

Association between polymorphisms in serine protease inhibitor, kazal type 5 and asthma phenotypes in a large German population sample

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Summary

Background Atopic diseases are characterized by immunoglobulin E (IgE)-mediated immune responses towards common allergens, many of which are proteases. Recently it has been suggested that a proteinase inhibitor gene, SPINK5, which is located on chromosome 5q31, may play a role in the pathogenesis of atopic diseases.

Objective We investigated the association between the polymorphism G1258A leading to a putative amino acid change (Glu420Lys) in serine protease inhibitor, kazal type 5 (SPINK5) and phenotypes of atopic diseases in a large general population sample of German children.

Methods Parental questionnaires were used and children underwent skin prick testing, pulmonary function testing and bronchial challenge. Blood was collected for serum IgE measurements and DNA extraction. In total, 1161 children were genotyped for the SPINK5 Glu420Lys polymorphism and association studies were performed.

Results A significant association between SPINK5 420Lys and the development of asthma was observed (OR 1.77; 95%CI: 1.02–3.06, $P = 0.041$ for 420Lys homozygotes). Atopic carriers of SPINK5 420Lys showed an increased risk for asthma and asthma symptoms (OR 2.06; 95%CI: 1.01–4.20, $P = 0.047$). When children with a combination of asthma and atopic dermatitis were compared with normal controls, the SPINK5 420Lys genotype was more prevalent in the disease group (OR 4.56; 95%CI: 1.370–15.12, $P = 0.007$). No association between SPINK5 420Lys genotypes and total serum IgE levels, skin prick test (SPT) reactivity or atopic dermatitis was observed.

Conclusion These results suggest that SPINK5 Glu420Lys polymorphism may be associated with certain asthma phenotypes characterized by the concomitant expression of asthma and atopic dermatitis or SPT reactivity.

Keywords asthma, atopic dermatitis, atopy, childhood, IgE, polymorphism, SPINK5

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Introduction

In recent years, the Th1–Th2 paradigm served as the main working hypothesis to disentangle the mechanisms by which an imbalance of the immune system may influence the development of atopic diseases such as asthma, atopic dermatitis and allergic rhinitis [1]. However, the Th1–Th2 paradigm seems not to sufficiently explain the onset of atopic diseases and the organ specificity of the disease. Rather, it may be changes in the barrier function of different surface organs such as skin, nasal and bronchial epithelial layers that confer the first step in the development of atopic diseases. A complex system of innate and adaptive immune defence mechanisms has been identified at the interface between the environment and the communicating body tissues. Changes in this delicate balance of interaction and defence by

alterations in the genetically conferred configuration of barrier proteins may have profound consequences. Thereby, a patho-mechanistic cascade may be initiated that finally leads to an imbalance of the Th1 and Th2 cytokine expression and the development of atopic diseases. Genes involved in barrier function mechanisms have been investigated for genetic variations, and their potential association with the development of atopic diseases has been reported recently [2].

Atopic diseases such as asthma, atopic dermatitis and allergic rhinitis are characterized by immunoglobulin E (IgE)-mediated immune responses towards common, ubiquitous allergens. A number of these allergens possess serine proteinase activity. Furthermore, mast cells contain proteases, which serve as mediators for inflammation. Thus, proteinase inhibitor molecules may play a role in modulating allergic reactions, and mutations in such genes may influence the susceptibility to develop atopic diseases.

Serine protease inhibitor, kazal type 5 (SPINK5) (alternatively named LEKTI (lymphoepithelial kazal-type-related inhibitor), a protease inhibitor protein, is encoded by the gene

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SPINK5, which is located on chromosome 5q31–32 [3]. SPINK5 consists of 33 exons coding for a 1064-amino acid precursor protein. Several mutations in SPINK5 gene such as frameshift mutations and splicing alterations have been linked to Netherton syndrome, a severe and rare autosomal recessive disorder characterized by defective cornification of the skin and immunological alterations resembling atopic dermatitis. In addition, allergic rhinitis, bronchial, elevated serum IgE levels and eosinophilia may be associated with this syndrome [4]. Within SPINK5, 32 single nucleotide polymorphisms (SNPs) have been described, six of which result in putative amino acid changes [2]. Polymorphisms reaching a minor allele frequency of greater than 10% (A1103G, G1156A and G1258A) were genotyped in two British population samples. An association between the SNP G1258A (Glu420Lys) and atopy, atopic dermatitis, elevated serum IgE levels and asthma was reported [2].

We present the results of an association study performed in a large German cross-sectional population sample with polymorphism Glu420Lys in the newly described asthma candidate gene SPINK5. This study was aimed at clarifying whether the previously reported association could be confirmed in other populations.

Materials and methods

Subjects

Between 1995 and 1996, a cross-sectional study was conducted in Munich, West Germany, to assess the prevalence of asthma and allergies in school children aged 9–11 years. Parental questionnaires for self-completion were sent through the schools to the families of 3830 children (response rate 87.6%). Children underwent skin prick testing, pulmonary function testing and bronchial challenge with hyperosmolar (4.5%) saline. Blood was collected for serum IgE measurements and DNA extraction. Only children of German origin were included in this study ($N = 2612$). The study methods have been described in detail elsewhere [5]. Therefore, only methods pertaining to this analysis are given below. Informed written consent was obtained from all parents and all study methods were approved by the ethics committee of the Bavarian medical council.

Questionnaire

Self-administered questionnaires included the International Study of Asthma and Allergies in Childhood (ISAAC) core questions on symptoms of asthma, allergic rhinitis and atopic eczema [5]. Children whose parents reported a doctor's diagnosis of either asthma, recurrent spastic or recurrent asthmatic bronchitis were classified as having asthma. The definition of 'wheeze ever' was based on the ISAAC question: 'Has your child ever had wheezing chest sounds?' Current wheeze was defined as wheezing in the last 12 months. The definition of atopic dermatitis and hayfever was based on a parental report of a doctor's diagnosis of atopic eczema and hayfever, respectively. High socio-economic status was assumed if either parent had at least 12 years of school education.

Skin prick test

The sensitivity to six common aeroallergens (*dermatophagoides pteronyssinus*, *dermatophagoides farinae*, *alternaria tenuis*, cat dander, and mixed grass and tree pollen) was assessed using highly standardized extracts and lancets (ALK, Horsholm, Denmark) according to ISAAC phase II protocols [5]. A child was considered sensitized if a weal reaction of more than 3 mm occurred to at least one specific allergen after subtraction of the negative control. Performance and reproducibility of this method were proven to be adequate and evaluation results were reported elsewhere [6].

Lung function testing and bronchial challenge

In 9–11-year-old children, lung function was measured by MasterScope Version 4.1 (Jäger, Würzburg, Germany). A minimum of two baseline spiograms was recorded and the highest of two reproducible (within 5%) measurements of forced expiratory volume in 1 s (FEV₁) was recorded as baseline FEV₁. Bronchial reactivity was assessed in a random sample of half the study population by changes in FEV₁ after inhalation of nebulized, hyperosmolar (4.5%) saline for increasing periods of time using ultrasound nebulizers (DeVilbiss Sunrise Medical, Langen, Germany). Bronchial challenge was stopped either if the FEV₁ had fallen at least 15% from baseline or if the total inhalation period of 15.5 min had been completed. All children were asked to withhold all asthma medications for at least 12 h before lung function testing. Children with a drop in FEV₁ of 15% or more from baseline were classified as having bronchial hyperresponsiveness.

Total and specific serum IgE measurements

Total serum IgE levels were measured using the Insulite system (DPC Biermann, Bad Nauheim, Germany). For statistic analysis, total serum IgE was transformed logarithmically and summarized descriptively with geometric means and respective 95% confidence intervals.

Molecular genetic methods

Genomic DNA was extracted from whole blood by a standard salting-out method [7]. All PCR reactions were carried out in a volume of 15 µL containing 20 ng of genomic DNA, using standard thermocyclers (Eppendorf, Cologne, Germany). Primers used to amplify the 304 bp region of interest were 5'-TGC AAT TGT GAG GAT TTC ACA G-3' and 5'-CCT GAA CAT GAT CTG TGG ATC-3'. A restriction endonuclease-based assay including an internal control cut with *HphI* (www.neb.com) was used to genotype the study population for polymorphism G1258A in the gene SPINK5 [2]. Enzyme-digested PCR products were run on agarose gels and visualized with ethidium bromide staining and ultraviolet illumination. A random sample of 5% was genotyped twice for quality control.

Statistical analysis

Hardy-Weinberg equations were calculated while *t*-tests were used to compare quantitative traits between groups. χ^2

statistics as well as Cochran–Armitage trend tests were used to compare qualitative traits between groups. All statistical analyses were carried out using the SAS software package (version 8.2).

Results

All available blood samples ($n = 1161$) from the Munich study population ($N = 2612$) were genotyped for the G1258A (Glu420Lys) polymorphism in exon 14 of the SPINK5 gene previously reported to be associated with asthma and atopic dermatitis [2]. With respect to descriptive and socio-economic factors, and the prevalence of atopic diseases, no selection bias could be detected between children genotyped for this study and those who were not (Table 1). In our unselected, cross-sectional study population, 26.2% of participants were GG homozygotes, 52.3% were heterozygous and 21.5% were AA homozygotes. Of these 1161 samples, only 13 samples could not be genotyped successfully. The frequency of the less common A allele at position 1258 was 0.48. This allele distribution is consistent with allele frequency previously reported in a British population [2]. Furthermore, the

distribution of genotypes in the population was in accordance with the expected Hardy–Weinberg equilibrium.

We assessed the relation between SPINK5 420Lys and atopic phenotypes in our study population of German school children as presented in Fig. 1. In the total study population, a significant association between homozygotes for SPINK5 420Lys and asthma was observed (OR 1.77; 95%CI: 1.02–3.06, $P = 0.041$). A borderline significant trend for an association with BHR was found (OR 1.75; 95%CI: 0.92–3.32, $P = 0.08$). In general, the presence of the A allele was associated with a strong increase in the prevalence of asthma, suggesting a dominant effect of the A allele. In atopic carriers of SPINK5 420Lys, an increased prevalence of asthma (OR 2.06; 95%CI: 1.01–4.20, $P = 0.047$) and current wheeze symptoms (OR 2.39; 95%CI: 1.07–5.36, $P = 0.034$) was identified (Fig. 2).

When children with a combination of asthma and atopic dermatitis were compared with normal controls ($n = 37$ vs. 415), the SPINK5 420Lys genotype was significantly more prevalent in the disease group (OR 4.56; 95%CI: 1.37–15.12, $P = 0.007$).

No statistically significant associations between SPINK5 Glu420Lys genotypes and total serum IgE levels

Table 1. Comparison between study populations: children included and not included in the study and those used in the sub-analysis of BHR

	German population with questionnaire ($n = 2612$)	Questionnaire and SPINK5 genotyped ($n = 1148$)	Questionnaire, SPINK5 genotyped, IgE, and BHR DNA ($n = 514$)
Male gender	49.3%	48.8%	49.0%
Mean age (years $\pm$ SD)	9.5 ($\pm$ 0.6)	9.5 ($\pm$ 0.6)	9.5 ($\pm$ 0.6)
Socio-economic status (high)	54.0 (52.1–55.9)	53.8 (51.0–56.8)	51.1 (46.8–55.5)
Asthma	10.3 (9.2–11.5)	11.4 (9.7–13.4)	11.2 (8.8–14.3)
Current wheeze	8.6 (7.6–9.8)	8.8 (7.3–10.6)	7.3 (5.3–9.9)
Atopic dermatitis	17.5 (16.1–19.1)	19.1 (16.9–21.5)	17.2 (14.2–20.9)
Current symptoms of atopic dermatitis	11.2 (10.0–12.5)	11.7 (10.0–13.7)	11.3 (8.8–14.4)
Hayfever	9.3 (8.2–10.5)	8.4 (6.9–10.2)	7.0 (5.1–9.6)
Current symptoms of hayfever	15.0 (13.7–16.4)	16.3 (14.3–18.6)	14.7 (11.9–18.0)

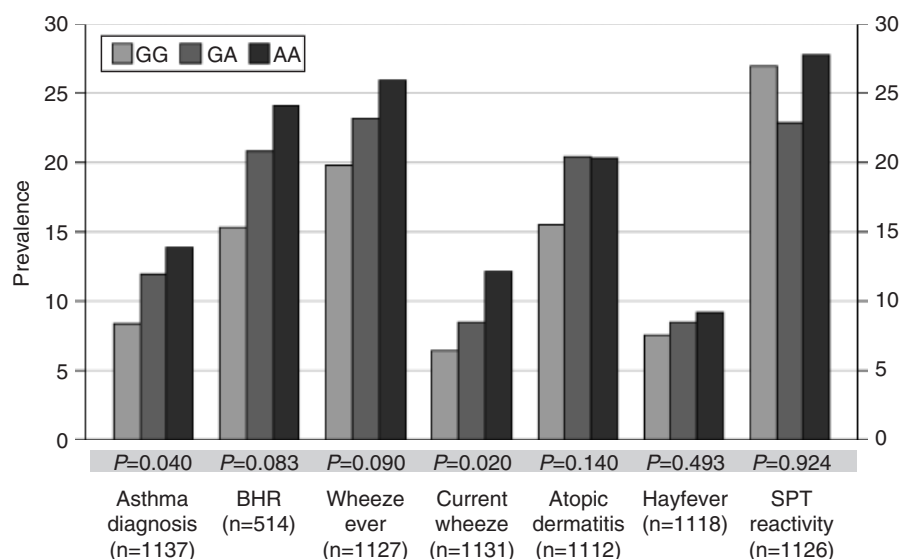


Fig. 1. Prevalence of asthma, bronchial hyper-responsiveness, atopic dermatitis, hayfever and skin prick test (SPT) reactivity by SPINK5 genotypes. * P for trend calculated by Cochran–Armitage trend tests.

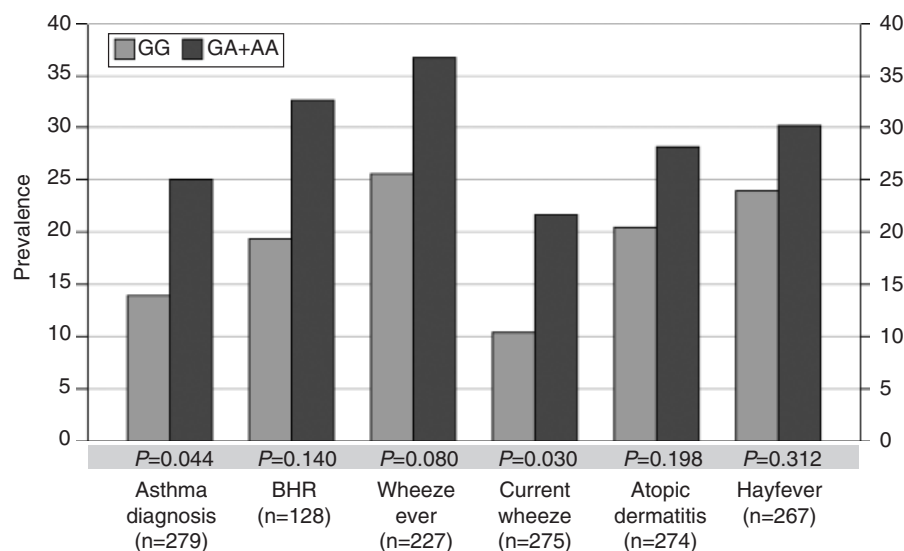


Fig. 2. Prevalence of asthma, bronchial hyper-responsiveness and different asthma symptoms by SPINK5 genotypes in atopic (SPT-positive) subjects. **P* calculated by χ^2 statistics to compare groups and phenotypes.

(GG = 62.0 IU/mL, AG = 64.1 IU/mL, AA = 67.5 IU/mL, $P = 0.519$), SPT reactivity or atopic dermatitis alone were observed (data not shown).

Discussion

This study presents evidence for an association between the SPINK5 polymorphism 420Lys and asthma. Especially in atopic children, an association between SPINK5 420Lys and an increased prevalence of ever and current asthma symptoms was identified. In children with a combination of asthma and atopic dermatitis, the SPINK5 420Lys genotype was significantly more prevalent. However, no significant association between SPINK5 Glu420Lys genotypes and total serum IgE levels, SPT reactivity or atopic dermatitis was observed. These results seem to confirm the previously reported association between the SPINK5 polymorphism Glu420Lys and asthma as well as with a concomitant occurrence of asthma and atopic dermatitis.

In the British population, a parent-of-origin effect was reported. There, SPINK5 420Lys from maternal origin showed a stronger association with atopic phenotypes than paternal alleles. As our study population consisted of unrelated individuals with no family data or parental DNA available, this analysis could not be reproduced in our population.

In our general population sample, the association of SPINK5 420Lys with asthma diagnosed by a physician and current wheeze was significant. Furthermore, a trend for an association between SPINK5 420Lys (A allele) and asthma symptoms as well as bronchial hyper-responsiveness was observed. As testing for bronchial hyper-responsiveness was only performed in a 50% random sample of the study population, the available sample size in this subgroup analysis was probably too small to reach statistical significance. Overall, homozygosity for the SPINK5 420Lys polymorphism was associated with a higher prevalence of all atopic phenotypes analyzed (Fig. 1).

When the study population was stratified by SPT reactivity, the association between SPINK5 420Lys and an increased

prevalence of atopic diseases reached statistical significance only in the atopic group (Fig. 2). This could indicate that the effect of the SPINK5 420Lys polymorphism is modified by atopy. As there seems to be no significant effect of the SPINK5 polymorphism on the occurrence of atopy *per se* as measured by SPT reactivity or total serum IgE levels, the mechanisms by which SPINK5 influences the expression of atopic diseases may be independent of classic Th1/Th2 pathways. A Th2-dominated immune system leading to the overexpression of total and specific IgE may represent a prerequisite for the development of atopic disease but, as multiple studies have shown, there seems to be no direct translation from an atopic status into the occurrence of atopic diseases such as asthma [8–10]. Even though asthma is very often associated with atopy, the amount of association between atopy and the presence of atopic disease such as asthma and atopic dermatitis may vary strongly in different populations [9, 11]. Thus, it could be hypothesized that apart from the atopy deviation of the immune system further, organ-specific factors have to be present to facilitate the development of the disease in different organs interacting with the environment (i.e. skin, nose and lung). These organ-specific factors could be changes in the epithelial membrane which decrease the ability of these organs to keep the barrier between the environment and the organism intact.

From the results presented in this and previous studies, it could be speculated that SPINK5 may just be such a factor that could be involved in the barrier function of at least two organs: the lung and the skin. The British study in which an association between SPINK5 polymorphisms and atopic phenotypes was initially reported was based on families recruited from a dermatology clinic through children affected by atopic dermatitis [2]. Thus, almost all asthmatics in this population sample were also affected by atopic dermatitis. To replicate these original study conditions, we selected children from our population that were concurrently affected by asthma and atopic dermatitis ($n = 37$). These children were compared with non-atopic children free of asthma and not diagnosed with atopic dermatitis ($n = 415$). In this subpopulation, the association with the SPINK5 420Lys polymorphisms was highly significant ($P = 0.007$). However, it has to be

taken into account that the number of subjects available for our subgroup analysis was rather low and thus has to be interpreted cautiously. Nevertheless, the same effect was found in differently recruited populations from Great Britain and Germany. Therefore, it seems quite unlikely that the positive association observed in both populations may have occurred by chance or due to stratification.

We have decided to study the polymorphism 420Lys because, out of 32 SNPs in the SPINK5 gene, it was identified by Walley et al. as the most promising [2]. In contrast to the second polymorphism tested in detail (368Ser), 420Lys was consistently associated with asthma in two independent British populations. Even though functional data on the effect of the 420Lys polymorphism are still missing, these data suggest that it is the most likely cause for the consistent association between SPINK5 and asthma. However, as a functional proof is missing, this association has to be interpreted cautiously. The possibility that other, not yet identified polymorphisms in linkage with 420Lys may be responsible for these findings cannot be excluded. Thus, the linkage disequilibrium between the SPINK5 420Lys polymorphism and other polymorphisms in the nearby candidate genes for asthma and atopy located on chromosome 5q31 (IL4, IL13 and CD14) was assessed but did not influence the associations presented here (data not shown). Therefore, the associations reported here seem to be most likely due to the effects of the SPINK5 420Lys polymorphism.

The relation between atopic dermatitis and asthma has been a matter of investigation in a number of studies [12–14]. It has previously been shown that atopic dermatitis is a strong predictor for the later development of asthma. However, only a certain percentage of children affected by atopic dermatitis develop asthma symptoms later in life and not all children with asthma are concurrently affected by atopic skin lesions. When these observations are interpreted in the context of the recent findings on SPINK5, it could be speculated that the SPINK5 420Lys polymorphism may help to identify those children with atopic dermatitis that are at a higher risk to develop asthma later on.

At this point, the biological role of the SPINK5 420Lys polymorphism on the function of the protein is not well known as functional data do not exist yet. However, from structural analysis of the protein it may be speculated that the SPINK5 420Lys polymorphism has some functional effects potentially affecting the epithelia's ability to perform its barrier function. Proteolysis of full-length SPINK5 by extraneous proteinases seems to release the KT domains, triggering a strong inhibitory response. It has been suggested previously that proteinase-activated receptors in the epithelium could serve as targets for mast cell proteinases and that SPINK5 may be important in balancing the inflammatory effects of mast cell lysis by inhibiting the proteinase-triggered activation [2, 15]. In this context, it may be relevant that the SPINK5 420Lys polymorphism introduces a basic residue into the linker sequence between SPINK5 domains 6 and 7, which can serve as an additional cutting point for proteinases, thus influencing the inhibitory effect of the protein. Apart from its role in epithelial barrier function, a number of possible mechanisms for SPINK5 to influence allergic disease have been proposed [2]. Proteinase-activated receptors can serve as targets for mast cell proteinases. In addition, the

expression of SPINK5 within the thymus may indicate a role for the gene in T and B cell maturation or in antigen handling within other thymic cells [16]. Furthermore, many allergens are also serine proteinases, and this proteinase activity may influence their allergenicity in the presence of variable proteinase inhibition. Functional analyses are needed to further assess the role of SPINK5 and the polymorphisms in this gene in epithelial barrier functions and the development of allergic diseases.

In conclusion, our data confirm the previously reported association between SPINK5 420Lys and asthma in a large, independent population. The strong association with asthma in children with concurrent atopic dermatitis or a positive SPT may indicate that SPINK5 plays an important role in certain subtypes of childhood asthma modifying the organ-specific occurrence of atopic diseases.

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References

- 1 Romagnani S. Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology. *Int Arch Allergy Immunol* 1992; 98:279–85.
- 2 Walley AJ, Chavanas S, Moffatt MF et al. Gene polymorphism in Netherton and common atopic disease. *Nat Genet* 2001; 29:175–8.
- 3 Magert HJ, Kreutzmann P, Standker L, Walden M, Drogemuller K, Forssmann WG. LEKTI: a multidomain serine proteinase inhibitor with pathophysiological relevance. *Int J Biochem Cell Biol* 2002; 34:573–6.
- 4 Chavanas S, Bodemer C, Rochat A et al. Mutations in SPINK5, encoding a serine protease inhibitor, cause Netherton syndrome. *Nat Genet* 2000; 25:141–2.
- 5 Weiland SK, von Mutius E, Hirsch T et al. Prevalence of respiratory and atopic disorders among children in the East and West of Germany five years after unification. *Eur Respir J* 1999; 862–70.
- 6 Nelson HS, Hirsch SR, Ohman JL Jr, Platts Mills TA, Reed CE, Solomon WR. Recommendations for the use of residential air-cleaning devices in the treatment of allergic respiratory diseases. *J Allergy Clin Immunol* 1988; 82:661–9.
- 7 Miller SA, Dykes DD, Poletsky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acid Res* 1988; 16:1215.
- 8 Illi S, von Mutius E, Lau S et al. The pattern of atopic sensitization is associated with the development of asthma in childhood. *J Allergy Clin Immunol* 2001; 108:709–14.
- 9 Yemaneberhan H, Bekele Z, Venn A, Lewis S, Parry E, Britton J. Prevalence of wheeze and asthma and relation to atopy in urban and rural Ethiopia. *Lancet* 1997; 350:85–90.
- 10 Britton WJ, Woolcock AJ, Peat JK, Sedgwick CJ, Lloyd DM, Leeder SR. Prevalence of bronchial hyperresponsiveness in children: the relationship between asthma and skin reactivity to allergens in two communities. *Int J Epidemiol* 1986; 15:202–9.
- 11 Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International

- Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. *Lancet* 1998; 351:1225–32.
- 12 Meijer A. Asthma predictors in infantile atopic dermatitis. *J Asthma Res* 1975; 12:181–8.
- 13 Gustafsson D, Sjoberg O, Foucard T. Development of allergies and asthma in infants and young children with atopic dermatitis – a prospective follow-up to 7 years of age. *Allergy* 2000; 55:240–5.
- 14 Anderson HR, Pottier AC, Strachan DP. Asthma from birth to age 23: incidence and relation to prior and concurrent atopic disease. *Thorax* 1992; 47:537–42.
- 15 Schechter NM, Brass LF, Lavker RM, Jensen PJ. Reaction of mast cell proteases tryptase and chymase with protease activated receptors (PARs) on keratinocytes and fibroblasts. *J Cell Physiol* 1998; 176:365–73.
- 16 Magert HJ, Standker L, Kreutzmann P et al. LEKTI, a novel 15-domain type of human serine proteinase inhibitor. *J Biol Chem* 1999; 274:21 499–502.