Immunological and Biological Characterization of *Blomia tropicalis* Allergens

Doctoral thesis at the University of Cartagena for obtaining the degree of

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Caracterización Inmunológica y Biológica de Alergenos de *Blomia tropicalis*

Tesis doctoral en la Universidad de Cartagena para obtener el título académico de:

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Abbreviations

2D: two dimensional

AscGST: GST from *Ascaris*

bp: base pairs

CB: cord blood

CBD: chitin binding domain

CBP: chitin binding protein

cDNA: complementary deoxinucleotide acid

CR: cross-reactivity

CRI: cross-reactivity index

CRD: component resolved diagnosis

ELISA: Enzyme linked immunosorbent assay

e.p.g.: eggs per gram

HDM: house dust mites

Ig: Immunoglobulin

LC-MS/MS: Mass spectrometry

GST: Gluthathione-S-transferase

LB: Luria bertani

LBA: LB supplemented with ampicillin

kDa: kilodalton

mAb: monoclonal antibody

MW: molecular weight

NBB: native binding buffer

nBlo t 12: natural Blo t 12

nBlo t 8: natural Blo t 8

ON: overnight

OR: odds ratio

OVA: ovalbumin

PAGE: polyacrylamide gel electrophoresis

pAb: policlonal antibody

pBlo t 12.0101: Blo t 12.0101 expressed in *Pichia pastoris*

PBS: phosphate buffer saline

PCR: polymerase chain reaction

PNGase: peptidoglycanase F

PVDF: polyvinylidene fluoride

rBlo t 8: recombinant Blo t 8

RNA: ribonucleic acid

ROC: receiver operator characteristics

RT: room temperature

r.p.m.: revolutions per minute

SDS: sodium duodecyl sulphate

SIT: allergen specific-immunotherapy

SPT: skin prick test

SSM: species specific-marker

Abstract

Aim: This doctoral thesis aimed to understand more aspects about the great allergenic potential of *B. tropicalis* through the characterization of purified allergens and the evaluation of their clinical impact.

Methods: Serum samples were obtained from three ongoing epidemiological programs from the Institute for Immunological Research in the University of Cartagena. Allergens were isolated from a cDNA library from *B. tropicalis* or, otherwise, their nucleotide sequences were synthesized by a commercial supplier (Genscript, USA). Natural allergens were purified by affinity chromatography. Anti-Blo t 12 mAbs and pAbs were obtained for allergen characterization. A murine model of asthma was done with Blo t 12 isoforms in the presence or not of chitin fragments. Immunochemical methods (ELISA, ELISA inhibition, WB and WB inhibition) and SPT were used for allergen characterization. In the FRAAT birth cohort study, IgE determination was done by ImmunoCap (*B.* tropicalis, *D. pteronyssinus, Ascaris spp.* and total IgE) and ELISA (Blo t 5, Blo t 12, AscGST, Asc l 3 and Asc s 1).

Results:

Chapter 3: IgE reactivity to Blo t 12.0101, a locally isolated isoform, was more frequent (36%) and intense than that to Blo t 12.0102 (23%), obtained from Singapore. The variant .0101, but not the .0102, was found to be glycosylated and this modification reduced antibody reactivity. Natural Blo t 12 could be isolated from *B. tropicalis* extract with antibodies raised in pBlo t 12.0101 immunized animals. IgE response to nBlo t 12 was more intense than that to any of the .0101 recombinants, *E. coli* or *P. pastoris*; however, both recombinants could diagnose most cases of Blo t 12 sensitization. There was not detected CR among Blo t 12 and other components from HDM and storage mites, other arthropods or *Ascaris lumbricoides*. In BALBc mice, both Blo t 12 isoforms induced airway inflammation (neutrophil-rich) and bronchial hyperreactivity without the need of an adjuvant (alum or chitin). However, chitin administration with .0101 isoform induced a more intense total IgE and methacholine response.

Chapter 4: Two new IgE reactive-components from B. tropicalis were isolated. Blo t 8, the GST from B. tropicalis, was detected as a common (45-80%), but mild, IgE sensitizer. It was predicted to contain surface exposed amino-acids clusters, shared with other GST from allergenic sources, including HDM and Ascaris. Specific IgE levels to nBlo t 8 or the recombinant isoform were highly correlated, but more intense to the former. Ubiquitin showed 100% amino acid sequence identity to human ubiquitin. IgE reactivity was found in 23.6% (n = 26) of asthmatic patients and

4.7% (n = 3) of controls. Mean specific IgE levels were higher in asthmatics (0.295 ± 0.15 vs. 0.150 ± 0.09, P = 0.001).

Chapter 5: In the FRAAT birth cohort (n=326) most children lived in poverty conditions. At 24 months, the prevalence of "wheezing ever" was 38.3% and 14.2% for recurrent wheezing. Maternal asthma was the main predisposing factor for wheezing [aOR 3.65 (95%CI 1.23-10.8), p = 0.01]. Clinical symptoms of milk/egg allergy or other food-induced allergies were scarce (1.8%) and no case of atopic eczema was observed. Maternal total IgE (aOR: 2.43, 95% CI: 1.09-5.43; p = 0.03) and poverty markers were associated with high cord blood (CB) total IgE. High CB total IgE was positively associated with *B. tropicalis* sensitization (Estimate: 0.42 p < 0.001) but protected from recurrent wheezing (aOR: 0.26, 95% CI 0.08-0.88, p = 0.03). Prevalence of IgE sensitization to HDM was high, especially to *B. tropicalis*, and increased with age. Unhygienic conditions were risk factors for *B. tropicalis* sensitization in children. HDM sensitization (and also *B. tropicalis* specific IgE) was weakly associated to ever-wheezing (aOR: 2.03, 95% CI 0.99-4.15, p = 0.05), with a population-attributable fraction of 19.7%.

Conclusions: Valuable information about the allergenic potential of *B. tropicalis* was obtained from the characterization of purified allergens:

- Blo t 12.0101 is a representative isoform of group 12. It is a species specific marker of *B. tropicalis*, thus, the developed two-site ELISA for its quantification can be useful to measure environmental exposition to *B. tropicalis*. Although less potent than the natural allergen, recombinant Blo t 12.0101 molecules can diagnose most cases of Blo t 12 sensitization. The chitin binding activity of this allergen influences total IgE production and bronchial hyperreactivity in mice.
- Blo t 8 is a frequent, but mild, IgE sensitizer in the characterized population. CR with other GSTs, including AscGST is supported by its molecular similarity.
- Ubiquitin is a new allergen from *Blomia tropicalis*. Our data suggest that it could be clinically relevant in asthma. Since the sequence of this allergen is identical to human ubiquitin, the possibility of an autorreactive response due to cross-reactivity should be further investigated.
- In this socioeconomically deprived population living in the tropics wheezing, but not atopic eczema, is a common problem. Total IgE levels were influenced by maternal IgE and were protective from wheezing. The frequency of sensitization to any HDM, especially to *B. tropicalis*, during the first three years of age is high and associated with wheezing. Although, HDM and Ascaris IgE levels are highly correlated, it could be discriminated that most double positive children are actually co-sensitized; positivity to nematode and *B. tropicalis* specific markers were very frequent.

Road map of this thesis

To help the reader here I describe the structure of this doctoral thesis. The conventional background, methods, results, and discussion format has been followed. All the investigations during my doctoral formation were intended to understand several aspects related to the allergenic potential of *B. tropicalis* and its relationship with other allergenic sources of relevance in the tropics; however, since their results can be grouped into three main topics, they were divided into individual chapters: the characterization of Blo t 12 allergen (chapter 3), description of novel allergens from *B. tropicalis* (chapter 4) and a comprehensive study about the evolution of IgE responses to *B. tropicalis* components in early infancy (chapter 5). The methods used in all these investigations were described in one single section (chapter 2) because most protocols for allergen characterization were similar for all the tested molecules, otherwise, important modifications were described. A general discussion about the most important findings of this thesis was presented in chapter 6. Future perspectives of these investigations were shown in chapter 7. The Appendix includes 2 tables with materials and equipment and also the list of publications from this thesis work. It is also important to declare that part of this data, related to the characterization of Blo t 12 and the development of an ELISA sandwich for allergen quantification, will be kept confidential due to our interest to patent the obtained product.

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Chapter 1. *General Introduction*

Allergy is a worldwide health problem, affecting about 25% of human population. For unknown reasons, it has increased during the last decades in several areas of the planet [1], reaching epidemic proportions. ISAAC studies have shown that in several areas of Latin America (Peru, Costa Rica, Brazil, Paraguay, Uruguay, and Panama) asthma prevalence is as high as described for UK, Australia, and New Zealand [2, 3]. In Colombia, the epidemiological surveys suggest that prevalence of atopic diseases has increased in the last decade. In 2010, disease rates were 12% for asthma and 32% for allergic rhinitis. Wheezing in childhood is another related problem, with a national prevalence of 25% in children younger than 5 years old [4]. Management of allergy is difficult; treatment is frequently inefficient, even in developed countries. The use of medicaments that only offer symptomatic relief is widespread. In contrast, administration of allergen specific immunotherapy (SIT) is less common, albeit being the only option able to modify the natural course of allergic diseases. Prophylaxis is not possible yet.

The tropics, a wide area of the planet where two thirds of humans inhabit, have particular conditions that promote allergy development. Its climate, warm and humid, favors mite growth and diversity of house dust acarofauna, which includes potent allergenic sources such as *Blomia tropicalis* and *Dermatophagoides pteronyssinus.* Most of its countries are underdeveloped and helminth infections are endemic. The predominance of African ancestry may also promote stronger IgE responses [5]. In addition, socio-demographical transitions [6], especially an accelerated and unorganized urbanism, migrations and health service problems difficult disease management. Basic scientific research may contribute to ameliorate these problems and probably its impact on the tropics will be more relevant because the knowledge about its allergenic sources is still scarce. The characterization of local allergens would aid to develop better reagents for diagnosis and treatment and the identification of environmental risk factors for IgE sensitization will help to determine the most appropriate prophylaxis measures.

The Institute for Immunological Research, in the University of Cartagena, has a long tradition in the study of the allergy, doing major contributions to the knowledge

about *Blomia tropicalis*, which together with *Dermatophagoides pteronyssinus* are the most important allergen sources in the tropics. This doctoral thesis aimed to understand more aspects about the great allergenic potential of *B. tropicalis* through the characterization of purified allergens and the evaluation of their clinical impact.

1. IgE responses and allergy

Type I hypersensitivity reactions, also named allergy, are characterized by the effect of specific IgE against molecules, commonly proteins from the environment, which, after antigen recognition elicit antibody- and cellular- mechanisms driving a distinct type of inflammation. A phase of sensitization occurs first; allergens are presented to naïve CD4+ T cells, which differentiate into allergen-specific Th2 cells. These lymphocytes orchestrate allergic inflammation, cooperating with B cells by promoting IgE class-switching and inducing proliferation of various cell populations that express FcεRI, a high affinity receptor for the IgE (i.e. mast cells, eosinophils and basophils). Allergen exposition, in sensitized individuals, results in a rapid effector response, mediated by IgE antibodies, that after its recognition activates immediate reactions, such as cell degranulation and release of pro-inflammatory mediators (histamine, TNF-α and others). IgE-mediated cell activation also triggers the synthesis of lipid mediators (i.e. prostaglandins and leukotrienes) and transcription of genes, among them, those coding for Th2 cytokines (IL-4, IL-5 and IL-13) that perpetuate allergic inflammation.

IgE responses can be clinically irrelevant or, on the contrary, underlie different allergic conditions, such as asthma, rhinitis and eczema. About 25% of humans suffer from any allergy. *Atopy* is the genetic predisposition to become sensitized and produce IgE antibodies in response to ordinary exposure to allergens [7].

1.1. Allergens

An allergen is any substance capable of inducing specific IgE production and effector immune response mediated by these antibodies [8]. Allergens are a heterogeneous group of proteins that do not share any structural motif or a general

biological mechanism related to allergenicity [9]. However, there are some allergens with intrinsic biological properties that promote IgE sensitization [10].

1.1.1. Isoallergens

Isoallergens is a term used to describe two related allergens with more than 67% of similarity in their sequence [6]. When only few numbers of variations are present, they could be also named as isoforms. Allergen polymorphisms could influence their allergenicity and their prevalence often varies among different environments [11, 12]. Therefore, identification of allergen variants should be followed by an evaluation of their IgE binding properties, especially, if they have a potential use as clinical reagents for allergy diagnosis or SIT.

1.1.2. House dust mite allergens

Allergens derived from house dust mites (HDM) are the most important cause of IgE sensitization in the world, but especially in the tropics. Although HDM allergy is not a problem restricted to this geographical area [13], the rates of sensitization, as well as the intensity of the IgE responses, are greater than in other latitudes [14]. This may be related to the climatic conditions, which are favorable for mite growth, but other factors, such as genetic background of the population and the co-exposure to helminths should be considered [15, 16]. Indoor levels of mite allergens remain high during the whole year in contrast to other regions such as Mediterranean Europe where mite growth has maximum peaks in the beginnings of autumn and a sharp decrease during the winter period [17]. Species from the *Dermatophagoides* genus (*D. pteronyssinus* and *D. farinae*) has a wide distribution in the planet, including temperate and tropical climates. Moreover, there are geographically restricted species, such as *B. tropicalis*, a potent source of allergen in the tropics.

1.1.2.1.1. The genus Blomia

Blomia belongs to the *Echimyopodidae* family. Three species have been identified in the house dust: *B. tropicalis, B. kulagini and B. tjibodas*. *B. kulagini* has been found in north-western Spain with an alpine climate and also in Egypt [18]; *B. tjibodas* has been reported in southern Chile and Germany [19], and it is an IgE

sensitizing source for German farmers [20]. Most research is focused on *Blomia tropicalis* because it is extensively dwelling in homes from tropical and subtropical regions. The fact that *B. tjibodas* IgE-reactive German farmers were also positive to *B. tropicalis*, even though there was not natural exposure to this species [21], suggest there is an important CR between this two closely related species. In the case of *B. kulagini,* the high degree of CR with *B. tropicalis* has been demonstrated experimentally [22].

1.1.2.1.1.1. Blomia tropicalis

Blomia tropicalis, previously recognized only as a storage mite, was first described by Van Bronswijk et al (1974) in house dust of tropical places [23]. In tropical countries, it habits in more than 90% of urban homes [24-26]. In subtropical regions, *B. tropicalis* is less frequent, about 30% of analyzed houses [27]. In Cartagena, Colombia *D. pteronyssinus* represents 37% of total mite population in the house dust, meanwhile *Blomia tropicalis* accounts for 40.1%. Although *D. pteronyssinus* is also a clinically relevant allergenic source, allergens derived from *Blomia tropicalis* are more specific of the tropical region.

In several countries of Latin America [14, 28-31], Asia [32-34] and Africa [35] it has been reported that *B. tropicalis* is an important cause of allergic sensitization and a risk factor for asthma development. Since the first descriptions of its allergenic relevance, there has been a considerable advance in the isolation of its allergenic components, addressed by research groups applying advanced genomic and proteomic approaches. From one dimensional electrophoresis separation, there have been identified 25 IgE binding components with sera from Colombian patients; some of them are already produced as purified recombinant proteins and are officially accepted as allergens [36, 37], the rest are poorly characterized, but there are evidence of *in vitro* IgE binding and homology to known allergens from other sources (Table 1.1). Screening cDNA libraries by clone sequencing, Chew FT found 18 homologous proteins from the 21current known HDM groups and 16 potential new allergens with homology to others from a source different to mites, mainly panallergens [38].

Table 1.1 *Blomia tropicalis* allergens

Allergen	MW (kDa)	IgE binding	Biochemical Activity
Blo t 1	25	61-92%	Cystein Protease
Blo t 2	14	7%	Lipid binding protein
Blo t 3	24-31	4.7-51%	Trypsine
Blo t 4	57	7.5-62%	α-Amilase
Blo t 5	14-17	10-100%	Unknown
Blo t 6	25	11%	Chimotrypsine
Blo t 8	26	80.2%	Glutathione-S-transferase
Blo t 9	30	ND	Serine protease
Blo t 10	33-37	10-29%	Tropomyosin
Blo t 11	98-110	10-86%	Paramyosin
Blo t 12	14	25-50%	Chitin binding protein
Blo t 13	14-15	13%-50%	Fatty acid binding protein
Blo t 14	117	ND	Vitellogenin
Blo t 15	62,5, 98, 105	ND	Chitinase
Blo t 18	60	ND	Chitinase
Blo t 19	7	3%	Antimicrobial peptide
Blo t 20	ND	ND	Arginin kinase
Blo t 21	13.2	57.9%	Unknown

*ND: Not described.

1.1.1.2.1.1.1. Cross-reactivity with other allergen sources

The IgE CR between *B. tropicalis* and other HDMs is variable among the species and although usually decreases as the distance in their phylogenetically relationship rises; it also depends on the pattern of exposition of those individuals whose sera are used for inhibition studies (Table 1.2). As mentioned, CR within allergenic species from Blomia genus is almost complete [22]. CR studies with other closely related mite species, members of the Glycyphagoidea super-family, has only been published in regard to *L. destructor* [39, 40], an important allergenic storage mite. Puerta *et al*., using serum pool from Colombian patients, found 82% of inhibition to *L. destructor* extract by *B. tropicalis* [39]. Johansson et al. detected that in the sera of *L. destructor* sensitized Swedish barners, *L. destructor* inhibited the IgE binding to 10 bands of the *B. tropicalis* extract [40]. Likewise, when inhibition studies with serum from Brazilian allergic patients were performed, *B. tropicalis* only inhibited IgE binding to 4 bands from *L.*

destructor extract. *Suidasia menadensis* is another mite found in house dust from the tropics, although in lower levels and frequency than *B. tropicalis* and *D. pteronyssinus* [41]. It has been reported an important degree of CR between *B. tropicalis* and this mite; *B. tropicalis* inhibited IgE binding to *S. menadensis* in solid phase (87.6%). Inversely, *S. menadensis* inhibited only 32% of IgE reactivity to *B. tropicalis*. This pattern of inhibition indicates that the main IgE antibodies are directed against *B. tropicalis* (primary sensitizer) and it is an illustrative situation when the detection of a high CR is dependent on the predominance in exposition to one antigen source.

CR between *D. pteronyssinus* and *B. tropicalis* is low to moderate [42]. At the level of purified allergens, there are some highly cross-reactive groups, such as tropomyosins, whose protein sequences are also conserved in other mites and invertebrates, including helminths; others with a low-moderate CR [43] and species-specific allergens. Most house dust mite allergen groups have been organized around *Dermatophagoides spp.* allergens. The identification of homologous in *B. tropicalis* is more recent in the timeline and their CR characterization should continue for a better evaluation of this phenomenon.

Table 1.2 Cross-reactivity of *B. tropicalis* with other mites

Source	Percent of IgE inhibition by *B. tropicalis*	Reference
Blomia kulagini	100	Cardona et al. [22]
Suidasia menadensis	87.2	Puerta el al. [41]
Lepidoglyphus destructor	83	Johansson et al [40]
Dermatophagoides pteronyssinus	40	Chew et al.

Blomia tropicalis also share allergenic epitopes with *Ascaris* spp. Analysis of this CR by Acevedo et al. showed that *Blomia tropicalis* and *D. pteronyssinus* inhibited 83.3% and 79% of IgE- binding to Ascaris, while Ascaris inhibited 58.3% and 79.3% to *B. tropicalis* and *D. pteronyssinus*, respectively. Tropomyosin [44] and Glutathione-S-

Transferase (GST) were identified as IgE cross-reactive groups. In addition to its potential impact on asthma pathogenesis, Ascaris infection and mite allergy diagnosis relying on the determination of specific IgE could be affected by this CR [16, 45].

1.1.1.2.1.1.2. Species-specific allergens

Various *B. tropicalis* allergens have been proposed to be species-specific. According to Kuo *et al* results, Blo t 5 and Der p 5 are not cross-reactive molecules [46]; however, Caraballo *et al* found that IgE binding to Der p 5 can be inhibited by BtM, an allergenic carboxy-terminal fragment of Blo t 5 [43]. Cheong *et al* cloned Blo t 1, a cystein protease with 34% of sequence similarity to Der p 1. Poor correlation between the specific IgE levels to these allergens was the only clue about their lack of CR [47]. The allergen Blo t 12 is another potential specific-marker of *B. tropicalis*. No homologous proteins have been described in the genus *Dermatophagoides*, other important allergen source in tropical countries, suggesting that it is a potential species-specific allergen; hence, it may be very useful in component-resolved diagnosis to identify *B. tropicalis* sensitization.

1.1.1.2.1.1.2.1. Blo t 12

Blo t 12 (Blo t 12.0101) was first identified by IgE immunoscreening in a *Blomia tropicalis* cDNA library constructed from mites collected in Cartagena [48], showing an IgE binding frequency of 50% when was tested in 32 individual sera by plaque assay. The analysis of its primary sequence revealed that this allergen has some degree of similarity to some chitin binding proteins. It contains a 90 amino acid domain, with key residues that confers the ability to bind this carbohydrate; however, this biological activity has not been demonstrated experimentally.

1.2. Adjuvants

1.2.1. Chitin

Important allergens sources such as mites, fungi and parasites contain chitin, the second most abundant biopolymer in nature. This carbohydrate seems to modulate immune response in mammals, especially, those of allergic type [49-52]. The first

finding about a promoting effect of chitin in allergic responses was published by Reese et al, who demonstrated that its nasal administration induce an inflammatory response in mice, characterized by basophil and eosinophil infiltration, a hallmark of allergic responses [53]. This effect, which was mediated by LTB4 released from activated macrophages, was not observed when using transgenic mice over expressing functional chitinases. Other interesting publications have found important results about chitin [49-51]. Intermediate size particles are highly immunogenic and pro-inflammatory, when they are administered with OVA; it is a potent adjuvant of allergic responses, similar to alum [51]. However, it also induced concomitantly other type of immune responses (Th1 and Th17), thus, it is considered a multifaceted adjuvant. In this regard, neutrophil infiltration in the lungs is also predominant when chitin is administered [50]. In other animal models, small chitin particles (<10 μm) has been found to promote Th1 response development against the sensitizing allergen [54, 55].

1.3 Risk factors for allergy and asthma

The causes of asthma and other allergic diseases remain unknown. Genetic and epidemiological studies suggest that for these multifactorial diseases the expression of different phenotypes depend on complex interactions between susceptibility genes and the environment [56]. This is reflected in the wide differences in prevalence and natural history of allergic diseases around the world [57].

1.3.1 Genetics

Asthma, allergic inflammation and IgE responses are inherited conditions, with unique and shared genetic determinants. More than 1.000 studies about the relationship of genetic variants and allergy susceptibility have been published [58]; however, disease prediction or clinical classification, based on the identification of individual genetic variants or combinations, is not possible yet. In spite that atopic phenotypes run in families, there are not known genetic variations that contribute substantially to their heritability. Several approaches (candidate genes [59], positional cloning [60, 61], genome wide associations [62, 63]) have been conducted to identify genetic risk factors for these conditions. Most susceptibility variants can be classified into these functional categories: epithelial barrier function [64], environmental sensing and signaling [65]

[66], Th2 responses and tissue response (repair, oxidative stress[67]). As in other complex diseases, few genetic associations with asthma/allergy have been replicated in several populations [68]. During the last years it has been a matter of great interest the epigenetic mechanisms determining asthma phenotypes and supporting the relationships between genomes and environments in this disease [69].

1.3.2. Environment

It is well known that environmental exposition to several factors influence allergy and asthma development [70]. Some risk factors seem to be universal (allergen sensitization, tobacco exposure [71, 72], air pollution [73-75]), it means, that its association with disease presentation or worsening is commonly replicated in different studies. For others, their effects on asthma and allergy are not clear (viral infections [76], helminthiasis [77-79]). Interestingly, some protective factors detected in developed countries, such as poor hygienic conditions, do not confer protection in deprived and overcrowded communities, where a high prevalence of asthma and early infections concur [6, 80, 81].

1.3.2.1. Helminth infections

Immune response against helminths share many features with that elicited by allergens in atopic subjects [82]. A Th2-polarized response, with high levels of total and specific IgE to nematode antigens, is induced in most of the exposed individuals. However, parasites also contain products with immunosuppressive properties [83]. The balance among these the two mentioned types of responses depends on several factors (nematode species, intensity of the infestation, host genetic background). Mild infections seem to promote allergic manifestations [78], in contrast to heavy infestation that reduce atopy presentation and of other related traits [79] [84]. Some helminths antigens cross-reacts with HDM allergens [44], confounding allergy diagnosis in infected populations. In the tropics, Ascaris sensitization is high and more frequent in atopic individuals. Due to the CR, it is unknown how many cases of sensitization truly correspond to HDM or nematode antigens; moreover, the biological relevance of this phenomenon on the inception of allergy in the tropics is not completely understood [85]. Birth cohort studies, exploring HDM and Ascaris sensitization with species-

specific and cross-reactive components, from early age, contribute enormously to solve these interrogants.

1.4. Allergy Diagnosis

Diagnosis of allergy is based on the clinical evaluation by a physician, and then, corroborated by *in vivo* or *in vitro* tests that determine IgE sensitization. Skin prick test is the most common and reliable diagnostic tool used by the allergist, but also, *in vitro* specific IgE determination is frequently used and under special clinical conditions (skin diseases, uncooperative patients, patients with high risk of anaphylaxis upon allergen exposure) is the first-line diagnostic option. Most currently used methods for IgE serology are based on the same biochemical principle: immobilization of the allergen in a solid phase, which is incubated with the serum or plasma sample, and then, detection by anti-human IgE coupled to an enzyme that permits signal generation and readout (i.e. by spectrophotometry, fluorometry or chemiluminiscence). Organ challenges are mostly used to define food and drug hypersensitivity [86]. *Ex vivo* tests, such as basophil activation assays [87], are also available, but it is more common its application on allergy research. Optimization of these procedures is an ongoing task, since adverse effects with *in vivo* testing can be avoided, especially in those patients with high risk of anaphylaxis.

1.4.1. *Component resolved diagnosis*

IgE sensitization has been usually explored with natural extracts, a complex mixture of proteins from the allergen source that contain allergenic and non-allergenic molecules. Standardization of natural extracts, to ensure a consistent composition of batches, has been always problematic; concentration of allergens is variable and affected by different factors [88]. Some allergens are highly susceptible to degradation or not easily extracted, occurring false negatives when the sensitizing allergen is not represented in the complete extract. When extracts are used for diagnosis, the cause of poli-sensitization to related-sources (co-sensitization or CR) cannot be ascertained in all cases. With the molecular cloning of many allergen genes and its production as recombinant proteins [89], it is possible to further refine diagnosis at the component level (Component resolved diagnosis, CRD). CRD allows a more precise definition of

IgE sensitization [90] and, thus, a better selection of those molecules for SIT to which a patient is actually sensitized. Advantages of this strategy have been widely discussed in several reviews [91]. Component-resolved diagnostic tests include species-specific marker allergens to diagnose the genuine sensitization to a given source and also cross-reactive molecules. Purified allergens can be natural or recombinant molecules.

1.4.2. Evaluation of recombinant allergens for diagnostic purposes

Production of allergens as recombinant proteins has been very useful for allergy research and clinics. It allows obtaining pure molecules in substantial quantities, a difficult task to achieve from natural sources. Cloning of allergens lets primary sequence identification and its classification into homology-based groups [38]. Molecular characterization is essential, especially if the recombinant allergen is intended for clinical use. Its resemblance to the natural counterpart must be assessed from the physicochemical, structural, and biological point of view. Immunological characterization is mandatory and it goes beyond IgE serology studies to know about its epidemiological relevance in a given population. CR with other allergens must be evaluated; it gives useful information for considering its inclusion in allergen batteries for CRD due to its high impact on clinical decisions for immunotherapy and allergen avoidance [92]. Its biological activity to induce IgE-mediated cell degranulation should be confirmed *in vivo* (skin prick tests) or *ex vivo* (basophil activation or histamine release assays). Epitope mapping provides also very valuable data [93]; it is expected that allergy diagnosis can be further refined to the epitope level, and in turn, the specificity of IT. On the other hand, identification of antigenic sites might be applied to create hypoallergenic engineered molecules for immunotherapy. Physico-chemical properties of the recombinant protein are also evaluated [94] (post-translational modifications, aggregation behavior and stability) due to its influence on the quality as pharmaceutical product.

1.5. Treatment

Allergies are difficult-to-treat conditions, with a limited number of therapeutic options, mostly offering symptomatic relief (corticosteroids, B_2 agonists, anti-leukotrienes). SIT, introduced in 1901 by Noon, is still the only treatment capable to modify the natural

history of atopic diseases. Mechanisms of action are partially understood, but include the induction of allergen-specific IgG4 and regulatory T cells [95, 96]. Although it has been demonstrated its effectiveness as a treatment for allergic rhinitis, asthma [97] and atopic dermatitis [98], safety and efficacy of SIT can be improved. SIT is usually performed with natural extracts. Since more allergens, than those required for desensitization, are administered, novel sensitizations can be induced [99, 100]. The allergenic content of extracts is difficult to standardize, especially because each patient can be sensitized to different allergen components. CRD and tailored immunotherapy aims for a more specific treatment, it means, administering only the precise components the patient needs to. With its production as a recombinant allergen, the difficultness to obtain purified allergens from the natural source is overcome. Currently, there is one recombinant allergen (Bet v 1) available for sublingual SIT, show good results. However, other options for treatment are genetically engineered allergen vaccines (hypo-allergens, hybrid or fusion proteins) where allergen sequence is modified in order to increase safety and/or efficacy of vaccination. Some of these molecules are under clinical trial evaluation [101].

Hypothesis

Characterization of purified allergens from *B. tropicalis,* including species-specific and cross-reactive molecules, improves the understanding of IgE sensitization and other allergy-related phenotypes in the tropics.

Thesis aims

To overall aim of the current doctoral thesis was to gain a further insight into various aspects related to *Blomia tropicalis* allergenicity – of current interest in Allergology research – through the production of its allergens as recombinant molecules and their immunological and biological characterization.

In Chapter 3: Immunological and biological characterization of group 12 allergens from *Blomia tropicalis* the allergen Blo t 12 was characterized at the immunological and biological level. Aspects such as IgE reactivity, isoform diversity, usefulness as a species-specific marker of *B. tropicalis* sensitization and the biological impact of its chitin-binding activity on allergenicity were evaluated. One manuscript [36] has already been published from these results and another is in preparation.

In Chapter 4: Identification of novel *Blomia tropicalis* allergens the aim was to isolate new allergens from *B. tropicalis,* obtained by PCR screening of a *B. tropicalis* cDNA library, and to describe their frequency of IgE sensitization in our population. Part of this data were presented in the most important international meetings on Allergy research (AAAAI 2010 and EAACI 2010 annual congresses) and were published in indexed journals in the form of abstracts.

In Chapter 5: Understanding the early immune response *to B.* tropicalis allergens and its clinical impact in children from the tropics**,** in a birth cohort from children born in Cartagena, a tropical city in Colombia, presentation of atopy-related phenotypes (wheezing and eczema) and the evolution of IgE responses were evaluated during the first three years of life. IgE determination to the HDM species, *B. tropicalis* and *D. pteronyssinus*, as well as to the nematode *Ascaris* spp. was performed to have an overall understanding of the dynamics of sensitization in early infancy. Moreover, sensitization to *B.* tropicalis purified allergens and to other from *Ascaris spp*. was evaluated to precise some aspects and interrelationships between these sources that can only be ascertained by using individual allergen components. One original publication [102], a submitted manuscript and a draft in preparation were derived from these results.

Chapter 2. *Material and methods*

2.1. Subjects

Serum and other biological samples that were used for evaluation of human immune responses to *B. tropicalis* allergens and other atopy-related traits (i.e. asthma, wheezing) were obtained from allergic patients or healthy subjects, belonging to any of the following epidemiological research programs of our Institution, all of them approved by Ethics Committees:

Candidate genes for asthma and atopy: The epidemiological relevance of *B. tropicalis* purified allergens as IgE sensitizers was evaluated in *B. tropicalis* allergic/asthmatic patients, belonging to a larger database of subjects recruited in an asthma case-control study (Grant numbers 331-2004 and 093-2007, funded by Colciencias), approved by the University of Cartagena Ethic Committee. Asthma was defined according to the Global Initiative for Asthma (GINA) criteria, using a standardized questionnaire tested previously in patients with a history of physician-diagnosed asthma [4]. The diagnosis was confirmed by a physician belonging to the research staff, sustained on a clear clinical history with clinical symptoms. Sensitization to *B. tropicalis* was confirmed by ELISA, as described previously [103].

FRAAT (Factores d**e R**iesgo par**a A**sma **y A**topia en e**l T**rópico) cohort: A birth cohort of 326 children from Cartagena, Colombia was recruited between 2007 and 2008 [104]. We created a community based birth cohort for a prospective follow up and collection of epidemiological data and biological samples. The majority of study participants belonged to the poorest communes and shared environmental conditions. The Ethic Committee of the "Fundación Santa Fe de Bogotá", Bogotá-Colombia, approved this study (CCEI-282-206). We explored at different time-points (between 0 and 42 months) during child life the evolution of IgE responses to *B. tropicalis* and other allergenic sources and its relationship with atopic-related phenotypes. Demographic details of this cohort were fully described in the publication #2 of this doctoral thesis [104]. The program was funded by Colciencias (Grant 325-2006).

Influence of Ascaris infection and/or sensitization on asthma severity (The ASA cohort): It is a well-characterized cohort of asthmatic patients (n=300) from Cartagena, Colombia. Asthma was defined according to the GINA criteria, using a standardized questionnaire tested previously in patients with a history of physician-diagnosed asthma. The diagnosis was confirmed by a physician belonging to the research staff, sustained on a clear clinical history with clinical symptoms as described previously. Patients also were evaluated by a complete clinical protocol that included: espirometry, skin prick tests and IgE serology using ImmunoCap and ELISA. Funded by Colciencias, Grant 602-2009 and approved by the University of Cartagena Ethic Committee.

2.2. Allergen obtention

2.2.1. Allergenic Extracts

Blomia tropicalis and *Dermatophagoides pteronyssinus* mite extracts were prepared as described here. Lyophilized mites were resuspended 1:20 in PBS pH 7.4 and incubated ON at 4 °C with continuous stirring. After centrifugation for 30 min at 6.000 r.p.m., the supernatant was filtered through 0.8, 0.45 and 0.2 μm filters. Extracts were dialyzed against water in a dialysis tube with a 3.4 kDa pore and then lyophilized and stored. Protein concentration was determined by Bradford method. Other extracts prepared from arthropod species of agricultural interest were kindly supplied by Dr. Krizkova-Kudlikova and Dr. J. Hubert [105] (Crop Research Institute; Praga, Czhec Republic).

2.2.2. Blo t 12 allergen group

2.2.2.1. The cDNA coding for Blo t 12 isoallergens

The cDNA coding for Blo t 12.0101 was isolated by IgE immunoscreening from a *B. tropicalis* cDNA library constructed with mites collected in Cartagena, Colombia, as described by Puerta *et al* [5]. Blo t 12.0102 was isolated from a cDNA library

constructed from mites collected in Singapore, and was kindly gift by Dr. YK Chua (University of Singapore, Singapore).

2.2.2.1.1. Allergen expression in Pichia pastoris

2.2.2.1.1.1. Cloning and expression

Subcloning of Blo t 12.0101 into pPIC9 vector for its expression in *Pichia pastoris* was accomplished by Dr. Puerta as fully described in the publication #1 of this thesis [36]. The recombinant pBlo t 12.0102 was also inserted in this same vector by Dr. Chua´s research group in Singapore. For protein expression, transformants were selected by the slow growing phenotype (Mut -) on the minimum methanol (MM) plates. In order to identify the high expression clones, an individual colony was inoculated in 50 ml of buffered glycerol-complex medium (BMGY, 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base with ammonium sulfate, 1% glycerol, 0.4 mg/l biotin, and 0.1 M potassium phosphate, pH 6.0) and grown at 30º C in a rotary-shaking incubator at 250 rpm. The cells were harvested by centrifuging at 1500 g for 5 min at room temperature when the OD_{600} nm reached 2.0 - 6.0. The cell pellet was resuspended in 10ml of buffered methanol-complex medium (BMMY, replacing the glycerol in BMGY with 0.5% methanol) for induction of the recombinant expression. The recombinant protein Blo t 12 was harvested after 24 hours of induction. It was secreted into the medium. Protein purification was done by anion-exchange chromatography.

2.2.2.1.2. Production in E. coli expression system and purification

A commercial service for synthesis and subcloning of Blo t 12.0101 and Blo t 12.0102 codon-optimized sequences – into two different vectors: pET32a+ (as a his tagged-thioredoxin fusion protein) or pET45b+ (as his-tagged protein) – was ordered to Genscript (Piscataway, USA). Plasmids were transformed into Origami (DE3) cells by electroporation. Competent cells were prepared by following the manual instructions. The plasmid (5 ng) was incubated on ice with competent cells for one minute. Then, the plasmid-cell reaction was added to an electroporation cell and received an electric pulse.

Rapidly, one milliliter of SOC medium was added to the cell, transferred to a 14 mL non-conical Falcon™ tube and incubated for one hour at 37° C. The transformation reaction was then plated onto LB agar plates, supplemented with ampicillin (100 µg/mL) as selective antibiotic, and incubated ON at 37° C. Next day, individual colonies were picked and grown in LBA medium (10 mL) ON at the same temperature. For induction of protein expression, the overnight culture was first diluted 1:20 in LBA and incubated at 37°C until reaching mid-log phase (OD_{600} 0.5), which usually lasted 3-4 hours. At this point, five milliliters of the culture was transferred to a new tube (non-induced control) and to the rest of culture it was added IPTG at 1 mM final concentration. Induction was done for 5 hours at 37°C.

Protein expression was evaluated by 5-15% discontinuous SDS-PAGE under reducing conditions. Five hundred microliters of non-induced and induced cultures were centrifuged at maximum speed for 1 minute, supernatants were decanted and pellets resuspended in 80 µL of Laemmli buffer, heated at 95°C and loaded (10 µL) onto the polyacrylamide gel. A successful protein expression experiment is generally observed as a protein band present in the induced-, but not in the non-induced- culture

The his-tagged fusion proteins were purified from the *E. coli* lysate in native conditions. Briefly, the induced culture was resuspended in native binding buffer (NBB) pH 8.0 (500 mM NaCl 50 mM NaH2PO4, pH 8.0) and incubated with lysozyme (1 mg/mL) for 30 min on ice, and then sonicated 6 times. The lysate was incubated with a Ni-NTA resin for one hour, washed with 20 mM imidazole - NBB, and eluted with 250 mM imidazole in NBB buffer.

2.2.2.2. Natural Blo t 12

Two different affinity columns were prepared to purify natural Blo t 12; one with polyclonal (pAb) serum from a pBlo t 12.0101 immunized rabbit and the other with a mixture of two anti-Blo t 12 monoclonal antibodies (mAbs, 3B11 and 1A6). The pAb serum (5 mL) was incubated with 1 mL of Protein A-Sepharose 4B Fast Flow beads for one hour at RT in an orbital shaker. In the case of the mAb affinity column, one milligram of each antibody was incubated with 1 mL of Protein A-Sepharose 4B Fast Flow. The following steps were similar for both columns. After centrifugation and

supernatant recovery, beads were washed with PBS pH 7.5 and then twice with 0.2 M sodium borate pH 9.0. To covalently couple antibodies to the matrix, fifty two milligrams of dimethyl pimelimidate dyhidrochloride (20 mM final concentration) were added to the beads and incubated for 30 minutes at RT. Later, the resin was precipitated by centrifugation and washed with ethanolamine 0.2 M pH 8.0 twice and then incubated for 2 hours with this same buffer to stop the coupling reaction. Finally, beads were washed in PBS.

For nBlo t 12 purification the affinity column was prewashed with glycine 100 mM pH 3.0 to remove non-covalent bound antibodies to protein A-beads. *B. tropicalis* extract was added to the column and incubated on ice for 2 hours in an orbital shaker. After recovery of flow throw, the resin was washed 6 times with PBS pH 7.5. Elution of antigens from the resin was done with 4 mL 100 mM glycine HCl pH 2.5, collected in 1 mL fractions, using tubes with 400 uL Tris 1 M pH 8.0 to neutralize the pH. The eluted fractions were dialyzed against water and lyophilized for protein concentration.

2.2.3. Gluthathione S transferase from B. tropicalis (Blo t 8) and other sources

2.2.3.1. Cloning, expression and purification of recombinant Blo t 8

Blo t 8 coding gene was amplified from a cDNA library of *B. tropicalis* constructed from local mites isolated in urban homes from Cartagena, Colombia. For amplification, primers 5'CACC- ATG GCA CCA TTG AAG ATT G 3' (forward) and 5' TTA TTC TTT GGC CCA ACG ATG G 3' (reverse) were designed according to a reported nucleotide *B. tropicalis* GST sequence (gi37958148). This fragment was amplified using *Taq* polymerase and purified with Qiaquick PCR purification kit. For cloning the amplified fragment into pET100 vector it was necessary to produce blunt ended amplicons. Thus, 45 ng of the amplified DNA were polished in a 10 uL reaction containing 2.5 U of *Pfu* polymerase and 1 mM dNTPs in an incubation step of 30 minutes at 72°C. Nucleotide sequences of cloned fragments were analyzed (Qiagen Genomic Services, Germany) from two different plasmids. For expression, Blo t 8/pET100 plasmid was transformed into BL21 (DE3) Competent Cells following the

protocol described in the manual. For protein expression, an overnight culture the clone was diluted 1:20 and after reaching mid-log phase (OD_{600}: 0.5), IPTG was added to a final concentration of 1 mM. The his-tagged Blo t 8 was purified from the *E. coli* lysate under native conditions, as previously described for Blo t 12 recombinants.

2.2.3.2. Natural glutathione-S transferase

Natural glutathione-S transferase from *B. tropicalis* was purified by affinity chromatography using Glutathione Sepharose 4B. Four milligrams of the protein extract were incubated with 1 mL glutathione beads for 90 minutes in a rotor. After centrifugation at 500 g for 5 minutes, the flow throw was collected, and beads were washes four times with PBS. After decanting the final wash, the resin was incubated for 10 minutes with 50 mM Tris pH 8.0, 10 mM reduced glutathione at room temperature. The beads were precipitated by centrifugation, and 1 mL eluate was harvested each time. Three more elution steps were done.

2.2.4. Blo t 5

Blo t 5 coding gene was amplified from a cDNA library of *B. tropicalis* constructed from local mites isolated in urban homes from Cartagena, Colombia. For the amplification of its coding region, primers 5'CAC CAT GAA GTT CGC CAT CGT TC (forward) and 5' CCT TAT TGG GTT TGA ATA TCC TTC ACT 3' (reverse) were designed according to the Blo t 5 nucleotide sequence (gi4204916), previously reported by Arruda K *et al*. The amplification reaction was prepared as described in Table 2.1.

Table 2.1 Polymerase chain reaction for amplification of Blo t 5

Reagent	[ ] stock	Vol.
dH2O		13.2
Enzyme buffer	10X	2.5
DNTPs	10 mM	0.8
Primer F*	25 pm/uL	3.0
Primer R*	25 pm/uL	3.0
Pfu polymerase		0.5
DNA (cDNA library)		2
Total (uL)		23.0 uL

After verifying the amplification of the expected fragment by electrophoresis in 1% agarose gel. Amplification reaction was cleaned using the QuiaQuick PCR purification kit and then, cloned into a pET100 vector as described above for Blo t 8. The protocol for protein expression was similar to those used above. The protein was purified from the bacterial lysate by Ni-NTA affinity chromatography under natural conditions.

2.2.5. Ubiquitin from B. tropicalis

The cDNA gene coding for ubiquitin was isolated by PCR using specific-primers, whose design was based on a *B. tropicalis* EST entry, with homology to the ubiquitin family. The amplification reaction was purified with QuiaQuick PCR purification kit and cloned into the pET100 vector following the guidelines recommended in the manual. Three microliters of the cloning reaction were used for transformation of Competent TOP 10' Cells. Plates were incubated ON at 37°C. At the next day, it was observed a good number of colonies in each plate (around 50-70 colonies). Seven of these colonies were picked up and analyzed by PCR. Two clones with different DNA fragment sizes were sequenced for identification of the correct insert. There was found that the clone with the expected sequence coded for two copies of ubiquitin (poli-ubiquitin), as normally occurs in eukaryotes. Since *E. coli* does not have enzymes to break the polyprotein into monomers; based on the obtained poli-ubiquitin sequence, it was ordered the synthesis of a sequence with just one copy of ubiquitin (Genscript, USA) and its subcloning into pET45b+ vector. Both plasmids were transformed onto BL21 (DE3) chemically competent cells for protein expression. The protocol for protein expression was similar to that described above for other allergens. The protein was purified from the bacterial lysate by Ni-NTA affinity chromatography under natural conditions.

2.2.6. Other recombinant allergens

Asc s 1 (ABA-1) and Asc l 3 were produced in our laboratory as previously described [44, 106]. Der p 2 and Der p 8 gene synthesis and subcloning into pET45b+

were ordered to Genscript™ (Piscataway, USA). Transformation into Origami (DE3) cells was done by electroporation. A GST-sigma like sequence from *Ascaris suum* was also synthesized and subcloned into pQE30 by Genscript™. The plasmid was transformed onto M15 competent cells by electroporation in our laboratories. Protein expression protocol was similar to that described for other allergens before. All allergens were soluble products, except Der p 2, that required denaturation with urea 8M before protein purification by the conventional Ni-NTA purification protocol. However, it could be eluted under native conditions.

2.3. Obtention of other biological samples

2.3.1. Polyclonal antibodies

Production of anti-Blo t 12 polyclonal antibodies (pAbs) was ordered to Vivotecnia (Madrid, Spain). Two New Zealand rabbits were immunized with pBlo t 12.0101 in a 2-week interval. Immunization success was monitored by ELISA. The final bleeding from each was used for allergen characterization experiments.

2.3.1.1. IgG purification

For some downstream applications IgG was purified from the complete serum using protein A-Sepharose. For 2 mL of serum it was used 1 mL of the resin. Antibodies were eluted with 0.1 M Glycine HCl pH 2.5 and dialyzed against PBS overnight. IgG reactivity was tested by ELISA, before and after purification.

2.3.2. Monoclonal antibodies

Monoclonal antibodies were produced by Dr. Martin Himly in the Christian Doppler laboratory for allergy research (University of Salzburg, Salzburg, Austria). Two mice were immunized with alum-adsorbed deglycosylated pBlo t 12.0101 in a 2-week interval. After hibridoma fusion, cells were grown for 9-10 days in selection medium (37°C, 7% CO2), and the supernatant from different wells were tested for antibody reactivity to Blo t 12 using ELISA. Single clones were obtained by a limiting dilution protocol. Subtype classification (IgG1, IgG2a, IgG2b and IgG3) was done by ELISA

with specific anti-mouse IgG antibodies. All the obtained mAbs were from the IgG1 subtype.

2.3.2.1. IgG Purification

Monoclonal antibodies were precipitated with $(NH_4)_2$ SO_4, and purified using a protein G column. The cells were harvested by centrifugation at 3000 g for 15 min at 4°C. The supernatant was transferred into Erlenmeyer flasks and neutralized using a 1 M Tris-HCl pH 8.0 until the medium color turned pink. The mAb was precipitated in a final concentration of 50% saturated $(NH_4)_2$ SO_4 at 0°C. After $(NH_4)_2$ SO_4 was grinded and added bit by bit every minute into the stirring Erlenmeyer flask, the suspension was centrifuged at 15,000 g for 20 min at 4°C. The pellet was resuspended in 50% saturated $(NH_4)_2$ SO_4 and centrifuged again at 15,000 g for 20 min at 4°C. This step was repeated once. The obtained pellet was dissolved in PBS pH 7.4 about 1/10 of initial volume. For further purification, a 2 ml protein G column was used. The column was washed with 30 mL PBS pH 7.4. After the antibody was loaded on the column, the flow through was stored at 4°C until further analysis and the matrix was washed again with 30 mL PBS pH 7.4. Elution was performed with 5 ml of 50 mM glycine pH 2.5 in a reaction tube filled with 700 µl 1 M Tris HCl pH 8 to neutralize the solution. The column was cleaned with PBS pH 7.4 and stored at 4°C in PBS pH 7.4 and 0.05 % NaN_3 to avoid contamination. Antibodies were dialyzed 3 times against PBS pH7.4 (MWCO: 6-8. kDa) and analyzed with UV detection at 280 nm and SDS-PAGE.

2.4. Physico-chemical characterization of proteins

2.4.1. SDS-PAGE

Proteins were separated by SDS-PAGE, mostly under reducing conditions. Discontinuous polyacrylamide gels (5-15%) were prepared according to the Mini-PROTEAN® Tetra Cell instruction manual (Bio-Rad). For reducing conditions, samples were treated as follows: Laemmli buffer (with β-mercaptoethanol) was prepared 2X and added in 1:1 proportion, heated at 95°C for 5 minutes, centrifuged by

15 seconds and loaded immediately onto the gel wells. SDS-PAGE was run at 200 VDC until the front dye reached the lower border of the gel (by approximately 45 min). When gels were stained with Coomasie blue, they were incubated for 30 min in a horizontal shaker with this solution, and then in Destain solution (1:4:5; acid acetic, methanol, water) for 2 hours or ON. Silver staining was especially used to visualize few amounts of loaded samples; it was performed according to the instructions of the kit.

2.4.2. Two dimensional electrophoresis

Proteins of *B. tropicalis* extract were precipitated using the ReadyPrep 2-D Cleanup kit (Bio-Rad, Calif., USA). The pellet was reconstituted in rehydration buffer (8 M urea, 4% CHAPS, 0.0002% bromophenol blue, 50 mM DTT, 0.2 BioLyte Carrier ampholytes, pH 3-10) by shaking at room temperature for 2 hours. Isoelectric focusing was performed using a 7 cm long immobilized gradient (from pH 3 to 10) strip (3-10 NL ReadyStrip™ IPG Strip, BioRad, Calif., USA). One-hundred and thirty micrograms of *B. tropicalis* extract were used for strip rehydration. Strips were then equilibrated in 2 buffers containing 6M urea 0.375 M tris pH8.8, 2% SDS with 2% DTT or 2.5% iodoacetamide, respectively. Isoelectric focusing was performed at 50mA/strip, 20 ºC as follows: step 1: 250 V for 250 V/hr, step 2: 4000 V for 2 hr and step 3: 4000 volts for 10.000 V/hr. Second dimension separation was done in 15% acrylamide gels. Two replicas of the separation were done, one for gel staining and another for PVDF electro transference and further western blot.

2.4.3. Glycosylation detection

PVDF-electrotransferred proteins were incubated in the dark for 20 minutes in a solution of 10 mM sodium metaperiodate dissolved in 100 mM acetate buffer, pH 5.5. The membrane was rinsed twice in PBS and washed three times for 10 minutes with PBS. Then, it was incubated for 60 minutes at RT with 0.125 mM biotin hydrazide (diluted 1:5000 in 100 mM acetate buffer pH 5.5). After washing, the membrane was blocked with 5% milk in PBS for 60 minutes. Then, three washes were done and streptavidine-horseradish peroxidase (1:5000 diluted in PBS) was added and incubated for 30 minutes. After washing again 3 times, membranes are exposed to ECL™ chemiluminiscence reagents. Images were obtained in a CCD camera.

2.4.4. Deglycosylation procedures

2.4.4.1. Metaperiodate oxidation

PVDF-electrotransferred proteins were incubated in the dark for 20 minutes in a solution of 10 mM sodium metaperiodate dissolved in 100 mM acetate buffer, pH 5.5. The membrane was rinsed twice in PBS and washed three times for 10 minutes with PBS. After this treatment, Western blot protocol is performed as fully described below.

2.4.4.2. PNGase F treatment

pBlo t 12.0101 was treated with PNGase F under native and denaturing conditions. For native conditions, 4 µg of the allergen was diluted in reaction buffer (50 mM sodium phosphate buffer containing 1% NP-40) and incubated with 2000 U of PNGase F for 3 h at 37°C. For denaturing conditions, the allergen was first heated for 10 minutes at 100°C with 0.5% SDS and 1% β-mercaptoethanol prior to addition of reaction buffer and PNGase F at 37°C.

2.4.4.3. Chemical method with trifluorometasulphonic acid (TFMS)

Pre-cooled 10% anisole in TFMS solution (100 uL) was added to 0.3 mg of lyophilized pBlo t 12.0101 or 1 mg of RNAse B (Glycoprotein standard as control). Protein samples were gently shaken for 3-5 minutes until dissolved and incubated for 3 hours at RT. In a methanol-dry ice bath, 60% Pyridine Solution and TFMS-treated samples were pre-cooled. Immediately after adding a pH indicator solution (0.2% Bromophenol Blue), inside the ice bath, the pre-cooled 60% Pyridine Solution was added, dropwise, to sample reaction vials until their color turned to blue, taking care to mix and cooling the reaction vial between drops. For chemical removal, proteins were purified in NAP-5 columns with a Sephadex G-25 resin.

2.4.5. SECTDA

High-performance size-exclusion chromatography (HPSEC) was performed using a 7.8 · 300 mm TSKgel-G2000$_{SWXL}$ column (Tosoh-Bioscience, Stuttgart, Germany) on a HP1100 system (Hewlett-Packard) equipped with a built-in UV detector

and online coupled with a right-angle light scattering, refractive index and viscosity detector array (TDA302; Viscotek, Houston, TX, USA). Samples were analyzed in duplicates using OmniSec4.2^{TM}. The protein amount was determined from the refractive index signal. Detector was calibrated with BSA.

2.4.6. LC MS/MS

Tryptic peptides were separated by reversed phase capillary high-performance liquid chromatography (HPLC, Nanoease Symmetry 300TM trap column and Nanoease Atlantis dC18TM separating column; Waters, Milford, MA, USA) directly coupled to an ElectroSpray ionization-quadrupole-time of flight mass spectrometer (Q-Tof Ultima Global; Waters). Data acquisition and instrument control was done with the Masslynx software V4.1 (Waters). Peptides were eluted with an acetonitrile gradient (Solvent A 0.1%v/v formic acid / 5% v/v acetonitrile, solvent B 0.1%v/v formic acid / 95% v/v acetonitrile; 5–45% B) and directly subjected to ionization and mass spectrometry. Data were acquired in the data-directed analysis mode in the mass range from 350 to 1900. Fragmentation time of the peptides was 4 s per ion, four ions were fragmented simultaneously. Peptide fragmentation data were acquired in the mass range from 50 to 1900. Survey and fragment spectra were analyzed using the software Proteinlynx Global Server version 2.2.5 (Waters) with automatic and manual data validation. For sequence identification, Swiss-Prot and TrEMBL database were used.

2.5. Immunochemical procedures

2.5.1. Total IgE determination

Determination of total IgE in humans was determined with two different methods. "Candidate genes for asthma", total serum levels were quantified by duplicate using a commercial enzyme-linked immunosorbent assay (ELISA) kit (RIDASCREEN® Total IgE; R-Biopharm, Darmstadt, Germany), following manual recommendations. In those subjects belonging to any of the two other epidemiological programs, total IgE was measured by ImmunoCAP (Phadia). In section 2.8, it will be fully described those methodological aspects regarding ImmunoCAP determinations, both for total and specific IgE.

2.5.2. Household indirect ELISA for specific IgE determination

Human IgE: Specific IgE against *B. tropicalis* was detected by ELISA as described previously [103]. For specific IgE determination to the recombinant allergens the same protocol was used but with some modifications. Briefly, microtiter plates were incubated with 100 µL of the antigen per well overnight (See Table 2.2 for details about each antigen coating). Then they were washed five times with PBS-0.1% Tween 20 and blocked with 100 µl of blocking buffer (1% bovine serum albumin, 0.02% sodium azide in PBS Tween 20) for three hours at room temperature in wet chamber. After washing again, 100 µL human sera (diluted 1:5 in PBS- 3% BSA) were added to each well with further overnight incubation at room temperature. Plates were then washed five times and 100 µL of alkaline phosphate conjugated anti-IgE (diluted 1:500 in buffer Tris pH 8.0 0.05 M, BSA 1%, $MgCl_2$ 1 mM, sodium azide 0.02%) were added and incubated for two hours at room temperature. After the final wash (5X), colorimetric reaction was done by adding 100 µL of para-nitrophenyl phosphate (pNPP) and then incubating at RT for 30 minutes. Absorbance at 405 nm was determined using a spectrophotometer. Results were expressed in optical densities (OD). The cut-off point was defined as the mean of 5 control negative sera plus 3 standard deviations. All samples were assayed in duplicate, and inter-assay and intra-assay variation coefficients were lower than 15% and 10%, respectively.

Table 2.2. Coating conditions for specific IgE determination to purified allergens

Allergen	Amount (µg)	Coating buffer
Blo t 12	0.5	0.2 M sodium carbonate/bicarbonate buffer pH 9.2
Blo t 5	0.5	0.2 M sodium carbonate/bicarbonate buffer pH 9.2
Ubiquitin	0.5	0.2 M sodium carbonate/bicarbonate buffer pH 9.2
Blo t 8	0.75	PBS pH 7.5
Der p 8	0.75	PBS pH 7.5
AscGST	0.75	PBS pH 7.5
ABA-1	1	64 mM sodium carbonate/bicarbonate, pH 9.6
Asc l 3	1	64 mM sodium carbonate/bicarbonate, pH 9.6

Rabbit or mouse IgG: Antigens were coated ON at 4 °C. Next day, wells were blocked with PBS-BSA 1% for one hour at RT, incubated with several dilutions of the rabbit serum, purified rabbit IgG or mAbs for one hour at RT, then with HRP-anti rabbit or mouse IgE (1:2000) respectively. A TMB substrate was used for reaction development.

2.5.3. ELISA inhibition

Blo t 12. To investigate the presence of shared and unique epitopes in Blo t 12 isoforms, ELISA inhibition assays were done. Pool 1 and 2 were used with Blo t 12.0101 and Blo t 12.0102 in solid phase, respectively. Pool 1 was prepared with sera from 6 Colombian allergic patients with higher IgE reactivity to Blo t 12.0101 than to Blo t 12.0102. Pool 2 was composed of 4 sera from Singaporean allergic patients; these sera were previously tested for IgE reactivity to their local isoform. The OD value of Pool 2 was higher for Blo t 12.0102 than that for Blo t 12.0101. These pools were incubated with each inhibitor (Blo t 12.0101, Blo t 12.0102 and BSA) at different concentrations for 10 hours at 4°C followed by the respective ELISA.

2.5.4. Sandwich ELISA

This technique was used for quantification of Blo t 12. Monoclonal antibodies (2 µg/mL), diluted in 0.1 M carbonate buffer pH 8.4, were coated on 96-well Maxi-sorp™ plates by ON incubation at 4 °C. Wells were blocked with PBS 1% BSA 0.05% Tween 20 for two hours at RT. Then, a standard curve was prepared with rBlo t 12.0101 in a range of concentrations from 0.78 ng to 1 µg/mL. Unknown samples (allergen extracts and dust samples) were also added at different dilutions. Standards and samples were incubated for 2 hours at RT. After washing, anti-Blo t 12 rabbit polyclonal IgG (diluted 1:500 in blocking buffer) was added to the wells and incubated for 1 additional hour at RT. For detection, alkaline phosphatase- rabbit anti-IgG (diluted 1:2000) was incubated with the plate for one hour. After the final wash (5X), colorimetric reaction was developed by adding pNPP (100 µL) and then incubating at RT for 20 minutes. The detection limit of the assay was calculated as the mean of blank readings (6 wells) plus 3 standard deviations.

2.5.5. Western blot

Separated SDS-PAGE proteins were electrotransferred to PVDF membranes at 180 mA for 90 minutes. Membrane was blocked with PBS-5% defatted milk and washed two times with 0.1% Tween 20 PBS. Subsequent steps varied according to the primary antibodies that were used:

Human IgE. Human serum was diluted 1:5 in PBS-BSA 3% and incubated overnight at RT with the strips. Membranes were rinsed twice and washed three times more with 0.1% Tween 20 PBS (10 minutes in each wash). Alkaline phosphate conjugated-anti human IgE (diluted 1:500 in blocking buffer) was used as secondary antibody and incubated for 3 hours at RT. Strips were washed four times by 10 minutes with 50 mM Tris 150 mM NaCl pH 7.5. Chromogenic reaction was developed with NTB-BCIP using standard recommendations from Harlow E. and D. Lane's book [107].

Mouse or rabbit IgG. Mouse mAb or pAb rabbit IgG were diluted 1:1000 in blocking buffer and incubated for one hour at RT. Membrane was rinsed twice and washed three times more with 0.1% Tween 20 PBS. Horseradish peroxidase conjugated anti mouse- or rabbit-IgG (diluted 1:100.000 in blocking buffer) was used as secondary antibody and incubated for 1 hour at RT. Membrane was rinsed twice and washed three times more with 0.1% Tween 20 PBS. Chemiluminiscence reaction was developed by addition of 1:1 mixture of the SuperSignal West Femto Maximum Sensitivity Substrate™ and incubation for 5 minutes in the dark. Images were obtained at different times in a specialized chemilumiscence image detector (G: Box, Syngene, UK). Exposition times for image capture varied according to the assay, but were in the range of 1 to 5 minutes.

2.5.6. Epitope mapping

2.5.6.1. Peptide synthesis

Ten peptides corresponding to the amino acids 20-39, 30-49, 42-61, 50-69, 60-79, 73-92, 88-108, 101-120, 111-130 and 124-144 of the Blo t 12.0101 protein were prepared by solid-phase synthesis with an automated peptide synthesizer, purified by high performance liquid chromatography and (HPLC) and analyzed by mass

spectrometry (SBS Genentech, China). Lyophilized peptides were reconstituted to 10 mg/mL in 2.5% dimethyl sulfoxide (DMSO) and then diluted in PBS before their use in ELISA inhibition assays.

2.5.6.2. Competitive inhibition

We used a competitive inhibition enzyme immunoassay to examine the ability of the 10 overlapping peptides to block human IgE binding against Blo t 12.0101. Five individual sera, which showed high specific IgE levels to Blo t 12.0101 but low or no reactivity to Blo t 12.0102, were used. End-point inhibition of IgE binding to this allergen was done with 1.5 mg/mL of each peptide for 5 hours at room temperature. BSA (1.5 mg/mL) and Blo t 12.0101 (0.1 mg/mL) were used as negative and positive controls, respectively. Peptides showing a significant level of inhibition were then tested at different concentrations to observe the dose-response relationship. The degree of inhibition was expressed as cross-reactivity index (CRI), using the following formula: (Peptide inhibition %/Homologous inhibition %) X 100. CRI values below 25% were considered as no inhibition, those between 25 and 50% as a low degree of inhibition.

2.6. Skin prick tests

Blo t 12. Skin prick tests were done in 48 patients for evaluating *in vivo* the allergenicity of recombinant molecules. For skin testing the appropriate concentration of recombinant Blo t 12 had been obtained by titration in an allergic patient. This concentration has been used in several studies involving *Blomia tropicalis* recombinant allergens [46, 108]. The test was performed in the volar side of the arm using 25 μg/mL of the purified allergens, 50% glycerinated. Histamine dyhidrochloride and glycerol were used as positive and negative control, respectively. A result was considered positive when the diameter of the wheal was greater than 3 mm. All these patients were also skin tested with *B. tropicalis* and *D. pteronyssinus* extracts (Leti, Madrid).

GST. Skin prick tests were done in 20 subjects for evaluating the *in vivo* the allergenicity of recombinant and natural isolated GST allergens (rBlo t 8, nBlo t 8, nAscGST, rAscGST and rDer p 8). All these persons were also skin tested with *B.*

tropicalis, *D. pteronyssinus* (Immunotek, Madrid) and *Ascaris lumbricoides* extracts. A similar technique and glycerinate preparation to that described above was used.

2.7. Bioinformatics analysis and allergen modeling

Blo t 12. PROSITE was consulted for prediction of functional domains in Blo t 12 isoallergens. BLAST analysis did not identify a homolog sequence with a solved tertiary structure for homology-based modeling. Thus, Metaserver (Bioinfobank) was employed to test 10 different folding-based methods and select the best template and secondary structure alignments for protein modeling. Only the predicted chitin binding domain in both Blo t 12 isoallergens could be modeled, based on spatial restraints from tachycitin, a type II chitin binding protein. Modeller 9v1 software was used for modelling purposes. Energy parameters were calculated with the GROMOS96 implementation of Swiss-Pdb viewer [109]. Prosa-web analysis indicated good overall and local quality for both theoretical models [110]. Imoltalk was used to draw the Ramachandran plots of the models. Procheck was employed to assess the geometry of models. HotPatch, an algorithm to detect protein-protein interaction sites, were used for prediction of IgE binding regions in Blo t 12 isoallergens [111]. Relative accessible surface area (ASA) for each residue was obtained with Asaview [112]. Pymol version 0.99 (Delano Scientific) was employed to visualize structures and generate images.

GSTs. Homology-based molecular modeling of *A. suum* GST, Bla g 5 (O18598), Blo t 8 (ACV04860) and Der p 8 (P46419) structure was done in the SWISS-MODEL server using as templates the following experimentally defined structures: 2on5H (*O. volvulus*), 1m0uA (*D. melanogaster*), 1gsuB (*G. gallus*) and 1c72d (*G. gallus*), respectively. Models were refined in Deep-View (energy minimization and rotamer replacements). Their quality was evaluated by several tools, including Ramachandran plots, WHATIF, QMEAN4 index and energy values (GROMOS96 force field). Relative values of accessible solvent area (r-ASA) were determined by ASA-view [112]. AscGST sequence was then aligned to each allergenic sequence to identify conserved residues. Those conserved and solvent accessible residues (rASA > 0.25) were then located in the 3D-model to identified clustered areas (>4 residues) as possible cross-reactive antigen binding areas.

2.8. Evaluation of the biological activity of Blo t 12

2.8.1. Affinity binding assays

Chitin binding assays were performed to evaluate the biological activity of Blo t 12 isoforms. Five micrograms of Blo t 12 proteins were incubated with 100 uL of chitin beads (New England Biolabs) or 10 mg of powdered chitin from crab shells in Tris 20 mM pH 8.0, NaCl 150 mM (buffer A), in a final volume reaction of 400 uL for 60 minutes in a orbital shaker at room temperature. After centrifugation (15.000 g x 5 minutes), the supernatants were harvested and the resulting chitin pellets were washed five times with buffer A. Proteins were eluted by incubating the chitin pellet with Laemmli buffer 1X, boiled for 5 minutes and run in a 15% SDS-PAGE. A chitin binding probe (New England Biolabs) was used as positive control.

2.8.2. Animal model of Blo t 12 sensitization

2.8.2.1. Preparation of Chitin Particles

Chitin fragments were obtained as described in other publications [50]. Chitin powder (derived from crab shells) was treated with NaOH 0.1 M (30 minutes at room temperature) for endotoxin removal [113]. Chitin was precipitated by centrifugation and washed with sterile PBS twice. The PBS- suspended chitin (10 mg/mL) was sonicated at 30% output for 5 minutes and filtered with nylon cell strainers (100, 70 and 40 um) to obtain fractions with different size fragments. Intermediate size chitin (40-70 um fraction) were used for immunizing animals [50].

2.8.2.2. Preparation of Allergens

Recombinant Blo t 12.0101 and Blo t 12.0102 were obtained in pET45b+/Origami (DE3) expression systems as described above. For endotoxin depletion, Triton X-114 was added to the allergen solution at 1% final concentration and incubated at 4°C in a rotor for 40 minutes, with occasional vortexing every 10 minutes. Samples were then incubated for 10 minutes at 37°C and centrifuged at 20.000 g for 20 minutes. After that, two liquid phases are visible and the upper one is recovered and treated again with this detergent under the same conditions for a total of three

cycles. Endotoxin depletion was confirmed by Limulus assay (Bio Whittaker, Walkersville, MD), following the manufacturer's instructions.

2.8.2.3. Immunization protocol

Six-week-old female BALB/c mice (from Instituto Nacional de Salud; Bogota, Colombia) were kept under standard conditions in the Animal Resource Center of the University of Cartagena. All experiments were performed in accordance with University guidelines and protocols were approved by the local ethical committee of this institution.

Mice received three intraperitoneal injections (Day 0, 7 and 14) of chitin (25 μg) plus Blo t 12.0101 or Blo t12.0102. Twenty micrograms of the allergen were administered each time. Seven days after the last immunization, mice were challenged daily (3X) with 10 μg of Blo t 12.0101 or Blo t 12.0101, respectively. The groups receiving chitin i.p. also received it intra-nasally. Other groups of mice received the isoallergens with the classical adjuvant: alum (2 mg). Each experimental group contained 5 or 6 mice.

2.8.2.4. Measurement of Airway Hyperreactivity

Lung function was assessed by methacholine-induced airflow obstruction, using a whole-body plethysmograph (Buxco Electronics, USA). Methacoline was diluted in water at different concentrations (50, 25, 12.5, 6.13 and 3.1 mg/mL) and administered in aerosol (90 seconds, 50 μL per chamber). Bronchial hyperreactivity was evaluated by means of enhanced pause (Penh) values [114], an indirect determination of this parameter that can be obtained in conscious animals.

2.8.2.5. Histology

Mice were killed 24 hours later and lungs were immersed in 10% neutral buffered formalin. Hematoxylin and eosin and periodic acid-Schiff stains were performed in the Histology Laboratory of the University of Cartagena (Dr. Ines Benedetti).

2.8.2.6. Serology determinations

Allergen specific IgE, IgG1 and IgG2a levels were determined in mice by ELISA. Maxisorp™ microtiter plates (were coated with the allergens at 5 µg/mL by overnight incubation at 4°C. Wells were then blocked with PBS 1% BSA 0.05% Tween 20 for 3 hours at RT. Plasma samples were incubated ON at 4°C. After 5 washes, wells were incubated with biotin labeled anti-mouse IgG1, IgG2a or IgE (diluted 1:1.000 in blocking buffer) for 1 hour at RT. After 5 washes, alkaline-phosphatase streptavidine (diluted 1:2.000 in 50 mM Tris 1% BSA 1 mM $MgCl_2$ pH 8.0) was added to the wells. Para-nytrophenil diphosphate, dissolved in 10% diethanolamine 0.05 mM $MgCl_2$ at 1 mg/mL, was used as substrate solution. After 30 minutes of incubation, the reaction was stopped with NaOH 3N. Optical densities were obtained by reading the plates in a spectrophotometer.

2.9. Birth cohort study

2.9.1. Epidemiological description of the cohort

For a fully detailed epidemiological description of this cohort, please refer to the methodology described in the Publication N°2 of this thesis [102].

2.9.2. Serum samples

During children's follow-up serum samples were obtained at different ages, whenever possible, either during planned clinical evaluations at the outpatient service or during domiciliary visits. The samples were grouped into four categories according to the age of collection: S1 (6-11 months), S2 (12-19 months), S3 (20-29months) and S4 (30-42 months). Six months was the minimum interval-time for serial serum collection.

Cord blood (CB) samples were obtained from the maternal portion immediately after delivery using a careful technique to avoid maternal-blood contamination[6]. Total immunoglobulin A levels (IgA) were quantified in all CB samples by immunoturbidimetry (Roche Diagnostic Systems, Berne, Switzerland). Only those with IgA below 4 mg/dL were included in the analysis. Blood samples from mothers were collected 12 hours after delivery to obtain sera for antibody determinations.

2.9.3. Quantification of Total and Specific IgE

IgE levels were determined using ImmunoCap system (Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden) according to the manufacturer's instructions. Total IgE and specific IgE to *B. tropicalis*, *D. pteronyssinus* and the nematode *Ascaris spp.* were measured in CB and at different time-points between 0 and 42 months. A low range assay was chosen for total and specific IgE determination; the minimum detection limit for both assays was 0.01 kU/L. In mothers, specific IgE to rDer p 2 (Accession number: ABG76196), rBlo t 5 (O96870), rBlo t 12.0101 and rABA-1 was also measured by ELISA. In mothers, specific IgE sensitization was also evaluated by skin prick test (SPT) against a battery of aeroallergens.

Specific IgE to Blo t 5, Blo t 12.0101, AscGST, Asc s 1 (ABA-1) and Asc l 3 was also determined in children at different periods (S1 to S4). Details about the ELISA protocol are described above (Section Household indirect ELISA for specific IgE determination). Cut-off points for recombinant allergens were chosen using receiver operator characteristic (ROC) curve analysis, defining as the state variable a positive result to *B. tropicalis* (for Blo t 5 and Blo t 12) or *Ascaris* (Asc s 1, Asc l 3 and AscGST) extracts by ImmunoCap.

2.9.4. Stool samples and parasitological examination

Parasitological analyses were done as described previously[6]. The results were expressed as egg per gram of feces (epg). The presence of eggs from geohelminths or parasite visualization was considered diagnostics of active infection.

To avoid false negatives, information about Ascaris infection was considered for those children with at least two parasitological examinations during follow up, including only those whose last examination was done at 24 months or older. In total, data from 162 children were used for analyses when the effect of Ascaris infection was evaluated. Ninety seven percent (n = 157) of children had more than three recorded stool examinations. The cumulative prevalence of a positive stool examination for A. lumbricoides was 10.5% and 1.8% for *Trichuris trichuria*.

2.9.5. Definitions of clinical phenotypes and other outcomes

Wheezing was defined as expiratory stridor with shortness of breath or whistling[6]. **Recurrent wheezing:** 3 or more episodes of wheezing in 12 months. **Maternal asthma and other allergic diseases:** The diagnosis of asthma and other allergic diseases among mothers were done using a questionnaire [4] and examination by a physician. **Sensitization**: A positive IgE value (> 0.35 kU_A/L, ImmunoCap) to any of the tested allergens. **HDM sensitization:** A positive IgE to B. tropicalis or D. pteronyssinus extracts. **HDM components' sensitization**: A positive IgE value to at least one of the recombinant allergens (rBlo t 5, rBlo t 12 and rDer p 2) in the mother. This variable was used to evaluate the influence of maternal HDM sensitization on child´s outcomes avoiding confusion due to nematode cross-reactive antigens. **High total IgE:** IgE value greater than the 70^{th} percentile in mothers and children. For CB total IgE, 80^{th} percentile was used as cutoff [115] .

2.9.6. Collection of dust samples

For measuring allergen and endotoxin levels, house-dust samples were collected at children homes using a vacuum cleaner (LG, Model V-CA241HT) adapted to 25 μM cellulose filters. The children's bed and bedroom's floor were aspirated during two minutes each over a 1 m2 area as described [29]. Dust samples were weighted and placed in sterile recipients. For endotoxin and allergen quantification the samples were extracted in 2 mL of pyrogen-free water-0.05% Tween 20 (1:20) per 100 grams of fine dust, mixed by rotation during 1 hour at room temperature and centrifuged at 3000 *g* during 10 minutes at 4°C. An aliquot of the supernatant (1/10 of the total volume) was kept at -20°C for endotoxin quantification. The remaining volume was mixed with PBS 10x-0.05% Tween 20 and PMSF at a final concentration of 1 mM and mixed by overnight rotation. The aliquots for measuring allergen concentrations were obtained after centrifugation at 3000 *g* during 10 minutes and kept at -20°C.

2.9.7. Statistical analysis

All analyses were performed using SPSS version 13.0 (Chicago, IL, USA). Total and specific IgE values were not normally distributed; descriptive information about these data for each age category was reported as the median value and its inter-quartile range. Mann-Whitney U test was used for comparison of continuous variables. Differences between proportions were analyzed by Pearson chi-squared test. A linear by linear association chi-squared test was employed to compare frequencies of IgE sensitization among age categories. Concordance between SPT results and maternal serology was evaluated by κ coefficient. Since frequencies of sensitization were higher when detected in vitro than by SPT, the first were chosen for analyses.

Univariate and multivariate binary logistic regression were used to analyze the relationships of exposures and outcomes. The predictor/exposures included: socio-demographic conditions, maternal IgE, level of CB total IgE and levels of children IgE. The outcomes were: CB total IgE, HDM sensitization in children, Ascaris sensitization in children, wheezing at 24 months and recurrent wheezing. Factors associated to the outcome in the univariate analysis were included in the multivariate model. Crude (OR) and adjusted odds ratios (aOR), 95% confidence intervals (95% CI (OR) and p-values were calculated.

Linear mixed models (LMM) [116, 117] were used to assess whether serial determinations of total IgE (log-transformed values) and mite- or Ascaris-specific IgE were affected by binary variables such as high CB total IgE, recurrent wheezing and Ascaris infection (treated as *fixed factors*). The indicator of a repeated measure was the variable *time* that grouped IgE serology data within the specified age-ranges (S1 to S4).

Population-attributable fractions (PAFs) were calculated by the formula: PAF = P_{ew} * (OR-1)/OR where P_{ew} is the HDM sensitization rates among wheezing children [118]. Power was calculated assuming an independent case-control design as described previously [119].

Results

Chapter 3. Immunological and biological characterization of group 12 allergens from *Blomia tropicalis*

Overview

Since the first description of Blo t 12 as an allergen [48], there was few information about the importance of this molecule in terms of its immunological and biological activity. However, in the preliminary phase of this work, bioinformatics analysis of its sequence revealed that Blo t 12 could represent an interesting allergen for molecular characterization and prompted to develop an important part of this doctoral thesis. There has not been found any homologous allergen in *Dermatophagoides spp.*, in spite that transcript sequences from these HDM species are fully represented in public gene databases due to the extensive searches of cDNA libraries that has been performed by molecular cloning methods [38]. Hence, Blo t 12 became a potential species-specific allergen from *B. tropicalis* species, which could contribute to the development of allergen batteries for component resolved diagnosis and tailored-immunotherapy of allergic diseases in the tropics. Chua YK and colleagues cloned another Blo t 12 isoform with 92% of sequence similarity to Blo t 12.0101. Since amino acids variations in isoallergens may influence its allergenicity [11, 120]; we compared the IgE immune responses to these two isoallergens in a sample population from Cartagena, Colombia and concluded that Blo t 12.0101 was the most important isoform in terms of IgE recognition - and fully representative of group 12 from *B. tropicalis* -. Then, other aspects related to the usefulness of Blo t 12.0101 as a tool for allergy diagnosis were investigated. Its lack of CR with other allergens sources, including various HDM species and other arthropods of agricultural research, was confirmed experimentally. Since the use of an allergen as a clinical reagent requires its production at substantial quantities, we explored the physico-chemical properties of this allergen expressed as a recombinant protein, in two different expression systems: *E. coli* and *P. pastoris*). Another product from this research was the development of an ELISA sandwich system with enough sensitivity to detect Blo t 12 in *B. tropicalis* extracts and house dust samples.

Blo t 12 is closely related to few known proteins, but conserves key residues for chitin binding activity, which are also found in certain proteins from the peritrophic membranes of arthropods [121]. The finding that this allergen was related to chitin coincided with an important moment in the study about the role of this carbohydrate in allergic responses. In the last five years, it has been described its adjuvant properties on allergic responses, but also its concomitant and heterogeneous effects in the immune system [49-51]. Motivated by this knowledge, we hypothesized that Blo t 12 allergenicity is promoted by its chitin-binding activity. With our experiments, we confirmed that, in fact, Blo t 12 isoallergens bind chitin as predicted by bioinformatics. However, in a murine model of allergen sensitization and airway hyperreactivity, it was observed that chitin binding activity had a moderate effect on the immune responses that Blo t 12 induced, specifically in the bronchial hyperreactivity and total IgE levels stimulated by the Blo t 12.0101 isoform. It can be also concluded from these experiments that both Blo t 12 isoallergens are highly immunogenic, because its administration without an adjuvant was enough to generate airway inflammation.

3.1. Evaluation of IgE immune response to Blo t 12 isoforms[1]

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3.1.1. Introduction

House dust mites exposure is a major risk for allergic sensitization and asthma development [122]. In tropical and subtropical places, *Blomia tropicalis* is one of the most important sources of clinically relevant aeroallergens [27, 123, 124]. Blo t 12 (Blo t 12.0101) was first identified by IgE immunoscreening in a *B. tropicalis* cDNA library constructed from mites collected in Cartagena [48], showing an IgE binding frequency of 50% when was tested in 32 individual sera by plaque assay. No homologous has been described for this allergen in the genus *Dermatophagoides*, the other important allergen source in tropical countries, suggesting that it could be included in allergen panels for CRD and SIT

Isoallergens is a term used to describe two related allergens with more than 67% of similarity in their sequence [125]. When only few numbers of variations are present, they could be also named as isoforms. Allergen polymorphisms could influence their allergenicity and their prevalence often varies among different environments [11, 12]. Therefore, identification of allergen variants should be followed by an evaluation of their IgE binding properties, especially, if they have a potential use as clinical reagents

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[1] These results were published in Clin. Exp. Allergy (2009) with the title: Immunological characterization of a Blo t 12 isoallergen: identification of immunoglobulin E epitopes.

for allergy diagnosis or SIT. In this study, we evaluated the IgE response to a locally isolated Blo t 12 isoallergen (Blo t 12.0101) in asthmatic patients from Cartagena, Colombia and performed linear epitope mapping. We also isolated, cloned and expressed another Blo t 12 isoform from mites collected in Singapore, Blo t 12.0102 and compared its IgE binding properties with Blo t 12.0101 to identify if their amino acid variations influence their allergenicity. To date, only these two Blo t 12 isoforms have been reported.

3.1.2. Results

Amino acid variations of Blo t 12 isoallergens could influence their tertiary structure

Figure 3.1 shows an alignment of the amino acid sequences of Blo t 12 isoallergens. Both proteins contain a predicted type II chitin-binding domain (CBD-II), similar to that found in other invertebrates and human chitinases.

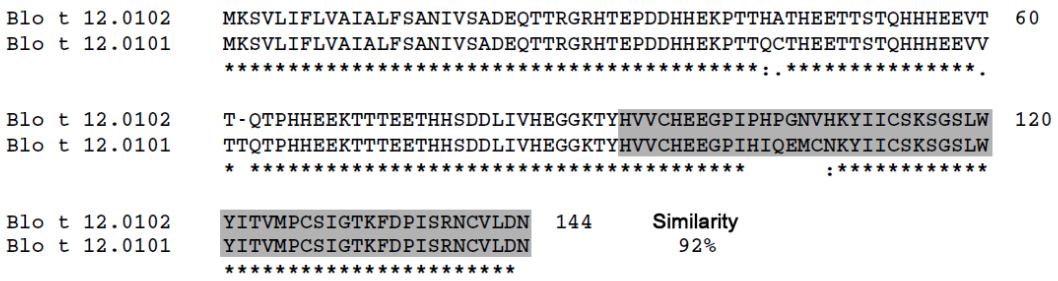

Figure 3.1 Amino acid sequences alignment of Blo t 12.0101 and Blo t 12.0102. Amino acid sequence residues are numbered at the end of each row. The first 20 residues correspond to a signal peptide. The predicted chitin binding domain type II (CBD-II) is shadowed.

Folding-based modeling of Blo t 12 isoallergens could only be carried out for the predicted chitin-binding domain. No reliable results could be obtained for the complete allergen. Superposition of both tertiary structures indicates that although the CBD-II from Blo t 12 isoallergens share a similar architecture (RMSD: 1.03 A°), amino acid

variations clearly influence secondary structure assignments. It is worth noting that loop and β-strand lengths are markedly different between these CBD-II domains (Figure 3.2). Although seven consecutive residues in this domain are different between these isoallergens, superposition of the two models showed that these variations did not affect local structural assignment; however, it does modify the spatial restraints of the loop region that connect this area with the next β-strand.

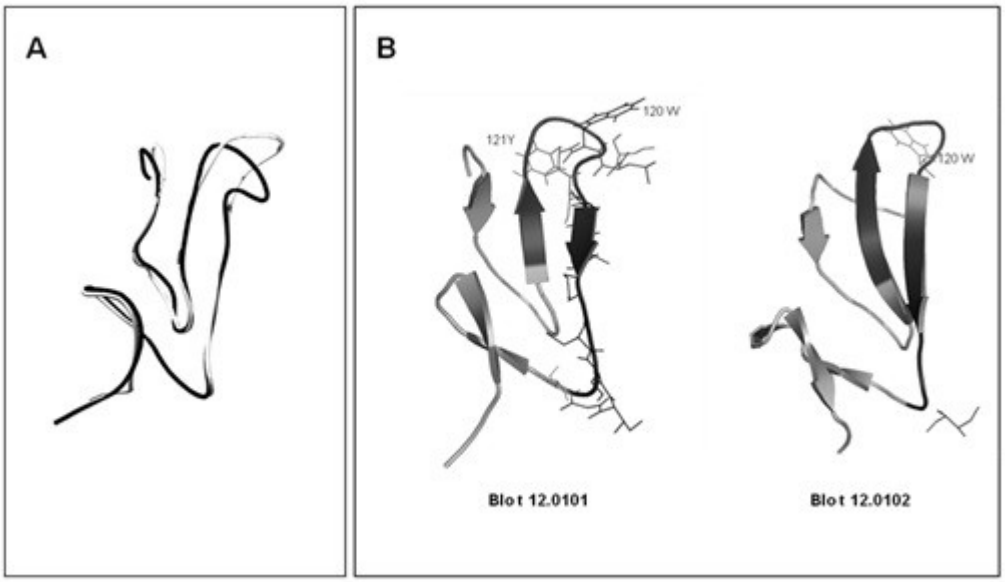

Figure 3.2. Molecular modeling of the chitin binding domain of Blo t 12 isoforms. (a) Superposition of CBD-II structural models from Blo t 12 isoallergens; Blo t 12.0101 (black), Blo t 12.0102 (gray). (b) In black, the location of E9 in chitin-binding domain type II (CBD-II) models. Residues identified by Hotpatch as part of this epitope are shown as sticks. Aromatic residues are labeled. (b) Superposition of CBD-II structural models from Blo t 12 isoallergens; Blo t 12.0101 (black), Blo t 12.0102 (gray).

Asthmatic patients from Cartagena showed a reduced Immunoglobulin E response against a naturally occurring isoallergen of Blo t 12

Specific IgE levels to Blo t 12 isoallergens were analyzed in sera from 107 asthmatic subjects. This population had a mean age of 36.23±18 and 46% were males. Eighty-one percent of them also had a physician diagnosis of allergic rhinitis. In the subgroup that was SPT, 95% were co-sensitized to *D. pteronyssinus*. The cut-off value for ELISA was 0.13 OD. The frequencies of IgE reactivity to Blo t 12.0101 and Blo t

12.0102 were 35.5% and 23%, respectively (p = 0.12). Twenty-four patients were positive to both isoforms, 14 to Blo t 12.0101 only, one patient was positive to Blo t 12.0102 only and 68 patients were negative for both isoforms. Most patients (10 out of 14) who reacted only to Blo t 12.0101 had lower specific IgE levels than those reacting to both isoforms (mean value, 0.147 vs. 0.535 OD). A high correlation is observed between the log-specific IgE values against these isoallergens (Figure 3.3). In contrast, specific IgE to any of the two Blo t 12 variants was not correlated with B. tropicalis-specific IgE levels, or total IgE.

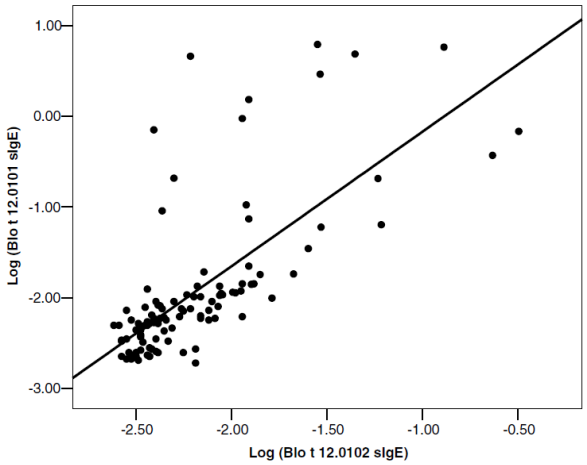

Figure 3.3. Correlation of specific IgE levels to Blo t 12 isoallergens. Scatter dot plot representing the correlation observed between log-specific IgE levels to Blo t 12.0101 and to Blo t 12.0102. Specific IgE values were measured by ELISA.

Specific IgE levels against Blo t 12.0101 were significantly higher than those to Blo t 12.0102 (0.25 ±0.43 vs. 0.124 ±0.08 OD, p = 0.001) (Figure 3.4) especially in those with a high response to Blo t 12.0101, which represents 50% of all positive cases. Similarly, the strength of the IgE response, as determined by the diameter of weal in SPT positive subjects to Blo t 12.0101, was greater than that to Blo t 12.0102 (mean values, 4.5 vs. 2.3 mm, p = 0.001. For both isoforms, a significant correlation was found between log-specific IgE levels and wheal diameter (Blo t 12.0101: r = 0.32, p = 0.027; Blo t 12.0102: r = 0.51, p=0.001). Blo t 12.0101 and Blo t 12.0102 inhibited 95% and 20% of IgE binding, respectively, when the former was in the solid phase. Inversely, Blo t 12.0102 in the solid phase was inhibited by Blo t 12.0101 as much as its homologous inhibition under both conditions, using a serum pool from Colombian patients and another from Singapore asthmatic individuals (Figure 3.5).

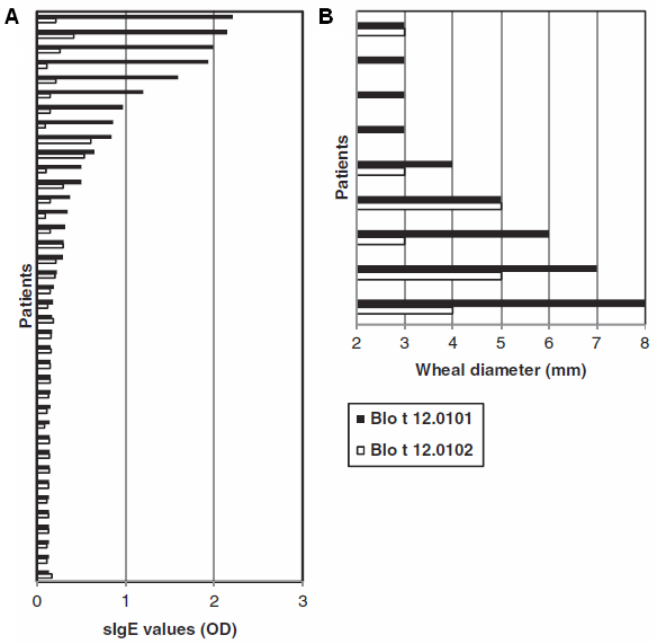

Figure 3.4 Specific IgE levels and skin prick test (SPT) results for Blo t 12 isoallergens. A) Horizontal bars represent specific IgE values against Blo t 12 isoallergens in each Blo t 12.0101 allergic patient; B) weal diameter values are induced by these isoallergens in SPT-positive subjects to Blo t 12.0101.

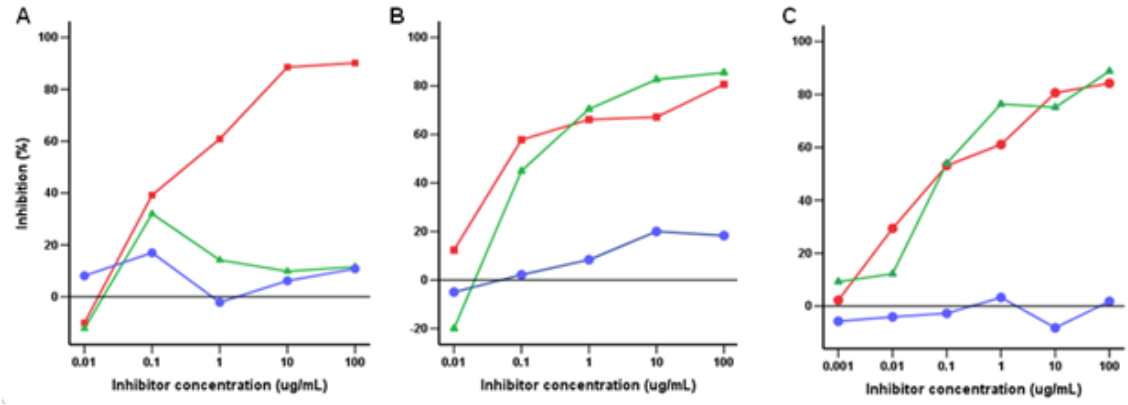

Figure 3.5. ELISA inhibition assays for assessment of Blo t 12 isoallergens cross-reactivity. A) Blo t 12.0101 in the solid phase, using a serum pool from Colombia patients who showed a high reactivity to Blo t 12.0101 but not to Blo t 12.0102. B) Blo t 12.0102 in the solid phase using a serum pool from Colombian patients or C) Singaporean allergic subjects. Blo t12.0101 (red), Blo t 12.0102 (green) and BSA (blue).

Linear epitope mapping reveals three IgE binding regions in Blo t 12.0101

Inhibition ELISA experiments with overlapping synthetic peptides identified three IgE binding areas in Blo t 12.0101. Peptide 9 inhibited IgE binding in 3 of 5 Blo t 12.0101 allergic sera, reducing OD values in more than 60% and identifying E9 epitope. Peptides 8 and 10, which share ten and seven residues with peptide 9 respectively, showed null or low inhibition of IgE binding to the full-length isoallergen, indicating that the three amino acids (Y121, I122 and T123) found only in E9 are critical for antibody recognition. Overlapping peptides 1, 2 and 3 also identified a region in Blo t 12.0101 that binds IgE (E2). In one patient, peptide 5 and 6 inhibited more than 50% of IgE binding from the complete allergen. These two peptides have an overlapping sequence of 6 amino acids and identify E6 epitope. Two amino acid variations between these isoallergens are included within this IgE binding epitope. Furthermore, a dose-response relationship was observed when different concentrations of peptides 2 and 9 were used for inhibition experiments (Figure 3.8). In two patients, none of these peptides inhibited IgE binding to the full length allergen. BSA, which was used as negative control, did not inhibit IgE binding to this allergen. Patients A550 and A368, who were also skin prick tested, showed a lower response to Blo t 12.0102 than to Blo t 12.0101; however, linear IgE epitopes were only recognized in one of this two patients (A550).

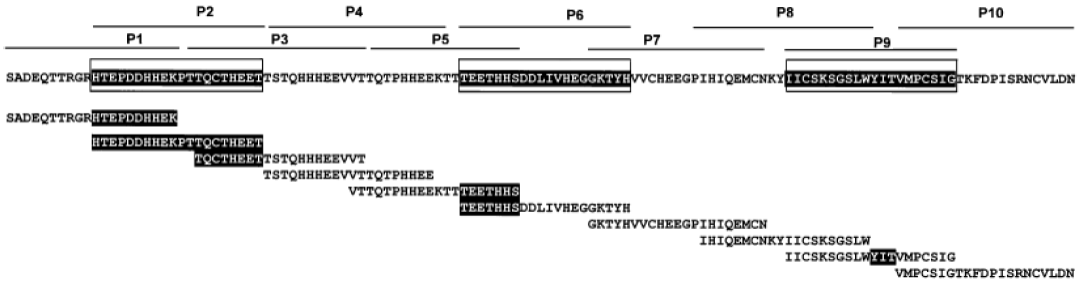

Figure 3.6 Amino acid sequences of overlapping peptides covering matured Blo t 12.0101. Antigenic regions identified in epitope mapping experiments are boxed and shaded in the sequence.

In silico structural analysis concurred with experimental data in identification of E9 as an epitope

Location of E9 in the tertiary structure of Blo t 12.0101 CBD-II showed that this region is a solvent accessible area composed by two beta strands connected by a loop. In Blo t 12.0101, the most extensive stretch of accessible residues is included in the epitope identified by peptide 9 (Figure 3.2). Using Hotpatch, an algorithm that predict sites in proteins that participate in protein binding (in this case, IgE antibodies); we identified two solvent accessible patches in the CBD-II of Blo t 12.0101 that correspond to most of P9 sequence. In contrast, the same analysis applied to Blo t 12.0102 identified only two common residues.

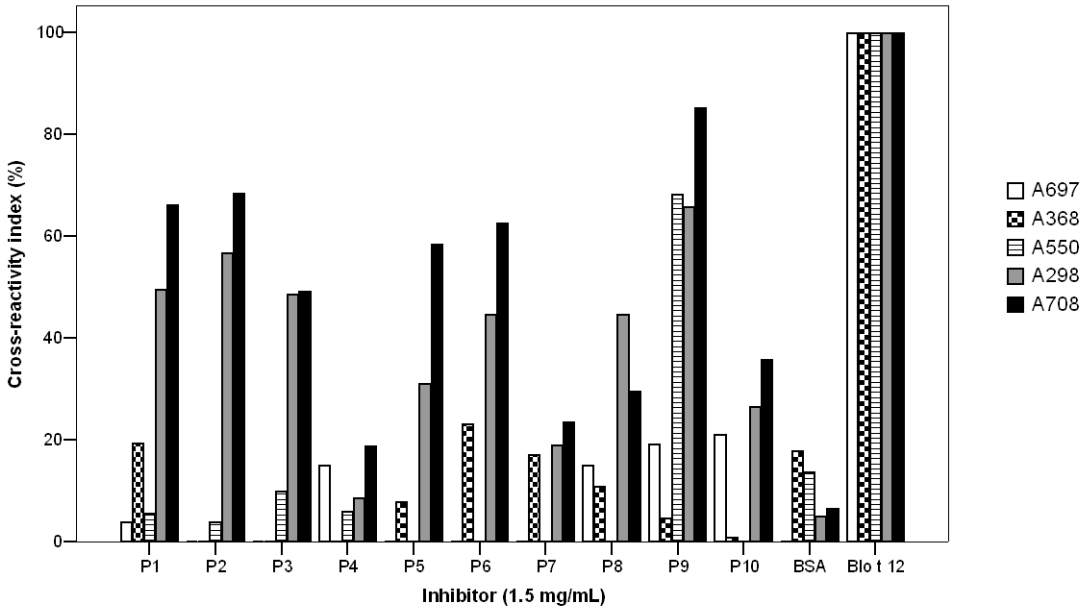

Figure 3.7 Cross-reactivity index between 10 synthetic peptides and complete Blo t 12.0101. Diluted sera were preadsorbed with each of the ten overlapping peptides, BSA or Blo t 12.0101 and reacted with the plate-bound Blo t 12.0101. A significant inhibition was considered when OD was reduced by more than 50%.

3.1.3. Discussion of results

In this study we detected that 35.5% of 107 asthmatic subjects living in Cartagena, Colombia were sensitized to Blo t 12.0101, the locally isolated isoallergen. In this population, higher IgE sensitization rates to *Blomia tropicalis* allergens have been observed only for the recombinant BtM, a partial clone of Blo t 5 [126]. According

to this, Blo t 12.0101 is an important *B. tropicalis* allergen in this tropical city where this mite, with *Dermatophagoides pteronyssinus,* are causes of allergic sensitization and a risk factor for emergency visits due to asthma exacerbations [14]. Since no homolog cross-reactive allergen has been reported in *Dermatophagoides spp*, it could be included as a component for allergy diagnosis and immunotherapy in this population.

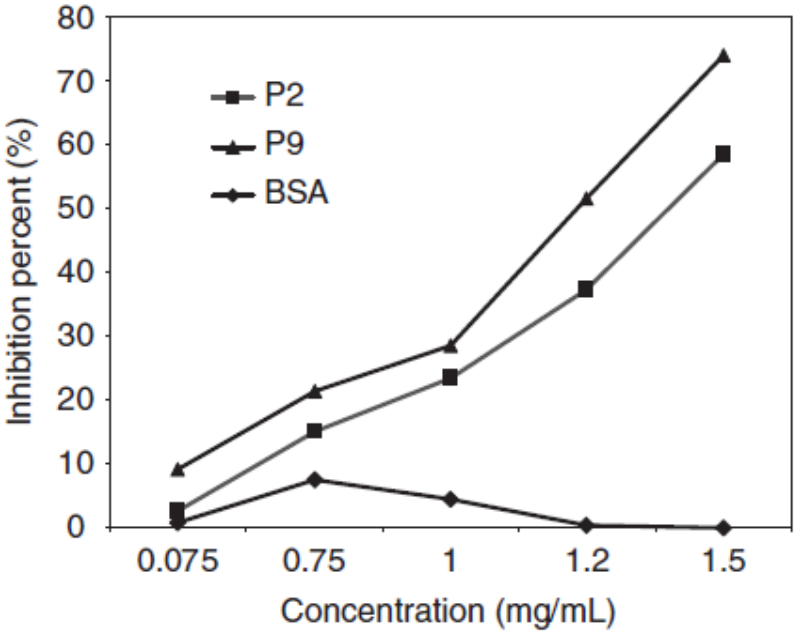

Figure 3.8 Dose-response curves for peptides 2 and 9 inhibiting IgE binding to Blo t 12.0101 isoallergen. Peptide 2 and 9 showed 58% and 74% of maximum inhibition respectively. BSA was used as negative control.

Genetic variation of isoallergens could influence their IgE binding, T cell response and clinical reactivity [127]. Variations in IgE reactivity to isoforms can be due to differences in exposition, since the distribution of isoallergens vary among environments. In the case of group 2 allergens from *Dermatophagoides pteronyssinus*, it has been showed that there are distinct prevalent isoforms in European and Asiatic countries, being the IgE response against them directly related to their presence in those regions [127, 128]. It is also possible that some amino acid changes affect the allergenic potential of the molecule. For example, Bet v 1 isoforms isolated from the

same birch extract showed a markedly different serological and clinical IgE response [11]. In this study, we compared the IgE response against two isoallergens isolated from different continents (America and Asia) because this could help to understand the importance of considering isoallergens when designing allergy diagnosis and treatment. We have detected that polymorphisms of Blo t 12 influence its IgE binding and reactivity in asthmatic patients, suggesting clinical relevance. Since Blo t 12.0101 inhibited Blo t 12.0102 as much as its homologous inhibition when using serum pool from Singapore, we hypothesize that all the Blo t 12.0102 epitopes are present in Blo t 12.0101, but unique epitopes exist in Blo t 12.0101, indicating that in spite of the immunological importance of Blo t 12.0102 in our population, Blo t 12.0101 is representative of both isoforms regarding to IgE epitopes. Thus, the locally isolated isoform is the most appropriate one for diagnosing IgE sensitization against Blo t 12 in Cartagena.

Blo t 12.0101 could also be clinically relevant in other populations, especially in America. Fifty percent of IgE reactivity against Blo t 12.0101 was observed in 36 allergic subjects from Tampa, USA [11]. Preliminary results from Havana, Cuba showed an IgE sensitization frequency of 37.2 % in the 42 tested patients (Puerta L. et al, unpublished). Although it is possible that the 0101 isoform is more relevant in this continent, IgE reactivity against Blo t 12.0102 has to be assessed to confirm it. Identification and quantification of these and other natural isoallergens by proteomic approaches must be done using allergen extracts to evaluate if there are differences in their geographical distribution. [129].

We further characterized at the epitope level the IgE response to Blo t 12.0101, the most important isoallergen in Cartagena. Three IgE binding regions were detected by means of ELISA inhibition: E2, E6 and E9. Two residues of E2 are not conserved in these isoallergens. Thus, E2 could be absent in Blo t 12.0102, explaining why we found a lower antibody reactivity to this isoform. E9 was the most immunoreactive epitope detected in Blo t 12.0101. By using overlapping peptides we could identify that Y121, I122 and T123 are critical residues for IgE binding to E9. Its amino acid composition is in agreement with a recent analysis about common residues in antigen-antibody complexes that found, as we observed in Blo t 12.0101, that tyrosines and tryptophans

are more frequent in antigenic sites than in no immunogenic surfaces [130]. Interestingly, mapped IgE epitopes from Hev b 6, another CBP, are also enriched with aromatic residues [93].

Conventional methods for B cell epitope prediction do not consider that aromatic amino acids have a high probability to participate in antigenic sites because of their hydrophobic character. In contrast, some structural-based algorithms assign high scores to those patches including hydrophobic solvent-accessible residues to predict protein-protein interaction interfaces. Hotpatch, one of them, predicted most residues from E9 as part of a potential protein binding site, but only for Blo t 12.0101. Relative ASA values analysis also supported our experimental finding: the most extensive stretch of solvent-exposed residues found in Blo t 12.0101 (greater than 8 amino acids) is included in the IgE binding region detected by peptide 9, however, in Blo t 12.0102 this region is less accessible, and some residues are buried in the molecular structure. Thus, it is possible that in spite E9 sequence is common in the two isoforms, the accessibility differences influence antibody recognition. IgE specific to the E9 epitope found in Blo t 12.0101 could bind with a lower affinity to the corresponding region in Blo t 12.0102.

Linear epitopes were not recognized by IgE antibodies from two patient's sera, suggesting that conformational epitopes are also present in this allergen. Identification of these epitopes with more refined technology is required to identify all clinically relevant IgE binding areas of Blo t 12. Noteworthy, a high molar excess of peptides with respect to the complete allergen were needed to achieve a significant IgE binding inhibition, indicating that Blo t 12 specific IgE antibodies have lower affinity to these reactive peptides than that to their respective epitopes in the complete allergen. A low affinity between complementary free peptides and antibodies could be explained by a loss of entropy on binding reaction due to a high flexibility and mobility of the former [9]. However, it is also possible that the identified linear epitopes are only a segment of larger conformational epitopes.

In conclusion, immunological characterization of two Blo t 12 isoforms helped us to identify Blo t 12.0101 as the most important isoallergen in Cartagena. Epitope mapping experiments identified three IgE binding regions in this molecule that could be

mutated to obtain hypoallergenic variants. Since IgE response to Blo t 12.0102 is lower than that to our local isoform, Blo t 12.0101 should be selected for diagnosis. Identification of the most important isoallergen in other populations should be also addressed locally.

3.2. Physico-chemical characterization of Pichia pastoris produced Blo t 12.0101

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3.2.1. Introduction

Blo t 12.0101 is a representative isoform of its allergen group[36]. IgE recognition of this allergen in various tropical populations is frequent. The interesting results obtained from the immunological characterization of this allergen in our population led us to investigate with more detail the physico-chemical properties of this recombinant allergen, produced in *Pichia pastoris,* in order to test its performance as a potential reagent for allergy diagnosis. Several aspects such as identity, aggregation behavior, post-translational modifications and their impact on immune reactivity were analyzed.

3.2.2. Results

Blo t 12.0101 in solution had a different behavior to that observed in SDS-PAGE

Blo t 12.0101 has a theoretical molecular weight of 14.2 kDa; however, in SDS-PAGE under reducing conditions, pBlo t 12.0101 was observed mainly as a 30-31 kDa band, but also as high MW aggregates (60 and 90 kDa) and in a lesser extent as a 17 kDa band. SECTDA analyisis revealed that pBlo t 12.0101 in solution was mainly a monomer (16-17 kDA, 59%), but also aggregated itself as dimers (16%), trimers (9%) and other multimers (13%). The heterogeneity of the sample and the higher MW than expected is suggesting that pBlo t 12.0101 has post-translational modifications retarding electrophoretic migration (Figure 3.9).

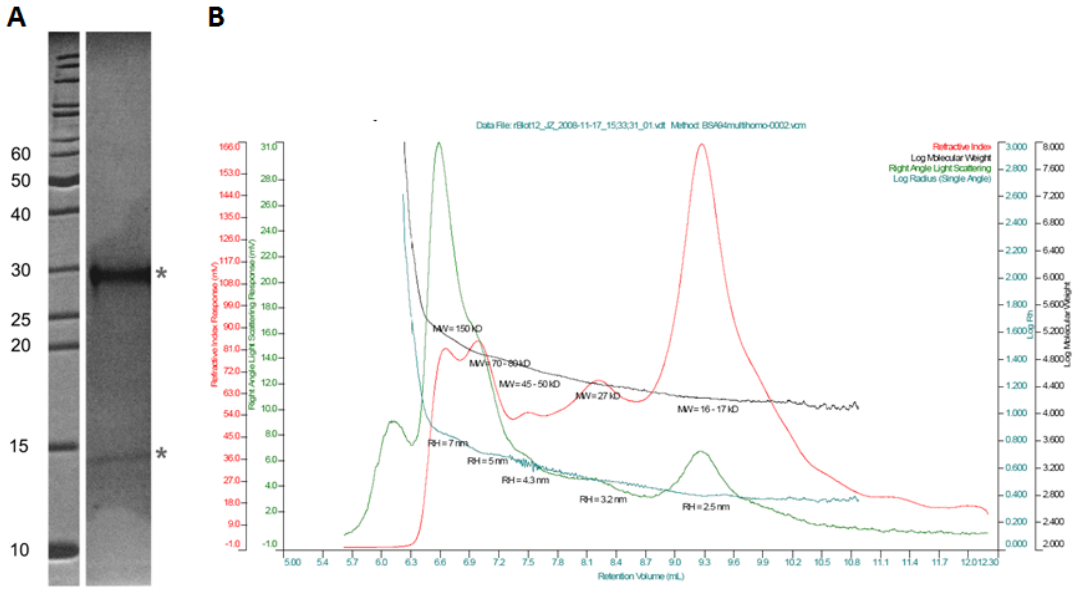


Figure 3.9. Aggregation behavior of pBlo t 12.0101. A) 15% SDS-PAGE showing electrophoretic migration of this allergen. Asterisks indicate the 14 and 29 kDa band. B) Results of SECTDA analysis

Hyperglycosylation of Blo t 12.0101 produced in Pichia pastoris could affect its diagnostic utility

A positive signal of glycosylation was detected in pBlo t 12.0101, but not in pBlo t 12.0102 (Figure 3.10a). Glycans modifying pBlo t 12.0101 were identified as high molecular weight mannoses (Figure 3.10b), typically observed in O-glycosidically linked mannoses from yeast glycoproteins. Moreover, MS-MS analysis of pBlo t 12.0101 detected glycan modifications in threonine residues of position 61-62, which are usually glycosylated at O-sites. Deletion of Thr61 in Blo t 12.0102 could cause its lack of glycosylation.

To analyze the influence of glycosylation on antibody recognition, pBlo t 12.0101 was deglycosylated and its IgE reactivity was tested by indirect ELISA. As expected for an O-glycosylated protein, treatment of pBlot 12.0101 with PNGase F did not change its molecular weight (Figure 3.11). In contrast, deglycosylation was successful by treating the protein with TFMS. As observed in Figure 3.11, the TFMS-treated allergen had a

lower molecular weight and it was not detected in the PAS staining. Although Blo t 12.0101 was used as immunogen to raise pAbs in rabbits, the obtained antiserum recognized pBlo t 12.0102 more strongly. However, after metaperiodate-oxidation, pBlo t 12.0101 recognition by anti-Blot 12.0101 rabbit pAb increased (Figure 3.12b). Human-IgE binding to pBlo t 12.0101 was also more intense after chemical deglycosylation of the allergen (Means, 1.59 vs. 2.17 O.D., p=0.03; Figure 3.12b).

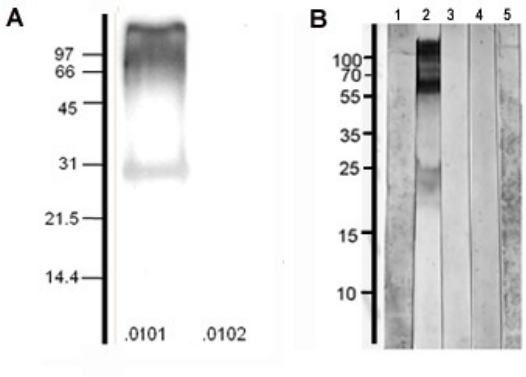


Figure 3.10 Glycosylation of Blo t 12 isoforms produced in *Pichia pastoris.* A. Western Blot of Blo t 12 isoforms using the ECL glycosylation detection module B. Detection of the sugar type of pBlo t12.0101 with the lectins DSA (Lane 1), GNA (Lane 2), MAA (Lane 3), PNA (Lane 4) and SNA (Lane 5).

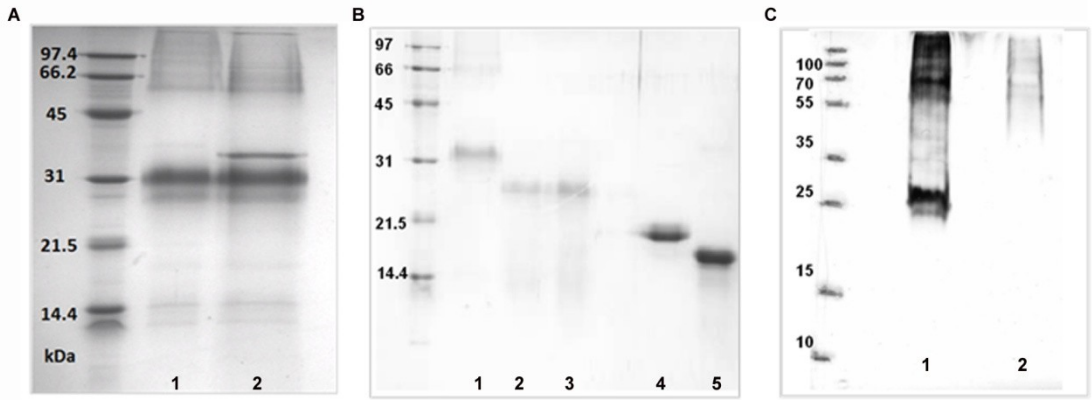
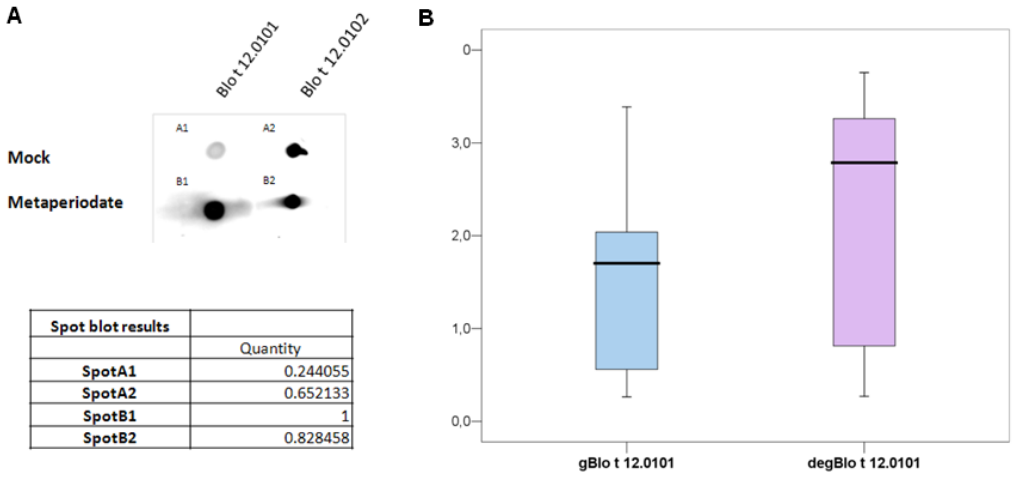


Figure 3.11. Deglycosylation of Blo t 12.0101. A) pBlo t 12.0101 treated (Lane 2) or not (Lane 1) with PNGase F B) pBlo t12.0101 before (Lane 1) and after treatment with TMSF (Lane 2 and 3); in lane 4 and 5, ribonuclease (positive control) before and after TMSF treatment, respectively. C) Schiff staining of pBlo t 12.0101 before (Lane 1) and after TMSF treatment.



Spot blot results	
	Quantity
SpotA1	0.244055
SpotA2	0.652133
SpotB1	1
SpotB2	0.828458

Figure 3.12. Influence of glycosylation on Blo t 12 antibody binding activity. A) Dot blots showing the effect of metaperiodate oxidation on pAb IgG binding. Densitometry analysis of the image is shown in the table below; B1, the most intense spot was taken as reference to calculate intensity value of the other spots B) Box plots of specific IgE determinations (n=6) against glycosylated pBlo t 12.0101 and the TMSF-deglycosylated allergen.

3.2.3. Discussion of results

Here we found very interesting aspects about the physico-chemical properties of the Blo t 12.0101 obtained in *Pichia pastoris*. Its unexpected relative MW in SDS-PAGE is not due to a natural process of protein dimerization, since in solution it is mainly observed as a monomer. A retarded migration seems to be the cause of its higher MW. This pattern has also been observed in peritrophic proteins, a particular type of proteins that Blo t 12 has sequence homology with. They are characterized by having a large number of negatively charged amino acids in the sequence that may cause a reduction in SDS-binding during electrophoresis, thus, hampering protein denaturation. Also, their relatively high number of prolines may result in extended structures that affect electrophoretic migration [131, 132].

We also detected that glycosylation of pBlo t 12.0101 affects its antibody binding activity. Although *P. pastoris* has the potential of performing many of the posttranslational modifications (disulfide bridge formation, and both O- and N-linked glycosylation) [133] that other eukaryotes do, a drawback of this expression system is

the possibility of an excessive glycosylation of proteins, even if they are not glycosylated when expressed in their natural hosts. Moreover, *P. pastoris,* as other yeasts, adds O-oligosaccharides solely composed of mannose residues, which is not sufficient to produce other variety of sugar motifs present in proteins from higher eukaryotes. Although Blo t 12 could be naturally glycosylated, it seems that O-glycan decoration by *P. pastoris* was not similar. There is no information about the glycosylation process in mite species; however, in other arthropods, such as insects, O-linked glycans varied from single monosaccharides (N-acetylgalactosamine, N-acetylglucosamine, mannose, glucose or fucose) to extensively modified glycosaminoglycan chains [134]. Der p 1 and Der p 2 are two examples of glycosylated allergens from mites that led to know about the diverse type of sugar motifs in this taxonomic order (i.e. mannosylation, fucosilation) [135]. The fact that pBlo t 12.0101 deglycosylation improved IgE reactivity indicates that its glycosylation by *P. pastoris* was rather inconvenient, probably by a steric hindrance effect to epitope antibody-binding, caused by the added long mannose chains.

In summary, expression of Blo t 12.0101 in *Pichia pastoris* does not let to obtain an optimal molecule for testing IgE reactivity. Protein hyperglycosylation reduced antibody binding activity.

3.3. Characterization of natural Blo t 12 and the representativeness of its recombinant counterparts

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3.3.1. Introduction

As a potential reagent for allergy diagnosis in the tropics, it is important to evaluate how representative is the recombinant Blo t 12.0101 of its natural counterpart. Monoclonal and polyclonal antibodies were obtained raised in animals immunized with pBlo t 12.0101; they were used for the isolation of natural Blot 12 (nBlo t 12) and its immunological characterization. Because hyperglycosylation of Blo t 12.0101 affected its antibody reactivity, the allergen was also produced in an *E. coli* system. The intensity of IgE recognition by allergic patients was compared among the obtained recombinants and the natural Blo t 12.

3.3.2. Results

Anti-pBlo t 12.0101 specific antibodies recognize different bands in B. tropicalis extract

Because we intended to purify Blo t 12 from the complete *B. tropicalis* extract by antibody affinity chromatography, the capacity of the obtained biological reagents (anti- pBlo t 12.0101 pAbs and mAbs) to recognize Blo t 12 in the natural extract was tested first. By ELISA, the rabbit pAbs reacted in a concentration-dependent manner to the *B. tropicalis* extract, and more intensely to the fecal-enriched fraction (Figure 3.10). By WB, it was observed that the anti-Blo t 12 pAbs recognized different bands on the extracts, which were not detected by the pre-immune serum, supporting the specificity of the recognition. Moreover, in collaboration with Dr. M. Himly (Christian Doppler laboratories, University of Salzburg, Austria) there were developed monoclonal antibodies from mice immunized with deglycosylated pBlo t 12.0101. In Figure 3.14, it

is observed the ability of each mAb to recognize various components on *B. tropicalis* extracts. As 3B11 and 1A6 mAb had the strongest recognition of bands in the extracts, they were selected for further experiments. Interestingly, they recognized an additional 23 kDa band in the fecal-enriched fraction, but not in the body-enriched extract. Also, it can be observed that rabbit pAbs and the mAbs 3B11 (Fig 3.10) and 1A6 had a similar pattern of recognition of bands in the natural extracts.

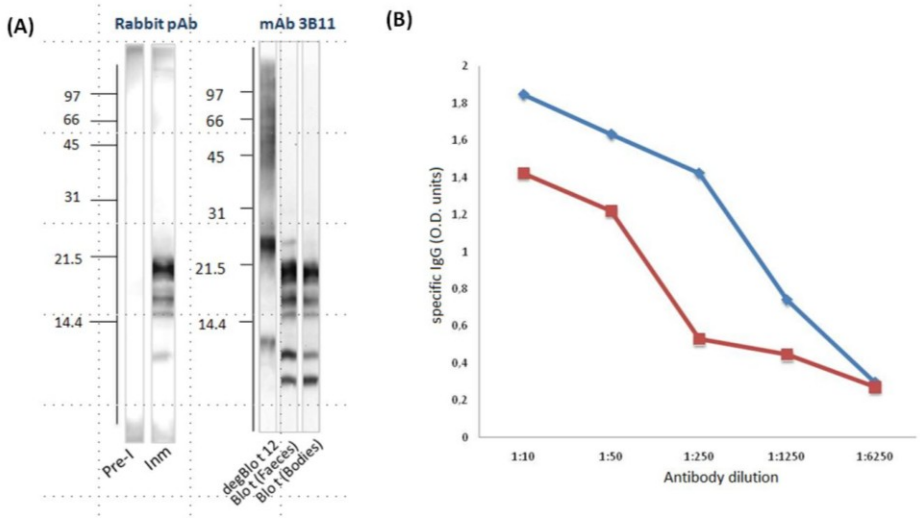

Figure 3.13 Anti-Blo t 12.0101 pAb can recognize the natural allergen in body- and faeces- enriched fractions of B. tropicalis extract. A) WB of B. tropicalis extracts using serum samples from a rabbit before (Pre-I) and after immunization (Inm) with pBlo t 12.0101. Band pattern recognized by the pAb agreed with that obtained using an anti Blo t 12.0101 specific mAb (3B11). B) ELISA results of testing anti Blo t 12 pAbs at different dilutions in 96-well plates coated with B. tropicalis extracts, prepared with bodies (red line) or faeces (blue line).

Identification of natural isoforms of Blo t 12 in B. tropicalis extract

Western blot using a mixture of anti-Blo t 12 mAbs (3B11 and 1A6) as primary antibodies led to the identification of two spots in a two-dimensional separation of *B. tropicalis* extract (Lot 28, house-hold prepared). Accordingly, it is confirmed that there are at least two isoforms of this allergen (Figure 3.15).

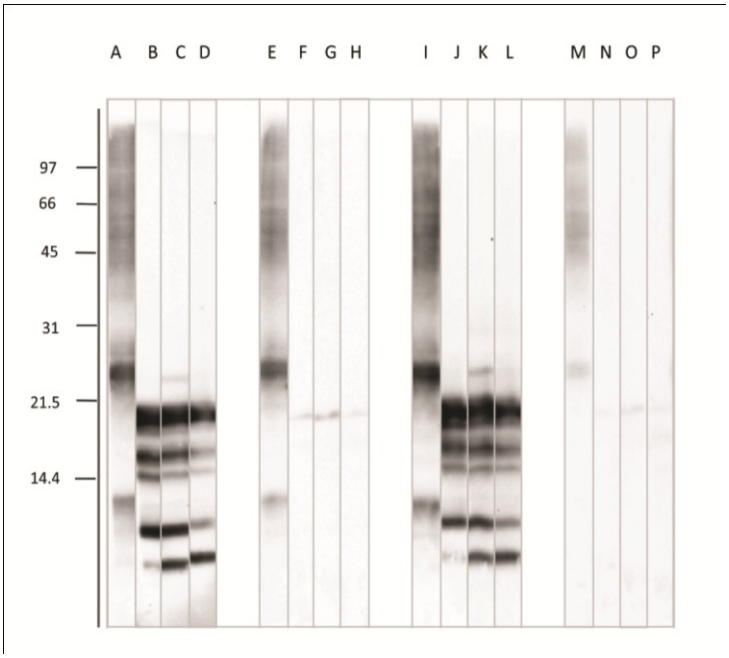

Figure 3.14. Immunoidentification of Blo t 12 in *B. tropicalis* extracts with specific monoclonal antibodies. Monoclonal antibodies 1A6 (A-D), 2J11 (E-H), 3B11 (I-L), 4I8 (M-P) detecting deglycosylated pBlo t 12.0101, B. tropicalis extract Lot JZS001, Heces enriched fraction of *B. tropicalis*, Body enriched fraction of B. tropicalis, respectively

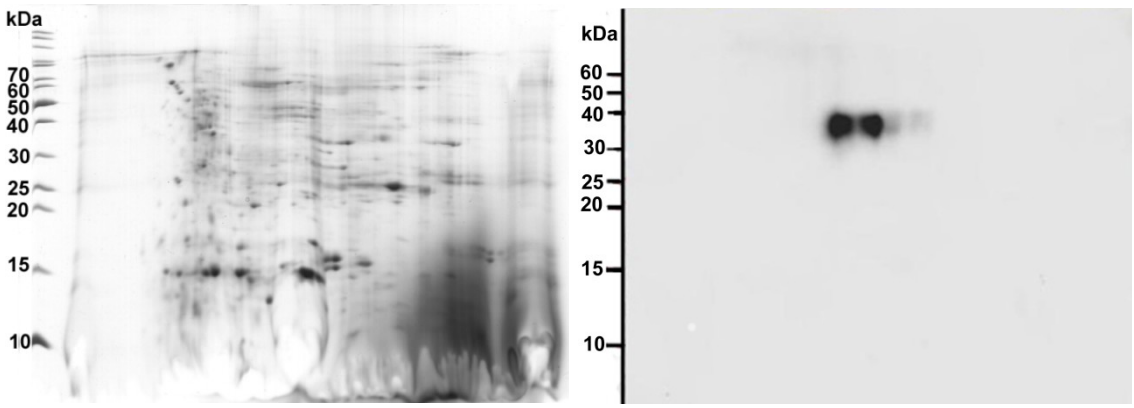

Figure 3.15. Identification of Blo t 12 isoforms in a two dimensional separation of *B. tropicalis* extract. Coomasie-stained 2D-PAGE (left) and WB results from a replicate gel (right) using 3B11 and 1A6 mAbs as detection antibodies.

Natural Blo t 12 can be isolated by immuno-affinity purification

Natural Blo t 12 could be isolated by antibody-affinity chromatography. It was first isolated with a polyclonal antibody column, in order to recognize a wider repertoire of epítopes, and thus, to be able to isolate various isoforms of the allergen. As shown in Figure 3.16, the pAb purified- nBlo t 12 had band pattern similarities with the deglycosylated pBlo t 12.0101, but the strongest band had a lower MW (20-21 kDa) than the recombinant protein (23 kDa). Human IgE recognition of the natural allergen was tested with a serum pool of *B. tropicalis* allergic patients that reacted to pBlo t 12.0101. As observed in Figure 3.17, the bands of 17 and 20 kDa were recognized by human sera. These results agreed with those from a previous experiment, where pre-adsorption of a serum from a Blo t 12-allergic patient with this allergen inhibited the IgE binding to the 17, 20 and 23 kDa bands (Figure 3.17b).

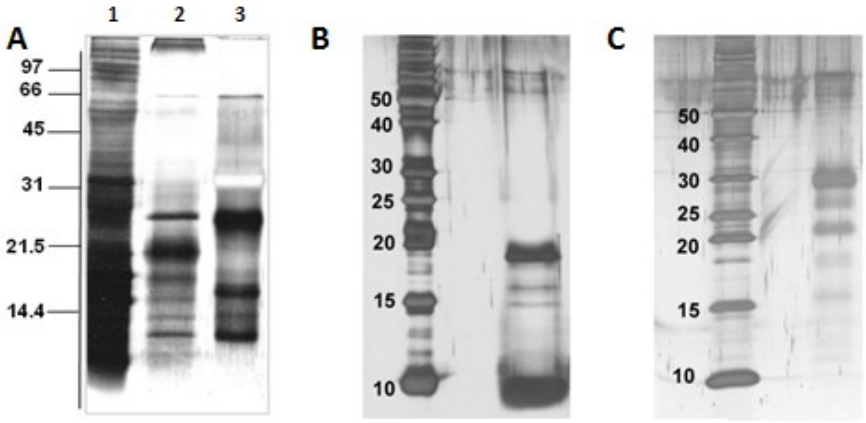

Figure 3.16 Purification of natural Blo t 12. A) First batch of nBlo t 12 purified with the anti-Blo t 12 pAb affinity column; Lane 1: *B. tropicalis* extract, L2: nBlo t 12 and L3: deglycosylated Blo t 12.0101. Purified natural Blo t 12 with 3B11-1A6 mAb column from household prepared extracts with mites from (B) Cartagena or (C) Immunotek (Spain).

The eluate from the mAb anti-Blo t 12 column shared similarities it is SDS-PAGE band pattern with the pAb-purified Blo t 12, but when isolated from *B. tropicalis* extracts from the same origin (*B. tropicalis* mites cultured by E. Fernandez-Caldas). The natural fraction isolated from household cultured-mites (Cartagena, Colombia),

although also shared 21 and 25 kDa, the strongest band had a MW of 30 kDa (Figure 3.16).

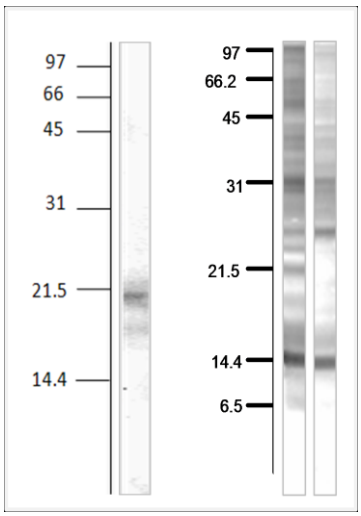

Figure 3.17. IgE recognition of natural Blo t 12. A) IgE recognition of purified nBlo t 12 by a serum pool from allergic patients. B) IgE recognition of *B. tropicalis* extracts before and after adsorption with pBlo t 12.0101

Glycosylation of natural Blo t 12 was evaluated by chemiluminiscence. As shown in Figure 3.18a, the band of 21 kDa was detected as glycosylated. Metaperiodate-oxidation of natural Blo t 12 did not affect its recognition by human IgE (Figure 3.18b).

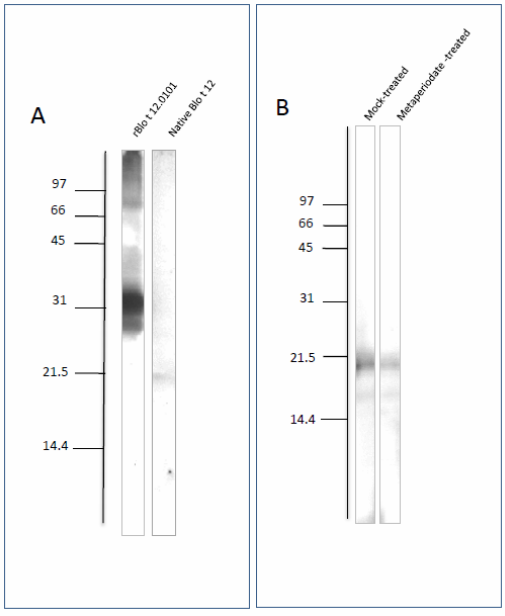

Figure 3.18 Characterization of the glycosylation state of native Blo t 12. A) Identification of glycosylated proteins by ECL Glyco-detection module B) Effect of metaperiodate oxidation on human IgE binding to natural Blo t 12.

Expression of recombinant Blo t 12.0101 in E. coli

Since hyperglycosylation of pBlo t 12.0101 affected its IgE binding, we further produced Blo t 12.0101 in *E. coli.* We first subcloned the gene sequence into pET32a, a vector that allows its co-expression with thioredoxin as a fusion protein, in order to ensure an appropriate folding when expressed in Origami (DE3) cells. Although protein expression into this system had a good yield and resulted in a soluble product (Figure 3.19), cleavage of thioredoxin with enterokinase was not very specific, causing excessive protein degradation. Thus, we decided to produce it in a pET45b+/Origami (DE3) expression system, which allowed its production as a His-tagged protein. As observed in Figure 3.19, this recombinant protein also ran at an unexpected molecular weight (23 kDa), higher than its theoretical determination. Its IgE reactivity was confirmed by WB using a serum pool from Blo t 12-allergic patients.

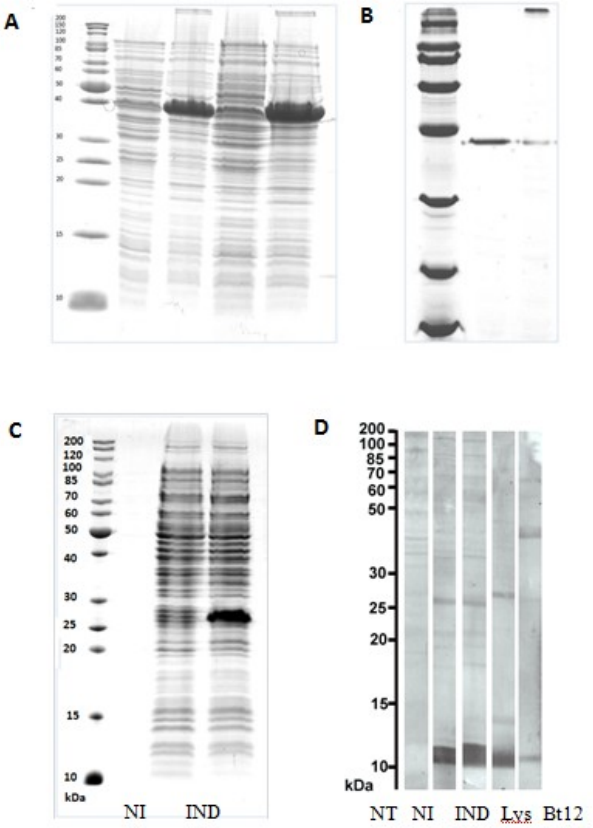

Figure 3.19. Expression of Blo t 12.0101 in Origami (DE3). Expression of Blo t 12.0101/pET32a: (A) SDS-PAGE of one expression experiment with two colonies(B) Purified protein in reducing and non-reducing conditions, respectively. Blo t 12.0101/pET45b+: (C) SDS-PAGE and (D) WB with human IgE of the expression experiment. NI: Non-induced; IND: induced-; NT: *E. coli* lysate from non-transfected cells; Lys: Bacterial lysate of an induced culture; Bt12: purified Blo t 12.0101/Trx (positive control).

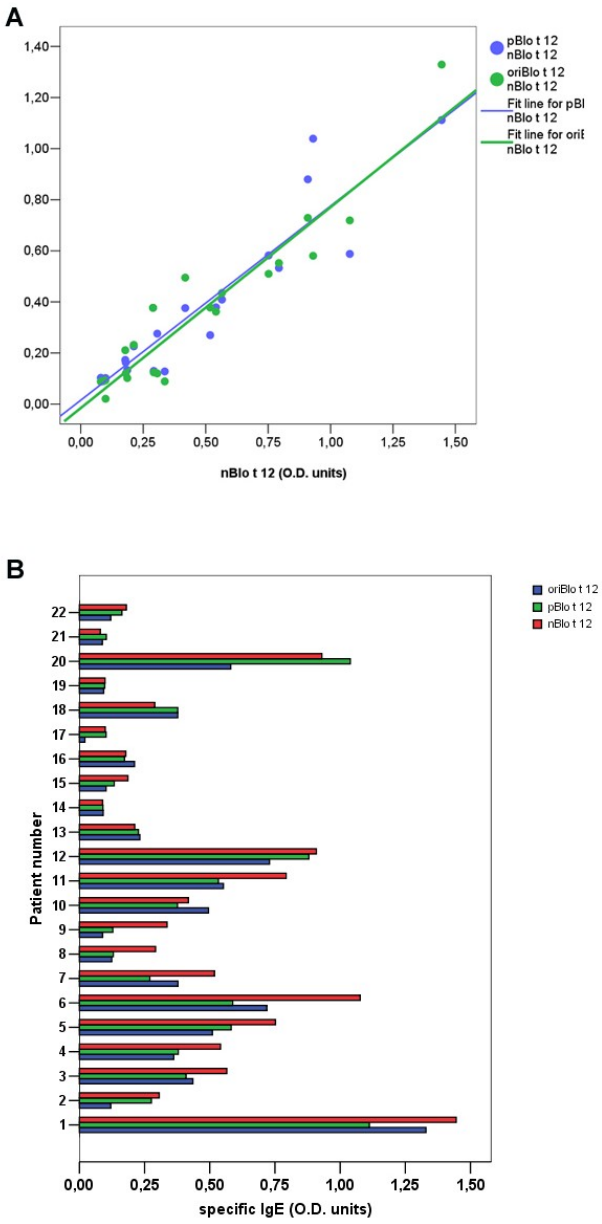

Figure 3.20 IgE responses to natural and recombinant Blo t 12. A) Correlation plot among natural Blo t 12 (x axis) with pBlo t 12.0101 or oriBlo t 12.0101. B) Bar chart with O.D. values to natural and recombinant Blo t 12 in 22 patients.

Comparison of IgE binding to natural and recombinant Blo t 12

The isolated natural Blo t 12 was compared to the recombinant Blo t 12.0101 isoforms in terms of its IgE binding capacity in 22 subjects. Although specific IgE values to pBlo t 12.0101 and *E. coli* Blo t 12.0101 were highly correlated to those

against the natural allergen (Spearman rho: 0.92 and 0.89, respectively); these were significantly lower (Wilcoxon-ranked test, p=0.06 in both comparisons). IgE reactivity to the two recombinant allergens did not significantly differ among them (Figure 3.20)

3.3.3. Discussion of results

Natural Blo t 12 could be successfully identified and isolated from *B. tropicalis* whole extract by affinity purification, using antibodies with strong reactivity to this allergen. Valuable information was obtained from these experiments: 1) since nBlo t 12 reacted to antibodies that were raised in animals immunized with pBlo t 12.0101, the recombinant isoform seems to be quite representative of the natural allergen in terms of epitope content. 2) Group 12 from *B. tropicalis* is in fact polymorphic; as demonstrated by 2D-PAGE and WB of the natural whole extract there are at least two isoforms of this allergen. However, it is probable that other isoforms exist since the purification was based on antibodies raised with only one isoform. Like in the GST family [136], it is plausible that other distantly related Blo t 12 variants to the known isoforms, displaying unique epitopes, were not detected. 3) As other important mite allergens [137, 138], we consider Blo t 12 is excreted in faeces since it was detected at higher concentrations in a fecal-enriched extract. Moreover, Blo t 12 has sequence homology with chitin binding proteins (CBP) that are expressed mainly in midgut epithelial cells and excreted to the intestinal lumen to be part and mediate arrangement of the peritrophic matrix, a membrane composed by chitin and proteins that surround food balls and participate in digestion and host defense against pathogens [139].

4) The bizarre electrophoretic migration of Blo t 12 is not exclusive of the recombinant allergens, but also of the natural counterpart. Natural Blot 12 migrated in SDS-PAGE with a higher MW than expected. Both, recombinant and natural Blo t 12 showed multiple bands in SDS-PAGE that were mostly visible with sensitive methods (silver staining and ECL WB). However, WB identification of Blo t 12 in 2D-PAGE indicated that there were two spots with the same MW, but different *pI*. Since protein denaturation protocols for 1D- and 2D- electrophoretic separation differed in several aspects (i.e. reducing agents and detergents) protein migration differences can result. It seems that Blo t 12 is a partially SDS-resistant protein, retaining still some degree of

secondary structure folding after treatment that affects electrophoretic migration. The sample buffer for 2D-PAGE contained another denaturant, urea, which led to appreciate the allergen more uniformly: two spots with a single MW in spite of its retarded migration.

Finally, although nBlo t 12 is glycosylated, this post-translational modification does not significantly influence its IgE reactivity. Thus, expression of Blo t 12 in *E. coli* was considered as an option to obtain the allergen in a glycosylated-free form. Allergen expression as a fusion protein with thioredoxin in Origami strain – a suitable host for disulphide bridge formation – was planned first. Unfortunately, cleavage from the fusion protein was problematic and inefficient. In contrast, expression of Blo t 12.0101 in pET45b+ yielded a soluble protein, whose IgE reactivity was further compared with the natural allergen. Both recombinant proteins, obtained in yeast or *E. coli*, had a similar behavior in terms of IgE reactivity by ELISA. IgE levels correlated very well with that against the natural allergen, but were significantly lower. The sensitivity of the recombinant allergens to detect Blo t 12 positive cases was~ 80%. The moderate representativeness of their natural counterpart is expected to be caused by the hyperglycosylation of pBlo t 12.0101 or the improper folding of the *E. coli* produced isoallergen.

In summary, we could efficiently isolate natural Blo t 12 and confirm it is a polymorphic group. IgE recognition to any the two tested recombinant Blo t 12.0101 isoforms was less intense than to the natural counterpart. Usage of other systems for recombinant protein expression should be contemplated.

3.4. Blo t 12 is a species-specific marker of Blomia tropicalis

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3.4.1. Introduction

Blomia tropicalis exhibits moderate CR with *D. pteronyssinus*, the other most important allergenic source in the tropics. Highly conserved proteins have been identified as cross-reactive allergens among mites and other invertebrates [108]. Others (Blo t 1 and Blo t 5) have been proposed to be species-specific. According to Kuo *et al*, Blo t 5 and Der p 5 are not cross-reactive allergens [46]; however, Caraballo *et al* found that IgE binding to Der p 5 can be inhibited by BtM, an allergenic carboxy-terminal fragment of Blo t 5. Cheong *et al* cloned Blo t 1, a cystein protease with 34% of sequence similarity to Der p 1. Poor correlation between the IgE levels to these two allergens was the only clue about its lack of CR [47]. The allergen Blo t 12 is another potential species specific-marker (SSM) of *B. tropicalis* since no homolog proteins have been described in other allergenic sources of relevance in the tropics.

Identifying a SSM from an allergenic source offers several advantages. As a part CRD, it helps to identify truly allergen sensitization to a defined source. For epidemiological studies about the role of allergen exposure on disease presentation is a useful tool, because environmental exposition to a certain source can be detected in a very specific manner, measuring an exclusive component. On the study about the evolution of IgE responses and allergen sensitization, evaluating a SSM is essential in those situations where co-exposition to CR sources is very common. Here we show the results of testing the reactivity of mAbs and pAbs obtained from Blo t 12.0101-immunized animals to different allergenic sources and agricultural pests.

3.4.2. Results

Blo t 12 is a species-specific marker of Blomia tropicalis

By ELISA, anti-pBlo t 12.0101 rabbit pAbs reacted to Blo t 12 recombinant isoforms and *B. tropicalis* extract, but not to *D. pteronyssinus*, storage mite (such as *T. putrescienceae* and *L. destructor*) and other arthropod (including agricultural pest and cockroach) extracts (Table 3.1).

Table 3.1. Testing cross-reactivity of Blo t 12 pAbs with allergenic mites and other arthropod species

Species	Anti-Blo t 12 pAb	Preimmune serum
T. castaneum	0.07	0.38
T. putrescientae	0.11	0.28
L. destructor	0.05	0.03
A. Ovatus	0.10	0.09
A. Sirus	0.04	0.13
rBlo t 12	**4.74**	**0.13**
B. Tropicalis	**1.15**	**0.07**
D. Farinae	0.08	0,11
A. sirus	0.15	0.17
B. germanica	0.11	0.17

We also explored cross-reactivity with *A. lumbricoides* and *D. pteronyssinus* testing mAb 1A6 and purified IgG from a Blo t 12 immunized rabbit. Neither the mAb 1A6, nor the pAbs recognized any component in *D. pteronyssinus* and *A. lumbricoides* extracts.

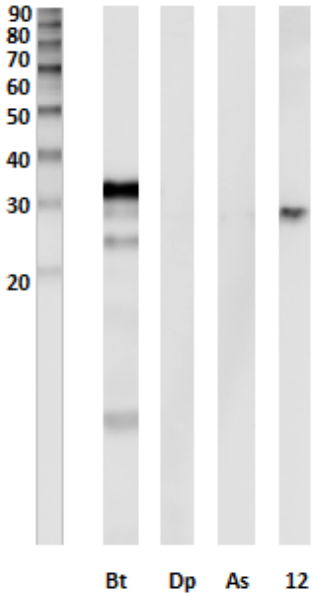

Figure 3.21 Testing cross-reactivity of Blo t 12 mAb 1A6 with *Dermatophagoides pteronyssinus* and Ascaris. WB of the following antigens in solid phase: *B. tropicalis* (Bt), *D. pteronyssinus* (Dp), *A. lumbricoides* (Asc) and Blo t 12.0101 (12)

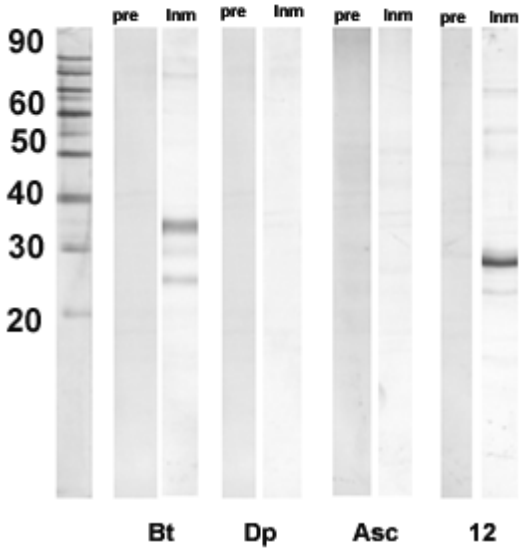

Figure 3.22 Testing cross-reactivity of Blo t 12 pAbs with *Dermatophagoides pteronyssinus* and Ascaris. WB of the following antigens in solid phase: *B. tropicalis* (Bt) *D. pteronyssinus* (Dp) *A. lumbricoides* (Asc) and Blo t 12.0101 (12) incubated with preimmunized (pre) and immunized (inm) serum.

3.4.3. Discussion of results

Of patients with HDM allergy in the tropics, more than 85% have serum-specific IgE antibodies to *B. tropicalis* and *D. pteronyssinus* [140]. The detected double positivity may be due to cross-reactivity or parallel sensitization. Distinction between these two possibilities is important for clinical decisions concerning SIT. Species-specific markers have been shown to be very important in this regard [141]. A truly species-specific allergen from *B. tropicalis* had not been found before [46, 47]. The idea of Blo t 12 as a SSM of *B. tropicalis* had been thought before since no homologous

allergen in *D. pteronyssinus* has been detected; but here with these results we confirm it experimentally.

3.5. Testing the biological activity of Blo t 12 and its influence of allergic sensitization

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3.5.1. Introduction

Several allergens bind to chitin [93, 144], a natural and abundant carbohydrate that exhibits immunomodulatory properties [49, 50]. It has been shown that chitin fragments can induce airway inflammation in exposed animals; also, when administered with OVA, it can potentiate its allergenicity, boosting IgE production in an IL-17 dependent manner [51]. This carbohydrate is sensed by different innate immune receptors, including mannose receptor. It is probable that coupling of allergens to this carbohydrate influence its capture by immune cells and in turn its immunogenicity. In this study, we confirm the ability of Blo t 12 isoallergens [36] to bind chitin and evaluate the influence of this activity on the development of airway inflammation in a murine model of asthma, immunizing animals with any of the two Blo t 12 recombinant isoforms –produced in *E. coli* – in presence or not of chitin. Taking advantage that Blo t 12.0102 in this expression system has a very low chitin binding activity; it was evaluated if the observed results of chitin on Blo t 12 immune responses were due to a bystander adjuvant effect of chitin [51] or more dependent of antigen binding.

3.5.2 Results

Blo t 12 isoforms bind chitin

Chitin affinity assays were performed with Blo t 12 isoforms to confirm its predicted biological activity. As noted in Figure 3.23, pBlo t 12.0102, but not pBlo t 12.0101 bound to chitin. Because it is possible that hyperglycosylation affects ligand affinity, the ability of TMSF-treated Blo t 12.0101 to bind chitin was tested, finding that this deglycosylated variant did not bind it either. Similar results were obtained when binding assays were done with another commercial preparation of chitin (alpha chitin obtained from crab shells).

As TFMS can alter lateral chains of amino acids and in turn protein biological activity, binding assays were repeated with the recombinant isoform produced in *E. coli.* As observed in Figure 3.24, *E. coli* produced Blo t 12.0101 could bind both chitin preparations, as evidenced by its recovery from the pellet. In contrast to the *Pichia* expressed variant, Blo t 12.0102, when produced in *E. coli*, showed lower affinity to this carbohydrate.

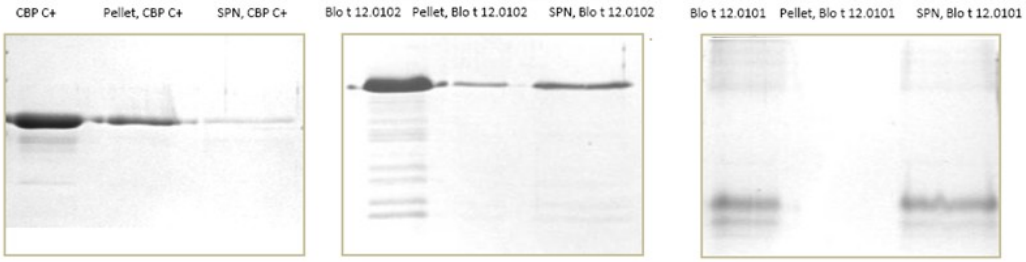

Figure 3.23 Chitin binding experiments with Blot 12 isoforms obtained in *Pichia pastoris*. CBP: Chitin binding probe (positive control). SPN: Supernatant.

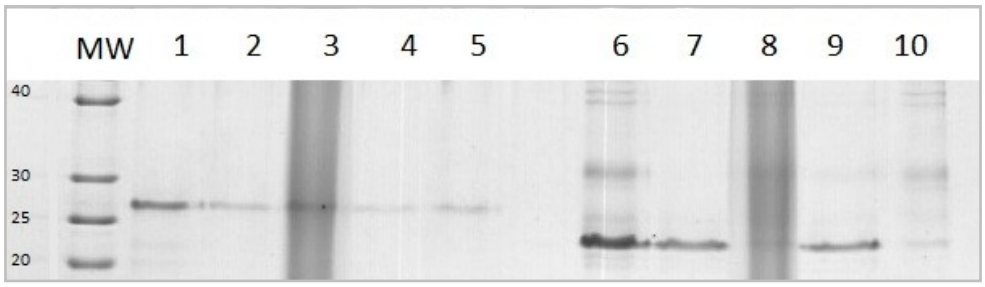

Figure 3.24 Chitin binding experiments with the Blo t 12 isoforms produced in *E. coli*. Lane (1) Blo t 12.0101 as a reference, supernatants (lanes 2 and 4) and pellets (lanes 3 and 5) of the experiments using powdered chitin or beads, respectively. Blo t

12.0102 (lane 6), supernatants (lane 7 and 9) and pellets (lanes 8 and 10) of the experiments using powdered chitin or beads, respectively.

Animal model of Blo t 12 sensitization

All groups immunized with Blo t 12.0101 or .0102 developed higher total IgE titers, compared to those receiving PBS or chitin alone (Figure 3.25). Administration of chitin with Blo t 12.0101 increased even more the levels of total IgE ($p=0.05$) compared to the allergen alone, but it did not have any raising effect when co-administered with Blo t 12.0102. Regarding Blo t 12 specific IgG1, there were not differences among levels in mice receiving any isoallergen alone or with chitin, neither between the mice immunized with the different isoforms. Interestingly, Blo t 12.0102 IgG2 levels were significantly greater than those to Blo t 12.0101 ($p=0.006$). The ratio IgG1/IgG2a was higher in Blo t 12.0101- (both, in those receiving the allergen alone or with chitin) than in Blo t 12.0102- immunized mice. Allergen-specific IgE was very low in all mice, including those receiving the allergen with alum.

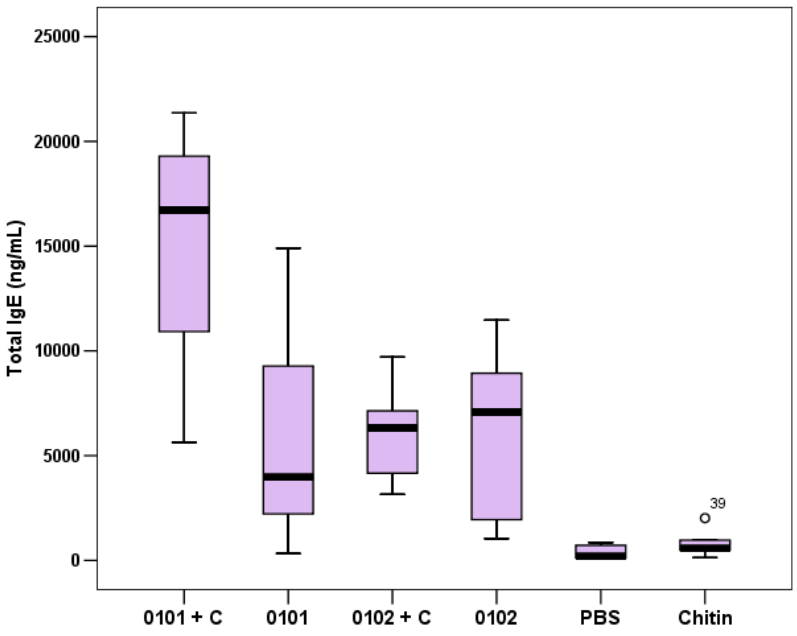

Figure 3.25 Total IgE levels in Blo t 12 immunized mice and controls.

Significant higher Penh values were observed in all allergen-immunized groups compared to the PBS injected mice, however, methacholine-induced airflow obstruction was more intense in those receiving Blo t 10.0101 than with Blo t 12.0102. At 12.5 mg/mL doses “Blot 12.0101 plus chitin” group had significantly higher Penh values.

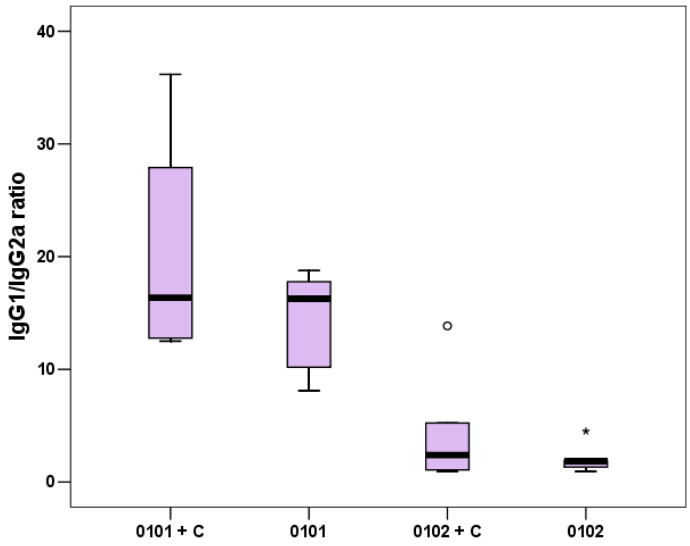

Figure 3.26 Blo t 12 specific IgG1/IgG2 ratio in allergen immunized mice. Box plots showing median and IQR in the different mouse groups.

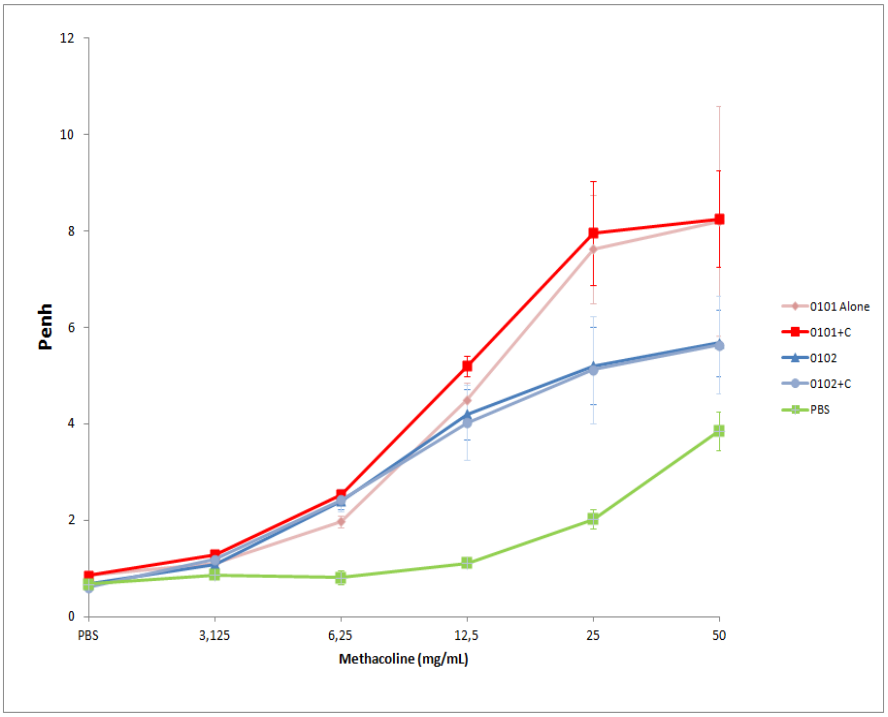

Figure 3.27 Airway responsiveness in Blo t 12 sensitization model.

Mice that were sensitized with any of the Blo t 12 isoforms (alone, plus chitin or alum) or alum-adsorbed *B. tropicalis* extract manifested a neutrophil rich tissue inflammatory response in their lungs (Figure 3.28). No eosinophils were observed in the H&E staining of lung sections. However, this inflammatory pattern was associated to airway mucus metaplasia, as evidenced by PAS staining (Figure 3.32). Chitin or PBS-injected mice did not have any sign of tissue inflammation.

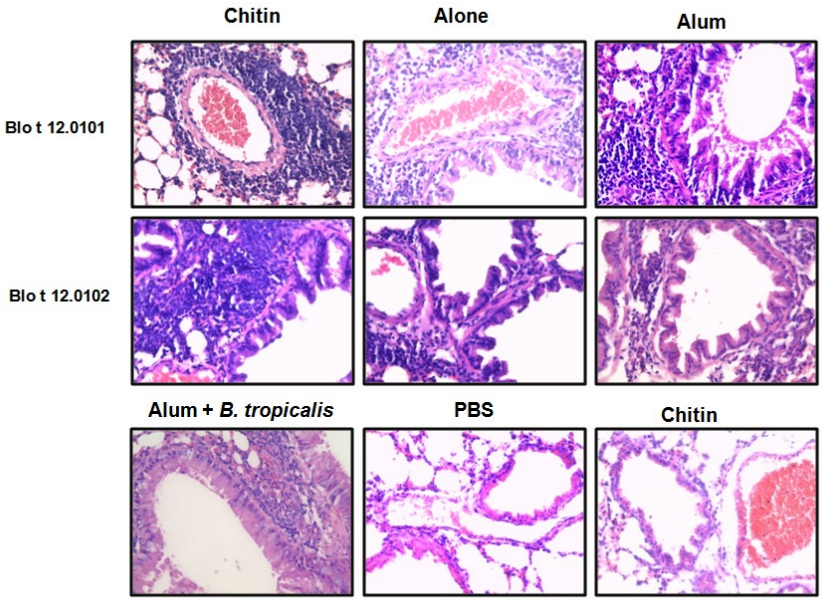

Figure 3.28 *In vivo* effects of Blo t 12 isoforms and chitin administration in mice. Hematoxylin and eosin staining of lung slides (x10 magnification). A neutrophil-rich perivascular and peribronchial infiltrate was observed in all groups of Blo t 12 and *B. tropicalis* immunized mice, but not in those receiving PBS or chitin.

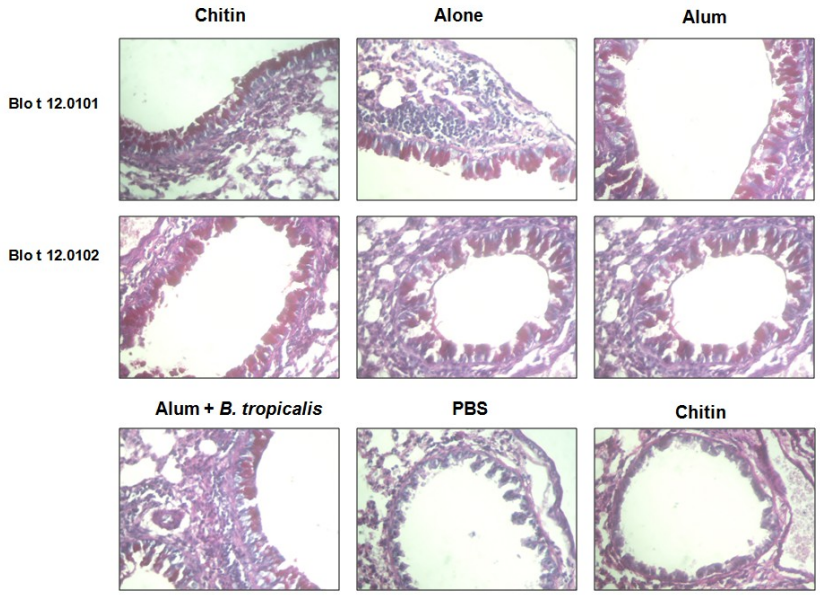

Figure 3.29 Effects on mocus production of Blo t 12 isoforms and chitin administration in mice. PAS staining of lung slides (x40 magnification).

3.5.3. Discussion of results

Here we found that Blo t 12 isoforms are CBPs with an intrinsic potential to induce airway inflammation. Chitin adjuvant effects on Blo t 12 immune responses were rather modest and only detectable when co-administered with the isoallergen .0101, the tested variant with the higher affinity to this carbohydrate.

Blo t 12 is a polymorphic group of allergens with chitin binding activity, but it is worth mentioning that selection of the system for protein expression influences its ligand affinity. Blo t 12.0101 hyperglycosylation in *P. pastoris* may explain its lack of chitin affinity when the assays were done with this recombinant isoform. In contrast, pBlo t 12.0102 showed good affinity to chitin, but this capacity was substantially reduced when the isoallergen was obtained in *E. coli*. We took advantage of this result to have two Blo t 12 isoallergens with different affinity to chitin for evaluating the influence of their binding activity on airway inflammation and allergen sensitization.

Both Blo t 12 variants had pro-asthmatic effects in exposed mice, but the elicited immune responses did not show all the classical hallmarks of an allergic process. Immunization with any of the two isoallergens induced mucus production, airway inflammation and bronchial hyperreactivity without the need of an adjuvant. Total IgE production was also raised in immunized animals; nevertheless, specific IgE sensitization was not detected in any of the groups receiving Blo t 12 and the triggered bronchial inflammatory process was not eosinophil-rich; instead, an important neutrophil infiltrate was predominant. These effects were not exclusive of Blo t 12; immunization with the whole *B. tropicalis* extract was also accompanied of similar inflammatory changes. The obtained results were not surprising since it had been previously observed that immunization of BALB/c mice with *B. tropicalis* induced a comparable inflammatory pattern [145]. It seems that although this mouse strain responds strongly to other antigens, such as OVA, in terms of allergic inflammation, it did not so to *B. tropicalis* allergens. Another published *B. tropicalis* sensitization model in A/S mice also caused neutrophil-rich lung inflammatory pattern, in contrast to *D. pteronyssinus,* which provoked a more classical eosinophil-rich airway cellular influx [146]. In our model, allergens were administered in concentrations that have proved to be useful for generating typical allergic responses after *B. tropicalis* immunization, but using different strains [145, 147, 148].

We explored if intermediate chitin fragments had a pro-allergenic adjuvant effect in the context of its co-administration with a recognized chitin binding allergen. In the present model, the observed findings differed in many aspects to that found by da Silva *et al* in an OVA airway hypersensitivity model in C57BL6 mice [51]. Chitin did not strongly potentiate the immune responses to any of the two isoforms; although it did induce a more intense bronchial methacholine-induced hyperreactivity and higher total IgE levels in those mice receiving Blo t 12.0101, the isoform with higher affinity to this carbohydrate. It is probable that the adjuvant effects of chitin were shadowed by the intrinsic inflammatory potential of Blo t 12 molecules, which is not observed in immunologically "inert" molecules such as OVA when administered alone [149]. In fact, allergen adsorption to alum (a classical Th2 adjuvant) neither reinforced any of the immune effects of these Blo t 12 isoallergens.

Other relevant findings were derived from the comparison of the biological effects caused by the two isoforms in sensitized mice. With or without chitin, the variant .0101 provoked a more intense degree of airway hyperresponsiveness than the .0102 isoform. In addition, a greater IgG1/IgG2a ratio in .0101 immunized mice indicated that this isoform elicited a more Th2 polarized response; unfortunately, cytokine production was not measured to confirm these results. Interestingly, a more frequent and stronger IgE response was detected to the .0101 than to the .0102 isoallergen in asthmatic subjects living in the tropics [36], suggesting then, both results, that the former isoform is intrinsically more allergenic. However, we consider this possibility with precaution because the *E. coli*-produced Blo t 12.0102 probably had folding differences (as suggested by its poor chitin affinity, contrary to the *P. pastoris* recombinant) that does not let to properly resemble the natural effect induced by this isoallergen. On the other hand, it may be also hypothesized that folding differences affecting the ligand binding domain influence the immune responses to these isoallergens in a chitin independent-manner. In fact, the existence of an intrinsic target in the host for mammalian chitin binding proteins is suspected since the participation of Th2 responses (up-regulation, activation of Th2 pathways) have been observed in chitin-free models [150, 151]. AMCase, a true mammalian chitinase that also contains an additional CBD without enzymatic activity conserves its pro-allergenic effects in the presence of chitin binding inhibitors. By PSI-BLAST, it was detected that Blo t 12 CBD is homologous to mammalian chitinases. Thus, it is theoretically plausible that in an analogous manner chitin binding allergens, such as Blo t 12, activate directly inflammatory immune-related pathways as mammalian CBP do.

Chapter 4. *Identification of novel Blomia tropicalis allergens*

Overview

One aim of this thesis was the isolation of new allergens from *B. tropicalis*. PCR-screening of a previously constructed cDNA library from *B. tropicalis* led to isolate potential and well-known allergens and isoallergens (Table 4.1). The isolation and characterization of two new allergens, GST and ubiquitin, is presented in this section.

Table 4.1 Isolated allergens from *B. tropicalis* cDNA library

Name	Isoallergens
Blo t 5	1
Blo t 21	2
Blo t 8	2
Poli-ubiquitin from *B. tropicalis*	1

As part of our investigations about the interrelationships between the immune responses elicited by *Ascaris* spp., *B. tropicalis* and other allergenic sources, in especial, those aspects derived from IgE CR, we focused on the characterization of Blo t 8, the gluthathione-S-transferase from *B. tropicalis*. Molecular modeling of its tertiary structure and comparison with other allergenic GSTs showed that CR among these molecules may be due to well conserved surface-exposed amino acid clusters. IgE sensitization to Blo t 8 is common in our population, but of low intensity. Although specific-IgE levels to the rBlo t 8 isolated isoform correlated with the natural allergen, they were significantly less intense. Sensitivity of the recombinant protein to detect sensitization was moderate. Since one species contains generally several GST isoforms, it is possible that this recombinant is not representative of a wider repertoire of epitopes that includes all GST isoforms from *B. tropicalis*.

Mercado *et al* had previously isolated a 6.5 kDa IgE binding peptide from the natural extract that was identified as ubiquitin by Edman degradation sequence analysis. Using sequence-specific primers, a cDNA coding for two copies of the ubiquitin ORF was isolated. A single copy of the ubiquitin was then obtained and expressed as a his-tagged peptide. Its sequence was identical to the human ubiquitin. IgE reactivity was found in 23.6% of asthmatic patients and 4.7% of healthy controls.

4.1. Immunological studies about Blo t 8, the Gluthathione-S-Transferase (GST) from B. tropicalis

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4.1.1. Introduction

Blomia tropicalis is an important allergenic source in the tropics [152], with around 30 IgE binding components [38], but a smaller number of properly characterized allergens. Cross-reactivity with other allergen sources has been experimentally demonstrated [45]. The impact of this phenomenon on allergy diagnosis is well recognized. Moreover, in regard to its CR with nematodes, it is also of current interest to understand its influence on the evolution of IgE responses in affected populations [153].

Tropomyosins are one example of allergens mediating CR between HDM and helminths [44, 154], but there are other IgE-reactive components to be investigated. Acevedo *et al* found that *B. tropicalis* extract inhibited the IgE binding to a 23 kDa band from *Ascaris suum*, identified by MS as a GST protein [45]. CR between filarial GSTs and Bla g 5, the homologous allergen in the cockroach [155], was also recently reported [156]. In this study, we sought to isolate and characterize the GST from *B. tropicalis* to understand more aspects about the immune response to HDM/nematode cross-reactive allergens in populations living in the tropics.

4.1.2. Results

Isolation, cloning and production of Blo t 8

It was isolated a 711 bp DNA fragment (Figure 4.1), coding for a 235 amino acid protein, with 96% identity to a previously reported Blo t 8 nucleotide sequence. The

new sequence was published in GenBank as Blo t 8.0102 (Access number GQ398117.1, mRNA; ACV04860.1, protein). The isolated isoform has a predicted *pI* of 5.24. Although its theoretical molecular weight is 27.5 kDa, Blo t 8 was observed as a 31 kDa band due to its histidine tag (Figure 4.1).

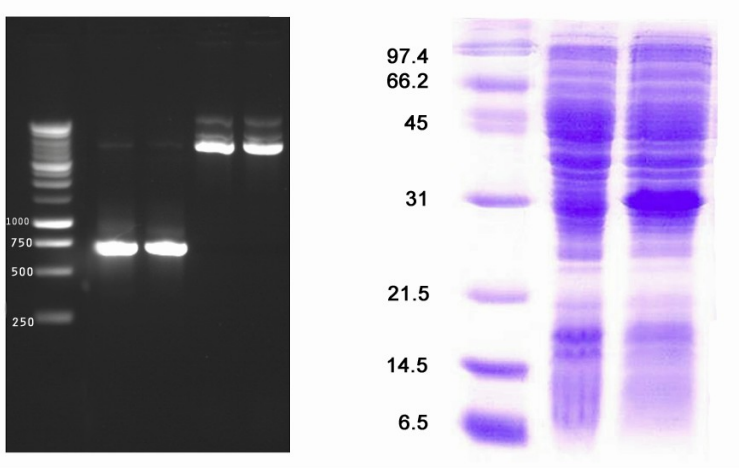

Figure 4.1 Cloning and expression of Blo t 8. Left, 1% agarose gel showing PCR fragments amplified with Blo t 8 specific primers (Lane 2 and 3) from two Blo t 8/pET100 purified plasmids (4 and 5). Right, 15% SDS PAGE showing results of Blo t 8 expression experiments. a) Non-induced control b) 1 mM IPTG -induced culture.

By sequence homology, this protein belongs to the μ class of gluthathione-S-transferase family. Phylogenetic analysis showed that Blo t 8 clustered with the rest of published mite GST sequences; the orthologous from *Ascaris suum* (AscGST) was more closely related to Bla g 5, both sigma-like GSTs. (Figure 4.2).

Molecular modeling of Blo t 8

The predicted Blo t 8 structure showed a typical GST architecture, with several alpha helices surrounding a beta-sheet sandwich domain. In spite of moderate sequence similarity (31-57%) mite-derived GSTs showed high tertiary structure conservation, as depicted in Figure 4.3 with a superposition of Blo t 8 and Der p 8 3D models. Using CONSURF, a surface-exposed patch, conserved among mite GSTs, was identified

(Figure 4.3). It comprised residues 71D, 72F, 73K, 76E, 79A, 82K, 83R and 105E, following Blo t 8 sequence positions.

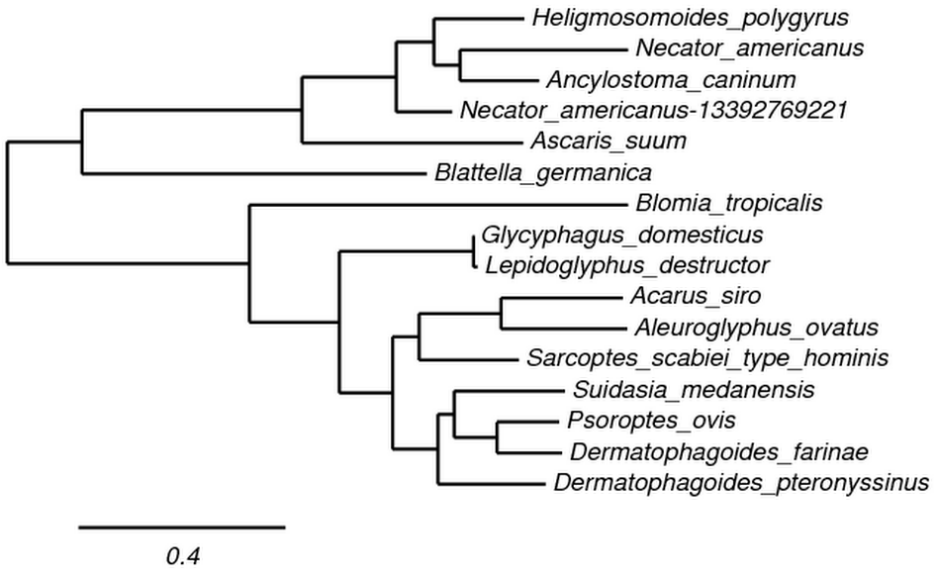

Figure 4.2 Phylogenetically tree of GST sequences from allergenic sources and nematodes.

It had been previously demonstrated that GST proteins mediate cross-reactivity between *Ascaris* and *B. tropicalis* extract [45]. We found that pre-adsorption of a serum pool with Ascaris extract was able to inhibit IgE binding to rBlo t 8 in a dose-dependent manner, reducing 70% Blo t 8-specific IgE values at the maximum inhibitor concentration [0.8 mg/mL]. Blo t 8 and AscGST have 43% of sequence similarity; comparison of their 3D structures led to identify two clusters of surface-exposed residues that could represent cross-reactive epitopes (Figure 4.4). One common region was identified on the C-terminal region of the molecules; composed by amino acids: K200, E204, P206, K209, E210, I212 and A213 (Patch #1, P1). Another conserved patch (P2) was located on the N-terminal region (D10, R33, N35, R36, D37, E38 and A41).

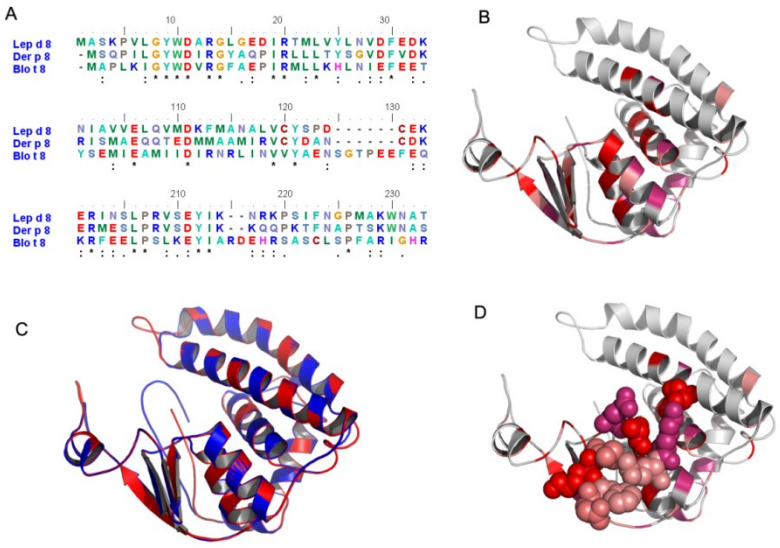

Figure 4.3 Sequence analysis and molecular modeling of Blo t 8. A) Alignment of Blo t 8 with its homologues from *L. destructor* (Lep d 8) and *D. pteronyssinus* (Der p 8), B) Theoretical model of Blo t 8. C) Superposition of Blo t 8 (in red) with Der p 8 (in blue) D) Conserved patch of superficial residues among GSTs from mites is shown as spheres.

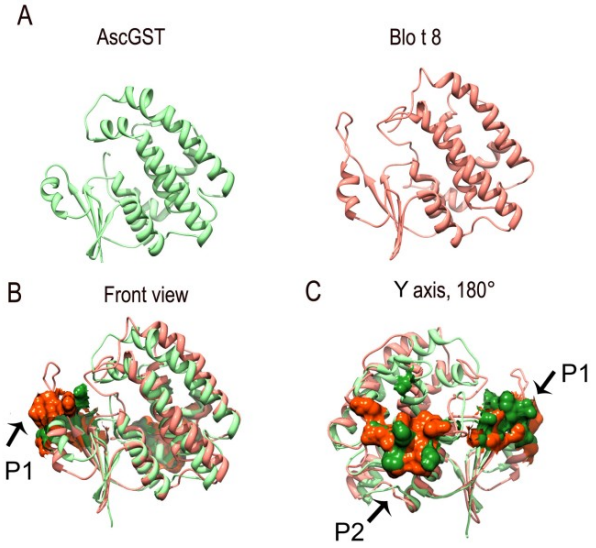

Figure 4.4 Prediction of cross-reactive areas between the GSTs from *B. tropicalis* and *A. suum.* A) Ribbon representation of AscGST and Blo t 8. B) Superposition of AscGST with Blo t 8. Patches of solvent-accessible and conserved residues are shown in surface view C) 180º rotation (Y axis) to visualize other conserved regions. Arrow indicating P1 and P2 conserved clusters.

IgE reactivity to Blo t 8 and other GSTs

A first screening to identify Blo t 8 sensitized subjects, in 91 sera from *B. tropicalis* allergic patients living in Cartagena, showed that 80.2% responded to this allergen; however, with low intensity in most cases (median 0.165, IQR: 0.145-0.195) The cut-off value to define a positive test was 0.13 O.D. units. Another group of asthmatic patients (n=127), part of the ASA cohort, was evaluated in terms of IgE recognition to different GSTs. IgE sensitization to Blo t 8 was 45%, 24% to AscGST and 17.9% to Bla g 5, the most potent GST allergenic isoform from *Blatella germanica.* Specific IgE values toward these three allergens were significantly correlated (Figure 4.5).

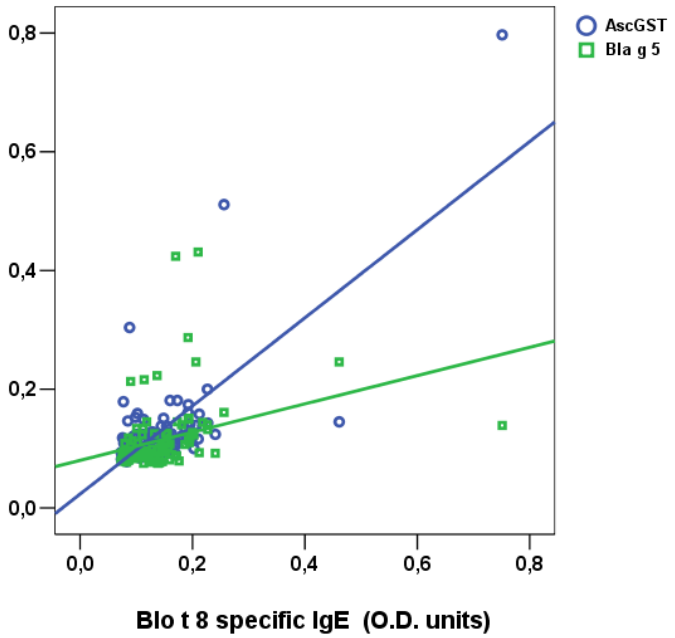

Figure 4.5 Correlation plot between specific IgE to Blo t 8, AscGST and Bla g 5. X axis, Blo t 8 specific IgE values; Y axis, AscGST- (blue) or Bla g 5- (green) specific IgE values. Lines represent linear regression slopes. Correlation coefficients: Bla g 5-Blo t 8, Spearman's rho: 0.47, p<0.0001; AscGST-Blo t 8, Spearman's rho: 0.51 <0.0001; Bla g 5-AscGST: Spearman's rho: 0.52, p<0.0001.

IgE reactivity to the natural and recombinant Blo t 8 was determined simultaneously in 36 subjects. Specific IgE values against these molecules were highly correlated (Spearman rho = 0.92, p<0.001) but were significantly higher to the natural

allergen (p=0.03; median: 0.148, IQR: 0.10-0.25 vs. 0.11, 0.10-0.17 O.D. units, respectively). The sensitivity of rBlo t 8 to detect IgE positive cases, with respect to the natural allergen, was 61.18% (15 out of 22 subjects).

Skin prick tests with different GSTs were done in a group of 20 subjects, including IgE-positive and –negative to Blo t 8. Five patients had a positive skin prick test to the natural allergen (3 mm papule in all of them); only one responded to the recombinant molecule (Figure 4.6).

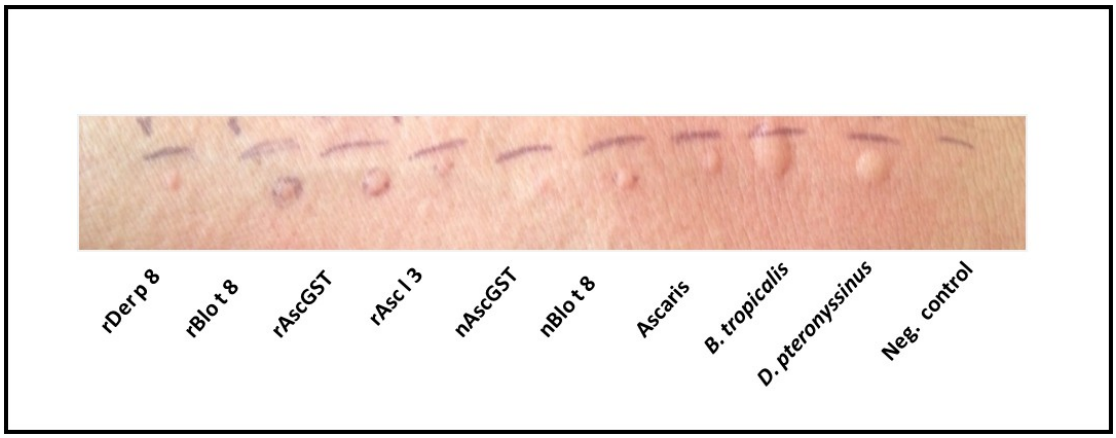

Figure 4.6 Skin prick tests with a battery of GST allergenic molecules. SPTs were done in a male 52 years-old asthmatic patient (A284).

Isolation of natural glutathione-S transferase from B. tropicalis

Using a glutathione-sepharose matrix we purified the natural GST from *B. tropicalis* extract. The eluate contained a 27 kDa band and another less intense migrating at 25 kDa (Figure 4.7). The yield of the purification was 12.5 µg per mg of extract. Natural Blo t 8 was detected as an unglycosylated protein (Figure 4.8).

We compared the specific IgE levels to the natural and recombinant allergen in 37 subjects. Its simultaneous IgE evaluation by ELISA showed they were highly correlated (r=0.92). IgE levels against the natural allergen was significantly higher than that to rBlo t 8 (p=0.03. 0.17 vs. 0.12 OD units, respectively).

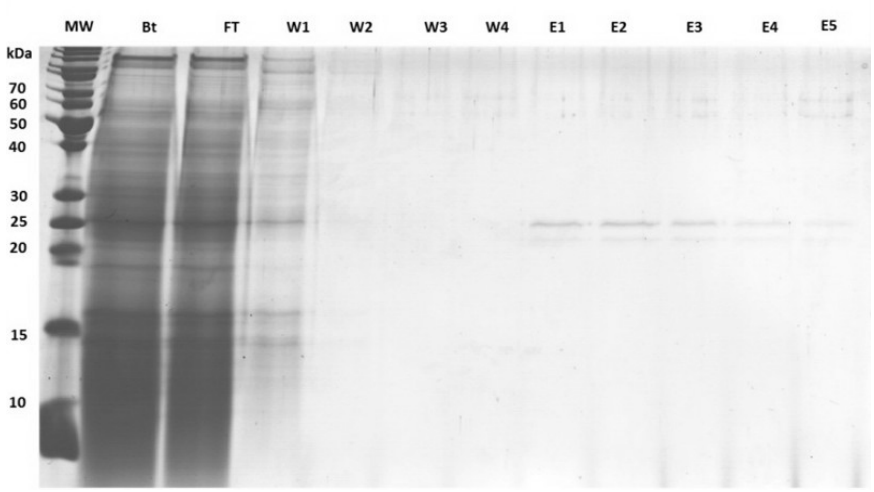

Figure 4.7 Purification of natural Blo t 8. The allergen was purified by affinity chromatography with gluthathione agarose. In this 15% SDS-PAGE: Bt, *B. tropicalis*; FT, flow throw; W, washes; E, eluates.

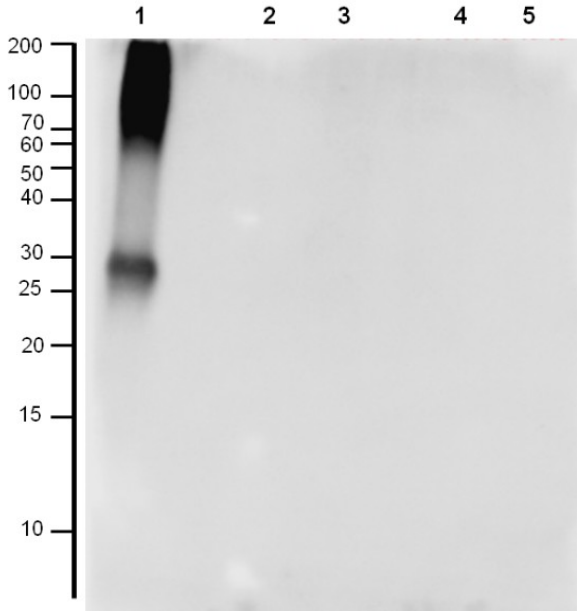

Figure 4.8 Glutathione -S- transferases from *B. tropicalis* and *A. lumbricoides* are not glycosylated. Western blot with ECL™ Glycodetection kit. 1) As positive control, pBlo t 12.0101, 2) rBlo t 8 and 3) rAsc GST as negative controls; 4) natural GST from *B. tropicalis* and 5) *A. lumbricoides*.

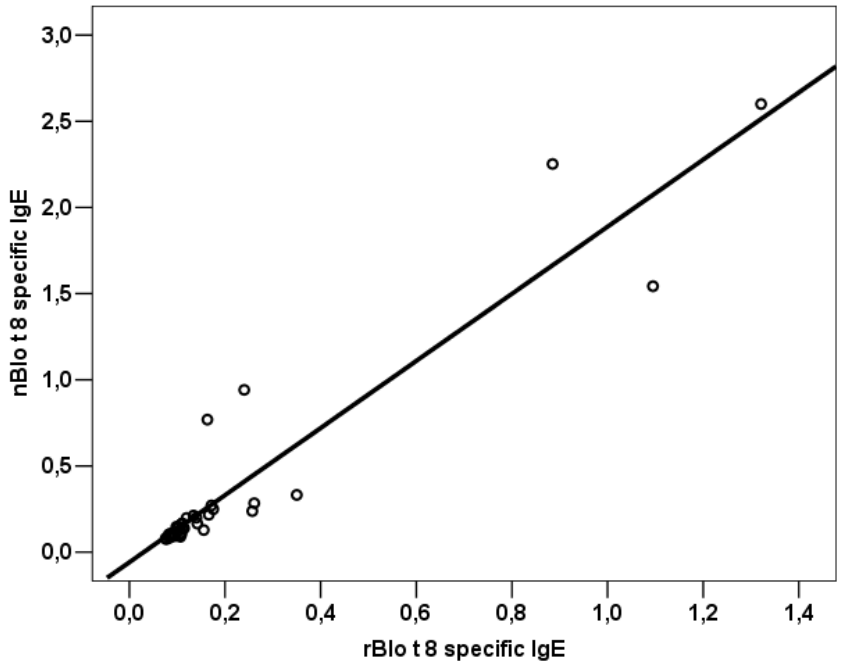

Figure 4.9 Correlation plot between specific IgE to natural and recombinant Blo t 8. Line represents linear regression slope.

4.1.3. Discussion of results

In this study we describe for first time the allergenic properties of Blo t 8, the gluthathione-S-transferase from *B. tropicalis*. As reported for Der p 8 [157], sensitization to this allergen was common, but mild, in an asthmatic population living in the tropics. Blo t 8 specific IgE levels were highly correlated to that against AscGST and Bla g 5. Although the overall similarity of GST primary sequences is moderate, they conserve clustered surface-exposed amino acids that may be part of cross-reactive epitopes.

There have been described several allergenic GSTs from different species, including fungi [157], cockroaches [155], mites [157, 158] and nematodes [156]. Cross-reactivity among different GSTs, in spite of moderate sequence conservation, has been also been experimentally demonstrated. For example, IgE specific-antibodies against Der p 8 cross-react with the GST from the cockroach *P. americana* [157]. Bla g 5 exhibits moderate CR with filarial GSTs; epitope mapping led to identify well-conserved linear

antigenic sites between the compared molecules [156]. Interestingly, one of the predicted cross-reactive epitope among AscGST and Blo t 8 involved various residues included in one N-terminal mapped epitope of Bla g 5, also conserved in the filarial nematodes.

The IgE responses to the natural Blo t 8 were more intense than to the isolated recombinant isoform. These results may be explained by different reasons; it is well known that in one species GST often exists as multiple isoenzymes. Low inter-class CR among GST has also been reported [159]. Huang *et al* found that nDer p 8 contains at least 8 different isoforms with different *pI* and the IgE reactivity of the produced recombinant isoform was not comparable to the native counterpart. In the case of *B. germanica* GSTs, another isoenzyme (BgGSTD1, δ-class) with low sequence identity (15%) and low CR to the sigma GST Bla g 5 was described [136]. Similarly, a wider repertoire of epitopes is expected in the natural GST *B. tropicalis* than those displayed on the isolated recombinant isoform; unique epitopes can be derived from other GST classes. It is probable that *B. tropicalis* also contain delta GSTs, as described in *D. pteronyssinus* [158]. On the other hand, we cannot discard that physico-chemical features of the produced recombinant allergen influence its lower reactivity. Its expression in a glycosylation-free system is not relevant since, in contrast to other GSTs, we did not find nBlo t 8 was glycosylated; however, it is possible that allergen folding was not optimal in *E. coli*. Interestingly, as with Der p 8 [157], rBlo t 8 had low affinity to a gluthathione-agarose resin (data not shown).

One special interest about this *B. tropicalis* allergen is the influence of CR with Ascaris homologous proteins on the elicited immune responses. With this preliminary data, it can be observed that IgE responses to the allergenic GSTs are different to tropomyosins, a very important cross-reactive group between allergenic arthropods and helminths with high allergenic potency [44]. Nor Blo t 8, neither the other tested allergenic GSTs, seem to be potent allergenic molecules in our population, as evidenced by low IgE titers in most Blo t 8 sensitized subjects and the induction of small papules by SPT. Santiago *et al* showed that filarial infection in mice induced cross-reactive antibodies to Bla g 5 that were able to induce IgE-mediated hypersensitivity reactions.

In humans, it was observed a strong IgG4 response to Bla g 5 in filarial-infected individuals. We hypothesize that early exposition to nematode infection would increase the chance to become sensitized to the GST from *B. tropicalis*, but induction of other immunomodulatory components as part of a Th2 modified response, such as IgG4, would hamper the development of hypersensitivity allergic reactions.

In summary, we have described some allergenic properties of Blo t 8, the GST from *B. tropicalis*. Allergen recognition was frequent, but IgE responses were of low intensity. The influence of cross-reactivity with another GST allergens, in especial those from nematodes, deserves further investigation.

4.2. Immunological studies about the ubiquitin from Blomia tropicalis

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4.2.1. Introduction

Blomia tropicalis is an important allergenic source in the tropics [152], with around 30 IgE binding components [38], but a smaller number of properly characterized allergens. Most searching strategies for allergen identification are focused on homologous proteins to the current mite allergen groups, usually created for classifying *Dermatophagoides spp.* components, the best characterized mite species in term of its allergenic potential. Proteomic analysis of allergen extracts can be very useful to identify novel allergen groups in mites. With this methodology, Mercado *et al* isolated a 6.5 kDa IgE binding peptide from the natural extract that was identified as ubiquitin by Edman degradation sequence analysis. Two from five sera of *B. tropicalis* allergic patients reacted to the protein. Based on this information, the aim of this study was to isolate the cDNA sequence of the ubiquitin from *B. tropicalis* for protein expression and characterization of its allergenic potential.

4.2.2. Results

Isolation of poli-ubiquitin cDNA

A 465 bp sequence, homologous to the poliubiquitin gene (with two identical copies of the ubiquitin sequence) was isolated from a *B. tropicalis* cDNA library using specific primers. Because *E. coli* cannot cleave the immature poliubiquitin product into monomers (as eukaryotes do), in order to obtain a protein product that resembles the natural peptide, a segment of 231 bp cDNA coding for a single ubiquitin peptide was codon optimized, subcloned into pET45b+ and expressed as His-tag fusion protein in

BL21-DE3 E. As shown in Figure 4.10, *B. tropicalis* ubiquitin is identical to the human orthologous.

```
Score =  137 bits (344),  Expect = 1e-32, Method: Compositional matrix adjust.
Identities = 67/67 (100%), Positives = 67/67 (100%), Gaps = 0/67 (0%)

Blo t  1   MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYN  60
           MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYN
Human  1   MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYN  60

Blo t  61  IQKESTL  67
           IQKESTL
Human  61  IQKESTL  67
```

Figure 4.10 BLAST results of the isolated *B. tropicalis* ubiquitin with the orthologous human gene.

Recombinant ubiquitin migrated as an 8.5 kDa band in 15% SDS-PAGE under reducing conditions. A pool of 2 sera that IgE-recognized the native ubiquitin was incubated with the 1 mM IPTG- induced culture lysate and the BL21(DE3) and non-induced culture lysates as negative controls. Different bands were detected in the induced-culture lysate but not in the negative controls (Figure 4.12).

Identification of ubiquitin in B. tropicalis extract

By Western Blot, the identity of the recombinant product was confirmed using a commercial monoclonal anti-ubiquitin antibody that recognizes bovine and human ubiquitin (clone 6C1**,** Sigma). This mAb bound to a 6.5 kDa band in *B. tropicalis* extract and to the recombinant ubiquitin. The immature poliubiquitin product was also recognized by this mAb as a 22 kDa band, although it runs as a 30 kDa band in SDS-PAGE (Figure 4.12).

IgE reactivity

The estimated cut-off for a positive IgE result to ubiquitin was 0.29 OD units. As Zeller et al [160], this value was three fold higher than the mean obtained with 10 sera from healthy, non-allergic subjects. In addition, we tested 10 cord blood serum samples and obtained similar O.D. values to the negative controls. IgE reactivity was found in

23.6% (n = 26) of asthmatic patients and 4.7% (n = 3) of controls. Mean specific IgE levels were higher in asthmatics (0.295 ± 0.15 vs. 0.150 ± 0.09, p = 0.001). After adjustment by sex, age and total IgE levels, sensitization to ubiquitin was associated with asthma (OR: 6.11; 95% IC: 1.01 – 37.1 p = 0.04).

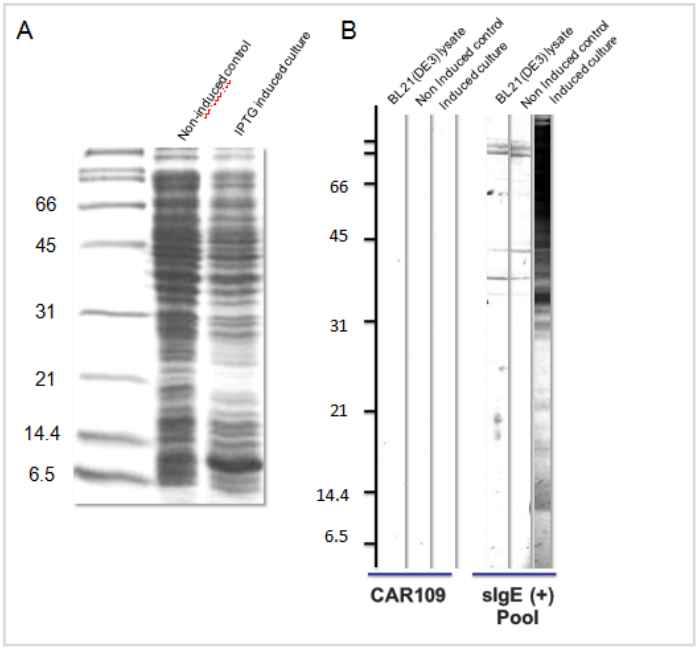

Figure 4.11 Expression of recombinant ubiquitin as a single copy. A) 15% SDS-PAGE showing results of Ubiquitin expression experiments. B) IgE Western Blot of the expression culture lysates with a negative control serum and nUbi IgE positive serum pool.

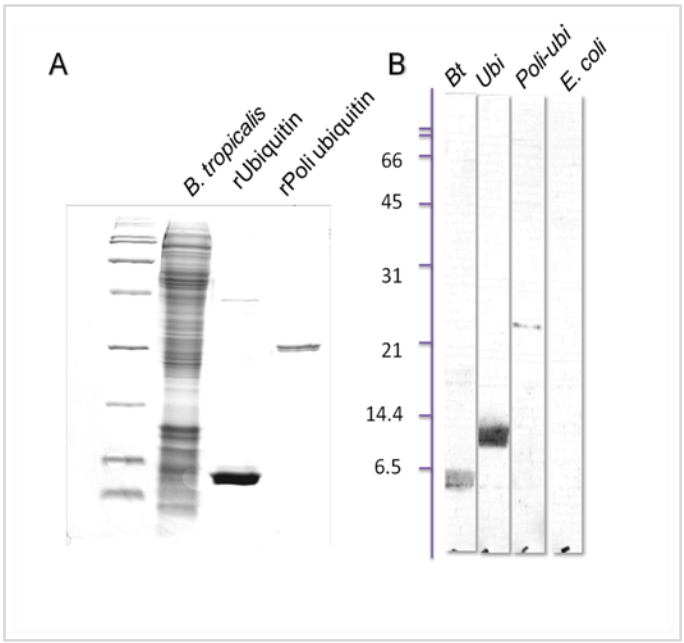

Figure 4.12 Identification of ubiquitin in *B. tropicalis*. A) SDS-PAGE of *B. tropicalis* extract, purified recombinant ubiquitin and poli-ubiquitin. B, Western Blot of these proteins with an anti-ubiquitin mAb. *E. coli* lysate was used as negative control

Table 4.2 IgE responses to ubiquitin in patients with asthma and healthy controls

	Asthma (n=110)	Controls (n=64)	p Value
Sex (Female)	56.4%	60.9%	0.334
Age	31.5 ±17.6	36.4 ±16.6	0.07
Total IgE	1112.43 ±1476	72.28 ±104.8	<0.0001*
B. tropicalis specific IgE	1.36 ±0.93	0.118 ±0.06	<0.0001*
D. pteronyssinus specific IgE	0.75 ±1.21	0.101 ±0.1	<0.0001*
Ubiquitin specific IgE	0.295 ±0.43	0.150 ±0.09	<0.0001*

Data are reported as mean ± standard deviation

4.2.3. Discussion of results

With these results we confirm, in a greater number of patients, that ubiquitin from *B. tropicalis* is an IgE- reactive molecule. Although there were healthy subjects responding to Bt-Ubi, frequency of IgE sensitization was significantly higher in asthmatic patients. Because this ubiquitin is identical to the human-derived peptide, the observed antibody response can also be considered as autoimmune. The recognition of intrinsic proteins as allergens (autoallergens) has been described before [161-163], cross-reacting some of them with heterologous molecules. For some of this group of "autoallergens", there have been demonstrated its capacity to induce IgE-mediated cell activation, especially in those individual co-sensitized to CR environmental allergens [164-166]. However, there are also intrinsic IgE binding proteins that do not activate basophils, but induced other type of immune effects. Hom s 2, for example, is a potent inducer of IFN-gamma secretion and epithelial cell damage [163]. Autoantibody responses to ubiquitin have been detected in patients with systemic autoimmune disorders [167], but its role of allergy have never been reported. These results only support the IgE reactivity towards ubiquitin; nevertheless, its capacity to induce allergic reactions must be demonstrated.

Chapter 5. *Understanding the early immune response to B. tropicalis allergens and its clinical impact in children from the tropics*

Overview

It is well-known that sensitization to *Blomia tropicalis* is common and intense in several populations living in the tropics. Although this mite inhabits most dwelling homes, this fact does not explain by itself its high allergenic potential. Helminth infections, especially that caused by *A. lumbricoides*, are common in socio-economically deprived regions of these latitudes and co-sensitization to this nematode is also very frequent. Cross-reactive antigens between Ascaris and the HDMs *B. tropicalis* and *D. pteronyssinus* are common IgE sensitizers among atopic and non-atopic subjects. According to our cross-sectional investigations, it seems that *Ascaris* sensitization is a risk factor for asthma and atopy, but a clear effect cannot be ascertained due to the CR phenomena. These limitations prompted us to initiate a prospective birth study, expecting to elucidate several aspects about allergy inception and early sensitization in the tropics. Analyzing species-specific markers of *B. tropicalis*, but also cross-reactive allergens, gave a descriptive overview about the origins of allergic responses in this area.

We designed the FRAAT birth cohort of children living in Cartagena, a tropical city of Colombia to investigate several aspects of allergy inception[6]. Recruitment of mother-newborn pairs began in 2007. This chapter will describe the socio-demographic characteristics of the entire cohort, the prevalence of atopy-related clinical phenotypes (Section 5.1, publication #2), the evolution of total and specific IgE responses during the first three years of life, evaluated at the level of complete extracts (Section 5.2, Submitted manuscript #3) and also by using purified allergens from *B. tropicalis* Blo t 5 and Blo t 12 and the *Ascaris* components Asc s 1, Asc l 3 and AscGST (Section 5.3, Manuscript in preparation, data presented at the CIA meeting 2012*).

In this cohort, most children lived under poverty conditions; *A. lumbricoides* was the most common nematode infection. Recurrent wheezing was common; atopic eczema

did not. The frequency of sensitization to HDM, especially *B. tropicalis*, during the first three years of age was high and associated with wheezing. Total IgE levels were influenced by maternal IgE and were protective from wheezing. Sensitization to *B. tropicalis* was genuine, since we also detected high sensitization rates to Blo t 5 and Blo t 12. Sensitization to Ascaris components was more frequent than to the purified *B. tropicalis* allergens in the beginnings of life (~6 months) but at the end of the follow-up (~ 3 years) *B. tropicalis* sensitization was slightly higher.

5.1. Epidemiological Description of the FRAAT Cohort[2]

5.1.1. Introduction

The causes of asthma and other allergic diseases remain unknown. Genetic and epidemiological studies suggest that for these multifactorial diseases the expression of different phenotypes depend on complex interactions between susceptibility genes and the environment [56]. This is reflected in the wide differences in prevalence and natural history of allergic diseases around the world [57]. In many regions of Latin America asthma is a public health problem affecting children and adolescents in urban areas; wheezing, asthma and allergic rhinitis are very frequent in some regions [168], with rates similar or even higher than in industrialized countries [169, 170]. Interestingly, anticipated protective factors, such as low hygienic conditions, do not confer protection in poor and overcrowded communities, where a high prevalence of asthma and early infections concur [4, 6, 80]. In addition, there are disparities among some phenotypes, such as allergic sensitization and prevalence of atopic eczema, when compared to those observed in industrialized countries [171]. In urban zones of Colombia, asthma is the most common chronic disease in children and IgE sensitization to mites is a hallmark in most patients [123, 172, 173]. The population of Cartagena, Colombia, has been previously studied to investigate genetic and environmental risk factors for asthma and

[2] The epidemiological description of the cohort was published as an original article in BMC Pulmonary Medicine with the title: **Particular characteristics of allergic symptoms in tropical environments: follow up to 24 months in the FRAAT birth cohort study**.

allergy, not only because asthma is common [174] but particularly because the city is in a tropical region where a warm and humid environment, facilitate the growth of a diverse mite fauna and the perennial exposure to high concentrations of their allergens [175]. Moreover, most of the population is poor and exposed to parasites, generating an interesting setting to study the influence of environmental factors on the susceptibility to allergic diseases.

There are few Latin American birth-cohort focused on allergic diseases [176-178] and they have explored several phenotypes and risk factors using different study designs. For example, Lopez et al. detected sensitization to D. pteronyssinus in 30% of wheezing and 11% of asymptomatic infants at 12 months of age in a prospective study (n = 102) in Brazil. They also found a weak association between wheezing and specific IgE to mites at 12 months. Environmental exposures and socioeconomic status were not evaluated [176]. Rullo et al. studied the role of respiratory infections, exposure to mouse allergens and breastfeeding on wheezing in 104 children living in a socially deprived community of Brazil. Analysis at 30 months showed strong association of wheezing with "respiratory infections requiring antibiotics". No association was found with endotoxin exposure or mite sensitization [177]. Cooper et al. in a study protocol presented the strategies for investigating the impact of early life exposure to geohelminth infections on the development of vaccine immunity, allergic sensitization and allergic inflammatory diseases in 2,403 neonates followed up to 8 years of age [179]. Our study population is an urban low-income community of admixed genetic background [5, 106], living in the tropics under limited sanitary conditions and exposed to mites and helminth allergens. We hypothesize that, for children growing up under these particular genetic and environmental conditions, the prevalence of some allergic phenotypes, as well as the nature and effects of risk factors are different to those found in cohorts from industrialized countries.

The aims of this study were: 1. To create a community-based birth cohort study for analyzing the effects of allergen exposure, early parasite infections and poor living conditions on the inception of allergic diseases, specially asthma; 2. To evaluate the effects of prenatal and other risk factors on the prevalence of wheezing and eczema and 3. To prospectively collect biological samples of children living in poor neighborhoods

of a tropical city for further immunological testing, molecular genetics and molecular microbiology screenings. Here we describe the study protocol, baseline characteristics, demographical observations and risk factors for wheezing up to 24 months, of the "Risk Factors for Asthma and Allergy in the Tropics" (FRAAT) study.

5.1.2. Results

Demographic characteristics of the population

Three hundred and twenty six mother-infants pairs were included. Fifty-two families were lost during follow-up (Figure 5.1). Reasons for exclusion were: moving out of city to rural villages (n = 23), lost of contact by inaccessible addresses or lack of telephones (n = 16), family declined (n = 7), social conflicts (n = 3), child dead (n =1), severe malnutrition (n = 1) and Rh isoinmunization (n = 1). Most families were lost in the interval of 0 to 6 months (n = 40), afterwards 12 families drop-out between 7 and 24 months. The sociodemographic characteristics of the excluded families were similar to those that continued in the study. Antecedents of allergic diseases were similar between excluded and non-excluded mothers and did not influence the willingness to participate. Maternal characteristics and pre-natal exposures to risk factors are summarized in Table 1 from the paper #2 of this publication. All families had similar environmental and living conditions. Ninety four percent of participants were clustered in the urban area of the poorest communes ("Comunas") of Cartagena and the rest in two rural villages, La Boquilla (3.1%, n = 10) and Pasacaballos (2.8%, n = 9). The ethnicity of the population was homogeneous, 74.8% of mothers were born in Cartagena, 24.6% in rural villages of the Northwest Coast of Colombia and 0.2% in the inner country. Most of the mothers were young (mean maternal age ± standard deviation, 23.2 ± 5.8 years), 58.9% multiparous, 59.5% house-wives and 62.5 with low level of education. Regarding prenatal exposures, 97.5% denied having smoked during pregnancy but 43.6% reported intradomiciliary secondary-exposure to a median of 35 cigarettes per week (IQR 14 – 70) and 1.36 ± 0.68 (mean ± standard deviation) smokers per household. Some families had habitual contact with fume from cooking with firewood and/or trash burning at homes or their neighborhoods. Intradomiciliary exposure to pets during pregnancy was

reported in 48.2% of mothers, being dogs the most common, and to poultry and pigs in 13.5%.

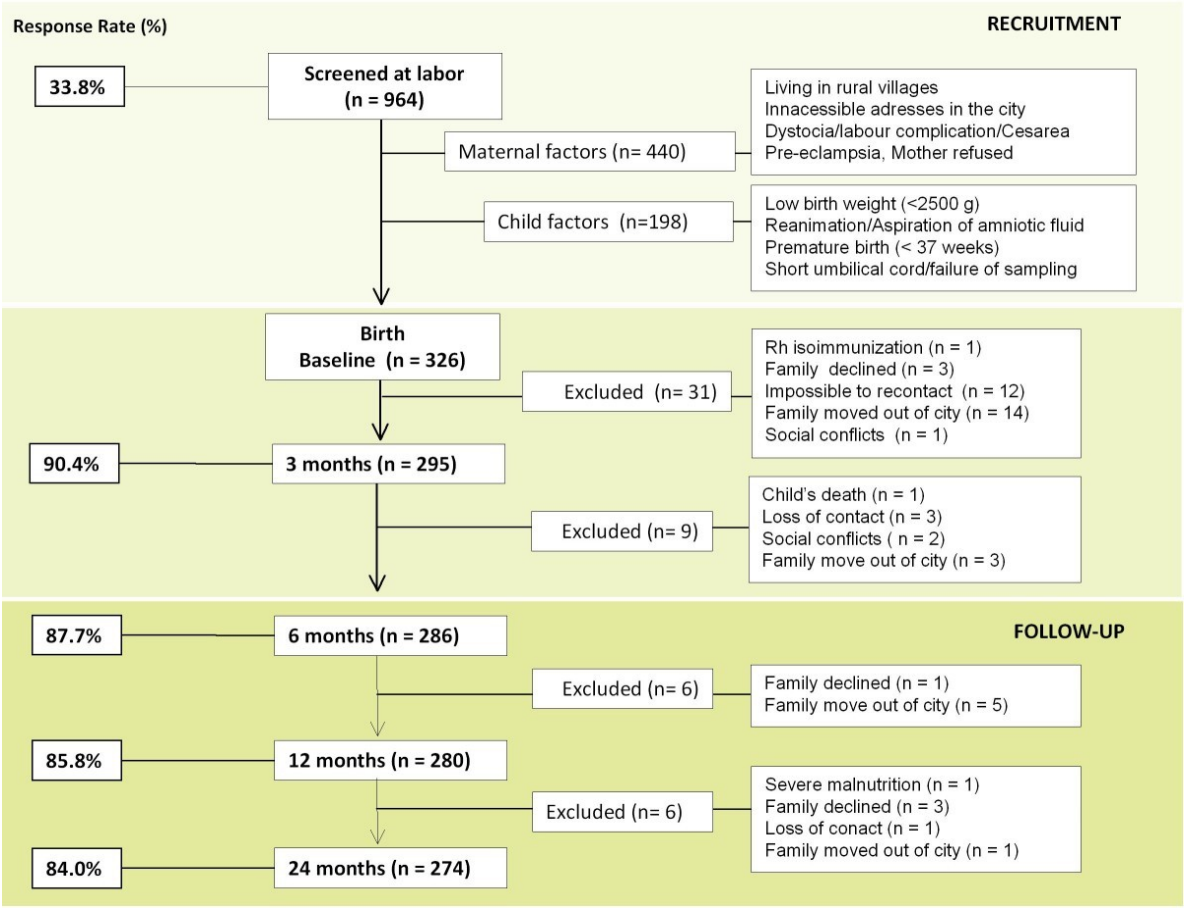

Figure 5.1 Flow chart of the data collection procedure in the FRAAT study

The prevalence of allergic diseases in mothers is presented in Table 5.1. Asthma and allergic rhinitis were the most common diseases and dust mite allergens the main sensitizers. Interestingly, cases of atopic eczema were not observed in mothers.

Table 5.1 Maternal antecedent of allergic diseases (n=326)

Phenotype	n (%)
Current asthma	21 (6.44)†
Duration of asthma (Mean, SD)	14.0 ± 9.01 years
Current rhinitis	84 (25.7)‡
Eczema	0 (0)

Phenotype	**n (%)**	
Food allergy	23 (7.1)	
Drug allergy	5 (1.5)	
Family history of asthma*	53 (16.3)	
Allergic sensitization (n = 265)**	**n (%)**	**Any allergy symptom (%)*****
Atopy (at least 1 positive test)	93 (35)	(50.5)
Mite sensitized	82 (30.9)	(54.8)
D. pteronyssinus	59 (22.3)	(59.3)
B. tropicalis	61 (23.0)	(54)
Cockroach	12 (4.5)	(58)
Cat	7 (2.6)	(42)
Dog	7 (2.6)	(57)
Molds (Pen, Asp, Alt)	8 (3.0)	(100)
Pollens (Art, Phl, Bet, Acacia)	7 (2.6)	(57)

†Probability of exposure in non-wheezers ($r_0 = 0.05$)

‡Probability of exposure in non-wheezers ($r_0 = 0.26$)

*Asthma in parents, grand-parents and/or siblings

** As defined by skin tests

*** Asthma, rhinitis, reported food allergy

SD: Standard deviation

Living conditions of children and particular environmental exposures

Children were visited at home by the research staff to investigate risk factors and to validate the information collected in baseline questionnaires. Post-natal socio-demographic conditions were similar to those described at baseline. The infant group included 139 females (48.6%) and 147 males (51.4%); most of them living in brick houses with floors of bare concrete. in non-paved streets. Forty per cent lacked sewage system, 20% toilettes and 11% tap water. Usually there were 6 people per household and most children lived with their parents, grand-parents, siblings and other relatives;

35% of them overcrowded, sharing bedrooms and mattresses with parents and older siblings. Furthermore, 35% of families had no fridge and 54% lacked sink for dishwashing. Early infections with parasites were detected as early as 3 months of age. The most common parasites were of the genera *Entamoeba spp*. Among nematodes, *Ascaris lumbricoides* was the most frequent, affecting 2.71% of children at 12 months and 5.22% at 24 months (Table 5.2). By 12 months, the median egg counts of *A. lumbricoides* were low (78 epg, IQR 76-1452) but increased between the first and the second year (3458 epg, IQR 975-9256), suggesting that in this age range *A. lumbricoides* infections are of low intensity (> 90% of subjects with egg counts < 50.000 epg) [180]. In addition, 101 had received anti-parasitic treatment and 95% at least one cycle of antibiotics (mean age of the first treatment 2 ± 3.4 months), due in part to the high prevalence of infectious diseases, e.g. pneumonia, urinary tract infections, bacillary dysentery.

Table 5.2 Prevalence of parasitic infection as determined by stool examination

Chapter 6.	0-6 months	0-12 months	13-24 months
Any parasite	17 (8.5%)	62 (21.3%)	58 (37.9%)
Polyparasitism	1 (0.5%)	2 (1.13%)	9 (5.88%)
Protoozoan			
Entamoeba ssp.	12 (6%)	34 (13.1%)	31 (20.2%)
Giardia lamblia	1 (0.5%)	12 (4.65%)	18 (11.7%)
Blastocystis hominis	1 (0.5%)	3 (1.16%)	3 (1.96%)
Endolinax nana	0 (0%)	1(0.38)	2 (1.30%)
Balantidium coli	0 (0%)	0 (0%)	1 (0.65%)
Helminths			
Ascaris lumbricoides	2 (1%)	7 (2.71%)	8 (5.22%)
Trichuris trichiura	0 (0%)	2 (0.77%)	2 (1.30%)
Ancylostoma duodenalis	2 (1%)	3 (1.16%)	3 (1.96%)
Strongyloides stercolaris	0 (0%)	0 (0%)	1 (0.65%)

Prevalence of wheezing and atopic eczema

The prevalence of "wheezing ever" was 17.5% at 6 months, 31.1% at 12 months and 38.3% at 24 months. Recurrent wheezing (3 or more episodes) was present in 7.1% of children at 12 months and 14.2% at 24 months. As confirmed by questionnaires and medical records. 94% of children wheezing between 0 and 24 months attended a medical center or emergency room at least once; 84% received salbutamol, 76% antihistamines and 43% oral steroids. Hospitalizations were documented in 23% of cases. In addition, half of children wheezing between 0 and 6 months continued wheezing until 24 months. At 24 months, 18.8% of children had experienced at least one episode of skin rash/hives, 11.8% of them treated with antihistamines by a physician. In 7.4% of cases no potential inducer could be identified. Drugs were commonly incriminated (6.9%), including antibiotics (amoxicillin and ampicillin in most cases) and anti-inflammatory (i.e. metamizole and ibuprofen). In 1.8% of cases the symptoms were related to food ingestion. Only two cases of rash/hives induced by egg ingestion and two cases by milk were clinically documented by the staff. According to our diagnostic criteria, no case of eczema was detected between 0 and 24 months.

5.1.3. Discussion of results

Birth cohort studies in different environments and populations are useful to understand the natural history of allergic diseases. Nowadays there are a number of such studies, most of them multicentric, with some common objectives and including a wide range of participants [181-184]. The results, mainly from Europe and US, have shown the importance of particular and common risk factors and their differential influences on allergic diseases, supporting the relevance of doing this type of investigations worldwide, to evaluate the great diversity of settings associated, not only with the climate, geographical location and genetic background, but also with low socio-economic conditions that generate particular risk factors and limit the access to health services. The prevalence of allergic diseases varies among regions and in Latin America, figures of asthma and other allergies in children are very high [185-190]. However, as mentioned, prospective birth cohorts in the region are still scarce, although a growing number are being reported [176, 177, 179].

A remarkable finding in our study is the high prevalence of wheezing ever and recurrent wheezing, higher than reported in some European cohorts [191-194], similar to data from the USA [195, 196] and lower than those found in some Latin American surveys [177, 189]. Considering that 12% of children of this cohort attended a hospital with bronchiolitis, one explanation of our findings is the effect of viral diseases, as has been shown in other studies from Latin America [177]. In fact, bronchiolitis was a high risk factor for wheezing in our cohort, with odds ratio similar to that found when laboratory diagnosis of viral infection was done [197]. Lower respiratory tract infections by Respiratory Syncytial Virus (RSV) are common in tropical developing countries causing 27 to 96% of all acute wheezing hospitalizations in children under 6 month of age [198]. In our children an important proportion of wheezing during this period might be caused by viruses and this related to the finding that only half of children wheezing before 6 months continued wheezing until 24 months. However, the existence of a group with recurrent wheezing (14.2%) suggests that other factors, including atopy, may be influencing this phenotype [176]. RSV infections can induce a change of the Th1/Th2 balance, expressed by an increase of the IL-4/IFN-gamma ratio and a persistent IgE response over the years [199, 200], and human rhinovirus-induced bronchiolitis carries a markedly risk of persistent wheezing and childhood asthma [201]. Besides, in the tropics, co-exposure to perennial high concentrations of mite allergens and helminth infections may induce an early allergic response with respiratory effects [202]. Also, living in socially deprived urban areas of underdeveloped countries lead to an early exposure to high levels of endotoxin, in turn associated with increased risk of wheezing during the first year of life [203, 204]. Allergy diagnosis by *in vitro* assays and skin prick tests will help to improve phenotype definition in our cohort and obtain data about risk factors for atopy associated-wheezing [205].

In this study, maternal asthma was consistently associated with wheezing and recurrent wheezing in the offspring, a finding already observed in different populations and considered an important risk factor for asthma in children before 5 years [206-209]. The mechanisms of this “maternal effect” and why it is more evident during early childhood are unknown. It has been reported that maternal asthma, as well as the child HLA-G genotype contribute to the presence of bronchial hyperreactivity [210]; in

addition, several factors may act in combination with maternal cytokines, antibodies and other mediators that act *in utero* or during the perinatal period [211, 212]. Furthermore, recent works in animals suggest that epigenetic mechanisms may play a role [213, 214], although the transmission of the maternal susceptibility via epigenetic changes or parental imprinting remains to be demonstrated. Remarkably, this association was detected at 12, from 13 to 24 and 0 to 24 months, suggesting that the maternal effect is on children that start wheezing after 6 months. Moreover, it was also evident with recurrent wheezing, a more severe phenotype and always held up after adjustments for covariates. These results are in agreement with a study in Brazil, were parental asthma was associated with atopic and non-atopic wheezing [215] but in contrast with other where maternal atopy was a risk factor for wheezing in poor-environments [216] because we found no association between maternal sensitization alone and wheezing.

Additionally, and in concordance with other surveys [217-219], we found that male gender was associated with wheezing in cases between 0 and 6 months, but the effect disappeared with increasing age. As it has also been reported, the prevalence and severity of asthma are higher in boys than in girls in the first 10 years of life, but this pattern is reversed after puberty [220]. Although the reasons of this observation remain unknown, there are interesting analysis and hypothesis [221, 222].

Contrary to expected, our data show no association of wheezing with active or passive maternal smoking, and the low prevalence of self-reported maternal smoking during pregnancy precluded further association analysis. Therefore, we investigated association between passive intra-domiciliary exposure and wheezing at 6, 12 and 24 months but the results were the same. The prevalence of wheezers was always higher in the group of children with prenatal passive exposure: 6 months (41.1% vs. 50.0%), 12 months (40.4% vs. 47.1%), and 24 months (40.0% vs. 45.2%) but this was not statistically significant. In addition to the type of housing and cultural patterns, another explanation for these results may be a Type II error because the sample is underpowered to detect the effects of smoking. Increasing the sample size and using biological markers of tobacco exposure could help to obtain a more accurate analysis.

The mechanisms of atopic dermatitis are not clear but hereditary factors and environmental exposures are supposed to play important roles [223, 224]. A main finding of this study is that in a socially deprived population of the tropics, the natural history of atopic conditions seems to be different to expected according to the “atopic march”, where eczema appears before the first two years followed by allergic respiratory symptoms [225, 226]. In fact, we found no cases of atopic eczema during the first two years of age, while wheezing was a frequent phenotype. Although we do not know yet if atopy is playing a role in the origin of wheezing in these children, it is clear that early respiratory symptoms predominate over cutaneous, atopy-associated, symptoms. In addition, asthma and allergic rhinitis were the most common allergic manifestations in mothers but there was no eczema among them. In a previous study we found a low frequency of atopic dermatitis in the general population of Colombia [4]. In developed countries, the prevalence of this disease is around 10 to 20% in children under 5 years [227, 228], but the ISAAC Phase III study in Latin America reports that, with some exceptions, the prevalence is less than 5% [229]. Under these rates we expected around 15 cases of eczema in our cohort.

Increasing evidence suggest that the "atopic march" includes only a subgroup of patients [230-232]. However, the reasons we found no cases of atopic eczema should be further analyzed. We hypothesized that the genetic background, with an important component of African ancestry and a different distribution of susceptibility alleles for eczema could be protective. Also, particular exposures of these children, with early infections as a hallmark, may confer protection, as has been suggested [204, 233]. The diagnostic criteria we used [234] are very important when aiming to study this disease in the tropics because infections and other medical conditions producing “itchy skin” are very frequent. Although cases of eczema may appear in our cohort after 2 years, it has been suggested that a great number begin within the first 12 months, disappearing around 3 years in a significant proportion of children [235]. Therefore, monitoring symptoms of this disorder, as well as other allergic diseases in this cohort is mandatory.

There are reports of associations between low income and asthma [236]. We found no relationship of allergy symptoms with socioeconomic strata, possibly because the study population is homogeneously poor and differences between strata are small.

Potential risk factors related to poverty, such as housing conditions, access to tap water and sewage system, exposure to fume from firewood and trash burning, intradomiciliary contact with poultry and pigs and large family size were not associated with wheezing. For most of them the prevalence of exposure among non-affected was high enough to detect an effect. Since the study population is not representative of all the social strata of the city the generalization of these results is limited. Further research is needed to evaluate the potential interactions that lead to allergy phenotypes in socially deprived environments.

Since our study was based in a prospective cohort design that included physician assessment of a broad range of factors, we think that accurate information was obtained. This strategy may reduce the number of participants and requires more resources but it is necessary to increase accuracy avoiding the bias from poorly defined phenotypes, as may occur when the information is obtained only from self-administered questionnaires, especially in populations with low education level. This is especially important for atopic eczema and wheezing because they may not be easily evaluated by parents. Two sources of ascertainment bias should be mentioned: one from the type of housing and living conditions that make it difficult to identify some exposures (e.g. pet ownership, smoke exposure) and other from the detection of phenotypes. The diagnosis of wheezing was made only when patients were evaluated by a physician or had documented history of medications and hospital admissions, as has been recommended [237] and this might bias our attention to the most severe cases, lowering the sensitivity of the survey. However, considering that the biologic samples are intended for serologic and molecular screenings we chose for well-defined phenotypes to reduce heterogeneity. Among the limitations of this study, one is the small number of children recruited, if compared to multicenter surveys. Although, according to the formerly reported prevalence of wheezing in this population our analyses have good power to detect some associations, increasing the number of participants could provide better information. However, many obstacles must be overcome to organize a prospective cohort in countries with high degree of poverty. Low education precludes the use of self-reported questionnaires, limiting the number of enrolled families. Another problem

affecting the response rate and the follow-up is the lack of well urbanized neighborhoods, which greatly increase the difficulty of the visits.

As in other birth-cohorts, a valuable selection of biological material has been done in this study [183]. Laboratory tests will help to assess genetic and environmental factors linked to asthma development, providing novel information about disease mechanisms in the tropics. An open question when studying allergies in this zone is the influence of *A. lumbricoides* infection on the process of IgE sensitization to common allergens, especially house dust mites [238]. Thus, IgE serology to *Ascaris spp.* and allergen extracts will be serially performed to explore primary sensitizers and how the infection status influences the frequency and strength of specific IgE response. Another aspect to be evaluated is the relationship between the immunological profile at birth and further wheezing in children. Immune- responsiveness gene expression will be monitored at different time-points. As differences in microbiota composition may influence immune response [239-241], stool samples has been collected from birth to 24 months, to identify gut microbiota composition and explore their potential relationship with the IgE responses and allergies.

5.2 Impact of cord blood IgE, maternal IgE and other prenatal factors on early sensitization to Blomia tropicalis

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5.2.1. Introduction

The patterns and evolution of IgE antibody responses in early life provide important information about the natural history of atopy and allergic diseases. Since the process is influenced by the environment and the genetic background of the population, birth cohort studies are valuable strategies to analyze early sensitization in humans [181, 182]. However, few of these investigations have been done in the tropics [176, 177], which is an important gap considering that about half of human beings live in this zone. The current concepts of diagnosis and management of allergic diseases are based on evidence collected in industrialized countries and temperate zones; then, knowledge of the evolution of IgE responses during infancy and its relationship with particular environmental factors in the tropics is essential for obtaining a more realistic view of the pathogenesis and treatment of allergy.

Studies in the tropics also help to analyze the universality of conceptual frameworks such as the hygiene hypothesis, the atopic march and the role of mite and parasite exposure in the inception of allergy [102, 242]. There is evidence that the prevalence and risk factors for wheezing are different in this region, and the effects of conventionally atopy-associated phenotypes such as cord blood total IgE and early allergen sensitization deserve further investigation. In addition, the role of particular conditions such as poverty, usually considered protective from IgE sensitization [243],

on the evolution of IgE responses in childhood is still controversial [244]. We designed the FRAAT birth cohort of children living in Cartagena, a tropical city of Colombia (South America), to investigate several aspects of allergy inception [102]. Here we describe the evolution of total and specific IgE responses in this population and their influence on wheezing at early infancy.

5.2.2. Results

Subjects and serology data

Serology data were obtained from 290 children 289 mothers and 285 CB samples. Ninety one percent (n = 265) began the study with serology information at S1. 3.3% at S2 (n = 9). 4.8% at S3 (n = 14) and 1% at S4 (n = 2). Two hundred and forty six children (84.8%) had 2 or more determinations of total and/or allergen specific IgE at different times between 6 and 42 months. The influence of prenatal factors on CB total IgE was evaluated in 277 children who had serology data and no evidence of maternal blood contamination. Immunoglobulin A was detected in five cord blood samples, which were excluded from the analysis. The effect of maternal IgE on recurrent wheezing was evaluated in a dataset of 261 children with determination of clinical phenotypes up to 24 months. The effect of IgE response on wheezing phenotypes at 2-3 years old was analyzed in 176 children. Demographic features and maternal allergic phenotypes of the sub-groups did not significantly differ from those at baseline (Table 5.3).

Maternal serology was characterized by high total IgE and frequent allergen sensitization

Maternal total IgE levels were high (Median 197.5 kU/L IQR: 81.1 - 633.0) and 59.4% of them were above a frequently used cut-off level to define atopy in other populations (150 kU/L) [245, 246]. We used the 70th percentile as cut-off point to define high total IgE in mothers (> 502 kU/L) [115]. Sensitization to *Ascaris spp.* was very frequent (49.7%). HDM were also common IgE sensitizers (Table 5.5). Frequencies of HDM sensitization were also assessed by skin prick tests; although they were lower (17.7% to *B. tropicalis* and 20.4% to *D. pteronyssinus)* than those detected by IgE serology, there

was concordance between the results obtained by the two type of measurements (κ = 0.42. $p < 0.001$ for *B. tropicalis*; κ = 0.392. $p < 0.01$ for *D. pteronyssinus*). HDM sensitization was more frequent in mothers sensitized to Ascaris (25.3% in non-sensitized vs. 72% in sensitized; $\chi^2 = 63.0$. $p < 0.001$). Total and specific IgE to all allergens were significantly higher in mothers with allergic rhinitis. However, only specific IgE levels to *B. tropicalis* and/or *D. pteronyssinus* were significantly higher in mothers with asthma (Table 5.4).

Table 5.3 Descriptive information about demographic factors. prenatal exposures and clinical phenotypes in the different data sub-sets analyzed in the study

Factor	Baseline (n=326)	Influence of prenatal factors on CB total IgE (n=277)	Influence of prenatal factors on wheezing (n=261)	Risk factors for sensitization at 2-3 years (n=176)
Gender [male. n (%)]	161 (49.3)	140 (50.5)	134 (51.3)	92 (52.3)
Child age (months). Mean ± SD	--	--	24 ±0	31.5±4.8
Ever wheezing [n (%)]	--	--	103 (39.5)	77 (43.8)
Recurrent wheezing [n (%)]	--	--	46 (17.6)	27 (15.3)
Maternal age at child birth (years. Mean ± SD)	23.2 ±5.83	23.3±5.9	23.2 ±5.9	23.2±5.9
Maternal Asthma [n (%)]	21 (6.44)	20 (7.2)	19 (7.3)	14 (8.0)
Maternal Rhinitis [n (%)]	99 (30.3)	83 (30)	78 (29.9)	52 (29.5)
Wood house [n (%)]*	83 (25.5)	70 (25.3)	68 (26.1)	51 (29)
Tap water	209 (89)	246 (88.8)	229 (87.7)	156 (88.6)
Sewage [n (%)]	191 (58.6)	164 (59.2)	150 (57.5)	94 (53.4)
Cockroaches at home [n (%)]	255 (78.2)	218 (78.7)	206 (78.9)	141 (80.1)
Dog at home [n (%)]	143 (43.9)	127 (45.8)	121 (46.4)	74 (42)
A pet (cat/dog) during whole pregnancy [n (%)]	107 (32.8)	92 (33.2)	89 (34.1)	53 (30.1)
Poultry/pigs during pregnancy [n (%)]	44 (13.5)	39 (14.1)	36 (13.8)	24 (13.6)

*Proportion in relation to brick house.

Maternal IgE and socio-demographic factors influence cord blood total IgE

No IgE sensitization was detected for any of the tested allergen/antigens in CB. Only one case of possible sensitization to *D. pteronyssinus* (specific IgE 0.2 kU_A/L) was found. CB total IgE levels in 220 samples were below the limit of detection, but were included in the analysis with an assigned 0.01 kU/L value. The range of CB total IgE was between 0.01 and 26 kU/L. The 80th percentile of CB total IgE (0.07 kU/L) was

used as cut-off to define high CB total IgE. The influence of maternal IgE and prenatal socio-demographic factors were analyzed by binary logistic regression. By univariate analysis, maternal high total IgE (OR: 3.83. 95% CI: 2.07-7.07; p<0.001). HDM- (OR: 2.75. 95% CI: 1.47-5.12; p < 0.001) or Ascaris- sensitization in the mother (OR: 3.14. 95% CI: 1.66-5.95; p < 0.001) were associated with high CB total IgE. Having a pet (cat/dog) at home during whole pregnancy was also positively associated with total IgE (OR: 2.33 95% CI: 1.28-4.25. p = 0.01) while socio-economic stratum was inversely related (OR: 0.41. 95% CI: 0.18-0.96; p = 0.04).

In the multivariate analysis, inclusion of predictors related to high CB total IgE showed that maternal high total IgE, but neither Ascaris nor HDM maternal sensitization, remained associated (Table 5.4, model 1). However, the interaction of maternal high total IgE with HDM sensitization (aOR: 2.87. 95% CI: 1.36-6.06; p < 0.01) or Ascaris sensitization (aOR: 2.59 95% CI: 1.24-5.46. p = 0.01) were also associated (Table 5.4, models 2 and 3). Similar results were obtained when maternal HDM components´ sensitization was assessed. Maternal high total IgE plus sensitization to any recombinant allergen was also strongly associated to high CB total IgE after adjustment for covariates (aOR: 5.88 95% CI: 2.47-13.98. p < 0.001).

Total and specific IgE values increase rapidly during early life

We assessed the influence of several prenatal and postnatal factors on the evolution of the IgE response. Using LMM, values of $\log_{10}$ total IgE (Estimate: 0.371; p < 0.001) and specific IgE to *B. tropicalis* (Estimate: 0.429, p < 0.001) and Ascaris (Estimate: 1.20, p < 0.05) during lifetime were higher in children who had high CB total IgE (Figure 5.1). No differences were found for *D. pteronyssinus* specific IgE. A tendency to have higher $\log_{10}$ total IgE (Estimate: 0.273, p = 0.07) was observed in children with positive *A. lumbricoides* stool examination and the association was significant when specific IgE to Ascaris was analyzed (Estimate: 0.23, p = 0.004). Under this analysis Ascaris infection had no effect on IgE responses to *B. tropicalis* or *D. pteronyssinus*; however, when data at S4 was evaluated independently. *B. tropicalis* IgE was higher in infected children (p = 0.05).

Table 5.4 Analysis of IgE levels and allergic sensitization in mothers

	All mothers (n=289)	n (%) >0.35 kU/l	n (%) >0.70 kU/l	Non AR (n = 202)	AR (n = 87)	P	Non asthma (n=266)	Asthma (n=23)	p
Ascaris spp.	0.35 (0.08-1.42)	143 (49.5)	108 (37.4)	0.24 (0.05-1.16)	0.57 (0.16-0.11)	0.03*	0.35 (0.07-1.5)	0.41 (0.1-0.88)	0.79
B. tropicalis	0.15 (0.01-0.83)	108 (37.4)	100 (27.7)	0.1 (0.01-0.46)	0.52 (0.07-3.18)	<0.001*	0.14 (0.01-0.73)	0.3 (0.15-4.33)	0.01
D. pteronyssinus	0.21 (0.06-0.7)	112 (38.8)	73 (25.3)	0.14 (0.06-0.51)	0.39 (0.13-2.61)	<0.001*	0.20 (0.06-0.63)	0.69 (0.14-7.32)	0.01
	Median (IQR)	n (%) >150 kU/l		Non AR	AR	P	Non asthma	Asthma	p
Total serum IgE levels	197.5 (81.1-633)	171 (59.4)		178 (65.7-569)	291(123-804)	0.001*	194 (76.3-627)	277 (106-744)	0.20

n (%): number and relative percent of sensitized mothers for each antigen/allergen.

AR: Allergic rhinitis

p-value obtained by comparing IgE levels between sub-groups (AR vs. Non-AR and Asthma vs. Non-Asthma) by Mann-Whitney test.

Total IgE increased progressively with age, from 0.02 KU/L in CB to 167.3 KU/L (similar to adult level) at S4. The influence of specific IgE on total IgE values was significant in the following order of relevance: *D. pteronyssinus* (Estimate: 347.7, p = 0.01). *B. tropicalis* (Estimate: 59.6, p = 0.01) and Ascaris spp. (Estimate: 20.9, p<0.001). In contrast to other reports[247], the contribution of specific IgE to HDM and Ascaris on the level of total IgE values, as determined after 24 age months were less than 1% in most cases (Mean: 0.64%. ± 0.89 SD; Range: 0.03 - 5.33%).

The prevalence of IgE sensitization in children is high

Frequency rates of IgE sensitization to *B. tropicalis. D. pteronyssinus* and Ascaris increased gradually with age (Figure 5.3, Table 5.6). Remarkably, the prevalence of IgE sensitization to *B. tropicalis* at S4 (median age 35 months) was significantly higher than that to *D. pteronyssinus* (p = 0.002) and similar to that found in mothers (33.3 and 37.4%. respectively; p = 0.44).

Unhygienic conditions and maternal IgE are risk factors for Ascaris sensitization in children

In a sub-sample of 176 children, with data on IgE serology after 24 months and information about the clinical phenotypes (Table 5.7), the effects of prenatal factors on IgE sensitization were analyzed. The frequency of sensitization to Ascaris, *B. tropicalis* and *D. pteronyssinus* was 23.6%. 27.3% and 14.2%, respectively. Median total IgE was 162 kU/L (IQR: 56.4 - 309.4).

Table 5.5 Logistic regression analysis of factors influencing high cord blood total IgE

Predictors	Univariate analysis			Multivariate Analysis								
				Model 1*			Model 2[⊥]			Model 3[§]		
	OR	95% C.I. (OR)	p	OR	95% C.I.(OR)	p	OR	95% C.I(OR)	p	OR	95% C.I (OR)	p
Maternal total IgE (>502 kU/L)	3.83[a]	(2.07-7.07)	<0.001	2.43	(1.09-5.43)	0.03						
HDM sensitization in the mother	2.75[b]	(1.47-5.12)	<0.001	1.51	(0.69-3.30)	0.30				1.85	(0.88-3.88)	0.10
Ascaris sensitization in the mother	3.14[c]	(1.66-5.95)	<0.001	1.57	(0.69-3.51)	0.28	1.85	(0.86-3.95)	0.11			
Cockroaches at home	0.52[d]	(0.27-1.01)	0.05	0.45	(0.22-0.94)	0.04	0.48	(0.23-0.99)	0.05	0.44	(0.21-0.91)	0.03
Exposure to a dog during pregnancy	1.86[e]	(1.02-3.38)	0.04									
Pet during whole/pregnancy	2.33[f]	(1.28-4.25)	0.01	2.74	(1.42-5.29)	<0.001	2.75	(1.42-5.30)	<0.001	2.70	(1.41-5.17)	<0.001
Socio-economical stratum**	0.41	(0.18-0.96)	0.04	0.46	(0.19-1.14)	0.1	0.47	(0.19-1.15)	0.10	0.43	(0.17-1.05)	0.07
Brick house (vs. wood)	0.41[g]	(0.22-0.77)	0.01									
Gender	0.75	(0.41-1.35)	0.33	0.79	(0.41-1.50)	0.46	0.77	(0.40-1.47)	0.43	0.78	(0.41-1.48)	0.44
High total IgE*Ascaris sensitization in the mother	3.59	(1.93-6.67)	<0.001							2.59	(1.24-5.42)	0.01
High total IgE*HDM sensitization in the mother	3.90	(2.09-7.27)	<0.001				2.87	(1.36-6.06)	0.01			

*Model 1: All significant predictors in the univariate analysis were included in the multivariate model; [⊥]Model 2: HDM sensitization and high total IgE in the mother are included as interaction terms; [§]Model 3: Ascaris sensitization and high total IgE in the mother are included as interaction terms. [§]Powers to detect association between each predictor and the outcome in the univariate analysis: [a] 0.99 (54.5% of exposed subjects in high CB total IgE vs. 23.9% in control group). [b] 0.91 (67.3% vs. 42.8%). [c] 0.96(70.9% vs. 43.7%). [d] 0.50(69.1% vs.81.1%). [e] 0.53 (58.2% vs. 42.8%). [f] 0.79 (49.1% vs. 29%). [g] 0.79 (60% vs. 78.4%) **Socioeconomic stratification ranges from 1 to 6. being 1 the lowest.

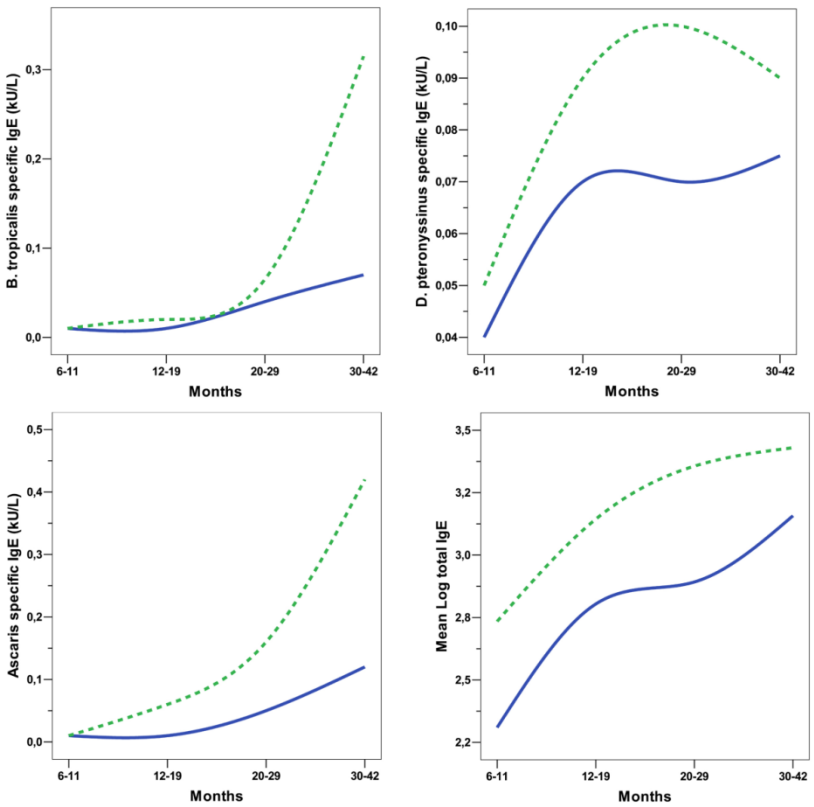

Figure 5.2 Difference in the dynamics of IgE response according to cord blood total IgE. Graph lines representing median IgE levels to *B. tropicalis*, *D. pteronyssinus*, *Ascaris spp.* and mean Log total IgE at the different time points during child life. discriminated by high CB total IgE (green dashed line) or not (blue line).

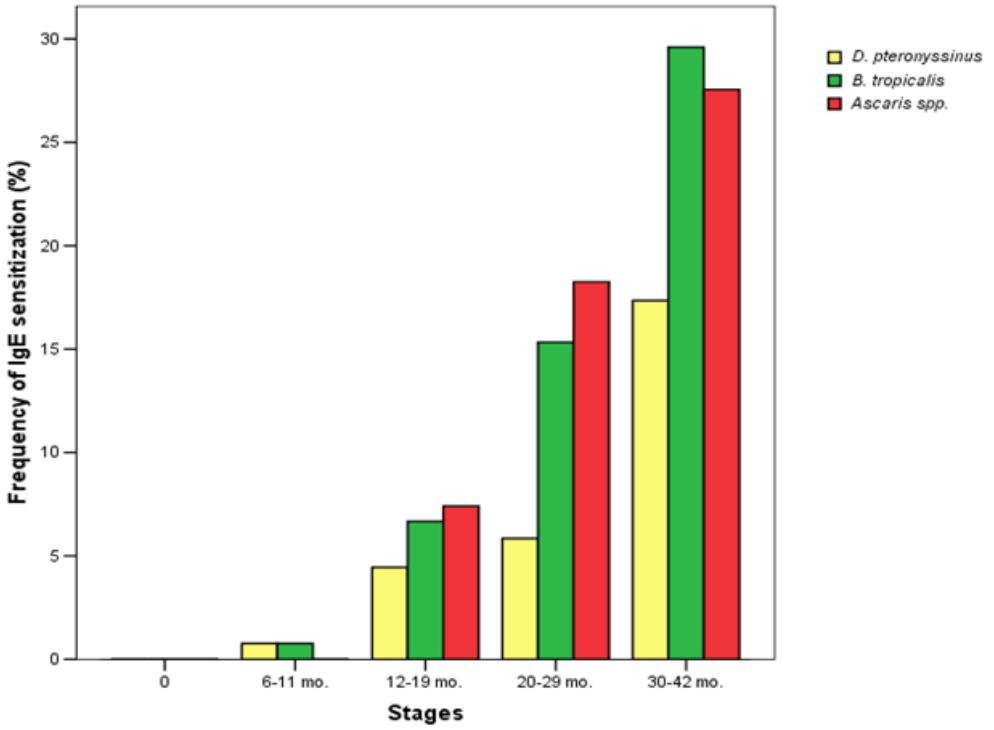

Figure 5.3 Frequencies of IgE sensitization to *D. pteronyssinus*, *B. tropicalis* and A*scaris spp.* at different time periods. Frequency of sensitization increases with age for all three allergens (Test for trend. p<0.001 for these three antigen/allergens)

Maternal IgE (high total IgE, Ascaris and HDM sensitization) and living under poverty and unhygienic conditions were positively associated to Ascaris sensitization in children (Table 5.7). As expected. a positive stool examination to Ascaris was also associated to this outcome (OR: 3.71, 95% CI: 1.14-12.06. p = 0.03). In addition, maternal sensitization to Ascaris (aOR: 5.34, 95% CI: 2.21-12.94; p < 0.001) and high CB total IgE (aOR: 5.12, 95% CI: 2.08-12.60; p < 0.001) were associated with Ascaris sensitization, all after adjustment by age, gender and building material of the house. Living in wood house (as a proxy of extreme poverty in this community) also remained as a strong risk factor for Ascaris sensitization in children (aOR: 3.76, 95% CI 1.63-8.68; p < 0.01).

Table 5.6 Descriptive of specific serum IgE levels in children from 6 to 42 months

	S1 (6-11 months) N = 265		**S2 (12-19 months) N = 137**		**S3 (20-29 months) N = 143***		**S4 (30-42 months) N = 103⊥**	
	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)
Ascaris spp.	--	0 (0)	0.82 (0.62-1.01)	10 (7.3)	0.79 (0.55-1.30)	25 (17.9)	1.06 (0.58-1.84)	27 (26.5)
B. tropicalis	0.57 (0.37-0.77)	2 (0.8	0.52 (0.48-0.74)	9 (6.6)	1.47 (0.70-2.72)	24 (16.9)	0.97 (0.55-1.65)	34 (33.3)
D. pteronyssinus	0.69 (0.36-1.02)	2 (0.8)	0.67 (0.48-0.99)	7 (5.1)	0.69 (0.48-1.55)	10 (7.0)	0.81 (0.48-1.35)	19 (18.6)
Total IgE	27.1 (8.73-66.3)		88.2 (25.2-200.5)		104 (34.6-254)		180.3 (61.1-375)	

Median (IQR) IgE levels to any antigen/allergen were calculated in the sub-group of sensitized children for each respective antigen. Median (IQR) total IgE was calculated for all children at each time point.

* Ascaris spp (n=140). *D. pteronyssinus* (n=142). *B. tropicalis* (n=142)

⊥IgE to *Ascaris* was measured in 102 children.

There is high correlation between the IgE responses to HDM and Ascaris

Considering the IgE CR between Ascaris and HDM allergens [45], the relationship between the IgE responses to these species was also analyzed. Ascaris specific IgE levels correlated with those to *B. tropicalis* (Spearman rho: 0.69 $p < 0.001$) and *D. pteronyssinus* (Spearman rho: 0.54 $p < 0.001$). Ascaris sensitization was associated to HDM sensitization when grouping *B. tropicalis* and *D. pteronyssinus* data in one category ($\chi^{2} = 37.3$. $p < 0.001$) or analyzed separately ($\chi^{2} = 40.1$. $p < 0.001$ for *B. tropicalis* and $\chi^{2} = 13.4$. $p < 0.01$ for *D. pteronyssinus*). Fifty two percent of Ascaris sensitized children. but only 10% of non-sensitized responded to any of the two HDM species. The odds ratio for responding to Ascaris in the presence of HDM co-sensitization was 9.4 (95% CI: 4.29-20.99. $p < 0.001$).

Table 5.7 Univariate analysis for HDM and Ascaris sensitization in children as outcomes

Predictor	**Ascaris**		***D. pteronyssinus***		***B. tropicalis***	
	OR 95% CI (OR)	p	OR 95% CI (OR)	p	OR 95% CI (OR)	P
Socio-economical stratum	0.20 (0.05-0.84)	0.03	0.77 (0.28-2.10)	0.61	0.66 (0.30-1.48)	0.31
Tap water	0.32 (0.12-0.84)	0.02	0.62 (0.19-2.04)	0.43	1.16 (0.42-3.23)	0.77
Sewage	0.35 (0.17-0.74)	0.01	0.53 (0.23-1.26)	0.15	0.66 (0.34-1.28)	0.22
Poultry/pigs at home during pregnancy	2.21 (0.89-5.52)	0.09	3.97 (1.48-10.66)	0.01	3.22 (1.33-7.79)	0.01
Wood house (vs. brick)	3.77 (1.80-7.88	<0.001	0.74 (0.28-1.99)	0.55	2.24 (1.11-4.51)	0.02
Cockroaches at home	0.68 (0.30-1.58)	0.37	0.75 (0.28-2.05)	0.58	0.78 (0.35-1.74)	0.54
Mouse pest at home	2.08 (0.76-5.68)	0.16	2.45 (0.80-7.53)	0.12	0.47 (0.18-1.25)	0.13
Pet (cat/dog) exposure during pregnancy	1.23 (0.61-2.50	0.56	0.93 (0.40-2.19)	0.87	1.57 (0.79-3.09)	0.20
Living with a pet during whole pregnancy	1.12 (0.53-2.38)	0.77	0.70 (0.26-1.86)	0.47	0.82 (0.39-1.71)	0.59

Predictor	Ascaris		*D. pteronyssinus*		*B. tropicalis*	
Exposure to a dog during pregnancy	0.97 (0.48-1.98)	0.94	0.91 (0.39-2.14)	0.83	0.60 (0.30-1.21)	0.15
Cooking at home with wood/coal	0.57 (0.21-1.53)	0.27	1.50 (0.46-4.90)	0.50	0.57 (0.22-1.46)	0.24
Exposure to trash fume at neighborhood	1.23 (0.61-2-.50)	0.56	0.86 (0.37-2.02)	0.74	0.95 (0.49-1.85)	0.88
Passive exposure to cigarette smoke	1.20 (0.59-2.43)	0.62	0.93 (0.40-2.19)	0.87	0.87 (0.45-1.70)	0.69
Exposure to cats during pregnancy	1.13 (0.39-3.25)	0.82	0.40 (0.14-1.15)	0.09	0.66 (0.26-1.68)	0.39
High CB total IgE (>0.07 kU/L)	6.15 (2.61-14.46	<0.001	1.47 (0.53-4.04)	0.46	2.76 (1.23-6.23)	0.01
High maternal total IgE (>502 kU/L)	4.50 (2.14-9.49)	<0.001	2.82 (1.18-6.72)	0.02	1.63 (0.80-3.33)	0.18
Maternal HDM sensitization defined by recombinants	2.54 (1.24-5.22)	0.01	1.07 (0.44-2.59)	0.88	1.34 (0.67-2.67)	0.40
Maternal HDM sensitization defined by ImmunoCap	2.35 (1.14-4.85)	0.02	1.20 (0.63-2.29)	0.59	1.06 (0.55-2.06)	0.86
Maternal *B. tropicalis* sensitization (>0.35 kU/L)	2.84 (1.38-5.82)	<0.001	0.97 (0.40-2.35)	0.95	1.19 (0.60-2.36)	0.61
Maternal *B. tropicalis* sensitization (>0.7 kU/L)	2.81 (1.35-5.86)	0.01	1.21 (0.49-3.01)	0.68	1.37 (0.67-2.80)	0.39
Maternal *D. pteronyssinus* sensitization (>0.35 kU/L)	2.47 (1.21-5.05)	0.01	1.06 (0.45-2.51)	0.90	1.18 (0.60-2.31)	0.64
Maternal *D. pteronyssinus* sensitization (>0.7 kU/L)	2.14 (1.00-4.58)	0.05	1.55 (0.62-3.89)	0.35	1.20 (0.56-2.56)	0.64
Maternal Ascaris sensitization (>0.35 kU/L)	5.77 (2.48-13.46)	<0.001	1.05 (0.45-2.46)	0.90	1.70 (0.87-3.35)	0.12
Maternal Ascaris sensitization (>0.7 kU/L)	5.53 (2.59-11.82)	<0.001	1.40 (0.59-3.29)	0.44	1.65 (0.84-3.25)	0.15
Maternal rhinitis	1.12 (0.53-2.38)	0.77	0.28 (0.08-0.99)	0.05	0.73 (0.35-1.56)	0.42
Maternal asthma	1.33 (0.39-4.49)	0.65	1.01 (0.21-4.80)	0.99	0.71 (0.19-2.66)	0.61
Maternal age	1.03 (0.97-1.09	0.30	0.98 (0.91-1.06)	0.60	1.01 (0.96-1.07)	0.66
Number of siblings	1.29 (0.99-1.66)	0.06	0.90 (0.63-1.28)	0.55	1.22 (0.95-1.56)	0.12
Gender (Female)	1.11 (0.55-2.23)	0.78	1.75 (0.73-4.22)	0.21	0.99 (0.51-1.92)	0.98
Ascaris infection	3.71 (1.14-12.06)	0.03	0.00 (0.00-0.00)	1.00	2.19 (0.70-6.91)	0.18

Predictor	Ascaris		*D. pteronyssinus*		*B. tropicalis*	
Child age (months)	1.05 (0.97-1.13)	0.22	1.10 (1.00-1.20)	0.04	1.07 (0.99-1.15)	0.05

Maternal IgE, cord blood IgE and child's sensitization show different behaviors with regard to wheezing

The relationship between maternal IgE and recurrent wheezing up to 24 months was evaluated. Maternal asthma was the most important predictor of recurrent wheezing up to 24 months (aOR: 3.02, 95% CI: 1.19-7.70. p = 0.02). Maternal IgE sensitization to HDM or Ascaris was not significantly associated with wheezing-ever or recurrent wheezing. Interestingly, high CB total IgE was inversely associated to recurrent wheezing (aOR: 0.26, 95% CI 0.08-0.88. p = 0.03). Also, the interaction of maternal total IgE and HDM sensitization was protective from this phenotype (aOR: 0.36, 95% CI 0.14-0.91. p = 0.03).

Sensitization rates in the first year of life were low and their relationships with wheezing were not further analyzed, although it should be mentioned that 2 out of 2 HDM-early sensitized children (detected at S1) developed recurrent wheezing. In the sub-sample that included children with serology data in the range of 24-42 months, frequencies of sensitization to Ascaris. *B. tropicalis* and *D. pteronyssinus* were not different among ever-wheezers or recurrent wheezers and their respective controls. However, HDM sensitization tended to be higher in children who had ever wheezed than in the nested controls ($\chi^2 = 3.22$, p = 0.07). Weak associations were found between *B. tropicalis* IgE levels and HDM sensitization when analyzed by logistic regression (Table 5.8). PAF for ever-wheezing associated to HDM sensitization was 19.7%. When analyzed as continuous variables, Log_{10} total IgE values during lifetime were lower in children with recurrent wheezing (Estimate: 0.209. p = 0.02). However, recurrent wheezers showed higher *B. tropicalis* specific IgE levels (Estimate: 0.021; p = 0.02). In this model, Ascaris infection was weakly associated to recurrent wheezing (aOR: 6.13 95% CI: 1.03-36.61. p = 0.05).

Table 5.8 Influence of child specific IgE on ever wheezing up to 2-3 years old

Predictor	Multivariate analysis							
	Model 1[§]		Model 2[§]		Model 3*		Model 4*	
	OR 95% C.I.	p	OR 95% C.I.	p	OR 95% C.I.	p	OR 95% C.I.	**p**
B. tropicalis IgE (kU/L)	1.47(1.00-2.16)	0.05	1.52(1.01-2.28)	0.04	--	--	--	--
HDM sensitization (Yes/No)	--	--	--	--	2.03 (0.99-4.15)	0.05	1.78 (0.88-3.59)	0.11
Age in months	1.05 (0.98-1.12)	0.20	1.04 (0.97-1.11)	0.31	1.03 (0.96-1.10)	0.42	1.04 (0.97-1.11)	0.26
Maternal rhinitis	--	--	2.22 (1.09-4.51)	0.03	2.29 (1.12-4.67)	0.02	--	--
Maternal asthma	2.86 (0.89-9.19)	0.08	--	--	--	--	2.65 (0.83-8.50)	0.10
CB total IgE (kU/L)	0.58 (0.33-1.02)	0.06	0.57 (0.33-1.02)	0.06	0.68 (0.36-1.31)	0.25	0.65 (0.37-1.15)	0.14
Tap water	1.84 (0.60-5.70)	0.29	1.85 (0.60-5.77)	0.29	0.51 (0.16-1.59)	0.25	1.95 (0.63-6.05)	0.25
Gender	0.80 (0.42-1.53)	0.50	0.72 (0.37-1.38)	0.32	0.62 (0.34-1.14)	0.12	0.76 (0.40-1.46)	0.41

[§]Model 1 and 2 included as predictor *B. tropicalis* IgE values in the multivariate analysis;

*Model 3 and 4. HDM sensitization as a binary variable. The influence of maternal asthma and maternal rhinitis was evaluated in separated models for each of the 2 predictors.

5.2.3. Discussion

Research focused on wheezing, asthma and atopy in the tropics suggest that although these are frequent conditions as in other latitudes, their origin and pathogenesis are different. Here we describe the evolution of the IgE response to common allergens and Ascaris and analyze its relationship with early wheezing. As expected, high serum maternal total IgE was associated with high CB total IgE [248], which in turn predisposed to stronger IgE responses during infancy. In agreement with other reports, we found that high CB total IgE was positively associated with atopy [115] but protected from wheezing [249]. Although in developed countries, high total CB IgE has been found associated with atopy-related phenotypes, such as recurrent wheezing and eczema [250, 251], our opposite finding replicates the results obtained by Sunyer *et al* in Tanzania [249], a tropical underdeveloped country. Why high CB total IgE protects from wheezing is unknown, but before speculating on it, some comments about CB IgE are pertinent. The source of CB IgE is controversial, both fetal and maternal production

have been described [252] and may influence IgE detection. We excluded maternal blood contamination and think that CB IgE was mainly a fetal product because an important role of placental-transfer is not supported by our results. First, in spite that in the tropics responses to HDM are strong, only one case of possible CB sensitization to *D. pteronyssinus* was found, being the mother negative for that allergen. Second, although total IgE values detected in mothers were higher than those reported in studies from developed countries [115, 253], the corresponding CB IgE titers were lower [252, 254]. In regard to mechanisms, we think it is possible that total IgE does not directly mediate protection [255] but may be associated to an ongoing protective process, probably induced *in utero* by parasite infections in mothers. This process could be extended during the first months (longitudinal total IgE values were lower in recurrent wheezers), modulating the pulmonary inflammation induced by viral or other agents and reducing the rate of wheezing. But, independently of possible mechanisms, our results confirm previous findings in a tropical country [249], suggesting that in these environments high levels of total IgE in CB are not predictors of atopy-related phenotypes.

Another important finding of this study is the association of wheezing with HDM sensitization; which is not in contradiction with the levels of total IgE observed in children with this phenotype. Allergen-specific and total IgE traits have shared but also unique genetic determinants [106, 256-258] and it has been shown that cognate and non-cognate IgE responses have different distributions in atopic families [259]. Furthermore, environmental stimuli and molecular mechanisms [260] are not necessarily the same for specific and total IgE. Our results are opposed to other studies and opinions. It has been suggested that, in the tropics, wheezing in infancy is mainly non-atopic and caused by low respiratory tract infections and some helminthiasis [6]. However, the influence of atopy in urban/poor settings could be higher than previously recognized. Cases of wheezing attributed to atopy may vary among populations, depending on genetic and socio-demographical factors. In our study about 20% of wheezing cases were attributable to HDM sensitization. This result is 10 times higher than that reported by Moncayo *et al* in children 6-16 years old from a rural area of Ecuador where helminth infections are more endemic [118]. Regarding the results of

other birth cohort studies in Latin America, only two have assessed this association. Lopez *et al* also found a positive relationship between allergic sensitization and recurrent wheezing during the first year of life in Brazilian children [176]. In the study of Rullo *et al* [177] in a low income Brazilian population with high risk for atopy, sensitization to *B. tropicalis* was not evaluated, which may explain the lack of association with HDM sensitization. Our results show that contrary to the opinion generated by the hygiene hypothesis, atopy may be very frequent in socially-deprived urban communities. The relationship of atopy and the inception of asthma in these settings needs to be further evaluated, knowing the differences between wheezing and asthma and the reported studies about the natural history of pediatric asthma in other populations [205, 261-263].

Fifty two percent of Ascaris sensitized children, but only 10% of non-sensitized, have positive IgE to any of the two HDM species. In addition, *B. tropicalis* IgE levels at S4 were higher in those children who had been infected by Ascaris. Genetic background of the host and unspecific boosting of the Th2 responses by the parasite may influence this finding but it is more likely that cross-reactivity plays the main role. The latter has already been recognized [45, 202], but here we found that it may start at early life. It is possible that some cases of HDM sensitization correspond to cross-reactive Ascaris-induced IgE antibodies rather than genuine atopy. Moreover, this response may have biological impact and act on different directions. For example, Ascaris specific IgE can induce allergic-symptoms after recognition of its own antigens and also those derived from HDM; furthermore, it can boost the responses to cross-reactive HDM antigens, to which population is perennially exposed.

In this cohort, frequencies of sensitization to *B. tropicalis* and *D. pteronyssinus* were markedly different, higher to *B. tropicalis*. Since the samples were analyzed at the same time for both allergens, technical errors can be ruled out. Limitation of *D. pteronyssinus* extract to detect sensitization in our population (e.g. different isoforms) was also ruled out because in mothers the expected results were found. In a work performed in wheezing children from Central Taiwan, it was also found higher sensitization rates to *B. tropicalis* than to *D. pteronyssinus* [264]. These data differ from those obtained in adults where frequencies of sensitization to these mites are similar

[123], a finding that was also shown in mothers of the present study. Interestingly, frequency of *B. tropicalis* sensitization, but not to *D. pteronyssinus,* at three years of age was similar to that in mothers. Since these HDM species are in similar proportions in homes of Cartagena [143, 265] it can be speculated about other origins of this finding. The sensitization process to each species may be independently controlled. Differences in the gene expression profile among these species, especially of "pro-allergenic" molecules [38], and their interactions with the genetic background of exposed individuals can shape the specific-IgE responses. Supporting this idea, individual genetic associations have been found with sensitization to each species. Although it is not known how some polymorphisms modulate IgE sensitization, in addition to HLA-alleles, other genetic variants may influence the response to *B. tropicalis* [103] or *D. pteronyssinus* sensitization [59, 266].

Although in our population poverty-associated conditions, such as wood houses and poultry/pigs at home, were not associated to wheezing, their effects on IgE responses were remarkable. Some predictors of poor and unhygienic living conditions were identified as risk factors for IgE sensitization, especially to Ascaris. This may be explained by a higher risk to become infected in the poorest population. The association with HDM sensitization could be due to the cross-reactivity with Ascaris antigens; however, it is important to mention that at this age sensitization to *B. tropicalis* was more frequent than that to Ascaris, so, it is possible that unhygienic conditions have a direct effect on this outcome.

Total IgE in children increased rapidly until reaching adult levels at S4, a dynamic not described in any of the published birth cohorts. This phenotype was not a risk factor for wheezing, instead, it was protective and may be explained by several reasons. High levels of total IgE have been consistently described in populations exposed to parasites, especially helminths [267, 268], which is expected in children living in the tropics. In addition, in our population and others, African ancestry has been strongly associated with high level of total IgE and asthma [5]. Therefore, it is possible that the genetic background also supports the tendency to acquire high levels of total IgE in early infancy. High total IgE has been associated with a number of conditions including asthma [269, 270], and has been used as an additional marker for atopy,

especially in children. However, although total IgE in parasited asthmatics is still higher than in non-asthmatics at the population level [106], in the tropics it is difficult to apply this criterion at the individual level. In this regard, our data support the idea that total IgE in children of low income communities living in the tropics has not the same diagnostic value as in industrialized parasite-free countries.

Several limitations of this study should be discussed. A birth cohort has advantages when evaluating the evolution of immune responses, but, in general, technical drawbacks are expected. Our study was hampered by particular socio-demographical conditions, such as frequent address changes, social conflicts, poverty and low education level [102]. Sample size was too small to accurately define some associations, affected by its *a priori* calculation, the response rate and missing data from active participants. Ascaris infection rate was lower than its *a priori* estimation, even though it was based on epidemiological information of near communities [271]. Thus, we cannot discard, for example, that the association we found between Ascaris infection and recurrent wheezing is actually stronger, as has been found in other surveys. Even though at 2 years of follow up response rate was 86%, there were missing data from active patients, for at least one time-period. However, having longitudinal data about IgE responses during 3 years of life was advantageous and helped to overcome some of these difficulties. LMM is a helpful statistic method for longitudinal analysis in the presence of missing data, also used in allergy research [116-118] due to its robustness, flexibility and capability of handling a variety of repeated measure problems. The concordance of the results derived from LMM with those obtained by cross-sectional analysis supports the validity of our findings.

Results of this study support several previous finding and contradict others. For example, contrary to expected according to the hygiene hypothesis, in this socioeconomically deprived, tropical population, the frequency of sensitization to any HDM during the first three years of age is high and HDM sensitization is positively associated to wheezing. The more evident reason to explain this dynamic toward high levels of total and specific IgE (in addition to the perennial exposure to HDM allergens), is parasite infections. However, other factors, such as air pollution, microbiota composition and genetic background deserve more investigation.

5.3. Evaluation of IgE response to Blomia tropicalis and Ascaris components

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5.3.1. Introduction

In the tropics, HDM sensitization begins early in life. In a socio-economically deprived population from Cartagena, *B. tropicalis* sensitization (determined with the whole extract) was detected in about 30% of ~ 3 years-old children from the FRAAT cohort study. However, Ascaris sensitization was also frequent, and a high correlation between specific IgE values to these sources was found. In these infants, cumulative prevalence of infection by Ascaris was 10%, but worm expulsion antecedents were high (about 50%). In addition, *B. tropicalis* IgE levels at 3 years of age were greater in children with Ascaris infected children, identified by stool examination. Genetic background of the host and unspecific boosting of the Th2 responses by the parasite may influence this finding but it is also likely that cross-reactivity plays the main role. It is possible that some cases of HDM sensitization correspond to cross-reactive Ascaris-induced IgE rather than genuine atopy. Moreover, this response may have biological impact and act on different directions. For example, Ascaris specific IgE may induce allergic-symptoms after recognition of its own antigens and also those derived from HDM, boosting the responses to cross-reactive HDM allergens, to which population is perennially exposed. The use of purified components for allergy diagnosis may help to identify when sensitization is due to cross-reactive molecules or rather to specific allergens from a defined source.

In this study, we evaluated the IgE responses to different allergen/antigens from Ascaris spp. and *B. tropicalis*. ABA-1 is a nematode-specific marker, since it has not

been found IgE CR with any HDM antigen. AscGST and Asc l 1 are cross-reactive with homologous proteins from other allergenic sources, including HDM. Blo t 5 and Blo t 12 are, both, HDM-species markers, being the last one merely representative of *B. tropicalis* sensitization. With this battery of allergens we aimed to evaluate more accurately *Ascaris*- and *B. tropicalis*- IgE responses and also their interrelationships, by exploring the dynamics of sensitization to cross-reactive and specific molecules in children from the FRAAT study at different time points during the first three years of life.

5.3.2. Results

Subjects and serology data

Serology data was obtained from 283 children at different time points between 6 and 42 months of age. Frequencies of IgE sensitization were determined in 232 children at S1, 79 at S2, 124 at S3 and 141 at S4. Maternal sensitization to ABA-1, Blo t 12 and Blo t 5 was also evaluated in their mothers; the estimated frequencies of sensitization were 14.4, 27.1 and 29.2%; respectively. There was no significant correlation between the maternal and child specific IgE levels to these allergens.

Estimation of cut-off values

Cut-off points to define sensitization were selected by ROC curves, using as state variables *B. tropicalis* or *Ascaris spp* sensitization (defined by ImmunoCAP results at the 0.35 kU/L level) in each respective case. Sensitivity and specificity of the obtained cut-off values for each allergen with respect to the complete extracts are shown in Table 5.9.

Sensitization to Ascaris and B. tropicalis antigens is high in early life

Sensitization to Ascaris and *B. tropicalis* allergenic components began early in life. At S1 (6-11 mo.) frequencies of sensitization ranged from 4 (to Blo t 12) to 20.6% (to AscGST) and were lower to the *B. tropicalis* allergens than to those derived from Ascaris (Table 5.10). Conversely, at the S4 period, the frequency of positive response to *B. tropicalis* allergen was comparable to those the Ascaris components; Blo t 5 was the

most frequent IgE sensitizer (53.3%) and 60.3% of children (73 out of 121) responded to any of the two HDM components.

Table 5.9 Performance of the cut-off values to define allergen sensitization with respect to the complete extracts

Allergen	Cut-off (O.D units)	Sensitivity (%)	Specificity (%)
Asc s 1	0.140	71.4	79.4
Asc l 3	0.140	59.5	77.9
AscGST	0.150	73.8	71.6
Blo t 5	0.125	62.5	79.5
Blo t 12	0.120	60.4	80.0

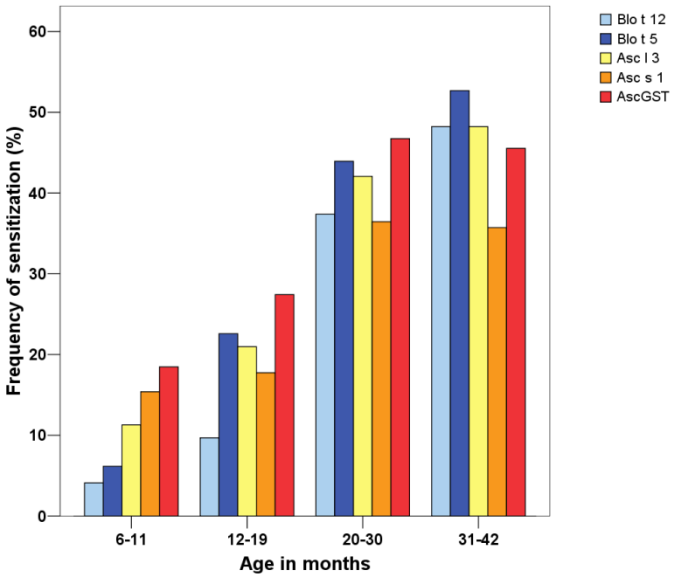


Figure 5.4 The frequency of sensitization to Ascaris and HDM allergenic components at different time periods. Frequency of sensitization increases with age for all three allergens

The strength of IgE responses also varied over time; a tendency to increase was observed in all of them (By LMM, $p<0.0001$ in all cases). Among the Ascaris antigens, at S1 period, ABA-1 IgE response was the least intense of all analyzed components, but it increased over time, becoming in the stronger IgE sensitizer at S4.

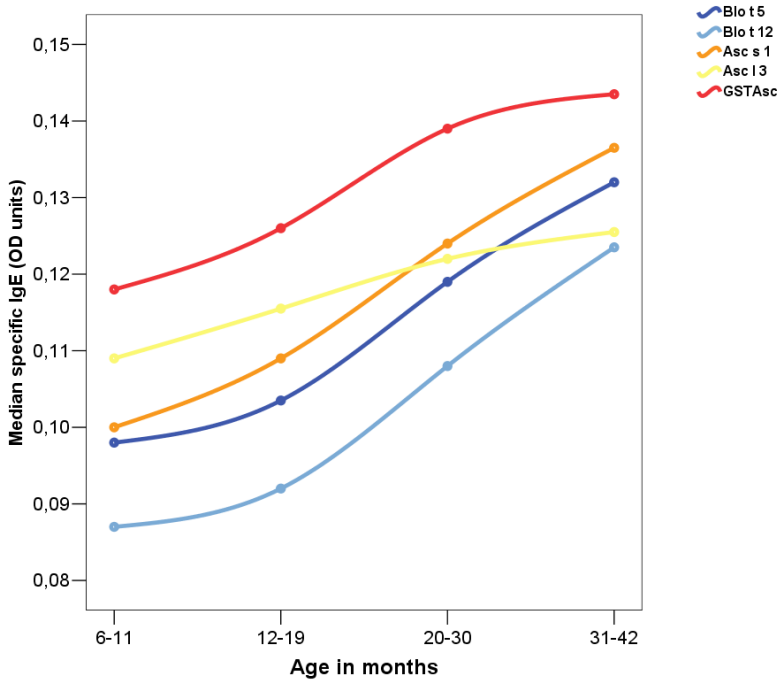


Figure 5.5 Evolution of IgE responses to HDM and Ascaris allergenic components during the first 3 years of life. Median IgE levels to Blo t 5, Blo t 12.0101, Asc s 1, Asc l 3 and AscGST in all evaluated children are shown for each time-period**.**

Asc s 1 sensitization is a good marker of Ascaris infection in early life

The cumulative prevalence of ascariasis (eggs in stool samples) was 10.5%. For detecting Ascaris infection, the 0.36 kU/L cut-off point for Ascaris sensitization had less sensitivity (33.3%) than the 0.14 cut-off point for Asc s 1 sensitization (67%). Regarding specificity, Ascaris IgE serology by ImmunoCap (85%) is 1.4 times more specific than Asc s 1 determination (60%). Since ABA-1 may be more sensitive to detect Ascaris infection than stool examination, we analyzed the patterns of IgE response to the *B. tropicalis* allergens in accordance to Asc s 1 sensitization. By LMM, Asc s 1 sensitized- showed longitudinally higher specific IgE values to Blo t 5 (Estimate 0.07 OD, $p < 0.0001$) and Blo t 12 (Estimate 0.13 OD, $p < 0.0001$) than non-sensitized children

Table 5.10 Descriptive of specific serum IgE levels to purified allergenic components in children from 6 to 42 months

	Stage 1 (6-11 mo.)		**Stage 2 (12-19 mo.)**		**Stage 3 (20-30 mo.)**		**Stage 4 (31-42 mo.)**	
	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)
GST	0.17(0.16-0.20)	46 (20.63)	0.21(0.16-0.27)	18(27.27)	0.23(0.17-0.39)	53(45.69)	0.28(0.20-0.64)	58(45.67)
Asc l 3	0.18(0.15-0.23)	45 (19.23)	0.19(0.16-0.37)	21(26.58)	0.20 (0.17-0.20)	46(37.40)	0.26 (0.17-0.50)	51(38.35)
Asc l 1	0.18(0.15-0.28)	37 (15.74)	0.17(0.16-0.32)	19 (24.05)	0.27(0.18-0.51)	51 (41.46)	0.36 (0.18-0.86)	65 (49.24)
Blo t 5	0.14 (0.13-0.15)	12 (5.9)	0.17 (0.14-0.19)	15(22.1)	0.20 (0.15-0.33)	51(43.2)	0.20 (0.16-0.28)	65(53.3)
Blo t 12	0.17 (0.14-0.35)	8(4.0)	0.18 (0.16-0.26)	7(10.3)	0.28 (0.16-0.51)	45(38.1)	0.22 (0.15-0.44)	58(47.9)

Median (IQR) IgE levels to any antigen/allergen were calculated in the sub-group of sensitized children for each respective antigen. Median (IQR) tIgE was calculated for all children at each time stage.

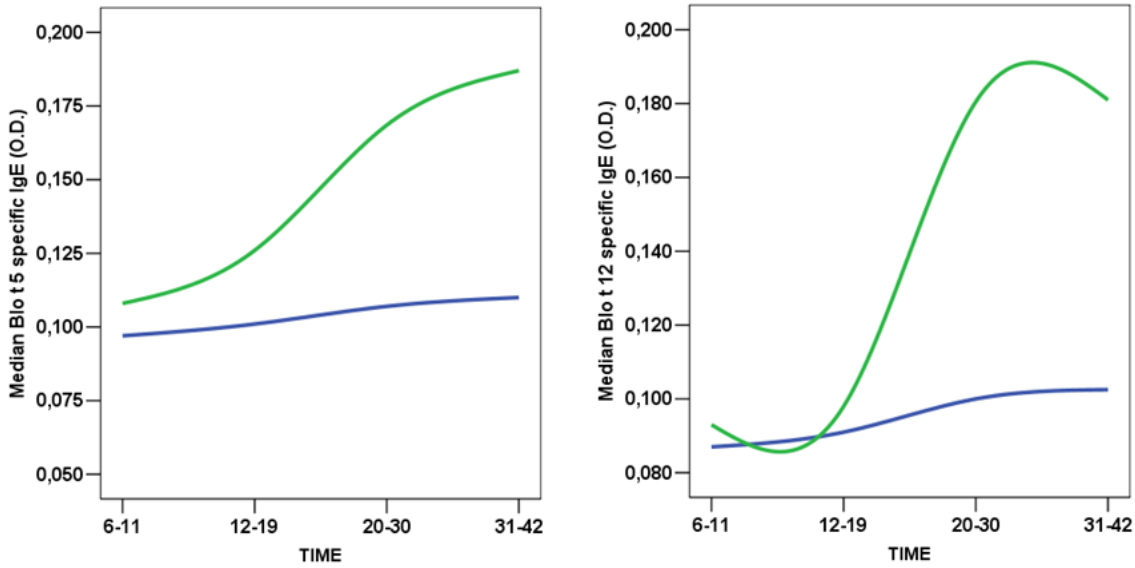
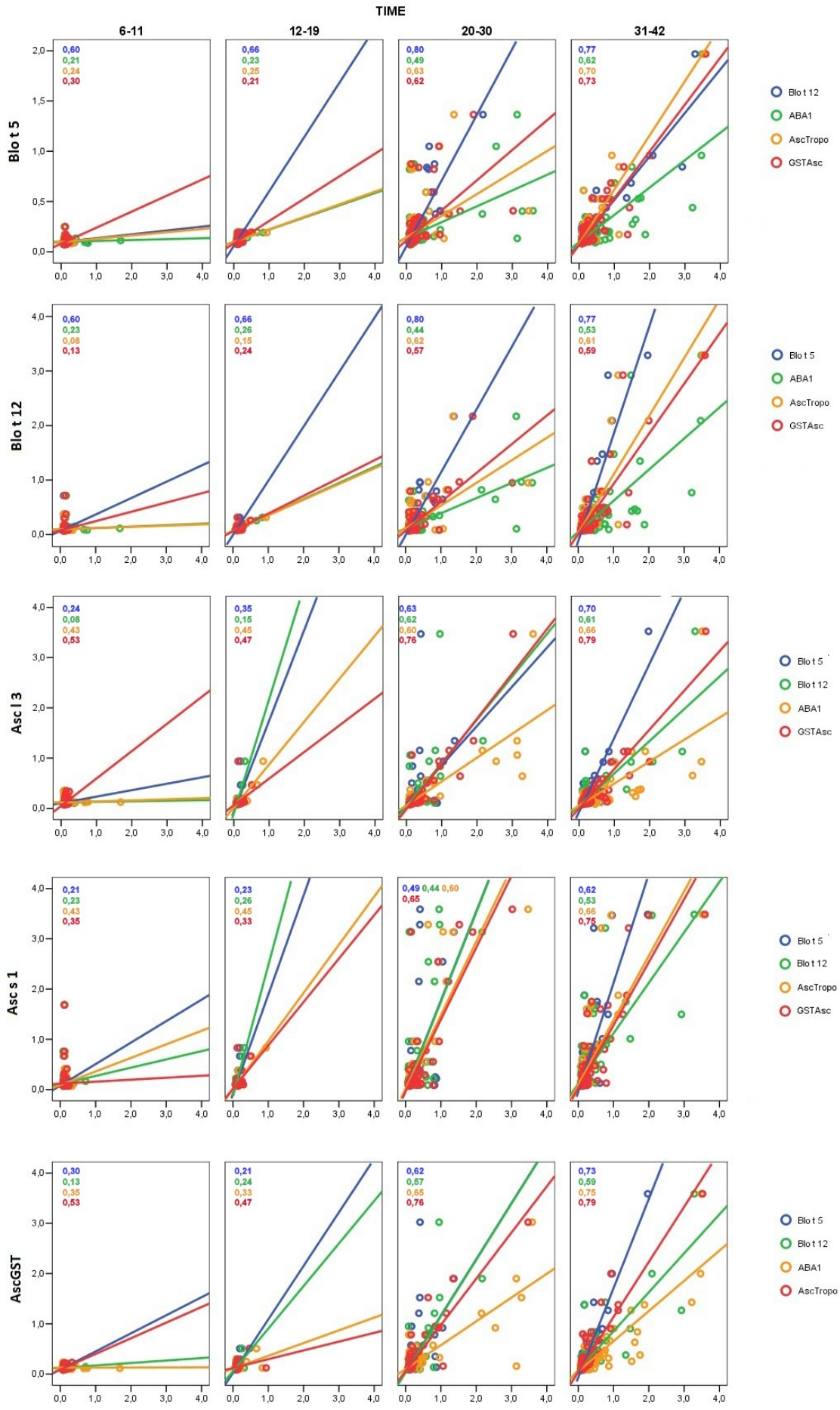


Figure 5.5 Difference in the dynamics of IgE response to Blo t 5 and Blo t 12 according to Asc s 1 sensitization. Graph lines representing median IgE levels to the mentioned allergen at the different time points during child life, discriminated by Ascaris sensitization (green line) or not (blue line).

Correlation among specific IgE to B. tropicalis and Ascaris components

For almost all allergens and time-periods, specific IgE values to one component were significantly correlated to rest of them. However, in the 6-11 mo. period this relationship was poor between *B. tropicalis* and Ascaris components, principally in the case of Blo t 12. As observed in Figure 5.6, the correlation coefficient of IgE values to Blo t 12 with that to Blo t 5 was high at S1 (Spearman rho =0.60), but low with Asc s 1a- (0.23) and insignificant with Asc s 3- (0.08) and AscGST- specific IgE (0.13). At the end of follow-up, correlation coefficient among all pairs of allergen-specific IgE values were significant and high (spearman rho coefficients range: 0.53 – 0.79).

Figure 5.6 Correlation plots among Ascaris and HDM recombinant allergens. In each row. Correlation among one allergen (Y axis) with the rest of them (X axis) is depicted in each row. Lines represent linear regression slopes. Numbers inside each plot correspond to Spearman rho coefficients for each comparison **(Figure in next page)**


TIME
6-11
12-19
20-30
31-42
Blo t 5
Blo t 12
Asc l 3
Asc s 1
AscGST
Blo t 12
ABA1
AscTropo
GSTAsc
Blo t 5
ABA1
AscTropo
GSTAsc
Blo t 5
Blo t 12
ABA1
GSTAsc
Blo t 5
Blo t 12
AscTropo
GSTAsc
Blo t 5
Blo t 12
ABA1
AscTropo

5.3.3. Discussion of results

Molecular diagnosis of allergy helps to define sensitization in a more precise manner [92]. Our previous investigations about the evolution of IgE responses in the FRAAT study had shown that *B. tropicalis* was the most important sensitizer in the first three years of life. However, keeping our observations about its CR with *Ascaris* antigens, we continue evaluating sensitization by using purified components from these two important sources with the aim to identify genuine responses and analyze the interrelationships between Ascaris and HDM specific IgE. With our findings, the possibility that *B. tropicalis* sensitization rates were overestimated in this cohort by the effect of nematode CR was ruled out.

Evaluating allergic sensitization with purified components indicated that truly responses to *B. tropicalis* are very frequent at early life, and not a matter of CR with Ascaris. It was interesting to observe that 60.3% of children responded to any of the two *B. tropicalis* allergens around 3 years old age, meanwhile the prevalence rates in mothers to these allergens, measured by the same ELISA protocol, were 27.1% to Blo t 12 and 29.2% to Blo t 5. Sensitization to the nematode-specific marker Asc s 1 was also high in these children, but not in mothers (14.4 %), and specific IgE values correlated very well with those to the cross-reactive components AscGST and Asc l 3, supporting then that early sensitization to Ascaris was neither a surrogate response of that elicited by HDMs. Taken this data together, it seems that IgE sensitization at this age is boosted, probably by light nematode infections; however, it is probable that many positive cases are transitional and disappear over time, as reflected by the lower frequency of IgE recognition to these components in adults from this same population. Although our conclusions about the lower prevalence rates in children than in adults can be skewed because the last are represented only by females (mothers from the FRAAT study), they are concordant with the prevalence rates obtained in other evaluations done in Cartagena. For example, in the ASA cohort, the IgE response to AscGST was close to 20%; in other reports we have observed that sensitization to Blo t 5 or Blo t 12 is lower than 50% of allergic subjects [36, 272], which represent a third part of the general population.

Primary sensitization to cross-reactive antigens in contexts of frequent co-exposition to both sources is difficult to define [45]. Although one weakness of this study was the use of only the representative CR components from Ascaris, we still consider that original approximations in this topic may derive from these analyses. In the first months of life (S1 period), sensitization to the *B. tropicalis* specific allergens, Blo t 5 and Blo t 12, was low in contrast to the more elevated rates of sensitization to the cross-reactive components from Ascaris. Moreover, specific IgE values against Ascaris and B. *tropicalis* components were poorly correlated. Since at this time period, IgE response to the nematode-specific marker Asc s 1 (ABA-1) was common and correlated with those to AscGST and Asc l 3, it may be proposed that primary sensitization to CR allergens in these children living in the tropics is due to helminth exposition.

Asc s 1 sensitization was associated with *A. lumbricoides* infection, but with low specificity. Since this type of fatty acid binding protein have been exclusively identified in nematodes, positive cases detected by IgE serology and not stool examination may be due to another infecting worm; however, since ascariasis is the most frequent helminth infection and polyparasited children usually were co-infected by this nematode [102], this explanation is less probable. Another possibility is that ABA-1 serology was more sensitive than the stool examination to detect parasite infection, which is not surprising finding due to the well-known limitations of the direct identification of involved pathogen to identify cases of active geohelmintiasis [273]. In fact, by questionnaire, mothers reported a worm expulsion antecedent in half of children, although the cumulative prevalence of Ascaris infection by stool examination was near 15%. On the other hand, Asc s 1 sensitization was associated to stronger responses to Blo t 5 and Blo t 12 in children. Unspecific boosting of the Th2 responses by the parasite may influence this finding; however, it can also be explained by a common genetic predisposition to become sensitized to aeroallergens and parasite antigens [274, 275].

In conclusion, by using purified allergenic components we could identify high sensitization rates to *B. tropicalis* and Ascaris in a very specific manner. Ascaris antigens seem to be the primary sensitizers in the IgE responses towards cross-reactive Ascaris-HDM components.

Chapter 7. *General discussion*

Development of this doctoral thesis contributed to the knowledge of *B. tropicalis* allergens, the most important cause of IgE sensitization in the tropics. With the production and characterization of three of its allergens, many issues of current interest in Allergology were investigated. The influence of isoform diversity on IgE responses, the identification of a SSM marker and the effects of chitin in the immune response to chitin-binding allergens are highlighted aspects from the molecular characterization of Blo t 12. With the identification of two new *B. tropicalis* allergens, a gluthathione-S-transferase isoform and ubiquitin, and its further characterization, we added novel examples in the study of two important topics: the cross-reactivity with nematode antigens and the relationship between allergy and autoimmune responses.

The group 12 of *B. tropicalis*, as other important allergens, is polymorphic. With the characterization of two isoallergens, obtained by molecular cloning, it was observed that in spite of its high degree of sequence similarity, their amino acid variations led to important differences in their biological and immunological behavior. In terms of IgE recognition, Blo t 12.0101 was identified as the most important isoallergen in Cartagena, concluding then that this variant should be the selected isoform for allergy diagnosis. Blo t 12 polymorphisms also influenced its production as recombinant allergens in different systems. Expression of Blo t 12.0101 in *Pichia pastoris* resulted in a hyperglycosylated product with lack of chitin binding affinity and a reduced antibody reactivity. In contrast, production of Blo t 12.0102 in the yeast was more convenient; it was obtained a soluble protein with an apparent proper folding since its biological activity was not negatively modified as occurred with the *E. coli* produced variant. Development of a murine model of asthma with the two isoforms – produced in *E. coli* – also showed differences in the elicited immune responses. The .0101 variant, with good affinity to chitin, induced a stronger hyperreactivity response to inhaled methacholine and a more Th2 polarized humoral response than the .0102 variant.

A truly species-specific allergen from *B. tropicalis* had not been found before [46, 47]. The identification of Blo t 12 as a *B. tropicalis* SSM will contribute enormously to the improvement of allergy diagnosis in the tropics. Detection of Blo t 12

sensitization will help to define if many cases of double positivity to *B. tropicalis* and *D. pteronyssinus* [140] are due to parallel sensitization. Moreover, we developed a sandwich ELISA system for Blo t 12 quantification in allergen extracts and environmental samples; a needed tool for detecting *B. tropicalis* exposition in our population. Although several kits for *B. tropicalis* allergen determination have been developed in academic research [142, 276, 277], only a Blo t 5 ELISA kit is commercially available. In spite that Blo t 5 is an abundant and epidemiologically relevant allergen; the mentioned assay showed a regular performance for its detection in house dust. In contrast, although Blo t 12 was found at lower concentrations than highly abundant allergens, such as Der p 1, it was detected in about 90% of dust samples. We also focused on testing the specificity of anti-Blo t 12 antibodies to recognize this allergen, but not other outstanding agricultural pests, because this Blo t 12 two-site ELISA system can be also very useful for the identification of mite-contaminated food products [278]. In this regard, the utility of the kit extends to temperate regions; *B. tropicalis* and other species in the Blomia genus are storage mites also in areas beyond the tropics. One study reported that *B. tropicalis* mites were found in 21% of cereal-based food products purchased at food retail outlets in United Kingdom [279]. Occupational allergy to *Blomia kulagini* present on cheese rind has been reported in Spain [280].

With the isolation of natural Blo t 12 it was realized that IgE responses to this allergen, at least in our population, are expected to be more intense and frequent than previously identified by using the recombinant molecule. Although any of the two produced recombinant versions are fairly good to diagnose Blo t 12 sensitization, it must be recognized that some positive cases will be missed. By the analysis of its sequence, it could be predicted that reproducing the physico-chemical properties of the natural allergen was a difficult task. Hence, *P. pastoris* was selected in first instance for protein expression due to its capability of disulphide bridge formation and other features that permit protein folding assistance. Unfortunately, a hyperglycosylated product with a reduced IgE binding activity was obtained. On the other hand, since an appropriate folding of cystein-rich proteins in *E. coli* have been successfully accomplished with new available modified strains, we tried to produce Blo t 12.0101 in Origami coupled to Trx,

with no good results in its purification that led to abandon the Trx-assisted folding and produce it directly as his tagged protein. The IgE binding properties of this *E. coli* recombinant did not exceed those from the *P. pastoris* molecule. In the presence of a wide number of options for recombinant protein production (i.e. usage of other eukaryotic systems, mutation of glycosylation sites or even production in *E. coli* but with a different cleavage system to separate the fusion proteins) more efforts can be placed on this area to produce a better recombinant.

A great part of this thesis was devoted to intensive research on one allergen (Blo t 12); however, this is just one example of the demanding task that implies a more general aim of our research group: the obtention of well-characterized allergenic components in the tropics. Thus, parallel to the characterization of Blo t 12, other allergens were sought in a *B. tropicalis* cDNA library and for others, although not included in this thesis; their nucleotide sequence was synthesized for further protein expression in our biotechnology laboratories. I contributed with my work to the description of the allergenic properties of Blo t 8 and the ubiquitin from *B. tropicalis*, two molecules whose importance as IgE sensitizers was unknown.

Ubiquitin from *B. tropicalis* is an IgE- reactive molecule. Although there were healthy subjects responding to Bt-Ubi, frequency of IgE sensitization was significantly higher in asthmatic patients. Because this ubiquitin is identical to the human-derived peptide, the observed antibody response can also be considered as autoimmune. The recognition of intrinsic proteins as allergens (autoallergens) has been described before [161-163], cross-reacting some of them with heterologous molecules. For some of this group of "autoallergens", there have been demonstrated its capacity to induce IgE-mediated cell activation, especially in those individual co-sensitized to CR environmental allergens [164-166]. However, there are also intrinsic IgE binding proteins that do not activate basophils, but induced other type of immune effects. Hom s 2, for example, is a potent inducer of IFN-gamma secretion and epithelial cell damage [163]. Autoantibody responses to ubiquitin have been detected in patients with systemic autoimmune disorders [167], but its role of allergy have never been reported. These results only support the IgE reactivity towards ubiquitin; nevertheless, its capacity to induce allergic reactions must be demonstrated.

Cross-reactive allergens between nematodes and HDMs are of special interest for our research group due to their influence on allergy diagnosis and, possibly, on the origins of IgE sensitization in the tropics. In this regard, a cDNA library was screened by PCR to identify the GST from *B. tropicalis*, a cross-reactive component between this mite and Ascaris [45]. Some allergenic properties of Blo t 8, the GST from *B. tropicalis*, were described here. Allergen recognition was frequent, but IgE responses were of low intensity. A similar response was detected to Bla g 5 and AscGST in the analyzed sample population, mostly composed of adults. However, it was interesting to observe that AscGST was the most frequent IgE sensitizer at early life in children from the same population, but also was the first component to reach a plateau in terms of intensity and frequency of recognition in the performed serial IgE determinations during the first three years of life. In accordance, the influence of CR with other GST allergens, in especial those from nematodes, deserves further investigation to understand the relevance of this allergenic component in tropical environments.

Understanding the differences of the allergenic potential of *B. tropicalis* and *D. pteronyssinus* is difficult. Perennial co-exposition to both sources – probably in similar amounts [143] – and the presence of cross-reactive allergens explains the higher rates of double sensitization in our population[123]. However, a small group of co-exposed, but mono-sensitized subjects [265] and also genetic associations with the IgE response to only one of the two species [59, 266] have reinforced the idea that sensitization processes to any of these HDMs are independently controlled. With the aim of identifying particularities of *B. tropicalis* allergenicity and understanding the evolution of early sensitization in the tropics, we decided to study these aspects in a sample of children living in Cartagena, followed from birth up to the age of 3 years. Due to its CR with HDM, among other reasons [153], evaluation of Ascaris sensitization was also essential for this purpose.

It is important to remark that in contrary to the hygiene hypothesis, although most families recruited in this cohort lived in poverty and unhygienic conditions, allergy was frequent. Half of mothers were sensitized to HDMs, 23% had allergic rhinitis and 9% reported current asthma symptoms. Clinical evaluation of children focused on previously reported atopy-related phenotypes of early presentation. In contrast to

populations from developed countries, wheezing, but not eczema, was common in children. Thus, relationships between IgE serology and clinical manifestations were limited to the former condition. In this cohort, frequencies of sensitization to *B. tropicalis* and *D. pteronyssinus* were markedly different, higher to *B. tropicalis*. Interestingly, frequency of *B. tropicalis* sensitization, but not to *D. pteronyssinus,* at three years of age was similar to that in mothers. Taken this data together it may be proposed that the allergenic potential of *B. tropicalis* is higher than *D. pteronyssinus*, but this effect is clearly visible only at early life since a continuous exposition to both allergen sources let susceptible individuals to become sensitized to both sources in similar proportions.

Although we identified that, uniquely, *B. tropicalis* IgE response, but not to *D. pteronyssinus* or Ascaris, was a risk factor for wheezing in children (supporting specific effects of this source) our concerning about the bias in allergy diagnosis due to CR between HDM and nematodes was also suspected in this case by the high correlation between the specific IgE values to the complete extracts of Ascaris and HDM, especially with *B. tropicalis*. In this sense, IgE serology at the extract level did not permit to discriminate when positive cases of HDM sensitization were due to cross-reactive Ascaris-induced IgE antibodies rather than genuine atopy. Therefore, this situation prompted us to analyze IgE sensitization by using purified components of *B. tropicalis* and Ascaris. Due to the limited amount of sera that can be collected in infants, we decided to explore from the wide repertoire of HDM allergens only two representative *B. tropicalis* components (Blo t 12 and Blo t 5), expecting to detect with them more sensitization cases than with *D. pteronyssinus* allergens (as detected at the level of extracts). IgE responses to Ascaris/HDM cross-reactive molecules were analyzed with the nematode component, guided by the hypothesis that antigen exposure from nematode infections boost IgE responses to the cross-reactive HDM allergens.

Evaluating allergic sensitization with purified components indicated that truly responses to *B. tropicalis* are very frequent at early life, and not a matter of CR with Ascaris. Sensitization to the nematode-specific marker Asc s 1 was also high and correlated with that to the HDM cross-reactive components AscGST and Asc l 3, supporting then, that early sensitization to Ascaris was neither a surrogate response of

that elicited by HDMs. Higher rates of sensitization with recombinant molecules than with complete extracts suggested that they were not sufficiently represented in the natural preparations. In the first study, for example, no cases of Ascaris sensitization were detected during the 6-11 months period; in contrast, positive results to any the three nematodes components accounted for approximately 20% of children.

In summary, with the characterization of three allergens from *B. tropicalis,* it was obtained a species-specific marker (Blo t 12), a nematode cross-reactive allergen (Blo t 8) and a potential autoallergen (ubiquitin). Analyzing IgE responses to *B. tropicalis* with extracts and purified allergens at early age led to know that this is the most important HDM allergenic source in our population and a mild risk factor for wheezing at early infancy. Furthermore, we got evidence that although IgE responses to *B. tropicalis* may be influenced by Ascaris infections, high frequencies of sensitization at early age are genuine and not a bias in diagnosis caused by the CR phenomenon.

Future perspectives

Although in this work Blo t 12 was extensively chacterized, there are still many issues to be evaluated in further investigations. Molecular characterization of the recombinant allergens indicated that other options for allergen production must be tried to obtain a more representative product of natural Blo t 12. Because we are also working in the experimental resolution of Blo t 12 tertiary structure as part of a scientific collaboration with Dr. Brian Smith from the University of Glasgow, the need of a recombinant molecule with a proper folding is mandatory. Thus, the production of Blo t 12 in fusion with thioredoxin, but with a different cleavage system for protein purification, is ongoing. We expect to get better results with this new recombinant molecule.

Research in murine models is restricted in Colombia due to availability of just few mouse strains. In our case, the use of only BALBc mice for developing a murine model of sensitization to *B. tropicalis* allergens gave us limited information about our initial interrogants. Collaborations with overseas partners are needed to test this sensitization model in other mouse strains.

More aspects are planned to be explored in the case of Blo t 8 and other GSTs. Parallel to the characterization of the *B. tropicalis* GST, we are currently studying the allergenic properties of AscGST since the immune response to this CR allergens is an important focus in our investigations about the characteristics of allergy in the tropics. Epitope mapping is necessary for identification of shared and unique antigenic determinants among these molecules. Another activity to be continued is the evaluation of their immunological potential using basophil activation tests and histamine release assays in a representative sample of IgE sensitized subjects. It seems very interesting for us to confirm and understand why these common IgE sensitizers induce only moderate to low allergic responses in this population. Similar experiments must be conducted to analyze the allergenic potential of ubiquitin; moreover, in this case is especially important to evaluate the cellular mechanisms possibly involved in the autorreactivity to this molecule.

The FRAAT cohort study is an ongoing project; we continue with the clinical follow-up of the recruited children and sample collection. We expect to evaluate more allergenic components of relevance in the tropics at older ages, using more efficient techniques such as allergen microarrays. Parallel projects in this regard are under performance. The number of allergen clones we produce in our laboratory includes 11 components from *B. tropicalis*, 5 from *D. pteronyssinus* and 3 from *Ascaris spp.* We are trying to take advantage of our strengths in the field of Biotechnology to provide us with tools for studying more aspects about the allergy in the tropics.

There have been tremendous advances in the field of allergy during the last 20 years; however, fundamental interrogates remain. One of them is the question about the inception and natural history of asthma, together with the role of allergens as a cause of this disease. Besides, being allergy a highly environmental-dependent condition, the inducers and phenotypes are not expected to be the same everywhere. Therefore, further research to clarify the patterns of clinical symptoms and allergen sensitization in the tropics is the main task in the collective perspective of the Institute for Immunological Research of the University of Cartagena.

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APPENDIXES

Appendix 1 Equipments

Equipment	Company
Orbital shaker	Thermo
Elisa plate reader Spectra MAX 250	Molecular Device
Microcentrifuge bench top model	Thermo
Incubator	Thermo
Thermocycler	BioRad
Lyophylizer	Labconco
Vortex mixer	
Electroporator	Biorad
Whole-body plethysmograph	Buxco
Qubit	Invitrogen
Isoelectrophocusing system	Biorad
Electrophoresis system Tetra cell	Biorad
Transblot system forelectrotransference	Biorad
Chemiluminiscence detector	Syngene
Sonicator	Thermo
Mesh	Resch
Optical microscope	Leica

Appendix 2 Reagents

Reagent	Company	Catalog number	Country
Bradford reagent	Sigma	B6916	St. Louis. MO, USA
Sodium tetraborate	Sigma	B9876	St. Louis. MO, USA
Dimethyl pimelimidate dyhidrochloride	Sigma	D8388	St. Louis. MO, USA
Ethanolamine	Sigma	110167	St. Louis. MO, USA
Glycine	Amresco	0167	Solon, OH, USA
QiaQuick PCR purification kit	Qiagen	28104	Hilden, Germany
Champion™ pET100 Directional TOPO® Expression Kit with BL21 Star™ (DE3) One Shot® Chemically Competent E. coli	Invitrogen	K100-01	Grand Island, NY, USA
Pfu polymerase	G Biosciences		VWR Interanational
Taq polymerase			
Lisozyme	Sigma-Aldrich	62971	Steinheim, Belgium
Ni-NTA resin	Qiagen	30230	Hilden, Germany
Imidazole	Sigma	I5513	
Glutathione Sepharose 4B	GE Healthcare		Uppsala, Sweden
Tris	Biorad	1610716	Hercules. CA. 94547
RIDASCREEN® Total IgE	R-Biopharm		
paranitrophenyl phosphate	Sigma	N2640	
ReadyPrep 2-D Cleanup kit	Bio-Rad		Hercules. CA. 94547
Urea	Sigma		
CHAPS detergent	Bio-Rad	161-0460	Hercules. CA. 94547
Bromophenol blue			
Dithiothreitol	Sigma	D-9779	St. Louis. MO, USA
ByoLyte Carrier ampholytes pH 3-10	Bio-Rad	163112	Hercules. CA. 94547
3-10 NL ReadyStrip™ IPG Strip	Bio-Rad	1632016	Hercules. CA. 94547
SDS	Bio-Rad	1610332	Hercules. CA. 94547
Iodoacetamide	Bio-Rad	163-2109	Hercules. CA. 94547
Acrylamide	Bio-Rad		Hercules. CA. 94547
Bisacrylamide	Bio-Rad		Hercules. CA. 94547
Ammonium persulphate	Bio-Rad	161-0700	Hercules. CA. 94547
Chitin powder	Sigma		St. Louis. MO. USA

Reagent	Company	Catalog number	Country
Chitin beads	New England Biolabs		
Triton X-114			
Limulus amebocyte lysate Kinetic-QCL™	Lonza	50-650U	Walkersville. MD. USA
Methacoline	Sigma		St. Louis. MO. USA
Maxisorp microtiter plates	Nunc		
Biotin mouse anti-IgE. Clone 23G3	eBiosciences	13-5992-81	San Diego, CA, USA
Biotin mouse anti-IgG1. Clone A85-1	BD Biosciences	553441	San Jose, California, USA
Biotin mouse anti-IgG2a	BD Biosciences	550332	San Jose, California, USA
ExtrAvidin®-phosphatase alkaline	Sigma	046K4761	St. Louis. MO. USA
Bovine serum albumin	Sigma	A7030	St Louis. MO. USA
Diethanolamine	Sigma	D8885	St. Louis. MO. USA
Mouse Total IgE OptIEA	BD Biosciences	555248	San Jose, California, USA
ECL™ glycoprotein detection module	GE Healthcare	RPN2190	
Sodium metaperiodate	Sigma	S1878	St. Louis. MO. USA
Sodium acetate	Sigma	S2889	St. Louis. MO. USA
SuperSignal West Femto Maximum Sensitivity Substrate	Thermo Scientific	34095	Rockford. IL. USA
Protein A-Sepharose® 4B. Fast flow	Sigma-Aldrich	127K0827	Steinheim. Germany
Protein G-Sepharose® 4B. Fast flow	Sigma	P3296	St. Louis. MO. USA
B. tropicalis CAP	Thermo	D201	Phadia AB, Uppsala, Sweden.