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Th2-associated immunity to bacteria in asthma in teenagers and susceptibility to asthma

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Abstract

Bacterial colonisation of the airways is associated with increased risk for childhood asthma. IgE against bacterial antigens has been reported in some asthmatics, suggesting a role for bacterial-specific Type-2 immunity in disease pathogenesis. We aimed to investigate relationships between bacterial-specific IgE amongst teenagers and asthma susceptibility.

We measured titres of IgE against *Haemophilus influenzae*(HI), *Streptococcus pneumonia*(SP) and *Staphylococcus aureus*(SA) in 1380 teenagers, and related these to asthma symptomatology and immunophenotypes.

IgE titres against SA-derived enterotoxins were highest amongst atopics and were associated with asthma risk. Surprisingly, IgE titres against HI and SP surface antigens were higher and not stratified by atopy, and independently associated with decreased asthma risk.

The positive association between Type-2 immunity to SA and asthma phenotypes likely reflects IgE-mediated effector cell activation via enterotoxin antigens which are secreted in soluble form . The contrasting benign nature of Type-2 immunity to HI and SP antigens may reflect their lower availability in soluble forms that can crosslink IgE receptors. We theorise that they may instead be processed by antigen presenting cells and presented to Type-2 memory cells leading to mucosal secretion of IL-4/IL-13, a mechanism widely recognised in other tissues to attenuate Th1-associated bacterial-induced inflammation.

Abstract word count: 200

Keywords

Asthma, Bacteria, IgE, Staphylococcal enterotoxin, Th2 immunity, Raine Study

Abbreviations

BHR: Bronchial hyperresponsiveness

HI: *Haemophilus influenzae*

P6: Outer membrane protein P6

PGF_2a: Prostaglandin F_2a

PspC: Pneumococcal surface protein C (also known elsewhere as CBPa and SpsA)

SA: *Staphylococcus aureus*

SAE: *Staphylococcus aureus* enterotoxin

SEB: *Staphylococcal* Enterotoxin B

SP: *Streptococcus pneumoniae*

Introduction

Lower respiratory infections have been linked in many studies to development and expression of asthma during childhood, especially amongst atopics [1]. The primary focus has been upon viruses, particularly rhinovirus [2, 3], and bystander airway tissue damage during host-anti-viral responses is widely believed to play a key role in driving wheezing disease pathogenesis towards chronicity. Considerably less attention has been paid to bacteria, with the exception of *Staphylococcus aureus* (SA) strains which produce soluble enterotoxins. These molecules function as superantigens and can directly activate a large proportion of peripheral T-cells [4], triggering polyclonal production of both Th1 and Th2 cytokines and synthesis of enterotoxin-specific IgE. Colonisation with superantigen-producing SA has been suggested to play a role in a range of atopy-associated diseases, including atopic dermatitis [5], rhinosinusitis and allergic rhinitis [6, 7] as well as adult asthma [8, 9] and childhood wheeze [10]. Recently, colonisation of the upper airways during early childhood with bacterial pathogens, including non-enterotoxin-producing strains, has been linked with subsequent development of persistent asthma [11], but the pathogenic mechanisms are unknown and the interpretation of these findings remains controversial [12].

The presence of underlying Type-2 immune responses to non-enterotoxin-producing bacteria in asthmatics, in particular bacterial-specific IgE, has been recognised for some time [13, 14]. Moreover these responses have recently been shown to be markedly boosted following severe asthma exacerbations in children [15] suggesting that antigens from these bacteria gain entrance to the immune system via the inflamed airway mucosa. The significance of these findings is unclear, and more detailed investigations of relationships between host immune responses including IgE to different classes of mucosal dwelling bacteria and susceptibility to

asthma are needed. In particular there is a requirement for baseline data on the spectrum of IgE responsiveness to bacteria in the normal population. We report below on IgE responses to three potential bacterial pathogens *Haemophilus influenzae* (HI), *Streptococcus pneumoniae* (SP) and enterotoxin-producing SA, in a community cohort of 1380 teenagers, and the positive and negative risk associations between antibody titres and underlying asthma-associated clinical phenotypes.

Material and Methods

Study subjects

The West Australian Pregnancy Cohort (RAINE) Study (WAPC) is a longitudinal birth cohort; mothers were enrolled from antenatal clinics from the main tertiary maternity hospital in Perth, Western Australia, and were not selected on any criteria other than having enrolled for antenatal care at the hospital [16]. Analyses presented here were based on data collected at the 14 year followup of this cohort, and did not utilise information collected at other ages. By studying asthma in 14 year old children we are biasing towards asthma that is more closely associated with atopy and more likely to persist into adult life. All aspects of the current study were approved by our institutional Human Ethics Committee and parents gave written consent, with assent provided by the teenagers.

Clinical phenotyping

Current asthma was defined as wheeze or cough without a cold in the last 12 months, plus use of any asthma medication in the last 12 months in children with a doctor diagnosis of asthma ever. Current rhinoconjunctivitis was assessed on the basis of parental response to a

standardised questionnaire regarding the child's symptoms (runny, blocked or itchy nose in the presence of runny or itchy eyes) over the preceding 12 months.

All spirometry measurements were performed in the morning (10-12pm) and conducted according to American Thoracic Society Guidelines with the subjects seated [17]. Standard variables, forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), the average forced expiratory flow between 75% and 25% of FVC (FEF_{75-25}) and FEV_1/FVC were calculated.

Subjects were eligible to participate in methacholine (MCh) challenge testing if they obtained reproducible spirometry, had an FEV_1 at least 80% of predicted, had no respiratory illness in the last 14 days and had withheld from their asthma medications for the standard period [17]. MCh challenges were performed with a Koko digidoser using a modified dosimeter technique [18]. Subjects were given an initial saline dose followed by doubling doses of methacholine (0.0625, 0.125, 0.25, 0.5, 1, 2, 4 & 8mg/ml) delivered via DeVilbiss 646 nebulisers. Testing was stopped once a patient's FEV_1 had fallen by $\geq$ 20% or the highest dose was administered. The dose response slope was calculated as the 2 point slope of the curve regardless of the fall in FEV_1 [19].

Lung function was represented in the statistical analysis by the FEV_1/FVC ratio. In a community population of adolescents where restrictive lung disease would be unexpected, this ratio is most likely to represent airway obstruction in a manner that is independent of body habitus, lung size and sex.

Immunological phenotyping

Atopic family history was obtained from the questionnaire completed by the primary caregiver. Questions were asked about first degree relatives (biological mother, biological father or sibling) seeking information about asthma, eczema, rhinoconjunctivitis and other allergies. Parents were asked to provide information about whether the allergies had been confirmed and by whom. Information was recorded about maternal and parental allergic history separately. A child was considered to have a positive family history if one or more first-degree relative have confirmed asthma, eczema, or rhinoconjunctivitis.

Current atopic status including serum titres of allergen-specific IgE was determined as detailed previously [20]. Subjects were considered atopic if they had total IgE ≥300 kU/L and/or specific-IgE ≥0.35 kU/L for any of the following allergens: HDM, rye grass pollen, cat, couch grass, mould mix, peanut or food mix. Total IgE as well as specific IgE to HDM *(Dermatophagiodes pteronyssinus)*, rye grass pollen *(Lolium perenne)*, cat, couch grass *(Cynodon dactylon)*, mould mix *(Penicillium notatum, Cladosporium herbarum, Aspergillus fumigatus, Candida albicans, Alternaria alternata, Helminthosporium halodes)*, peanut and food mix *(egg white, milk, fish, wheat, peanut and soybean)* to a mixture of *S. aureus* enterotoxins (SEA, SEC & TSST-1), were measured by ImmunoCAP (Phadia AB, Uppsala, Sweden).

IgE to mixed SA enterotoxins were measured by Phadia ImmunoCAP [8]. IgE antibodies to outer membrane protein P6 from HI and Pneumococcal surface protein C (PspC) from SP were assayed by modifications to the procedure described to use humanised IgE antibody for absolute quantitation [21]. A microtitre plate dissociation-enhanced immunofluoresence

assay (DELFIA^{TM}) was performed where antigen coating was standardised by capturing His-tagged recombinant antigens with anti-His monoclonal antibody coated as described [22]. The assay was calibrated by interpolating the results from a titration curve constructed with recombinant Der p 2 captured by the same procedure and a standardised (IU/ml) humanized IgE anti-Der p 2 (Indoor Biotechnolgies VA USA). The lower limit of detection was 0.1 ng/ml (0.041 kU/L) for P6 and PspC. The P6 antigen was prepared as described [15] and the PspC (aa 1 to 445) antigen as previously shown by Brooks-Walter et al [23].

Peripheral blood mononuclear cells (PBMC) were cryopreserved and subsequently cultured as described previously [20]. PBMC were cultured for 48h in AIM-V medium with $4x10^{-5}$M 2-mercaptoethanol alone or with 200ng/ml SEB (Sigma-Aldrich, Castle Hill N.S.W., Australia). $0.6x10^6$ cells were cultured in round-bottomed 96-well plates (Nunc, Roskilde, Denmark) at a concentration of $1.2x10^6$ cells/ml. Measurements of cytokines and other inflammatory mediators was as detailed previously [20] and in Online Depository.

Statistical analyses

Univariate analysis of group differences in IgE titres were examined using Mann Whitney U test, and bivariate relationships were assessed using Spearman's correlation. Continuous variables were $\log_{10}$-transformed for use in regression analyses. To allow $\log_{10}$ transformation, cytokine delta values ≤0 were converted to 0.01; antibody values below the limits of detection were ascribed a value equivalent to half the limit of detection. Variables in multivariate analyses were selected based on univariate association with outcome at $p<0.10$. Bronchial hyperresponsiveness (BHR) dose slope was analysed with linear regression,

asthma risk and BHR risk with logistic regression, and asthma severity with ordinal regression. Analyses were performed using SPSS software.

Results

IgE responses to Staphylococcal enterotoxins (SAE) in atopic and non-atopic teenagers

IgE responses against SAE are common amongst teenagers (19.3% of cohort; Supplementary Table 1) and more frequent amongst atopics (26.8% versus 8.0% in non-atopics). Moreover as shown in Figure 1A the mean SAE-IgE titres are also preferentially increased amongst atopics, suggesting that responsiveness is linked to the atopic phenotype. This gradation is similar if results are expressed as percentage with IgE titres above assay detection limits (52.7% versus 22.3% respectively; Supplementary Table 1).

SAE-IgE and risk for asthma

Figure 1B shows SAE-specific IgE titres within the cohort after stratification by atopy and clinical phenotype, and illustrates the consistent relationship between SAE-IgE titre and the presence of respiratory symptoms in atopics. Further analysis by univariate logistic regression (Table 1) confirmed this association. In particular when we stratified by atopy status SAE-IgE was a significant risk factor amongst atopics for asthma and also for BHR, while amongst non-atopics SAE-IgE was a risk factor for BHR only. Fig 1C further examines relationships between SAE-specific IgE and asthma. For this analysis the population was grouped on the basis of serum SAE-IgE levels (undetectable plus population terciles amongst responders), and asthma risk was computed for each group by univariate logistic regression; this risk appears to increase progressively with antibody titre. This association was explored further via logistic regression modelling, focusing on the atopics identified in Table 1 as the "at risk" group. In addition to bacterial-specific IgE titres, these analyses utilised a range of data derived from the study cohort relating to respiratory, inflammatory and atopy-associated functions [20]. A two-step process was undertaken as shown in Table 2. Firstly, univariate

logistic regression was performed to identify individual asthma risk variables (Left panel, Table 2), and these included a range related to intensity of underlying atopy. Of note they also include SAE-specific cytokine responses (IL-5, IL-10 and IL-13). The final multivariate regression model combined these significant variables as shown in Table 2. The list of variables identified as independently associated with asthma risk was dominated by measures related directly or indirectly to conventional atopy, notably aeroallergen-specific IgE and a marker of eosinophil activation. The IgE component of the SAE-response did not remain as a significant risk variable but was supplanted by the SAE-specific cytokine response in the form of IL-10.

SAE-IgE and risk for BHR

A potential association between SAE-IgE responses and risk for BHR development is also suggested by the results in Table 1. Accordingly multivariate linear regression analyses to predict methacholine dose slope were performed employing variables that were significant in initial univariate regressions. In contrast to asthma, univariate analyses showed that SAE-IgE was a risk factor for BHR for both non-atopic and atopic subjects (Supplementary Table 2), so these were assessed separately in multivariate analyses. Within the atopic population SAE-IgE does not survive as a significant risk factor for BHR in the multivariate model (Table 3), however IL-5 responsiveness to Staphyloccocal enterotoxin displays a strong positive correlation with degree of BHR. In contrast SAE-IgE survives as an independent risk factor in the non-atopics.

IgE responses to HI and SP antigens and risk for respiratory symptoms

Supplementary Table 1 and Figure 2A illustrate the spectrum of IgE titres to HI P6 and SP PspC antigens within the study cohort. IgE responses to these antigens are more frequent and

stronger than those against SAE and (in contrast to the pattern seen for SAE) the atopic and non-atopic subpopulations display comparable responses to P6 and PspC. Moreover, IgE responses to these antigens are strongly intercorrelated within both atopics (rho 0.658; p=0.000) and non-atopics (rho 0.623; p=0.000) but are unrelated to responses to SAE (Supplementary Table 3).

Univariate analyses on the whole population stratified by P6- and PspC-IgE titre (Fig 2B) and by atopic status (Table 4) revealed relationships markedly different from those observed for SAE-IgE. In particular IgE titres against these non-enterotoxin antigens associated inversely with asthma risk (Fig 2B versus Fig 1C, and Table 4 versus Table 1). Of note, amongst the atopics both P6-IgE and PspC-IgE remained as significant protective factors in relation to asthma risk in multivariate analyses which included allergen specific IgE measures as confounders (Table 5).

Discussion

The most intensively studied bacteria in relation to pathogenesis of inflammatory airway diseases are enterotoxin-producing SA. Their secreted products can trigger production of T-cell cytokines including IL-5, IL-10 ,IL-13 and also IFNγ [24, 25], and can also lead to inhibition of T-regulatory cell activity [26].

In this cohort IgE responses to SAE were higher and more frequent amongst atopics, in particular those with airway symptoms (Figure 1A). Moreover in univariate models SAE-specific IgE titre was a significant risk factor for asthma and BHR, particularly amongst atopics (Table 1). SAE-IgE titres in atopics correlate strongly with those against

conventional aeroallergens and total IgE (not shown), and the SAE-IgE effects in these subjects relating to asthma risk were no longer significant after adjusting for overall IgE levels and the potential confounders identified in initial univariate analyses (Table 2). This suggests that the direct disease promoting effects of IgE against SAE may be secondary to those resulting from IgE against the common aeroallergens, and/or may be restricted to subjects with severe wheeze as suggested elsewhere [8-10]. Alternatively SAE-IgE may be a surrogate for other covert aspects of the Th2-associated response to SA enterotoxin, including effects of SAE on polyclonal stimulation of IgE production and/or on IgE-independent mechanisms. It is pertinent to note here our earlier findings that T-cells from a subset of atopic children are hyperresponsive to stimulation with the archetypal SA enterotoxin B (SEB; [27, 28]) which triggers cytokines including IL-5, IL-10 and IL-13. We accordingly included assessment of PBMC responses to SEB as part of the immunophenotyping of this cohort. As noted in Table 2 SEB-induced IL-5, IL-10 and IL-13 responses were associated with asthma risk in atopics in univariate analyses, and inclusion of these variables in the final multivariate model identified the IL-10 component of the SEB response as an independent associate of asthma risk. Despite the anti-inflammatory action of this cytokine in relation to allergen-specific Th2 responses [29] its production in excess and/or in the wrong context has been associated with increased susceptibility to wheezing-related symptoms [20, 30]. The present findings are also consistent with a recent report linking susceptibility to asthma symptoms following occupational LPS exposure to levels of LPS-induced IL-10 production by PBMC [31].

The association between SAE-IgE and risk for BHR was further examined by multiple linear regression, incorporating potential confounders identified by univariate linear regression. As found for asthma, SAE-IgE was not a significant risk factor for BHR in atopic teenagers

(Table 3). However, this model does contain SEB-induced IL-5 which can drive eosinophilia, in addition to Eosinophil Protein X, a product of activated eosinophils. Heightened eosinophil-related responses were previously identified as the strongest risk factor for wheeze and BHR in atopic school children [32].

In contrast to these findings relating to atopics, the positive association observed amongst non-atopics between BHR and SAE-IgE by univariate analysis (Table 1) remained significant after adjustment for potential confounders by logistic regression (Table 3). This suggests that SAE-specific IgE may function as an independent risk factor for BHR in non-atopics, implying that despite its low titre this IgE may contribute to airways inflammation in these subjects in whom significant IgE of other specificities is extremely rare.

IgE antibodies to nasopharyngeal bacteria have been reported in a number of studies and a recent investigation by some of the present authors detected a steep rise of anti-P6 IgE titres in children in the wake of severe asthma exacerbations [15], which implies that sufficient bacterial antigen penetrated the mucosa during this period to stimulate underlying bacterial-specific immunity. These responses are unlikely to be restricted to antibody, given that bacterial products exemplified by HI P6 antigen are also potent inducers of proinflammatory cytokines from human macrophages [33] including those at mucosae. The present findings do not identify the precise contribution of anti-bacterial Th2 immunity to the local cytokine milieu in the airways, but this contribution is clearly more complex than a straightforward addition to ongoing inflammation. In particular, HI- and SP-specific IgE is *inversely* associated with asthma risk in healthy atopic teenagers (Table 4; Fig 2B) despite the fact that respective (mean) production levels are *higher* than corresponding responses to SAE which are positively associated with risk (Fig 1C vs Fig 2B). This discrepancy may be due in part

to the active secretion of soluble SA enterotoxins [34]; in contrast, PspC antigen of SP would be expected to be mainly encountered covalently attached to the cell wall [35] and although HI do secrete vesicles that contain the outer membrane antigen P6s [36] these are 200nm structures, which may not be readily available for cross-linking IgE-FcR-armed receptors.

Additionally, the finding of circulating specific IgE antibody against HI and SP also automatically denotes the presence of additional forms of underlying Th2 immunity against these antigens, In particular the presence of IL-4 and/or IL-13-secreting T-helper cells [37]. Indeed, IL-13 production by HI-stimulated PBMC from both atopics and non-atopics has been reported previously by the authors for P6 [38] and IL-4 production against other undefined antigens in HI extracts has also been reported [39]. It is noteworthy in this regard that IL-4 and IL-13 are highly pleiotrophic cytokines with multiple regulatory functions beyond IgE regulation, including an important role in modulation of macrophage activity. On the one hand, pre-treatment of macrophages with IL-4 can induce "alternative activation" leading to potentiation of cytokine responsiveness to subsequent microbial exposure [40]. Conversely, as demonstrated with human cells from a variety of sources including synovium, blood, peritoneum and CNS [41-44], IL-4/IL-13 can also play an *anti-inflammatory* role via attenuation of bacterial-induced production of pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 by macrophages. P6-specific and PspC-specific Th2-memory cells of the type required for stimulation of IgE production could thus serve as an inducible source of such "homeostatic" IL-4/IL-13 in response to transmucosal leakage of these antigens and their uptake by local antigen presenting cells [45], thus helping to attenuate the proinflammatory effects of ligands released by the organisms. Followup studies are required to elucidate the potential dualistic nature of these poorly understood Th2 responses to mucosal dwelling bacteria.

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Table 1. Relationship between serum titres of SAE-IgE and disease risk: univariate logistic regression

Clinical outcome	Cohort subgroup	N (n)[#]	*SAE-IgE*		
			OR[*]	(95% C.I.)	*P*
Asthma	Whole population	1337 (140)	1.95	(1.39 - 2.76)	<0.001
	Atopics	799 (113)	1.47	(1.01 - 2.16)	0.048
	Non-atopics	537 (27)	1.83	(0.61 - 5.52)	0.286
Rhino-conjunctivitis	Whole population	1336 (527)	1.93	(1.50 - 2.48)	<0.001
	Atopics	798 (413)	1.21	(0.91 - 1.62)	0.188
	Non-atopics	537 (114)	1.79	(0.93 - 3.44)	0.080
BHR	Whole population	1286 (236)	2.06	(1.54 - 2.76)	<0.001
	Atopics	767 (182)	1.53	(1.11 - 2.12)	0.010
	Non-atopics	518 (54)	2.41	(1.04 - 5.58)	0.041

#N=total cases in analysis, n=cases positive for clinical outcome
* odds ratio per $\log_{10}$(kU/L)

Table 2. Logistic regression modelling of risk for asthma in atopics.

Log of Variables	Univariate regressions			Multivariate regression		
(except*)	p	OR	95% CI	p	OR	95% CI
SAE IgE	0.048	1.47	(1.01 – 2.16)	ns		
HDM-IgE	<0.001	1.71	(1.44 – 2.03)	0.002	1.40	(1.14 – 1.72)
Cat-IgE	<0.001	1.53	(1.25 – 1.87)	ns		
Mould-IgE	0.040	1.21	(1.01 – 1.45)	ns		
Total IgE	0.001	1.87	(1.30 – 2.70)	ns		
Atopic family history*	<0.001	4.47	(2.13 – 9.38)	0.002	3.71	(1.61 – 8.55)
Eczema*	0.059	1.70	(0.98 – 2.94)	ns		
Rhinoconjunctivitis*	<0.001	2.88	(1.85 – 4.49)	0.011	1.98	(1.17 – 3.36)
FEV_1/FVC*	0.004	0.96	(0.94 – 0.99)	ns		
BHR dose slope*	<0.001	4.46	(2.91 – 6.83)	<0.001	3.21	(1.98 – 5.20)
Eosinophils	<0.001	4.58	(2.24 – 9.37)	ns		
Neutrophils	0.045	0.36	(0.13 – 0.98)	ns		
Eosinophil Protein X	<0.001	6.37	(2.84 – 14.28)	0.002	4.65	(1.78 – 12.11)
SEB induced IL-5	0.033	1.98	(1.06 – 3.71)	ns		
SEB induced IL-10	0.011	2.56	(1.24 – 5.28)	0.006	3.43	(1.42 – 8.30)
SEB induced IL-13	0.011	3.08	(1.29 – 7.34)	ns		

Univariate regression analysis utilised measures of total and specific-IgE (HDM, rye, cat, couch grass, and mould), gender, BMI, presence of eczema and/or rhinoconjunctivitis, lung function (FEV_1/FVC ratio, and BHR dose slope), blood eosinophil and neutrophil numbers, urinary eosinophil protein X, PGF_2a, soluble CD14, and responses of PBMC to SEB which elicited moderate – high level cytokine production (IL-5, IL-10, IL-13, IFNγ) in all members of the cohort.). Only significant variables are shown and included in multivariate regression; the sample comprised 94 asthmatics and 547 non-asthmatics. "ns" indicates not significant.

Table 3. Multiple linear regression for BHR dose slope.

	Atopics				Non-atopics			
Variables	***P***	**B**	**S.E.***	**t**	***P***	**B**	**S.E.***	**t**
SAE-IgE	ns				0.005	0.149	0.052	2.8
HDM-IgE	<0.001	0.096	0.013	7.5	-			
Mould-IgE	0.033	0.034	0.016	2.1	-			
FEV_1/FVC	<0.001	-0.015	0.002	-6.3	<0.001	-0.010	0.002	-4.5
Eosinophils	ns				0.047	0.075	0.038	2.0
Eosinophil Protein X	0.007	0.185	0.068	2.7	-			
Sex	<0.001	-0.235	0.036	-6.6	<0.001	-0.141	0.030	-4.7
Atopic family history	0.040	0.080	0.039	2.1	-			
SEB IL-5	0.002	0.102	0.033	3.1	-			

Variables included in regression were significant in univariate analysis.
*S.E.: standard error
"-" not in regression
"ns" not significant

Table 4. P6-IgE and PspC-IgE titres and disease risk : univariate logistic regression.

Clinical outcome	Cohort subgroup	N (n)	*P6-IgE*			*PspC-IgE*		
			OR	**(95% C.I.)**	***P***	**OR**	**(95% C.I.)**	***P***
Asthma	Whole population	1337 (140)	0.82	(0.66 – 1.02)	0.080	0.76	(0.62 - 0.92)	0.006
	Atopics	799 (113)	0.75	(0.59 – 0.97)	0.027	0.73	(0.58 - 0.91)	0.006
	Non-atopics	537 (27)	1.14	(0.70 - 1.84)	0.606	0.86	(0.56 - 1.33)	0.498
Rhino-conjunctivitis	Whole population	1336 (527)	1.06	(0.93 - 1.21)	0.397	1.09	(0.96 - 1.23)	0.179
	Atopics	798 (413)	1.13	(0.95 - 1.33)	0.161	1.10	(0.94 - 1.29)	0.224
	Non-atopics	537 (114)	0.92	(0.71 - 1.20)	0.554	1.10	(0.87 - 1.38)	0.440
BHR	Whole population	1286 (236)	1.09	(0.92 - 1.30)	0.308	1.11	(0.95 - 1.30)	0.193
	Atopics	767 (182)	1.11	(0.91 - 1.35)	0.312	1.17	(0.97 - 1.42)	0.092
	Non-atopics	518 (54)	1.03	(0.73 - 1.47)	0.866	0.95	(0.69 - 1.30)	0.745

#N=total cases in analysis, n=cases positive for clinical outcome

Table 5. Identification of asthma risk factors in atopics by multivariate logistic regression.

Variables	*P6-IgE*			*PspC-IgE*		
	OR	**(95% C.I.)**	***P***	**OR**	**(95% C.I.)**	***P***
P6-IgE	0.72	(0.53 - 0.97)	0.032		-	
PspC-IgE		-		0.75	(0.57 - 0.98)	0.034
HDM-IgE	1.35	(1.09 - 1.66)	0.006	1.39	(1.13 - 1.71)	0.002
Rhinoconjunctivitis	2.00	(1.18 - 3.37)	0.010	1.98	(1.17 - 3.34)	0.011
BHR Dose Slope	3.15	(1.93 - 5.15)	<0.001	3.52	(2.16 - 5.72)	<0.001
Eosinophil Protein X		ns		3.92	(1.51 - 10.16)	0.005
Atopic family history	3.99	(1.73 - 9.19)	0.001	3.67	(1.60 - 8.45)	0.002
Eosinophils	2.72	(1.01 - 7.34)	0.048		ns	

Details of variables included in regressions are in Table 2 except SEB-induced cytokine responses. The sample comprised 95 asthmatics and 549 non-asthmatics. "-" indicates not in regression, and "ns" not significant.

Figure Legends

Figure 1 SAE-IgE titres within subgroups of the cohort. Comparison between groups were assessed by Mann Whitney U test,*: P≤0.05, ***: P≤0.005. **A,** Data shown are geometric means (95% CI) of SAE-IgE titres (kU/L) stratified by atopy. **B,** Data shown are geometric means (95% CI) of SAE-IgE titres stratified by clinical outcomes (asthma, rhinoconjunctivitis, and bronchial hyperresponsiveness) in the whole population, atopic and non-atopic subgroups. **C,** Subjects with detectable SAE-IgE titres were split into terciles according to SAE-IgE; T_1: 0.15-0.23, T_2: 0.24-0.50, T_3: 0.51-30.9. Risk of asthma was computed from univariate logistic regression, and presented as mean risk of asthma amongst subjects in each tercile relative to subjects with undetectable SAE-IgE titres.

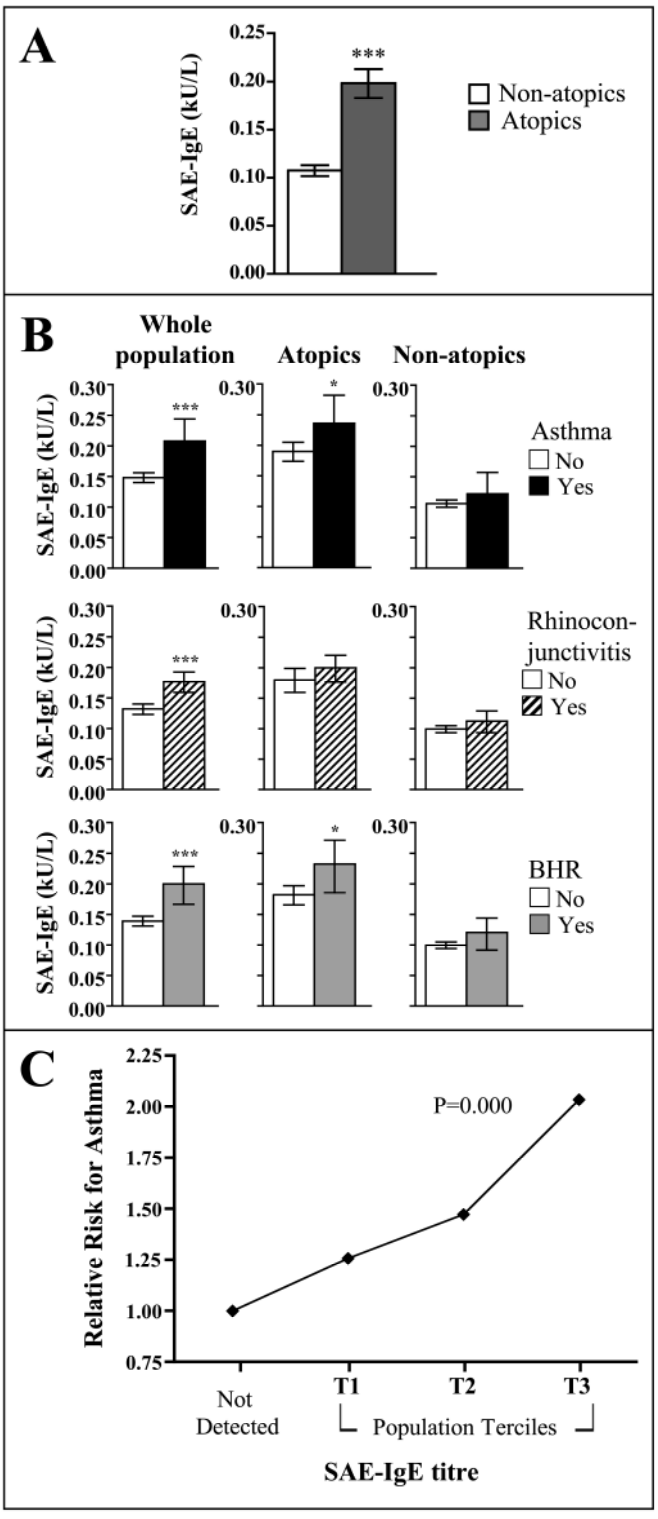

Figure 2 P6-IgE and PspC-IgE titres within subgroups of the cohort. **A,** Geometric means (95% CI) of specific IgE titres (kU/L) are shown for the atopic and non-atopic subgroups.

Comparison by Mann Whitney U test found no significant difference between titres for atopic and non-atopic subgroups. **B,** Subjects were split into terciles on the basis of detectable PspC-IgE, T_1: 0.04-0.65, T_2: 0.66-1.78, T_3: 1.79-12.67, and P6-IgE titre, T_1: 0.04-0.29, T_2: 0.30-0.99, T_3: 1.00-15.51. Univariate logistic regression models employing PspC-IgE and P6-IgE titres were used to calculate risk of asthma, which are presented as mean risks amongst subjects in each tercile relative to subjects with undetectable IgE-titres.