Positionally cloned genes and age-specific effects in asthma and atopy: an international population-based cohort study (ECRHS)

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ABSTRACT

Background Several genes identified by positional cloning have been associated with asthma and atopy, but few findings have been replicated. Age at onset of asthma has been associated with different phenotypic characteristics, and with variants at chromosome 17q21 identified through genome-wide association. This study examined the associations and age-specific effects on asthma, atopy and bronchial hyper-responsiveness (BHR) of five candidate genes previously identified by positional cloning (*ADAM33, PHF11, NPSR1, DPP10, SPINK5*).

Methods 51 polymorphisms from 2474 participants from 13 countries who took part in the European Community Respiratory Health Survey (1990–2000) were studied. Asthma and age at onset of asthma were assessed by questionnaire data, BHR by methacholine challenge and atopy by specific immunoglobulin E to four common allergens.

Results Significant associations with asthma, atopy and particularly for asthma with atopy were observed for a large region of 47 kb in the *NPSR1* gene, even after Bonferroni correction for multiple comparisons ($p<0.001$). The associations with *NPSR1* were stronger in those reporting a first attack of asthma before the age of 15, with statistically significant interactions with age of onset found for three SNPs. The evidence for *ADAM33* and BHR and for an age-specific effect of two SNPs in *DPP10* and asthma was weaker.

Conclusion This study provides further evidence for an effect of *NPSR1* on asthma, atopy and atopic asthma. In addition, this analysis suggests a role for *NPSR1* in early-onset asthma driven by the strong effect of this gene on atopic asthma.

Asthma is a complex disease which shows phenotypic heterogeneity and is related with atopy, bronchial hyper-responsiveness (BHR) and increased immunoglobulin E (IgE) levels.[1] Linkage analysis followed by positional cloning has been a powerful design to identify the genetic basis of asthma.[2] Eight susceptibility genes to asthma, BHR or atopy have been identified by this technique, although their function remains unclear. ADAM metallopeptidase domain 33 (*ADAM33*) on chromosome 20p was initially identified as an asthma and BHR susceptibility gene.[3] Neuropeptide S receptor 1 (*NPSR1*, also known as *GPRA* or *GPR154*), which is located on chromosome 7p, encodes a G-protein-coupled receptor that is upregulated in epithelial cells in inflamed airways and was initially identified as an asthma and atopy-related gene.[4] PHD finger protein 11 (*PHF11*) on chromosome 13 was reported as a gene associated with atopy and total IgE.[5] Dipeptidyl-peptidase 10 (*DPP10*) located on chromosome 2q may modulate the activity of various proinflammatory and regulatory chemokines and cytokines, and could also be important in neural regulation of smooth muscle tone.[6] Serine protease inhibitor kazal type 5 (*SPINK5*, chromosome 5q) encodes a protease inhibitor protein and was identified as a gene involved in high serum IgE levels and atopic manifestations.[7] Other genes identified by positional cloning are HLA-G histocompatibility antigen class I G (*HLA-G*),[8] cytoplasmatic FMR1-interacting protein 2 (*CYFIP2*),[9] prostanoid DP receptor (*PTGDR*)[10] and interleukin-1 receptor antagonist (*IL1RN*).[11] Studies on these candidate genes have not always replicated the initial findings.[2 12]

Longitudinal studies have shown that age at onset of asthma is associated with differences in phenotypic characteristics.[13] Information on age at onset may help to identify genetic determinants and understand the underlying pathophysiology that contributes to particular phenotypes.[1] Bouzigon *et al*[14] recently reported that the risk of asthma conferred by 17q21 variants is restricted to early-onset asthma. Only one recent study has evaluated whether a similar pattern by age at onset occurs for asthma genes identified by positional cloning.[15]

The aim of this study is to replicate genetic associations with asthma, BHR and atopy and to identify age-specific effects of five candidate genes previously identified by positional cloning (*ADAM33*, *PHF11*, *NPSR1*, *DPP10*, *SPINK5*) using data from a large international population-based cohort (the European Community Respiratory Health Survey, ECRHS).

METHODS

The methodology of the ECRHS has been described elsewhere.[16 17] Briefly, the ECRHS is a random population-based multicentre cohort of subjects aged 20–44 years at time of recruitment (1990, ECRHS I) and then followed up approximately 10 years later (ECRHS II). Subjects completed a long questionnaire on respiratory symptoms and underwent a clinical evaluation including lung function, atopy and bronchial responsiveness. A complementary sample of

subjects with asthma symptoms at recruitment was also included in the study and followed up.

The presence of asthma was based on a positive response to one of two questions: attack of asthma during the 12 months preceding the interview or current use of asthma medication. Most patients with asthma defined by these two questions (93%) also reported a doctor diagnosis of asthma. Age at onset of asthma was assessed by the question "How old were you when you had your first attack of asthma?" The long-term reliability of this question was reported to be high in the ECRHS.[18] Since analyses of other studies have defined asthma as a report of "doctor diagnosed asthma", we also used this definition in an alternative analysis. BHR was defined as a 20% fall in forced expiratory volume in 1 s (FEV_1) from the highest FEV_1 post-diluent value during methacholine challenge with an accumulated dose of 1 mg.[16] Specific IgE levels to four common aeroallergens (cat, timothy grass, *Dermatophagoides pteronyssinus* and *Cladosporium herbarum*) were measured with the Pharmacia CAP system (Pharmacia Diagnostics, Uppsala, Sweden). Atopy was defined as sensitisation (specific IgE levels >0.35 kU/l) to any allergen.

In order to increase the accuracy of the classification of asthma, we considered subjects as cases if they reported any of the three mentioned traits both at baseline (ECRHS I) and at follow-up (ECRHS II). Our comparison group included all subjects who had negative tests for atopy or BHR or who did not report ever having had asthma at both stages of the ECRHS. In addition, subjects recruited in the symptomatic sample (and who did not qualify as cases) were also excluded from the comparison group. Figure 1 shows the selection process of subjects in the ECRHS study and those available for the present analysis.

A description of the selection and genotyping of single nucleotide polymorphisms (SNPs) is given in the online supplement and the SNPs examined are listed in table 1 in the online supplement. SNPs were genotyped using the SNPlex platform (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions.

Statistical analysis

Analyses were adjusted for country, body mass index, smoking status and sex. The analysis of single marker effects was performed assuming an additive genetic model, considering the most frequent allele as the reference category and using logistic regression analysis implemented in the SNPassoc package Version 1.5–1 from R Statistical Software Version 2.6.1 (R Foundation for Statistical Computing, Vienna, Austria, 2007).[19] To correct for multiple comparisons, we applied a Bonferroni correction for the 51 independent loci genotyped (significant $p = 0.001$). Haplotypes were estimated using haplo.em function from haplo.stats package Version 1.3.8.[20] D', r^2 and χ^2 p values for marker independence were estimated to determine linkage disequilibrium between genetic markers. We used a sliding window approach to construct haplotypes in windows up to four adjacent SNPs using the haplo.score.slide command from HaploStats package. The population attributable risk (PAR) for haplotypes was calculated from the odds ratios (ORs) using the formula $PAR = 100 \times (P \times [OR - 1]/[P \times (OR - 1) + 1])$, where P is the prevalence of the haplotype in the population.

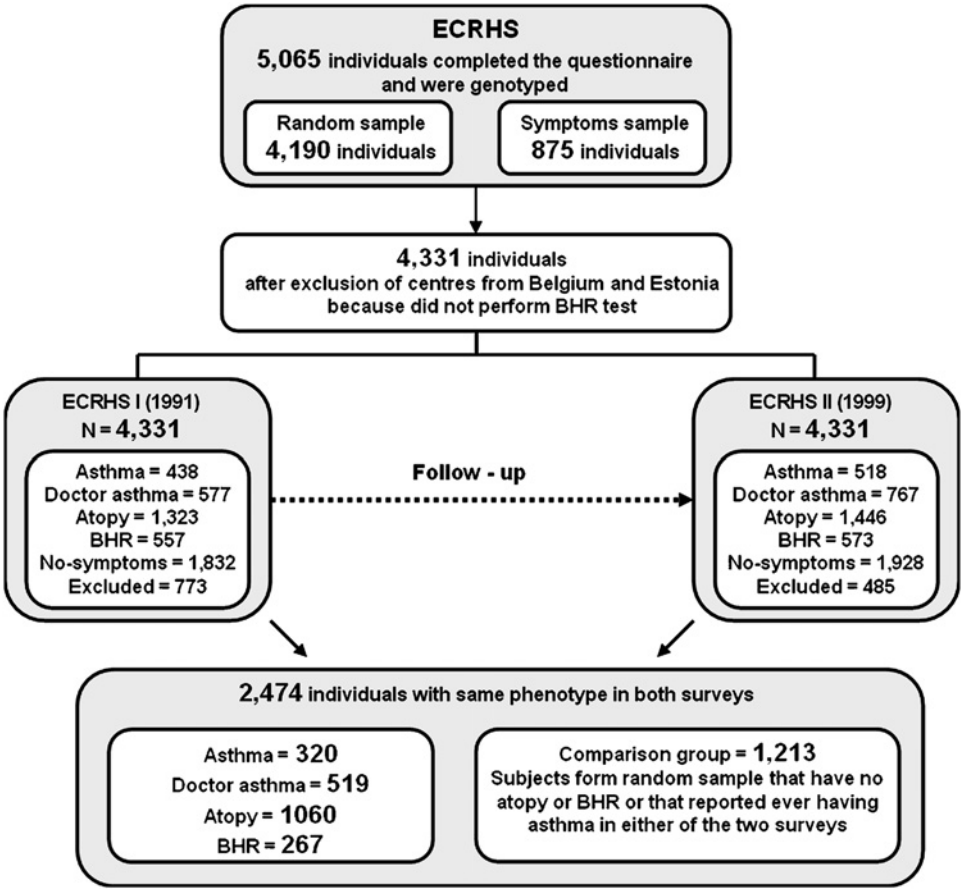

Figure 1 Flow chart showing the selection process and numbers of subjects available for analysis. Definitions for asthma, atopy and bronchial hyper-responsiveness (BHR) are specified in the Methods section. ECRHS, European Community Respiratory Health Survey.

Table 1 Characteristics of population (includes cases and subjects from the comparison group and cases)

Variable	Total population (n = 4331)	Population in this analysis (n = 2474)
Country		
Australia	341 (7.9%)	239 (9.7%)
France	549 (12.7%)	330 (13.3%)
Germany	439 (10.1%)	256 (10.3%)
Norway	436 (10.1%)	254 (10.3%)
Spain	1165 (26.9%)	571 (23.1%)
Sweden	643 (14.8%)	414 (16.7%)
Switzerland	400 (9.2%)	202 (8.2%)
UK	358 (8.3%)	208 (8.4%)
Sex		
Men	2089 (48.2%)	1271 (51.4%)
Women	2242 (51.8%)	1203 (48.6%)
Smoking		
Never	1855 (42.8%)	1124 (45.4%)
Ex	1144 (26.4%)	670 (27.1%)
Current	1348 (31.1%)	692 (28%)
Mean (SD) age (years)	42.8 (7.2)	42.4 (7.1)
Comparison group	1213 (28%)	1213 (49%)
Asthma	320 (7.4%)	320 (12.9%)
Atopy	1060 (24.5%)	1060 (42.8%)
Atopic asthma	195 (4.5%)	195 (7.9%)
Non-atopic asthma	55 (1.3%)	55 (2.2%)
Atopy in non-asthmatics	865 (20%)	865 (35%)
BHR	267 (6.2%)	267 (10.8%)
Other*	1307 (30.2%)	422 (17.1%)

*Subjects who reported any of the outcomes examined or other respiratory symptoms in either of the surveys, but not in both.
BHR, bronchial hyper-responsiveness.

We identified the most appropriate cut-off for age at onset of asthma following a modification of the method described by Bouzigon *et al*.[14] For each specific age from 1 to 40 years, associations between genetic polymorphisms and asthma were assessed by multinomial regression analyses stratifying cases using the specific age at onset of asthma as a cut-off (0 = control; 1 = onset of asthma on or before specific age; 2 = onset of asthma after specific age). By using this model we compared each group of cases with the whole set of non-asthmatic individuals. Tests for linear trend of OR were calculated using the categorised variable quantitatively after assigning a linear score to each ordered category. We then identified the age at onset for which the p value of interaction between the polymorphisms and age at onset was maximum. Interaction was assessed using the likelihood ratio test by comparing the models with and without the interaction term.

Population stratification analysis of the ECRHS was assessed using a set of ancestry-informative markers in addition to oculocutaneous albinism II (*OCA2*) and lactase (*LCT*).[21 22] Genomic control[23] and principal component analysis using EIGENSTRAT[24] (see figure 1 in online supplement) fail to evidence population stratification.

Additional information on material and methods can be found in the online supplement.

RESULTS

The characteristics of the population are shown in table 1. Our analysis was restricted to 4331 subjects of ECRHS II whose DNA was available and who had a genotyping rate over 80% (mean 98.5%) and complete evaluation on asthma, atopy and BHR (figure 1). The prevalence of asthma was 7.4% (n = 320), of atopy 24.5% (n = 1060) and of BHR 6.2% (n = 267). Overlapping between these three phenotypes is shown in figure 2. The comparison set was restricted to the 1213 subjects (28.0%) who fulfilled the a priori criteria. Only one SNP (rs765023) was found to deviate from Hardy-Weinberg equilibrium in the comparison group ($p<0.01$, see table 2 in online supplement).

Significant associations (nominal p value ≤0.05) with asthma, atopy and BHR are summarised in table 2. Nominally significant associations for asthma (six SNPs) or atopy (eight SNPs) were observed only for the *NPSR1* gene. Multiple test correction (Bonferroni $p = 0.001$) yielded three SNPs associated with asthma (rs184448, rs324957, rs324981) and one with atopy (rs324396). For BHR, one significant association was found with a polymorphism of the *ADAM33* gene (rs2787095), although this did not pass Bonferroni correction ($p = 0.04$). Complete information for all the evaluated polymorphisms is shown in table 3 in the online supplement.

To exclude possible overlapping effects of asthma and atopy, we evaluated in a stratified analysis (figure 3) those *NPSR1* SNPs more strongly associated with either atopy or asthma (p value for any of two outcomes <0.01, see table 2). To evaluate these effects we compared ORs for each subgroup. Independent effects were observed for both atopy and asthma but, mainly, a joint effect was seen with the highest ORs in subjects with both atopy and asthma (figure 3).

Multiple marker analysis was performed in those genes that showed significant associations in the single marker analysis. In *NPSR1* we identified three blocks with high linkage disequilibrium (see figure 2 in the online supplement), one of 6 kb, one of 12 kb and a large block of 47 kb which contains the risk polymorphisms identified for asthma and atopy (table 2). Through haplotype sliding windows analysis (table 3), we identified a four marker region in the 47 kb block of the *NPSR1* gene (rs184448, rs324396, rs324957, rs324960) significantly associated with asthma ($p = 0.001$), atopy ($p = 0.001$) and atopic asthma ($p = 0.004$). One of the SNPs previously identified in the single marker analysis for asthma was not in this block (rs324981). However, this polymorphism is in strong linkage disequilibrium with three of these four markers ($D' > 0.95$, $r^2 > 0.60$, χ^2 p value $<2\times10^{-16}$). The PAR for this haplotype was 9.15% for asthma, 6.05% for atopy and 9.75% for the combination of asthma and atopy.

Additional analyses using different outcome definitions supported these findings (see table 5 in online supplement). A less restrictive definition of asthma was defined as subjects

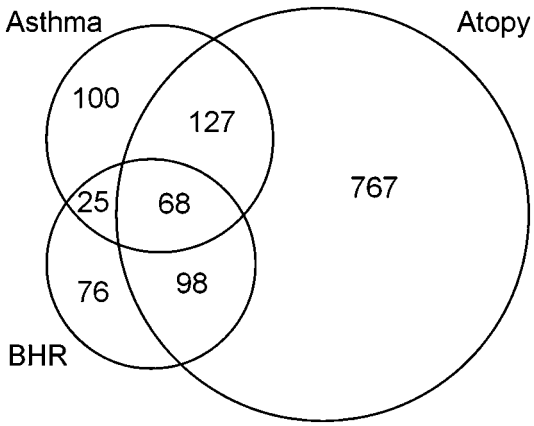

Figure 2 Proportional Venn diagram showing the overlap of asthma, atopy and bronchial hyper-responsiveness (BHR) in this population.

Table 2 Nominal significant associations (p<0.05) between single nucleotide polymorphisms (SNPs) and persistent asthma, persistent atopy and persistent bronchial hyper-responsiveness (BHR) under the additive genetic model

		Asthma		Atopy		BHR	
Gene	SNP	OR (95% CI)	p Value	OR (95% CI)	p Value	OR (95% CI)	p Value
ADAM33	rs2787095	1.06 (0.87 to 1.29)	0.5403	1.12 (0.99 to 1.27)	0.0626	1.23 (1.01 to 1.5)	0.0382
NPSR1	rs714588	1.04 (0.86 to 1.27)	0.6709	0.87 (0.77 to 0.99)	0.0285	0.90 (0.74 to 1.11)	0.3245
NPSR1	rs1345267	0.81 (0.66 to 1.00)	0.0436	0.91 (0.80 to 1.03)	0.1326	0.94 (0.76 to 1.16)	0.5622
NPSR1	rs184448	1.48 (1.20 to 1.82)	0.0002	1.20 (1.06 to 1.36)	0.0049	1.14 (0.93 to 1.4)	0.2111
NPSR1	rs324396	1.21 (0.97 to 1.51)	0.0868	1.25 (1.09 to 1.43)	0.0010	1.16 (0.94 to 1.45)	0.1721
NPSR1	rs324957	1.41 (1.15 to 1.74)	0.001	1.20 (1.05 to 1.36)	0.0051	1.14 (0.93 to 1.40)	0.1999
NPSR1	rs324960	0.74 (0.60 to 0.93)	0.008	0.85 (0.74 to 0.97)	0.0140	0.93 (0.75 to 1.16)	0.5270
NPSR1	rs324981	0.69 (0.56 to 0.85)	0.0004	0.84 (0.74 to 0.95)	0.0064	0.87 (0.71 to 1.06)	0.1591
NPSR1	rs325462	0.76 (0.62 to 0.93)	0.0085	0.88 (0.78 to 0.99)	0.0367	0.86 (0.71 to 1.05)	0.1356

Model adjusted by country, body mass index, age, sex and smoking status.

reporting asthma in either of the two contacts. The same analysis was done for atopy. The results support the effects observed for permanent phenotypes (ie, subjects reporting outcomes in both cohorts), with the most significant effects on asthma and atopy observed for the SNPs located in the second haplotype block. An additional analysis for "physician diagnosed asthma" showed the same pattern of associations as those observed for the main analyses.

We then investigated age-specific effects for these genetic variants. Multinomial logistic regression for each cut-off of age at onset was performed for the six significant SNPs for asthma (see figure 3 in online supplement). A significant interaction was observed for SNPs rs324981 and rs325462 for age at onset between 9 and 14 years. For rs324981 (figure 4a), the maximum p value was observed for a cut-off at age 14 (p for interaction = 0.01), while for rs325462 it was at age 10 (p for interaction = 0.02). The cut-off was set at the maximum age for which interaction was significant (14 years). From the 320 individuals reporting persistent asthma, 141 reported the first attack of asthma before the age of 14 and 146 after the age of 14. Eleven subjects did not report age at onset in both surveys and 24 had different categories between the two surveys and were therefore excluded from the analysis of age at onset. The strength of the association (OR) between these *NPSR1* polymorphisms and asthma was more pronounced in patients with early-onset asthma (≤14 years, table 4). The analysis for the remaining *NPSR1* polymorphisms (see table 6 in online supplement) showed a significant interaction for another *NPSR1* variant, rs324381 (p for interaction = 0.05), with a pattern of association according to age at onset similar to the two above *NPSR1* SNPs (OR 0.66 for early-onset asthma and OR 0.86 for late-onset asthma). Differences in the prevalence of atopy were observed between early-onset and late-onset asthma (75% vs 49%).

Asthma and atopy are strongly correlated in children. The evaluation of age-specific effects for atopic asthma did not show significant interactions for the rs324981 SNP (figure 4b) or the remaining *NPSR1* SNPs (data not shown). The stratified analysis using the cut-off age of 14 years (table 4) shows that the association of these variants with early-onset asthma is similar in atopic asthma to that in general asthma. Overall, the effect for late-onset asthma is increased for atopic asthma, narrowing the differences between early and late onset of asthma.

The evaluation of age at onset of asthma using the cut-off at age 14 years for the other genes (see table 6 in online supplement) shows significant interactions for two polymorphisms in the *DPP10* gene, rs1430090 (OR 0.81 for early-onset asthma vs OR 1.17 for late-onset asthma, p for interaction = 0.04) and rs10496465 (OR 0.85 for early-onset asthma vs OR 1.36 for late-onset asthma, p for interaction = 0.03), although the effect of these polymorphisms was not significant in either early-onset or late-onset asthma.

DISCUSSION

This study confirms the association of *NPSR1* gene with atopy and asthma, and identifies for the first time that the effect of *NPSR1* on asthma could be age-dependent, being stronger for asthma with onset at early ages. Weaker evidence was observed for an age-specific effect of *DPP10* on asthma. In addition, we reported a weak association between *ADAM33* and BHR that did

Table 3 Haplotype analysis of *NPSR1* with sliding windows

Outcome	Window size	Best global p value	Loci	Best individual p value	Best individual haplotype	OR (95% CI)	PAR
Asthma	2	0.0005	10, 11	0.0004	GC (15%)	1.66 (1.25 to 2.21)	9.01
	3	0.002	10, 11, 12	0.002	GCA (15%)	1.57 (1.18 to 2.10)	7.88
	4	0.001	10, 11, 12, 13	0.001	GCAC (15%)	1.67 (1.22 to 2.29)	9.13
Atopy	2	0.004	10, 11	0.001	GT (30%)	1.27 (1.10 to 1.46)	7.49
	3	0.012	10, 11, 12	0.0012	GTA (30%)	1.26 (1.10 to 1.45)	7.23
	4	0.019	10, 11, 12, 13	0.001	GTAC (28%)	1.23 (1.11 to 1.53)	6.05
Atopic asthma	2	0.0007	10, 11	0.001	GC (15%)	1.78 (1.26 to 2.51)	10.47
	3	0.0006	10, 11, 12	0.004	GCA (15%)	1.66 (1.18 to 2.35)	9.01
	4	0.0003	10, 11, 12, 13	0.004	GCAC (15%)	1.72 (1.19 to 2.51)	9.75

Model adjusted by country, body mass index, age, sex and smoking status.
Locus 10: rs184448; locus 11: rs324396; locus 12: rs324957; locus 13: rs324960.
PAR, population attributable risk.

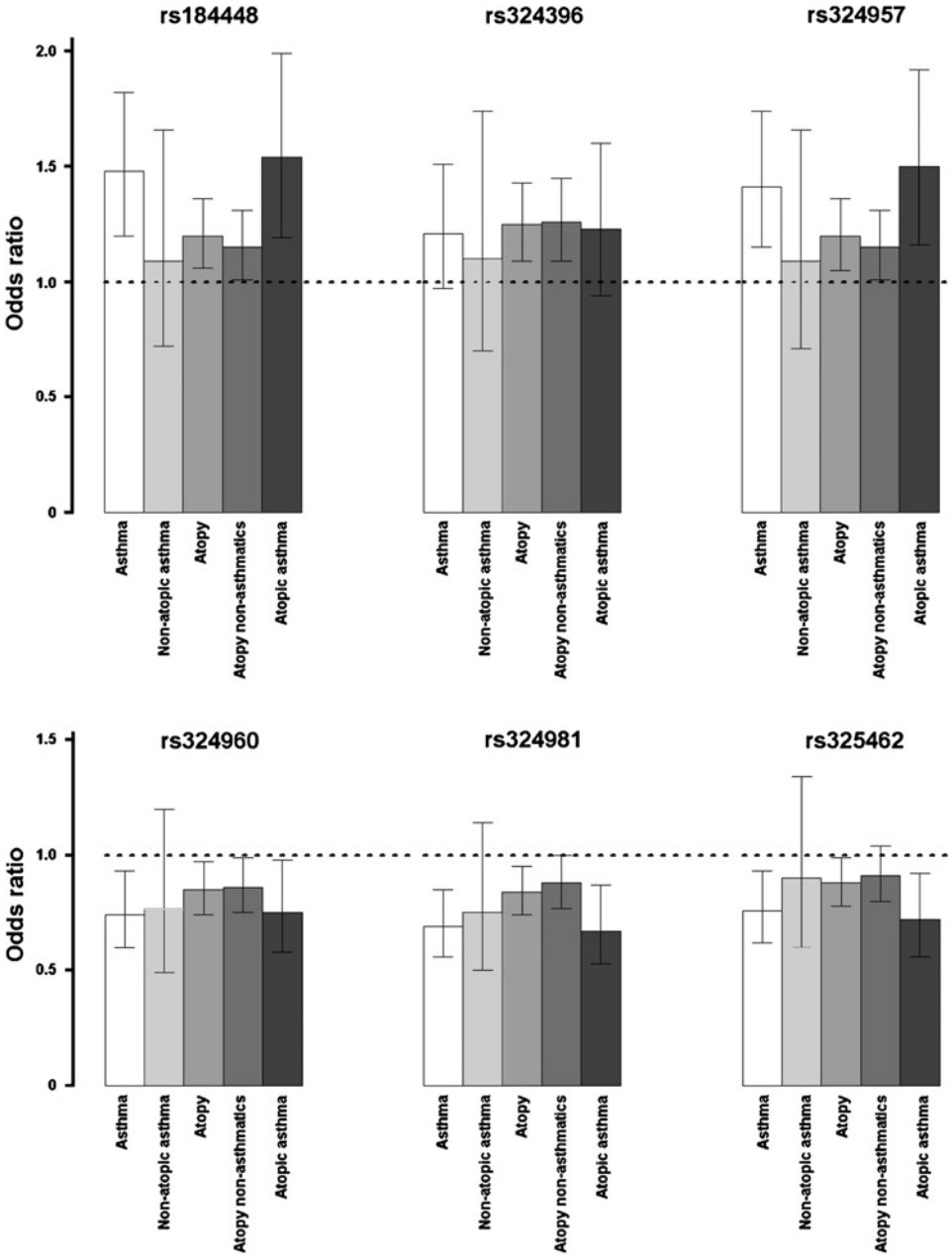

Figure 3 Odds ratios (bars) and 95% confidence intervals (lines) for the stratified analysis of the combinations of atopy and asthma for selected *NPSR1* polymorphisms. The logistic regression model was adjusted by country, body mass index, age, sex and smoking status. Exact values for odds ratios, confidence intervals and p values can be found in table 4 in the online supplement.

not pass Bonferroni correction. No statistically significant associations were found for *PHF11* and *SPINK5*. The failure to replicate can be caused by several factors such as phenotype heterogeneity, small sample size, different populations and population stratification.[25] *PHF11* was initially reported as a gene associated with total IgE and asthma.[5] We failed to replicate the results for asthma and atopy, although we evaluated specific IgE instead of IgE levels. Only one replication study reported a weak association with asthma.[12] *SPINK5* was associated with high serum IgE levels and atopic manifestations,[7] and replication studies have confirmed these findings.[26 27] To date, only two studies have evaluated the association with asthma and reported inconsistent findings.[26 27]

Our results support previous evidence for the role of *NPSR1* in asthma and atopy and, in addition, suggest that the effect is stronger for the combination of asthma and atopy. The *NPSR1* gene was identified by positional cloning as an asthma and atopy (high IgE levels) susceptibility gene.[4] While some subsequent studies have replicated this finding in asthma, atopy (high serum IgE) or allergic symptoms,[12 28–32] other studies did not find a significant effect.[15 33–35] The similar effect on asthma and atopy was observed in the initial study[4] and in most further replication studies.[28–31] The effect on the combination of asthma and atopy (allergic asthma) has also been reported by previous studies.[12 28 30 32]

Polymorphisms of *NPSR1* significantly associated with asthma and atopy in this study are located in the region comprising intron 2, exon 3 and intron 3. This region is within the same 133 kb genomic segment identified by positional cloning as a susceptibility locus for asthma-related phenotypes.[4] Among these polymorphisms, only the rs324981 (located in the exon 3) is a non-synonymous substitution, resulting in an amino acid replacement in the protein (I107N). This change is located in the first exoloop of the putative ligand-binding protein[4] and produces a gain of function, increasing the intrinsic efficacy (the ability of neuropeptide S to activate the receptor) as well as intracellular

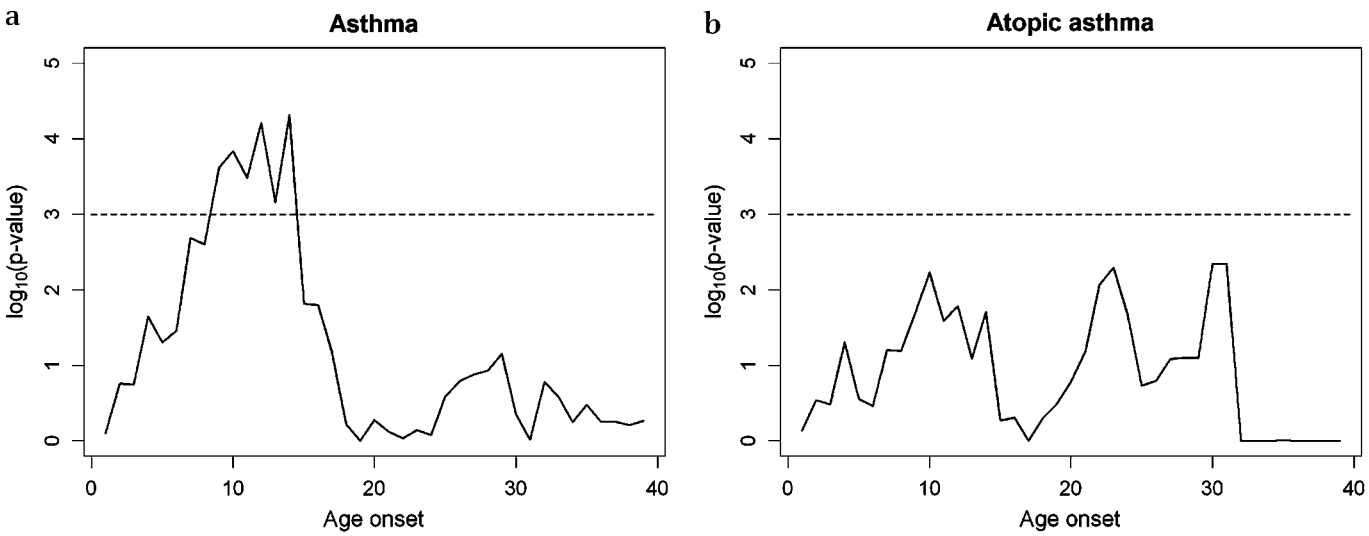

Figure 4 p Values for interaction from the multinomial logistic regression for each cut-off of age at onset and the rs324981 SNP from *NPSR1* for (a) asthma and (b) atopic asthma. Dotted lines indicate the nominal p value (0.05). Model adjusted by country, body mass index, age, sex and smoking status.

trafficking of the receptor to the cell membrane.[36] Association of the other polymorphisms (mainly located in the second intron) may be explained by an effect on alternative splicing or linkage with an untested variant.

The age of asthma onset may differentiate phenotypic characteristics.[13] We identified a cut-off point for age at onset at 14 years. This cut-off point is consistent with the age of puberty, and a similar age has been used in previous studies for discerning childhood and adult asthma.[37 38] Early-onset asthma is more likely to be linked to a family history of asthma, an indicator of a stronger genetic component, and is a more homogeneous disease, both of which increase the probability of identifying genetic effects.[1] The stronger effects of *NPSR1* in early-onset asthma suggest that the pathophysiological mechanisms may vary according to age. In children, asthma is strongly correlated with atopy.[1 13] We have shown that the effect of *NPSR1* is stronger for atopic asthma and, in addition, the differences between early-onset and late-onset asthma disappeared when atopic asthma was evaluated. This suggests that the stronger effects observed at early ages are due to an effect of these genes on atopic asthma. The putative role of *NPSR1* in the modulation of innate immunity and its implication in atopic sensitisation[28] supports the hypothesis that this gene could be more closely linked to early-onset asthma than to adult asthma through its implication in atopic asthma. Recent work found that *NPSR1* may modulate the effect of farm exposure in allergic symptoms in children.[39] Although previous studies have reported an effect of *NPSR1* polymorphisms in both childhood and adult asthma, our study is the first to discern the role of *NPSR1* by age of onset. We also observed an interaction between age at onset and two polymorphisms in *DPP10*. The lack of a significant association of these variants with asthma, however, makes interpretation of the interaction difficult. A recent analysis conducted on the British 1958 birth cohort[15] showed that, similar to our study, the

Table 4 Multinomial analysis of age at onset of asthma and *NPSR1* SNPs under the additive genetic model

SNP	OR (95% CI)	p Value	OR (95% CI)	p Value	p Value interaction
Asthma	Onset asthma ≤14 (n = 141)		Onset asthma >14 (n = 146)		
rs1345267	0.83 (0.62 to 1.12)	0.2163	0.77 (0.58 to 1.02)	0.0655	0.5927
rs184448	1.71 (1.26 to 2.32)	0.0005	1.36 (1.03 to 1.78)	0.0281	0.1604
rs324957	1.75 (1.29 to 2.35)	0.0003	1.27 (0.97 to 1.67)	0.0850	0.0674
rs324960	0.64 (0.46 to 0.88)	0.0067	0.79 (0.59 to 1.05)	0.1070	0.1431
rs324981	0.54 (0.40 to 0.74)	0.0001	0.81 (0.62 to 1.06)	0.1331	0.0134
rs325462	0.60 (0.44 to 0.81)	0.0007	0.86 (0.66 to 1.13)	0.2786	0.0243
Atopic asthma	Onset asthma ≤14 (n = 106)		Onset asthma >14 (n = 71)		
rs1345267	0.87 (0.62 to 1.20)	0.3931	0.66 (0.45 to 0.97)	0.0348	0.3142
rs184448	1.69 (1.20 to 2.38)	0.0025	1.45 (1.00 to 2.10)	0.0481	0.2887
rs324957	1.72 (1.23 to 2.40)	0.0015	1.46 (1.01 to 2.11)	0.0469	0.314
rs324960	0.66 (0.46 to 0.94)	0.0203	0.88 (0.60 to 1.28)	0.4957	0.0765
rs324981	0.59 (0.42 to 0.83)	0.002	0.72 (0.50 to 1.04)	0.0806	0.181
rs325462	0.61 (0.44 to 0.84)	0.003	0.76 (0.53 to 1.09)	0.1398	0.1683

Model adjusted by country, body mass index, age, sex and smoking status.

effect of *DPP10* was specific for asthma onset after the age of 17 years, while they did not observe an effect of *NPSR1*. This study differs in the main outcome of asthma used because it included wheezing in the definition of asthma.

Our results indicate an effect of *ADAM33* on BHR. Although the significance of this association was weak ($p = 0.04$) and did not pass Bonferroni correction, other studies have also reported this association (reviewed by Hersh *et al*[12]). These findings support the hypothesis that *ADAM33* is more related to airway wall remodelling rather than being restricted to asthma.[40]

Despite the strong evidence for *NPSR1* reported in this study, several issues in the interpretation of the findings should be considered. The first is related to the international nature of the ECRHS cohort and a potential concern for population stratification. Previous analysis in the ECRHS has shown little evidence of population stratification, but the number of markers evaluated could be insufficient.[22] Even though there is only a low level of genetic variation among Europeans, there is some relation between genetic and geographical distances.[41] To take into account this limitation, all the analyses were adjusted by country.

Another issue is related to phenotype definition. For this analysis we selected persistent traits at the time of evaluation to have a more reliable evaluation of the phenotypes. This implies that, irrespective of age at onset, all had asthma in adulthood. Persistence of asthma from childhood to adulthood could be related to a different phenotype of asthma than that occurring in childhood and not recurring later. Longitudinal cohort studies have shown that patients with persistent asthma have a higher prevalence of allergic sensitisation, airway responsiveness, lower lung function and more severe asthma in childhood than those in whom asthma did not persist.[38 42] We have already discussed the strong association of *NPSR1* with atopic asthma, but we cannot exclude the possibility that this could also be due to an effect of this gene on other specific phenotypes such as more severe asthma.

Multiple testing is an important source of false positive results. In the analysis of the genetic main effects we considered a Bonferroni correction by the number of SNPs tested and not by the number of outcomes assessed since there is a high correlation among the phenotypes. Some polymorphisms from *NPSR1* remained significant after Bonferroni correction, which highlights the strength of this association. However, the Bonferroni test is over-conservative since polymorphisms within a gene are not completely independent and selection of the genes was based on strong pre-established hypotheses. For these reasons, the reported association between *ADAM33* and BHR using a threshold p value of 0.05 may be considered as a positive replication. In the analysis of the interaction with age at onset, we also applied a threshold p value of 0.05 because the a priori statistical power to detect an interaction with age at onset was relatively low.[43] These results should therefore be interpreted with caution.

In conclusion, this study gives independent support to *NPSR1* as a risk factor for asthma, atopy and atopic asthma. In addition, this analysis adds evidence to the importance of considering age-specific effects in evaluating the genetic risk factors in asthma, suggesting a role for *NPSR1* in early-onset asthma driven by the strong effect of this gene on atopic asthma. To a less extent, this study replicates previous evidence for the role of *ADAM33* on BHR and suggests an age-specific effect of *DPP10* on asthma.

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