1 GENERAL INTRODUCTION

Partly based on:
Helminth-induced IgE and protection against allergic disorders

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INTRODUCTION

Over the past few decades, the prevalence of allergic disease has been on the rise in both developed and developing countries[1]. There is strong evidence for differences in the prevalence of atopic disorders between urban and rural areas in many parts of the world, with higher prevalence of allergic diseases reported in urban areas[2-7].

The observed urban-rural differences may be influenced by socio-economic status (SES)[4]. The measurement of a child's SES can be approximated by consideration of household income, parental occupation and education level, living conditions, or habitation in geographic locations with a predominantly poor population[8,9]. These variables have each been linked with childhood asthma[9,10]. However, some studies of SES and asthma have reported contradictory results. Some have reported that children in families with low SES have an increased asthma risk[11-14], while others have found an inverse relation between low household income and asthma[15] or no relationship between asthma and parental SES levels[16-18]. These conflicting results may stem from the complexity of the meaning of SES, which can vary between studies, locations and culture[10].

Apart from exposure to allergens, altogether environmental factors that may encompass or be related to SES, along with genetics, play a key role in the development of allergic diseases. Environmental influences can include exposures to pollution, microbes, parasites and lifestyle[19,20]. Of particular interest have been infections with parasitic helminths that are highly prevalent in tropical regions of the developing world. It is estimated that a quarter of the world's population is chronically infected with helminths such as *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), *Necator americanus* or *Ancylostoma duodenale* (hookworms), schistosomes and filarial worms[21].

Despite the close parallels between immune responses that characterize helminth infections and allergic diseases, namely increased levels of immunoglobulin (Ig)-E and eosinophils along with T cells that preferentially secrete T helper type 2 (Th2) cytokines, the clinical outcome with respect to immediate hypersensitivity and inflammation is not the same[22]. Moreover, there is little geographical overlap worldwide between helminth infections and allergies. In fact, several studies have reported a negative association between the presence of helminth infections and atopic disorders[23]. However, the relationship between atopic disorders and helminth infections is not always consistent since there are also studies that show that helminth infections have no effect or are associated with increased atopic disorders[24,25].

HELMINTH INFECTIONS AND ATOPIC DISORDERS

The prevalence of allergic diseases has been increasing mainly in the developed world where changes in lifestyle and environment have taken place, characterized by increasing sanitation, hygienic measures and urbanization including pollution. In less developed countries, the prevalence of atopic disorders is relatively low but the so-called "allergic march" is starting in these geographical areas due to ongoing dramatic changes in lifestyle

and the environment. Although not as pronounced as in western countries, atopic disorders are prevalent in developing countries with the tendency for prevalences in urban centers to approach those seen in affluent countries.

In developed countries, the presence of IgE antibodies to allergens increases the risk of atopic disorders. These antibodies which bind to high affinity IgE receptors present on effector cells of the allergic response, i.e. to mast cells and basophils. These IgE antibodies can be cross linked by allergens, an event that leads to effector cell degranulation including histamine release. The mast cell degranulation leads to inflammation and if the target organ is the airways it can result in symptoms which can be recognized as an asthmatic attack. One of the diagnostic methods for allergy is to perform a skin prick test[26]. This is based on applying allergens to the skin, pricking the skin with a lancet and assessing whether a reaction develops to the allergen within 15 minutes. A wheal swelling and flare (redness) reaction induced by degranulation of skin-resident mast cells is considered as proof of sensitization to the allergen extract applied. In developed countries, the skin prick test (SPT) is often interchangeable with the measurement of IgE antibodies to allergens by *in vitro* tests[27], i.e. both tests usually show quite close correlation, with a positive test frequently (but not always!) being associated with clinical allergy. However, the relationship between IgE, SPT and symptoms of allergy can be different in different geographical areas. For example, the proportion of SPT reactivity in allergic asthma appears to be much smaller in some rural areas compared to urban centers in Europe[28] or in many developing nations compared to developed ones[29,30]. Helminth infections have some interesting effects on the relationship between IgE, SPT and clinical symptoms.

Several epidemiological studies have shown that helminth infections can be negatively associated with allergic outcomes[23]. Some studies have shown that having schistosome or filarial infections decreases the risk of SPT positivity[31,32]. Similarly, studies in Ecuadorian and Vietnamese children have demonstrated that having soil transmitted helminth infections decreases the risk of SPT positivity[33,34]. In addition, Cooper *et al.* found that the presence of serological markers of chronic helminth infections (elevated levels of total serum IgE or anti-*A. lumbricoides* IgG4) were independently negatively associated with allergen SPT reactivity[35]. Araujo *et al.* reported a strong and inverse association between skin responses to allergens and infection *with Schistosoma mansoni*, among persons living in an area endemic for this helminth[36]. Similarly, a study in Gabonese children observed that the risk of a positive SPT was reduced by 72% if a child was infected with *S. haematobium*[32]. Moreover, an investigation conducted among 1385 urban and rural Ghanaian children aged 5-16 showed a strong negative association between schistosome infection and SPT reactivity to mite but not with reported wheeze or asthma[37]. However, in regard to clinical symptoms of asthma, a case-control study in urban and rural Ethiopians aged 16 to 60 years, indicated that active hookworm infection reduced the risk of reported wheeze[38].

Although the majority of studies have shown a negative association between helminth infection and SPT, there are studies that show that helminths may increase the risk of asthma and other atopic disorders. Obihara *et al.* found *Ascaris*-specific IgE may be a risk

factor of atopic disease in populations exposed to mild *A. lumbricoides* infection[24]. In line with this, a study in China, in an area with a low burden of *A. lumbricoides*, demonstrated a positive association between helminth and allergen skin test reactivity as well as asthma risk[25]. Finally, there are also studies showing no significant association. A study in Brazil among patients aged 12 to 30 years with asthma or rhinitis living in an urban area endemic for geohelminths showed that individuals infected with a low intensity of *A. lumbricoides* did not differ in the frequency of positive SPT to dust mites from those negative for *A. lumbricoides* living in the same area[39]. A birth cohort study of children from Ethiopia, which investigated the effect of geohelminths on allergic symptoms, at 3 years of age, found that there was no association between helminth infections and wheeze nor with eczema[40].

Most studies on helminth infections and allergy mentioned above had a cross sectional design. Intervention studies using anthelmintic treatment are an important to investigate whether helminth infections play a role in atopic disorders. So far, excluding the study in this thesis, two randomised placebo-controlled trials with albendazole have been conducted but they showed conflicting results. One study conducted in Ecuador, comprising a school-cluster randomised trial of one year albendazole treatment, reported no change in either sensitization to allergens or allergic symptoms[41], while another trial in Vietnam showed that the prevalence of skin reactivity increased after one year of albendazole treatment but, consistent with the Ecuadorean study, clinical allergy did not change significantly[42]. It is difficult to explain these conflicting observations, which may relate to differences in treatment regimens (incomplete and/or short-lived helminth eradication), intensity of infection, co-infections, reinfections or acute vs chronic infections[43,44].

MECHANISMS BEHIND THE ASSOCIATION BETWEEN HELMINTHS AND ALLERGIES

Given that most studies seem to show a negative association between chronic helminth infections and SPT reactivity, researchers have been looking for the mechanisms that could explain this. Although the mechanisms associated with this inverse relationship are not fully understood, it has been suggested that strong immune regulatory networks might be involved. This means that high levels of suppressory cytokines such as interleukin (IL)-10 and transforming growth factor-beta (TGF-β) as well as regulatory T and B cells[45], which seem to expand during chronic infections with helminth parasites, might down regulate allergic responses. The question as to where in the allergy cascade they exert their down regulatory activity is still unanswered. It is possible that regulatory responses affect Th2 and thereby IgE. However, immune regulation could also down-regulate the effector phase of an allergic response which involves inflammation induced by mast cell degranulation[46]. This notion is supported by reports showing that IL-10 could inhibit basophil degranulation[47] and by the negative association between IL-10 and SPT[48,49]. Regarding antibodies, in the 1970s the idea that helminths might induce polyclonal stimulation of IgE-producing plasma cells with many different specificities that would compete with allergen specific IgE for binding

to high affinity IgE receptors on mast cells was first proposed[50,51]. This competition would reduce the chance of an allergen dependent mast cell degranulation and therefore explain the absence of strong allergic responses in helminth infected subjects[52]. However, Mitre *et al.* have shown that the high ratio of polyclonal IgE to allergen specific IgE did not inhibit basophil degranulation[53]. Moreover, it has been shown that increases in IgE result in the up-regulation of IgE receptors and that anti-IgE treatment is often accompanied by down-regulation of IgE receptors which argues against the ability of high total IgE to compete out specific IgE[54]. An alternative hypothesis that helminth infections may be associated with increased levels of allergen-specific IgE that are functionally poor and therefore cannot lead to basophil or mast cell degranulation[55] is gaining support from several recent studies that indicate cross-reactive IgE might be associated with poor biological activity[56,57].

CROSS-REACTIVITY BETWEEN ALLERGEN AND HELMINTH

In general, cross-reactivity reflects the phylogenetic relationship between organisms that results in a high degree of homology in the primary structure of proteins[58]. Cross-reactivity occurs when antibodies elicited to one epitope also recognize similar epitopes in other homologous molecules[59]. In allergy, the allergen that is supposed to induce the original allergic responses is named the primary sensitizer, and the others are considered cross-reactive allergens[59]. Two types of IgE cross-reactivities have been described, one due to homologous proteins, and the other due to sugar moieties (complex N-glycans on plant and invertebrate glycoproteins) known as cross-reactive carbohydrate determinants (CCDs)[58].

Cross-reactive carbohydrate determinants (CCDs) and helminths

The asparagine-linked carbohydrate components of plant and insect glyco-proteins are highly cross-reactive and are known as cross-reactive carbohydrate determinants (CCDs)[60]. Two typical non-mammalian substitutions to N-glycans of plant glycoproteins are an α(1,3)-linked fucose on the proximal N-acetyl glucosamine and a β(1,2)-linked xylose on the core mannose[61]. Bromelain is a protein from pineapple stem which carries N-glycan structure that is often used as a marker for CCDs reactivity.

These CCD epitopes are also found in helminth parasites. The existence of IgE antibodies directed to CCDs was first reported by Aalberse *et al.* in the early 1980s[62]. The study demonstrated that serum IgE from European pollen or venom allergic patients cross-reacted with extracts from various allergenic foods. However, treating the extracts with periodate, which destroys the carbohydrate structures abolished IgE binding, indicating the involvement of carbohydrates in this cross-reactivity[62]. Another investigation by the same group observed elevated levels of IgE against peanut extract among grass pollen-sensitized European patients without peanut SPT reactivity or clinical symptoms of peanut allergy[63]. Furthermore, among 91% of those with a discrepancy between specific IgE to peanut and

SPT, IgE against CCDs could be detected[63]. In some of these patients, almost complete inhibition of IgE to peanut (as measured by competitive radioallergosorbent test) was possible with CCD. In addition, cross-reactive IgE directed against CCDs in this study was demonstrated to have poor biologic activity in terms of inducing basophil degranulation[63].

The most convincing proof of the complete lack of clinical relevance of CCD-specific IgE was reported by Mari *et al*. in a study where grass pollen allergic patients with high titers of CCD-specific IgE were skin tested and subjected to an oral challenge with human lactoferrin expressed in rice kernels. This transgenic molecule was substituted with multiple IgE-binding glycans, but both SPT and oral challenge were completely negative[64]. Therefore, this study shows that CCDs, although having (poor) biological activity (histamine release from basophils) this does not translate into clinical allergy. It has not yet been elucidated what the explanation of this lack of clinical relevance is, but the polyvalent conjugation of lactoferrin with CCDs demonstrates that it cannot simply be explained by monovalency.

With regard to helminth-induced IgE cross-reactivity, a recent study among schoolchildren (aged 5-16 years) in Ghana, West Africa demonstrated how cross-reactivity between helminth antigens and allergens can affect IgE sensitization patterns and clinical expression of allergy[65]. In this study the overall prevalence of peanut-IgE sensitization was 17.5% (233 out of 1328). However, none of the peanut-IgE sensitized children had either SPT reactivity to peanut or reported adverse reactions to peanut. In this study, the presence of *S. haematobium* infection was positively associated with an increased risk of having peanut specific IgE. In a subset of this study population, both the CCD marker bromelain and *S. haematobium* soluble egg antigen (SEA) inhibited IgE binding to peanut extract. This study also showed that peanut-specific IgE was strongly correlated with CCD-specific IgE. These results indicate that much of IgE to peanut in Ghanaian children could be directed against CCD which is also present in the schistosome SEA. The basophil histamine release assays demonstrated that the IgE directed against peanut in this population had low biologic activity[65].

This study provides a model which proposes that parasite-induced IgE against CCDs that are carried by parasites, might account for high IgE levels to food allergens and the finding that this IgE does not lead to reactivity to allergenic extracts either *in vitro* (in basophil release assay) or *in vivo* (in skin prick testing) further confirms that these IgEs to CCDs are clinically irrelevant[66].

Peptide cross-reactivity and helminth

Cross reactions between allergens from invertebrates such as for example mite and snail; cockroach and *Ascaris*; mite, shrimp, and cockroach; and mites and schistosomes[58] have been reported and involve protein cross reactivity. Three of the proteins that are involved in these examples are tropomyosin, glutathione S-transferase (GST) and paramyosin[58].

Tropomyosins are proteins involved in the contraction of muscle cells along with actin and myosin[67]. Not only are tropomyosins major allergens of seafood, mite and cockroach but are also highly immunogenic helminth proteins[68]. Tropomyosins from invertebrates

are strong inducers of IgE antibody responses in humans[69]. Santiago *et al*. demonstrated that there is 72% identity at the amino acid level between the tropomyosin from the filarial parasite *Onchocerca volvulus* (OvTrop) and the house dust mite tropomyosin Der p 10[70]. They showed a strong correlation between specific IgE to Der p 10 and IgE to OvTrop. In addition, histamine release from basophils sensitized with the sera of individuals IgE-positive to Der p 10 could be triggered by either the OvTrop or Der p 10. It is however important to realize that such biological activity is not a proof of clinical allergy. In the study by Mari *et al*. with transgenic lactoferrin, histamine release was reported at relatively high protein concentrations, but SPT and oral challenge with the same molecule were negative[64]. The study by Santiago *et al*. does however confirm that the anti-tropomyosin antibodies induced in filarial infection are cross-reactive with those allergenic tropomyosins of invertebrates (mite) that may affect sensitization[70]. As expected, no clinical mite allergy was reported for the subjects studied, indicating that these cross-reactive responses are of no clinical relevance.

In another investigation, Santos *et al*. showed that the predicted structure of *A. lumbricoides* tropomyosin was similar to that of *Periplaneta americana* tropomyosin[71]. The same study compared IgE responses to these proteins in Brazilian children aged 3-6 years living in an area endemic for helminths to responses in cockroach allergic patients aged 2 to 52 years also from Brazil[71]. A strong correlation was also found for IgE antibodies to tropomyosin from *A. lumbricoides* and from *P. americana* in sera from both populations. Seventy-six percent (90 out of 119) of subjects from the parasite endemic area had positive IgE antibodies against cockroach tropomyosin without allergy to cockroach[71]. A study conducted by Acevedo *et al.* has also demonstrated high allergenic cross-reactivity between *Blomia tropicalis* tropomyosin (Blo t 10) and *Ascaris* tropomyosin in Colombian asthmatic patients[72].

The glutathione S-transferases (GSTs) are detoxification enzymes found in most living organisms[73]. The important known sources of GSTs are cockroaches, house dust mites and molds, however, those GSTs from invertebrates including helminths are known to be strong inducers of IgE[74]. Moreover, *Blattella germanica* GST caused positive immediate skin tests in cockroach-allergic asthmatic patients, suggesting that GST from cockroach is a clinically relevant allergen[75]. Regarding helminth-induced IgE cross-reactivity, Santiago *et al*. showed that the GST from the filarial worm *Wuchereria bancrofti* (WbGST) and cockroach GST (Bla g 5) were 30% identical at the amino acid with marked similarity in the N-terminal region[76]. Interestingly, mice infected with *Heligmosomoides bakeri*; a parasite that contains a GST that is 32% identical to Bla g 5, developed immediate hypersensitivity reaction in the skin to cockroach GST (Bla g 5), suggesting that some parasite-induced cross-reactivity may induce *in vivo* reactivity to the cross-reactive allergen in a common allergen source like house dust mite[76].

Paramyosin is another allergen family from invertebrate muscle that are targeted in IgE responses against helminths[77]. A study among patients reporting symptoms of allergy and another group of *Ascaris*-infected subject in the Philippines showed evidence of cross-reactivity between paramyosin from mite (*B. tropicalis*) and paramyosin from *A. lumbricoides*[78]. This study observed that IgE to mite extract among allergic patients can be inhibited, up to 92%, by *Ascaris* antigen while mite extract could inhibit up to 54% of *Ascaris*-sIgE among *Ascaris*-

infected subjects. Of note, IgE responses to the recombinant form of the paramyosin *Blomia* allergen (Blo t 11) were seen in 80% of allergic patients and 46% of *Ascaris*-infected subjects[78].

In general, IgE cross-reactivity between helminth antigens and allergens demonstrates a limited to diagnostic value of examining IgE responses to whole allergen extracts in helminth-endemic populations. Establishing the molecular basis of cross-reactivity between helminths and common allergen sources is essential to evaluate whether sensitization to the latter is true primary sensitization or cross-reactivity induced by helminths.

COMPONENT-RESOLVED DIAGNOSIS (CRD) IN ALLERGY DIAGNOSIS

For the past few decades, *in vitro* allergy diagnostics has been largely based on the detection of specific IgE to whole extracts comprised of allergenic and non-allergenic components. However, this approach has been problematic since the allergenic content of whole extracts is often difficult to standardize and also the specific allergic reaction inducing components in whole allergen extracts can be hard to identify. Such issues in *in vitro* allergy diagnostics led to the development of component-resolved diagnostics (CRD) in which purified natural or recombinant allergens are used to detect IgE sensitization to individual allergen molecules[79,80].

The use of molecular techniques and recombinant DNA technology has allowed the sequencing, synthesizing and cloning of allergenic proteins leading to the production of recombinant allergens for CRD[81]. Recombinant allergens can be generated as defined molecules with consistent quality and without biological variation[82].

The molecular biological techniques underlying CRD were initially employed for the determination of the primary structures and molecular identities of allergens[83]. The sequence analysis of allergens allowed the identification of structurally related allergens and also revealed how closely linked cross-reactive molecules may not be differentiated by the immune system[83]. CRD involves the use of specific marker allergens to identify primary sensitization towards a particular allergen source and to discriminate it from sensitization to CCD or to homologous allergens as a result of cross-reactivity[84]. CRD can therefore facilitate the differentiation between clinically important and less relevant specific IgEs[85].

In terms of the application of CRD to research questions in helminth-endemic populations, the report by Amoah *et al.* on peanut allergy among Ghanaian school children found that in study subjects with elevated IgE to whole peanut allergen, responses to recombinant major peanut allergens (rAra h 1, rAra h 2 and rAra h 3) were generally undetectable or very low (<1 kU_A/L)[65]. In addition, among Brazilian children living in a helminth-endemic urban area, Carvalho *et al.* evaluated the use of IgE responses to *B. tropicalis* allergens (rBlo t 5 and rBlo t 21) in improving the specificity of determining mite allergy in this population[86]. The study showed that the assays using these recombinant marker allergens for primary *B. tropicalis* sensitization, exhibited no IgE cross-reactivity with *A. lumbricoides* antigens and therefore conferred higher specificity in detecting primary mite IgE sensitization than crude mite extract.

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SCOPE OF THE THESIS

In recent years, much research from the developed world has looked at risk as well as protective factors for childhood allergy. The sharp rise in atopic disorders in the developed world within a relatively short period of time indicates that environmental factors, in addition to the already known genetic pre-disposition, play an important role in development and control of these diseases. However, there are no conclusive answers on which are the most important environmental changes that underlie the increase in atopic disorders. Interestingly, investigators are shifting their attention to the developing world for clues on how changes in the environment, influenced by socio-economic status (SES), affect the development of atopic disorders. One of these environmental factors is helminth infections. Indonesia is one of the low-to-middle income countries which is in epidemiological transition with increasing level of urbanization accompanied by changing disease patterns; where helminth infections are highly prevalent in rural areas but in urban centers there is a rise in atopic disorders. Therefore, this thesis set out to address the following four questions in Indonesia:

- Are the risk factors of atopic disorders different between children belonging to low or high socio-economic status living in the same urban area?
- What are the risk factors for atopic disorders in children living in rural and semi urban areas where helminth infections are highly prevalent?
- What is the effect of long term anthelmintic treatment on atopic disorders?
- What is the profile of allergen specific IgE antibodies in children from helminth-endemic areas?

In **Chapter 2** and **Chapter 4**, we show how environmental factors and socio-economic status effect atopic disorders in schoolchildren in different areas of a developing country.

Chapter 3 provides an overview of the study population used in the following chapter 4, 5 and 6. It has been published as a study protocol paper.

Chapter 5 provides the result of a household-based-cluster-randomized, double-blind placebo-controlled anthelminthic trial on allergy.

In **Chapter 6**, we look at the characteristics of allergen specific IgE antibodies in order to better understand the molecular basis of IgE recognition of allergens in a helminth-endemic area.

Chapter 7 summarizes our findings and provides directions for future research to understand the link between helminth infections, socio-economic status and atopic disorders.

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