 x 12.5 mg/ml	3.69		
7	2 x 50 mg/ml	7.37		
8	4 x 50 mg/ml	14.7		
9	2 x 200 mg/ml	29.5		
10	4 x 200 mg/ml	59.0		
11	4 x 400 mg/ml	118.0		
12	8 x 400 mg/ml	236.0		
13	16 x 400 mg/ml	472.0		
14	32 x 400 mg/ml	944.0		

FEV1 for saline – 20% = ……

PD20AMP = ……

***Do NOT perform if baseline FEV1 < 40% predicted or < 1.0 litres or if subject is pregnant or breastfeeding**

Appendix F:

Juniper Allergic Rhinoconjunctivitis Quality of Life Questionnaire

RHINOCONJUNCTIVITIS QUALITY OF LIFE QUESTIONNAIRE

NAME: _____ **DATE:** _____

VISIT 1: IDENTIFY 3 ACTIVITIES THAT HAVE BEEN LIMITED BY NOSE/ EYE SYMPTOMS DURING THE PREVIOUS WEEK AND WRITE THEM ON THE LINES BELOW.

EXPLAIN THE YELLOW AND GREEN RESPONSE CARDS TO THE PATIENT.

PLEASE SCORE EVERY ITEM: CIRCLE THE NUMBER THAT BEST DESCRIBES HOW THE PATIENT HAS BEEN DURING THE LAST WEEK.

ASK EVERY QUESTION EXACTLY AS WORDED.

ACTIVITIES (YELLOW CARD)

How troubled have you been by each of these activities during the last week as a result of your nose/ eye symptoms?

a)	Activity 1	0	1	2	3	4	5	6
b)	Activity 2	0	1	2	3	4	5	6
c)	Activity 3	0	1	2	3	4	5	6

SLEEP (YELLOW CARD)

How troubled have you been by each of these sleep problems during the last week as a result of your nose/ eye symptoms?

a)	Difficulty getting to sleep	0	1	2	3	4	5	6
b)	Wake up during the night	0	1	2	3	4	5	6
c)	Lack of a good night's sleep	0	1	2	3	4	5	6

NON-HAYFEVER SYMPTOMS (YELLOW CARD)

How troubled have you been by these problems during the last week as a result of your nose/ eye symptoms?

a)	Fatigue	0	1	2	3	4	5	6
b)	Thirst	0	1	2	3	4	5	6
c)	Reduced productivity	0	1	2	3	4	5	6
d)	Tiredness	0	1	2	3	4	5	6
e)	Poor concentration	0	1	2	3	4	5	6
f)	Headache	0	1	2	3	4	5	6
g)	Worn out	0	1	2	3	4	5	6

PRACTICAL PROBLEMS (YELLOW CARD)

How troubled have you been by each of these problems during the last week as a result of your nose/ eye symptoms?

a)	Inconvenience of having to carry tissues or handkerchief	0	1	2	3	4	5	6
b)	Need to rub nose/ eyes	0	1	2	3	4	5	6
c)	Need to blow nose repeatedly	0	1	2	3	4	5	6

NASAL SYMPTOMS (YELLOW CARD)

How troubled have you been by each of these symptoms during the last week?

a)	Stuffy/ blocked	0	1	2	3	4	5	6
b)	Runny	0	1	2	3	4	5	6
c)	Sneezing	0	1	2	3	4	5	6
d)	Post nasal drip	0	1	2	3	4	5	6

EYE SYMPTOMS (YELLOW CARD)

How troubled have you been by each of these symptoms during the last week?

a)	Itchy eyes	0	1	2	3	4	5	6
b)	Watery eyes	0	1	2	3	4	5	6
c)	Sore eyes	0	1	2	3	4	5	6
d)	Swollen eyes	0	1	2	3	4	5	6

EMOTIONAL (GREEN CARD)

How often during the last week have you been troubled by these emotions as a result of your nose/ eye symptoms?

a)	Frustrated.....	0	1	2	3	4	5	6
b)	Impatient or restless.....	0	1	2	3	4	5	6
c)	Irritable	0	1	2	3	4	5	6
d)	Embarrassed by your symptoms.....	0	1	2	3	4	5	6

ACTIVITY SHEET

1. BICYCLING
2. READING
3. SHOPPING
4. DOING HOME MAINTENANCE
5. DOING YOUR HOUSEWORK
6. GARDENING
7. WATCHING TV
8. EXERCISING OR WORKING OUT
9. GOLF
10. USING A COMPUTER
11. MOWING THE LAWN
12. PLAYING WITH PETS
13. PLAYING WITH CHILDREN OR GRANDCHILDREN
14. PLAYING SPORTS
15. DRIVING
16. SINGING
17. DOING REGULAR SOCIAL ACTIVITIES
18. HAVING SEXUAL RELATIONS
19. TENNIS
20. TALKING
21. EATING
22. VACUUMING
23. VISITING FRIENDS OR RELATIVES
24. GOING FOR A WALK
25. WALKING THE DOG
26. OUTDOOR ACTIVITIES
27. CARRYING OUT YOUR ACTIVITIES AT WORK
28. SITTING OUTDOORS
29. TAKING CHILDREN TO THE PARK

RHINOCONJUNCTIVITIS
& RHINITIS
QUALITY OF LIFE QUESTIONNAIRE
GREEN CARD

0. NONE OF THE TIME
1. HARDLY ANY TIME AT ALL
2. A SMALL PART OF THE TIME
3. SOME OF THE TIME
4. A GOOD PART OF THE TIME
5. MOST OF THE TIME
6. ALL OF THE TIME

Hookworm Study

RHINOCONJUNCTIVITIS
& RHINITIS
QUALITY OF LIFE QUESTIONNAIRE
YELLOW CARD

0. NOT TROUBLED
1. HARDLY TROUBLED AT ALL
2. SOMEWHAT TROUBLED
3. MODERATELY TROUBLED
4. QUITE A BIT TROUBLED
5. VERY TROUBLED
6. EXTREMELY TROUBLED

Hookworm Study

Appendix G:

Daily diary for Allergic Rhinoconjunctivitis study

Daily diary Hookworm and rhinitis

Daily Diary **Next Appointment**_____

Initials ☐☐ **ID** ☐☐ **Week** ☐☐

Record the best of THREE peak flows measurements for Morning and Evening

	Date	Day of the week	Morning peak flow	Evening peak flow
Day 1			☐☐☐	☐☐☐
Day 2			☐☐☐	☐☐☐
Day 3			☐☐☐	☐☐☐
Day 4			☐☐☐	☐☐☐
Day 5			☐☐☐	☐☐☐
Day 6			☐☐☐	☐☐☐
Day 7			☐☐☐	☐☐☐
Day 8			☐☐☐	☐☐☐
Day 9			☐☐☐	☐☐☐

Daily diary Hookworm and rhinitis

Symptoms (graded from 1(very mild) to 10 (very severe)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Date									
Day of the week									
Antihistamines (tick if used)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Nasal steroids (tick if used)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Skin itching	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Skin redness	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Nausea or sickness	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Diarrhoea	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Abdominal pain	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Flatulence or wind	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Indigestion	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Loss of appetite	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Wheeze	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Cough	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Breathlessness	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Tiredness	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐

University of Nottingham Johanna Feary 0115 823 1946 Out of hours 0115 823 1947

USEFUL INFORMATION

For 12 hours prior to the study visit:

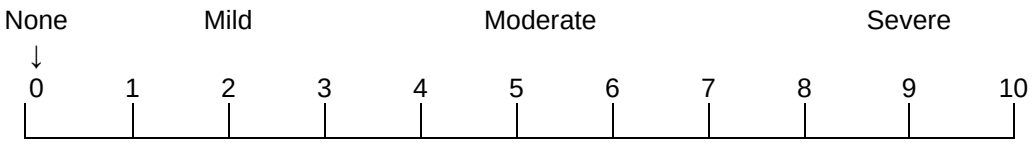
- No strenuous exercise
- No caffeine containing food and drink (tea/coffee/cola/chocolate)

For 24 hours prior to the study visit:

- No antihistamines
- No nasal sprays

SYMPTOM SCALE

Use this to help you when you are filling in the daily diary. You need to grade each of your symptoms on a scale of 1 to 10.
For example, indigestion which is mild would score 2 out of 10.



CONTACT TELEPHONE NUMBERS

- Dr Johanna Feary 0115 8231936
- 24 hour contact number 0115 8231937
  (Leave a message and telephone number including area code)

PLEASE WRITE ANY OTHER SYMPTOMS YOU MIGHT EXPERIENCE HERE WITH THE DATE AND DAY AND YOUR SYMPTOM SCORE (OUT OF 10) NEXT TO THEM

Appendix H:

Extended hookworm infection information sheet

Extended Hookworm Infection

For subjects in hookworm treatment group who decide not to take mebendazole

As you are now aware, you received 10 hookworm larvae at the beginning of the study looking at the effects of hookworm infection on asthma. At this point, and in accordance with the study protocol, now that the experimental period is over, we advise you to take a course of mebendazole tablets to eradicate the infection. However, you have expressed a desire not to take these tablets so that you keep the hookworm infection.

We would not expect you to have any serious adverse effects with a dose of 10 hookworm larvae and they will die naturally within a period of approximately 5 years. However there is a chance of developing complications due to long-term hookworm infection and some other issues which you should be aware of and which are listed below. We would therefore ask you to read this document carefully and sign where indicated at the bottom of the page. Please ask if you are uncertain of anything.

Possible adverse effects of long-term infection are:

- Anaemia (low blood haemoglobin) due to the hookworm removing small amounts of blood from your gut – this may cause you to feel tired or breathless and sometimes needs treatment with iron tablets. For someone who is otherwise well nourished however, and has no other cause of anaemia, anaemia is unlikely to occur.

- Endomyocardial fibrosis, which is an extremely rare complication of some long-term worm infections. Inflammation of the heart muscle occurs in this condition, which can cause scarring and in turn lead to heart failure. However this condition usually occurs with worms of a completely different species to the one that we are using, and generally, only in longer term infections. This complication is therefore extremely unlikely.

Further advice:

- If you are a blood donor, you should inform the blood transfusion service that you have hookworm infection, so they can give you up-to-date advice about when you can start to donate again.

- Females should be aware that mebendazole tablets should not be taken during pregnancy or whilst breastfeeding.

Please read the following and initial on the dotted line to show you have read and understand each statement:

- o I am aware that I have been infected with hookworm as part of the asthma study…….
- o I am aware of the long term risks of having hookworm infection outlined above……..
- o I have been provided with a course of mebendazole tablets to eradicate the hookworm infection but at this point in time, I have decided not to take them……...

Signed ……..

Name/ID……

Date……

Witness signature…….

Witness name ……

Date……

Appendix I:

New hookworm infection for those receiving placebo in trial

Hookworm Infection

As you are aware, you received placebo at the beginning of the study looking at the effects of hookworm infection on hay fever. The results of our study showed no strong evidence that giving people hookworm had a beneficial effect on their hay fever although a few individuals did report an improvement. The study also showed that there were no negative effects on breathing and so we are in the process of performing a study looking at the effects of hookworm infection on asthma symptoms. We will send you the full results of both these studies when they are published.

You have now expressed an interest in receiving a dose of 10 hookworm larvae to see if it improves your hay fever symptoms. It is important that you are aware that this will be outside the context of a study.

We would not expect you to have any serious adverse effects with a dose of 10 hookworm larvae and they will die naturally within a period of approximately 5 years. However there are some symptoms which can occur as a result of having hookworm infection and some other issues which you should be aware of and which are listed below. We would therefore ask you to read this document carefully and sign where indicated at the bottom of the page. Please ask if you are uncertain of anything. If you wish to eradicate the hookworm at any time in the future, you will need to take a 3 day course of a tablet called mebendazole which you can obtain on prescription from your GP. Your GP will be sent a copy of this document.

Short term adverse effects of hookworm infection:

- There is likely to be an itchy red skin rash which lasts up to a week but can return 7-14 days later before settling completely.
- About half of people given hookworm larvae in our studies, will experience some ‘stomach upset’ such as abdominal cramps, diarrhoea or indigestion. These vary from person to person and can last from a couple of days to a few weeks.

Possible adverse effects of long-term hookworm infection:

- Anaemia (low blood haemoglobin) due to the hookworm removing small amounts of blood from your gut – this may cause you to feel tired or breathless and sometimes needs treatment with iron tablets. For someone who is otherwise well nourished however, and has no other cause of anaemia, anaemia is unlikely to occur.

- Endomyocardial fibrosis, which is an extremely rare complication of some long-term worm infections. Inflammation of the heart muscle occurs in this condition, which can cause scarring and in turn lead to heart failure. However this condition usually occurs with worms of a completely different species to the one that we are using, and generally, only in longer term infections. This complication is therefore extremely unlikely.

Further advice:

- If you are a blood donor, you should inform the blood transfusion service that you have hookworm infection, so they can give you up-to-date advice about when you can start to donate again.

- Females should be aware that mebendazole tablets to eradicate the hookworm should not be taken during pregnancy or whilst breastfeeding.

Please read the following and initial on the dotted line to show you have read and understand each statement:

- o I have requested infection with hookworm ……..

- o I am aware of the adverse effects of having hookworm infection outlined above……...

Signed ……..

Name…….

Date…….

Witness signature……

Witness name …….

Date…….

If you have any questions in the future regarding the hookworm, then you can contact the following doctors/researchers who are based in the Division of Epidemiology and Public Health, Clinical Sciences Building, City Hospital, Nottingham:

Dr Johanna Feary
0115 823 1936
johanna.feary@nottingham.ac.uk

Dr Andrea Venn
0115 8231721
andrea.venn@nottingham.ac.uk

Professor John Britton
0115 8231054
j.britton@virgin.net

Appendix J:

Patient Information Sheet (Asthma study)

Patient Information Sheet

Placebo-controlled study of hookworm infection in people with asthma

Investigators: Dr Johanna Feary, Dr Andrea Venn, Professor D Pritchard & Professor J Britton

You are invited to take part in a research study. Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with a friend or relatives if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. You can keep this information sheet. If you take part in the study, you will be given a copy of the signed consent form to keep.

Thank you for taking time to read this.

Why are you doing this study?

Asthma and hay fever have become increasingly common in more affluent societies over the last few years but the reason for this increase is uncertain. One possible explanation is that we live in an increasingly clean environment, and are therefore exposed to fewer infections; our immune systems start to respond to normally harmless things like dust. It is this inappropriate immune response that may contribute to asthma.

One infection that is now rare in more affluent societies is hookworm. This is a small worm which has evolved alongside humans and lives in the bowel. Millions of people in the developing world have hookworm and studies in Ethiopia suggest that infection with hookworm is associated with a lower risk of asthma and allergic disease. This raises the intriguing possibility that deliberate hookworm infection may be of benefit in the treatment of asthma and allergy. To investigate this properly we need to do a clinical study in a similar way to how new drugs are tested. This study will look at the possible benefits and side-effects of hookworm infection in people with asthma.

We recently completed a pilot study of hookworm infection in people without hay fever or asthma. Ten people (including the investigators for this study) received 10, 25, 50 or 100 hookworm larvae to work out which was the lowest dose that stimulated an immune response but caused a minimum of side effects. Both the 10 and 25 doses were well tolerated but we have chosen 10 larvae because this dose stimulated a good immune response and we want to use the lowest possible dose. We have also completed a study looking at hookworm infection in people with hay fever where people were given either 10 hookworm larvae or placebo and found the hookworms were well tolerated.

Why have I been chosen?

You have asthma and have expressed an interest in assisting with research in Nottingham.

Do I have to take part?

No, you don't. It is up to you whether or not to take part. If you do decide to take part, you will be given a copy of this information sheet to keep and asked to sign a consent form. Also, if you decide to take part, you will be free to withdraw at any time, without giving a reason if you don't want to. This will not affect the standard of any medical care you may be receiving.

What will happen to me if I take part?
The study will involve visits to the Division of Respiratory Medicine at Nottingham City Hospital over 18 weeks. These will be on a fortnightly basis at first and then every 4 weeks towards the end of the study. There will also be one week when we will ask you to attend for an extra blood test. The first visit will take around 1½ hours, however each subsequent visit lasts about 40 minutes. We realise this is a considerable time commitment but we would like to keep a close eye on you.

At the first visit, we will take about 20ml of blood to make sure you are not anaemic and have no evidence of having had hookworm infection in the past. Female volunteers will be asked to give a urine sample for a pregnancy test. We will also do skin tests for allergy to cat, dust and grass extracts: this involves a small scratch on the skin and a drop of each extract to see if this causes an itchy reaction. If you do have some itchiness from this test it should settle in about 20 minutes. We will repeat this skin prick test at the end of the study to look for any change in your skin reaction. We will then measure your sensitivity to an inhaled aerosol called adenosine monophosphate. This involves inhaling adenosine monophosphate given by us through an inhaler device and then doing a simple lung function test. We will then repeat the inhalation at stronger doses until the lung function test shows that you are responding. This is the end of the test and you can take a reliever inhaler (salbutamol, Ventolin) if you wish. At all times during the tests, you can stop whenever you wish.

At the second visit, we will put a gauze pad on your arm under a plaster. This will either contain a placebo solution containing histamine or a solution containing 10 hookworm larvae. In both cases, there may be some skin itching and redness where the solution is applied. The larvae then find their way through the skin, via the lungs to the intestine, where they stay until eradicated. You should keep the plaster on for 24 hours after which time you can remove it and put it in a special container which we will provide.

At each of the visits over the next 16 weeks, we will ask you to complete a questionnaire about your asthma symptoms, we will take about 20ml of blood to measure your blood count, inflammatory and allergic responses and repeat the lung function tests with adenosine monophosphate. We will also need a stool sample to look for hookworm eggs. Between visits we will ask you to complete a diary of your asthma symptoms, any side effects you experience, a record of your peak flow (we will show you how to do this) and a record of which inhalers you have used.

After 16 weeks, or if you choose to withdraw from the study, we will tell you whether you have received the hookworm or placebo. If you are received hookworm, we will then eradicate the infection with mebendazole tablets (100mg twice each day for three days). These are very effective at treating hookworm infection but are only effective once hookworm is in the bowel (about 4 weeks from the start). If you decide to withdraw from the study before this point we will give you tablets with clear instructions about when to start them to ensure they work. Female volunteers will have another urine test to ensure they are not pregnant before taking the tablets.

We would like to see you again two and four weeks after treatment to perform a blood test and to collect a stool sample. This is to make sure the infection has cleared and that you are well. All information gained during the study will be confidential. We will reimburse reasonable travel expenses incurred during the study.

What is the drug or procedure being tested?
We are assessing the effects of hookworm infection in people with asthma.

What are the possible disadvantages and risks of taking part?
There is likely to be some itching and redness of the skin where we give the histamine placebo or hookworm larvae. This should settle within a week but may return 7-14 days later before settling completely.

Some worm infections, but not hookworm, have been shown to cause an increase in airway responsiveness when the larvae pass through the lungs on their way to the bowel. This may lead to symptoms of cough, wheeze or breathlessness. Therefore, before giving hookworm to people with asthma, we wanted to check that there would be no negative effect on airway responsiveness with hookworm and so we performed a safety study where we gave 30 people with hay fever either hookworm or placebo and measured their airway responsiveness. Our results confirmed that having hookworm infection did not increase airway responsiveness when compared with the placebo and so we would not expect participants to have any increase in asthma symptoms during the study.

When the hookworm arrives in the bowel, some 'stomach upset' may occur. The side effects which are possible are abdominal cramps, nausea, diarrhoea and flatulence. If you experience these symptoms and find them unacceptable, you are free to finish the study at any point and we can give you tablets to get rid of the infection. Hookworm infection is very common in many parts of the world and other than in people who are badly nourished, or in very young children, in whom it can cause anaemia or weight loss; no other major common adverse effects are known to occur.

One extremely rare complication of some long-term worm infections is called endomyocardial fibrosis. Here, inflammation of the heart muscle occurs and can cause scarring which can, in turn, lead to heart failure. This condition usually occurs with completely different species of worms from the one that we are using, and only in people who have very long term infections. In view of this, and the fact that we are eradicating the worm infection after 4 months, we do not expect it to be a problem in this study.

If there is a chance you may be pregnant (or become pregnant in the next four months), you should not take part in the study. If you are a blood donor, you should not donate during the study. Before donating again after the study you should inform the blood transfusion service that you have taken part in this study so they can give you up to date advice about when you can start to donate again. Hookworm infection is not contagious in developed countries with normal standards of hygiene and sanitation. Therefore, if you wash your hands carefully after going to the toilet, you will not be contagious to others.

Mebendazole can cause abdominal pain, diarrhoea or rashes and should not be taken in pregnancy. Its effects can also be increased by an anti-acid drug, cimetidine: we will ask you to change this if you are on it.

We suggest that you inform your private health insurance company (if you have one) that you are taking part in this study.

What are the possible benefits of taking part?
The information we get from the studies may help to improve treatment of allergies and asthma in the future.

What will happen to the results of the research study?
We will send you a summary of the final results.

Who is funding the research?
The Wellcome Trust are funding the research

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Appendix K:

Juniper Asthma Quality of Life Questionnaire

INTRODUCTION

THE ASTHMA QUALITY OF LIFE QUESTIONNAIRE HAS BEEN TESTED AND VALIDATED USING THE WORDING AND FORMAT THAT FOLLOWS. IT IS IMPORTANT THAT INTERVIEWERS ADHERE TO THE EXACT WORDING WHEN ADDRESSING THE PATIENT (REGULAR TYPE) AND FOLLOW THE INSTRUCTIONS (ITALIC TYPE). DEVIATION FROM BOTH WORDING AND INSTRUCTIONS MAY IMPAIR THE RELIABILITY AND VALIDITY OF THE QUESTIONNAIRE.

THE QUESTIONNAIRE

This questionnaire is designed to find out how you have been feeling during the past two weeks. You will be asked about ways in which your asthma has limited your activities, the symptoms you have experienced as a result of your asthma, and how these have made you feel.

HAND THE RESPONSE SHEET TO THE PATIENT. EXPLAIN THAT YOU WANT THE PATIENT TO RESPOND TO EACH QUESTION BY WRITING THE NUMBER OF THE RESPONSE IN THE APPROPRIATE ROW AND COLUMN. AT THE FIRST VISIT, THE RESPONSES WILL BE IN THE FIRST COLUMN.

AT EACH FOLLOW-UP VISIT, PATIENTS ARE INSTRUCTED TO TAKE NOTE OF THE SCORE THAT THEY RECORDED AT THEIR PREVIOUS VISIT.

BEFORE READING EACH QUESTION, MAKE SURE THAT THE PATIENT IS LOOKING AT THE CORRECT COLOURED RESPONSE CARD.

How limited have you been ***during the last 2 weeks*** in these activities as a result of your asthma?

A 1. Please indicate how much you have been limited by your asthma in **STRENUOUS ACTIVITIES** (such as hurrying, exercising, running up stairs, sports) during the past two weeks. [GREEN CARD]

A 2. Please indicate how much you have been limited by your asthma in **MODERATE ACTIVITIES** (such as walking, housework, gardening, shopping, climbing stairs) during the past two weeks. [GREEN CARD]

A 3. Please indicate how much you have been limited by your asthma in **SOCIAL ACTIVITIES** (such as talking, playing with pets/children, visiting friends/relatives) during the past two weeks. [GREEN CARD]

A 4. Please indicate how much you have been limited by your asthma in **WORK-RELATED ACTIVITIES** (tasks you have to do at work*) during the past two weeks. [GREEN CARD]

**If you are not employed or self-employed, these should be tasks you have to do most days*

A 5. Please indicate how much you have been limited by your asthma in **SLEEPING** during the past two weeks. [GREEN CARD]

S 6. How much discomfort or distress have you felt over the past two weeks as a result of **CHEST TIGHTNESS?** [RED CARD]

EM 7. In general, how much of the time during the past two weeks have you **FELT CONCERNED ABOUT HAVING ASTHMA?** [BLUE CARD]

S 8. How often during the past two weeks did you feel **SHORT OF BREATH** as a result of your asthma? [BLUE CARD]

EN 9. How often during the past two weeks did you experience asthma symptoms as a result of being **EXPOSED TO CIGARETTE SMOKE?** [BLUE CARD]

S 10. How often during the past two weeks did you experience a **WHEEZE** in your chest? [BLUE CARD]

A 11. How often during the past two weeks did you feel you had to **AVOID A SITUATION OR ENVIRONMENT BECAUSE OF CIGARETTE SMOKE?** [BLUE CARD]

S 12. How much discomfort or distress have you felt over the past two weeks as a result of **COUGHING?** [RED CARD]

EM 13. In general, how often during the past two weeks did you feel **FRUSTRATED** as a result of your asthma? [BLUE CARD]

S 14. How often during the past two weeks did you experience a feeling of **CHEST HEAVINESS?** [BLUE CARD]

EM 15. How often during the past two weeks did you feel **CONCERNED ABOUT THE NEED TO USE MEDICATION** for your asthma? [BLUE CARD]

S 16. How often during the past two weeks did you feel the need to **CLEAR YOUR THROAT?** [BLUE CARD]

EN 17. How often during the past two weeks did you experience asthma symptoms as a result of **BEING EXPOSED TO DUST?** [BLUE CARD]

S 18. How often during the past two weeks did you experience **DIFFICULTY BREATHING OUT** as a result of your asthma? [BLUE CARD]

A 19. How often during the past two weeks did you feel you had to **AVOID A SITUATION OR ENVIRONMENT BECAUSE OF DUST?** [BLUE CARD]

S 20. How often during the past two weeks did you **WAKE UP IN THE MORNING WITH ASTHMA SYMPTOMS?** [BLUE CARD]

EM 21. How often during the past two weeks did you feel **AFRAID OF NOT HAVING YOUR ASTHMA MEDICATION AVAILABLE?** [BLUE CARD]

S 22. How often during the past two weeks were you bothered by **HEAVY BREATHING?** [BLUE CARD]

EN 23. How often during the past two weeks did you experience asthma symptoms as a result of the **WEATHER OR THE AIR POLLUTION OUTSIDE**? [BLUE CARD]

S 24. How often during the past two weeks have you been **WOKEN AT NIGHT** by your asthma? [BLUE CARD]

A 25. How often during the past two weeks have you had to **AVOID OR LIMIT GOING OUTSIDE BECAUSE OF THE WEATHER OR AIR POLLUTION?** [BLUE CARD]

EN 26. How often during the past two weeks did you experience asthma symptoms as a result of being **EXPOSED TO STRONG SMELLS OR PERFUME?** [BLUE CARD]

EM 27. How often during the past two weeks did you feel **AFRAID OF GETTING OUT OF BREATH?** [BLUE CARD]

A 28. How often during the past two weeks did you feel you had to **AVOID A SITUATION OR ENVIRONMENT BECAUSE OF STRONG SMELLS OR PERFUME?** [BLUE CARD]

S 29. How often during the past two weeks has your asthma **INTERFERED WITH GETTING A GOOD NIGHT'S SLEEP?** [BLUE CARD]

S 30. How often during the past two weeks have you had the feeling of **FIGHTING FOR AIR?** [BLUE CARD]

A 31. Think of the **OVERALL RANGE OF ACTIVITIES** that you would have liked to have done during the past two weeks. How much has your range of activities been limited by your asthma? [YELLOW CARD]

A 32. Overall, among **ALL THE ACTIVITIES** that you have done during the past two weeks, how limited have you been by your asthma? [GREEN CARD]

DOMAIN CODE:

S	**=**	**Symptoms**
A	**=**	**Activity Limitation**
EM	**=**	**Emotional Function**
EN	**=**	**Environmental Stimuli**

ASTHMA
QUALITY OF LIFE QUESTIONNAIRE
GREEN CARD

1. TOTALLY LIMITED, COULDN'T DO ACTIVITY AT ALL
2. EXTREMELY LIMITED
3. VERY LIMITED
4. MODERATE LIMITATION
5. SOME LIMITATION
6. A LITTLE LIMITATION
7. NOT AT ALL LIMITED

ASTHMA
QUALITY OF LIFE QUESTIONNAIRE
RED CARD

1. A VERY GREAT DEAL OF DISCOMFORT OR DISTRESS
2. A GREAT DEAL OF DISCOMFORT OR DISTRESS
3. A GOOD DEAL OF DISCOMFORT OR DISTRESS
4. A MODERATE AMOUNT OF DISCOMFORT OR DISTRESS
5. SOME DISCOMFORT OR DISTRESS
6. VERY LITTLE DISCOMFORT OR DISTRESS
7. NO DISCOMFORT OR DISTRESS

ASTHMA
QUALITY OF LIFE QUESTIONNAIRE
BLUE CARD

1. ALL OF THE TIME
2. MOST OF THE TIME
3. A GOOD BIT OF THE TIME
4. SOME OF THE TIME
5. A LITTLE OF THE TIME
6. HARDLY ANY OF THE TIME
7. NONE OF THE TIME

ASTHMA
QUALITY OF LIFE QUESTIONNAIRE
YELLOW CARD

1. SEVERELY LIMITED - MOST ACTIVITES NOT DONE
2. VERY LIMITED
3. MODERATELY LIMITED - SEVERAL ACTIVITIES NOT DONE
4. SLIGHTLY LIMITED
5. VERY SLIGHTLY LIMITED - VERY FEW ACTIVITIES NOT DONE
6. HARDLY LIMITED AT ALL
7. NOT LIMITED AT ALL - HAVE DONE ALL ACTIVITIES THAT I WANTED TO DO

Appendix L:

Daily diary for asthma study

Hookworm and Asthma

Initials ☐☐ **ID Number** ☐☐☐ **Week** ☐☐

Record the best of THREE peak flows measurements for Morning and Evening

		Morning *Please complete this section when you wake up in the morning*			**Evening** *Please complete this section before you go to bed*		
Date	**Day of the week**	**Morning peak flow**	**Night time symptoms score**	**Number of puffs of reliever inhaler during the night**	**Evening peak flow**	**Daytime symptoms score**	**Number of puffs of reliever inhaler during the day**

Hookworm and Asthma

Other Symptoms (graded from 0(none) to 10 (very severe))

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Date									
Day of the week									
Skin itching at plaster site	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Skin redness at plaster site	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Nausea or sickness	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Diarrhoea	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Abdominal pain	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Flatulence or wind	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Indigestion	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Loss of appetite	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Wheeze	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Cough	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Breathlessness	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐
Tiredness	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐

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Night time symptoms

Assess each morning on waking (symptoms such as chest tightness, wheezing, breathlessness and cough)

0=No symptoms during the night
1=Symptoms on waking but not causing you to wake early
2=Symptoms causing you to wake up once or to wake early
3=Symptoms causing you to wake twice or more (including waking early)
4=Symptoms causing you to be awake most of the night
5=Symptoms so severe that you did not sleep at all

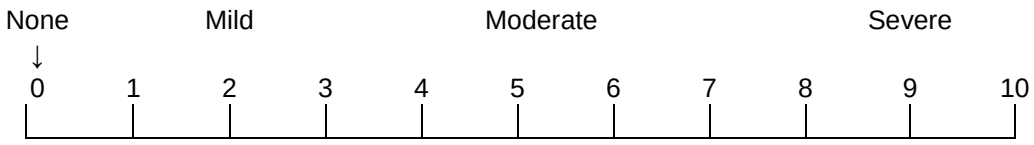
Daytime symptoms

Assess each evening, just before going to bed (symptoms such as chest tightness, wheezing, breathlessness and cough)

0=No symptoms during the day
1=Symptoms for one short period during the day
2=Symptoms for two or more short periods during the day
3=Symptoms for most of the day which did not interfere with usual activities
4=Symptoms for most of the day which did interfere with usual daytime activities
5=Symptoms so severe that you could not perform your usual daytime activities

Other Symptoms Scale

Use this to help you when you are filling in the daily diary. You need to grade each of your symptoms on a scale of 0 to 10. e.g. indigestion which is mild would score 2 out of 10.



Please write any other symptoms you might experience here with the date and day and your symptom score (out of 10) next to them

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USEFUL INFORMATION

For 4 hours prior to the study visit:

- No salbutamol (ventolin)

For 12 hours prior to the study visit:

- No strenuous exercise
- No caffeine containing food and drink (tea/coffee/cola/chocolate)
- No salmeterol (serevent), symbicort or seretide

For 24 hours prior to the study visit:

- No antihistamines
- No nasal sprays

CONTACT DETAILS

- Dr Johanna Feary 0115 8231936

- 24 hour contact number 0115 8231937
  (Leave a message and telephone number including area code)

Appendix M:

Data extraction form and Newcastle-Ottawa quality assessment scale

DATA EXTRACTION FORM **Reviewer:** **Study ID:**

DESCRIPTION OF STUDY

Study design	Cohort ☐ Case-control ☐ Cross-sectional ☐ RCT ☐
Timing of study	Prospective ☐ Retrospective ☐ N/A ☐
Measurement of Parasite exposure	Stool ☐ Perianal tape ☐ Urine ☐ IgE ☐
Specific parasites	Ascaris ☐ Trichuris ☐ Hookworm ☐ Enterobius ☐ Schistosoma ☐ Giardia ☐ Other ☐ Combined ☐
Measurement of Atopy	Skin prick test ☐ RAST ☐ Both ☐ Other ☐
Aeroallergen	D. pteronyssinus ☐ House dust ☐ Grass ☐ Cockroach ☐ Cat fur ☐ Dust allergens ☐ Mite ☐ Other ☐ Combined ☐
Setting (e.g. country, number of centres)	

PARTICIPANTS

Inclusion criteria		
Exclusion criteria		
Number of participants studied		
Number lost to follow up		
Final number of participants evaluated		

METHODOLOGICAL QUALITY OF STUDIES

Newcastle – Ottawa Scale	**Case-control/cross-sectional studies**	*	**Cohort studies**	*
1. Selection	a) Case definition b) Representativeness of cases c) Selection of control d) Definition of controls		a) Representativeness of exposed cohort b) Selection of non exposed cohort c) Ascertainment of exposure d) Outcome occurred	
2. Comparability	a) Matching / adjustment for age b) Matching / adjustment for other factors Factors=……		a) Matching / adjustment for age b) Matching / adjustment for other factors Factors=……	
3. Ascertainment	a) Ascertainment of exposure b) Same method of ascertainment for cases and controls c) Non response rate		a) Ascertainment of outcome b) Was follow up long enough for outcomes to occur c) Adequacy of follow up of cohort	

Note: if retrospective cohort study, then no * to be given for Ascertainment b) and c)

DATA EXTRACTION FORM **Reviewer:** **Study ID:**

RESULTS

Primary Outcomes	Group= N=	Group= N=

Comments: