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

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ABSTRACT

Rationale. 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. We examined associations and age-specific effects on asthma, atopy and bronchial hyperresponsiveness of five candidate genes previously identified by positional cloning (*ADAM33, PHF11, NPSR1, DPP10, SPINK5).*

Methods. We studied 51 polymorphisms from 2474 participants from 13 countries who took part in the European Community Respiratory Health Survey (1990–2000). Asthma and age at onset of asthma were assessed by questionnaire data, bronchial hyperresponsiveness 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 kilobases in the *NPSR1* gene, even after Bonferroni correction for multiple comparisons (p-value<0.001). The associations with *NPSR1* were stronger among those reporting a first attack of asthma before age of 15, with statistically significant interactions with age of onset found for three SNPs. Weaker evidences were observed for *ADAM33* and bronchial hyperresponsiveness and for an age-specific effect of two SNPs in *DPP10* and asthma.

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

INTRODUCTION

Asthma is a complex disease that shows phenotypic heterogeneity and is related with atopy, bronchial hyperresponsiveness (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 up-regulated 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 to 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 to 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 phathophysiology that contributes to particular phenotypes.[1] Recently, Bouzigon et al.,[14] reported that 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 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 (European Community Respiratory Health Survey, ECRHS).

METHODS

The methodology of ECRHS has been described elsewhere.[16,17] Briefly, the ECRHS is a random population-based multicentre cohort of subjects aged 20-44 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. Ethical approval was obtained for each centre from the appropriate institutional ethics committee and written consent was obtained from each participant.

The presence of asthma was based on a positive response to either of two questions: attack of asthma during the 12 months preceding the interview or current use of asthma medication. Most asthmatics defined through these two questions (93%) also reported a doctor diagnosis of asthma. Age at onset of asthma was assessed through 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 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. Bronchial hyperresponsiveness (BHR) was defined as a 20% fall in FEV1 from the highest FEV1 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 sensitization (specific IgE levels >0.35 kU/L) to any allergen.

In order to increase accuracy in 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 negatives test for atopy, BHR or did not reporting 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 describes the subject selection process of the ECRHS study and subjects available for the present analysis.

A description of the SNP selection and genotyping can be found in the supplementary material. The list of SNPs examined can be found in supplementary table 1. SNPs were genotyped using the SNPlex™ platform (Applied Biosystems, Foster City, CA) according to manufacturer instructions.

Statistical analysis

Analyses were adjusted for country, body mass index (BMI), smoking status and sex. 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)[19] from R statistical software (version 2.6.1).[20] To correct for multiple comparisons we applied a Bonferroni correction for the 51 independent loci genotyped (significant p value = 0.001). Haplotypes were estimated using haplo.em function from haplo.stats package (version 1.3.8).[21] 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 4 adjacent SNPs using the haplo.score.slide command from HaploStats package. Population attributable risk (PAR) for haplotypes was calculated from the odds ratios (OR) using the formula PAR=

100*(P*[OR-1]/ [P*(OR-1)+1]) , where P is the prevalence of the haplotype in the population.

We identified the most appropriate cut-off for age at onset of asthma following a modification of the method described by Bouzigon et al.[22] 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 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*).[23,24] Genomic control[25] and principal component analysis using EIGENSTRAT[26] (supplementary figure 1) fail to evidence population stratification.

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

RESULTS

Population characteristics are shown in table 1. Our analysis was restricted to 4,331 subjects of ECRHS II whose DNA was available, that 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 1,213 subjects (28.0%) who fulfilled the a priori criteria. Only one SNP (rs765023) was found to deviate from Hardy Weinberg Equilibrium (HWE) in the comparison group (P<0.01, supplementary table 2).

Table 1. Population characteristics. Population in this analysis includes cases and the subjects from the comparison group and cases.

Variable	Total Population (n=4,331)	Population in this analysis (n=2,474)
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	1,165 (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		
Males	2,089 (48.2%)	1,271 (51.4%)
Females	2,242 (51.8%)	1,203 (48.6%)
Smoking		
Never	1,855 (42.8%)	1,124 (45.4%)
Ex	1,144 (26.4%)	670 (27.1%)
Current	1,348 (31.1%)	692 (28%)
Age (mean±sd)	42.8 (7.2)	42.4 (7.1)
Comparison group	1,213 (28%)	1,213 (49%)
Asthma	320 (7.4%)	320 (12.9%)
Atopy	1,060 (24.5%)	1,060 (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*	1,307 (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.

Significant associations (nominal P value ≤ 0.05) with asthma, atopy and BHR are summarized in table 2. Nominally significant associations for asthma (six SNPs) or atopy (eight SNPs) were observed only for *NPSR1* gene. Multiple test correction (Bonferroni P-value 0.001) yield 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). The complete information for all the evaluated polymorphisms is shown in supplementary table 3.

Table 2. Nominal significant (P < 0.05) associations between the SNPs and persistent asthma, persistent atopy and persistent BHR under additive genetic model. Model adjusted by country, body mass index, age, sex and smoking status.

		Asthma		Atopy		BHR	
Gene	SNP	OR (95%CI)	p-value	OR(95%CI)	p-value	OR(95%CI)	p-value
ADAM33	rs2787095	1.06(0.87-1.29)	0.5403	1.12(0.99-1.27)	0.0626	1.23(1.01-1.5)	0.0382
NPSR1	rs714588	1.04(0.86-1.27)	0.6709	0.87(0.77-0.99)	0.0285	0.9(0.74-1.11)	0.3245
NPSR1	rs1345267	0.81(0.66-1)	0.0436	0.91(0.8-1.03)	0.1326	0.94(0.76-1.16)	0.5622
NPSR1	rs184448	1.48(1.2-1.82)	0.0002	1.2(1.06-1.36)	0.0049	1.14(0.93-1.4)	0.2111
NPSR1	rs324396	1.21(0.97-1.51)	0.0868	1.25(1.09-1.43)	0.0010	1.16(0.94-1.45)	0.1721
NPSR1	rs324957	1.41(1.15-1.74)	0.001	1.2(1.05-1.36)	0.0051	1.14(0.93-1.4)	0.1999
NPSR1	rs324960	0.74(0.6-0.93)	0.008	0.85(0.74-0.97)	0.0140	0.93(0.75-1.16)	0.5270
NPSR1	rs324981	0.69(0.56-0.85)	0.0004	0.84(0.74-0.95)	0.0064	0.87(0.71-1.06)	0.1591
NPSR1	rs325462	0.76(0.62-0.93)	0.0085	0.88(0.78-0.99)	0.0367	0.86(0.71-1.05)	0.1356

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 odds ratios for each subgroup. Independent effects were observed for both atopy and asthma, but mainly, a joint effect can be seen with the highest odds ratios among subjects with both atopy and asthma (figure 3).

We performed multiple marker analysis in those genes that showed significant associations in the single-marker analysis. In *NPSR1*, we identified three blocks with high linkage disequilibrium (supplementary figure 2), one of 6kb, 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 *NPSR1* gene (rs184448, rs324396, rs324957, rs324960) significant 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, $\chi 2$ p-value < $2x10^{-16}$). The population attributable risk for this haplotype was 9.15% for asthma, 6.05% for atopy and 9.75% for the combination of asthma and atopy.

Table 3. Haplotype analysis of *NPSR1* with sliding windows. Model adjusted by country, body mass index, age, sex and smoking status.

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

Locus 10 : rs184448; Locus 11: rs324396; Locus 12: rs324957; Locus 13: rs324960.

Additional analyses using different outcome definitions supported these findings (supplementary table 5). A less restrictive definition of asthma was defined as subjects reporting asthma in either of the two contacts. The same analysis was done for atopy. 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 effect for these genetic variants. Multinomial logistic regression for each cut-off of age at onset was performed for the six significant SNPs for asthma (supplementary figure 3). 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 old). From the 320 individuals reporting persistent asthma, 141 reported first attack of asthma before age of 14 and 146 after 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 for the analysis of age at onset. The strength of the association (odds ratio) between these *NPSR1* polymorphisms and asthma is more pronounced among early onset asthmatics (≤14 years) (table 4). The analysis for the rest of *NPSR1* polymorphisms (supplementary table 6) shows significant interaction for another *NPSR1* variant, rs324381 (p for interaction = 0.05), showing a pattern of association according to age at onset similar to the two above *NPSR1* SNPs (OR = 0.66 for early onset and OR = 0.86 for late onset). Differences in the prevalence of atopy were observed between early and late onset asthma (75% versus 49% respectively).

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 rest of *NPSR1* SNPs (data not shown). The stratified analysis using the cut-off of at 14 years (table 4) shows that the association of these variants with early onset asthma is similar in atopic asthma compared to general asthma. Overall, the

effect for late onset is increased for atopic asthma narrowing the differences between early and late onset of asthma.

Table 4. Multinomial analysis of age at onset asthma and *NPSR1* SNPs under the additive genetic model. Model adjusted by country, body mass index, age, sex and smoking status.

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

The evaluation of age at onset of asthma using the cut-off at age of 14 on the other genes (supplementary table 6) reveals significant interactions for two polymorphisms in the *DPP10* gene, rs1430090 (OR = 0.81 for early onset versus OR = 1.17 for late onset, p for interaction = 0.04) and rs10496465 (OR = 0.85 for early onset versus OR = 1.36 for late onset, p for interaction = 0.03), although the effect of these polymorphisms was not significant in either early or adult onset of 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 evidences were observed for an age specific effect of *DPP10* on asthma. In addition, we reported a weak association between *ADAM33* and BHR that do not pass Bonferroni correction. In this study, 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.[27] *PHF11* was initially reported as a gene associated to total IgE and asthma.[5] We failed to replicate 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 manifestation[7] and replication studies have confirmed this findings.[28,29] Up to date only two studies have evaluated the association with asthma and reported inconsistent findings.[28,29]

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 through 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,[12,30-34] other studies did not reported significant effect.[35-38] The similar effect on asthma and atopy was observed in the initial study[4] and in most of further replication studies.[30-33] The effect on the combination of asthma and atopy (allergic asthma) has been also reported by previous studies.[12,30,32,34]

Polymorphisms of *NPSR1* significantly associated to 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 aminoacid 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 trafficking of the receptor to the cell membrane.[39] 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.

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 articles for discerning childhood and adult asthma.[40,41] Early-onset asthma is more likely to be linked to family history of asthma, an indicator of stronger genetic component, and is a more homogeneous disease, both increasing the probability to identify genetic effects.[1] The stronger effects of *NPSR1* in early onset suggest that pathophysiology mechanisms may vary according to age. In children, asthma is strongly correlated with atopy.[1,13] We reported that the effect of *NPSR1* was stronger for atopic asthma and, in addition, the differences between early and late onset disappear when atopic asthma was evaluated. This suggests that the stronger effects observed in early ages are due to an effect of these genes on atopic asthma. The putative role of *NPSR1* in modulation of innate immunity and its implication in atopic sensitization,[30] supports the hypothesis that this gene could be more closely linked to early asthma onset than 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.[42] Although previous studies have reported an effect of *NPSR1* polymorphisms in both childhood and adult asthma, our study is the first analysis discerning 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 the interpretation of the interaction difficult. A recent analysis conducted on the British

1958 birth cohort[38] showed, similar to our study, that the effect of *DPP10* was specific for asthma onset after 17, while they did not observe an effect of *NPSR1*. This study differs in the main outcome of asthma used because they include 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 do 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.[43]

Despite strong evidences 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.[24] Even though there is only low level of genetic variation among Europeans, there is some relation between genetic and geographical distances.[44] To take into account this limitation all the analysis 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 with a different phenotype of asthma as compared to that occurring in childhood and not recurring later. Longitudinal cohort studies have shown that persistent asthma had a higher prevalence of allergic sensitization, airway responsiveness, lower lung function and more severe asthma in childhood compared to those whose asthma did not persist.[41,45] We have already discussed the strongest effect of *NPSR1* on atopic asthma but we can not exclude that the effects observed are due to an effect of these genes 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 also by the number of outcomes assessed since there is a high correlation among the phenotypes. Some polymorphisms from *NPSR1* remain significant after Bonferroni correction which highlights the strength of this association. However, Bonferroni 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 P-value threshold of 0.05 may be considered as a positive replication. In the analysis of the interaction with age at onset, we also applied a P-value threshold of 0.05 because the a priori statistical power to detect interaction with age at onset was relatively low.[46] For these reason these results should 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 on the evaluation of the genetic risk factors in asthma, suggesting a role of *NPSR1* on early onset asthma driven by the strong effect of this gene on atopic asthma. To a less extent, this study replicates previous evidences of the role of *ADAM33* on BHR and suggests an age-specific effect of *DPP10* on asthma.

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COMPETING INTEREST

The authors declare they have no competing of interests.

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FIGURE LEGENDS

Figure 1. Flow chart describing the subjects selection process and showing the numbers available for the analysis. Definitions for asthma, atopy and BHR are specified in the methods section.

Figure 2. Proportional Venn diagram, showing the overlap of asthma, atopy and BHR in this population.

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 at supplementary table 4.

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 line indicates the nominal p-value (0.05). Model adjusted by country, body mass index, age, sex and smoking status.

Supplementary Material

SUPPLEMENTARY METHODS

The methodology of ECRHS has been described elsewhere.[1-3] Briefly, the ECRHS is a random population-based multicentre cohort of subjects aged 20-44 at time of recruitment (1990, ECRHS I) and then followed approximately 10 years later (ECRHSII). Subjects completed a long questionnaire on respiratory symptoms and exposures and underwent a clinical evaluation including lung function, atopy and bronchial hyperresponsiveness. A complementary sample of subjects with asthma symptoms at recruitment was also included in the study and followed-up. Ethical approval was obtained for each centre from the appropriate institutional ethics committee and written consent was obtained from each participant.

The presence of asthma was based on a positive response to either of two questions: attack of asthma during the 12 months preceding the interview or current use of asthma medication. Age at asthma onset was assessed through 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 ECRHS.[4] Since analyses of other studies have defined asthma as a report of "doctor diagnosed asthma", we also used this definition in an alternative analysis. Bronchial hyperresponsiveness (BHR) was defined as a 20% fall in FEV1 from the highest FEV1 post-diluent during methacholine challenge with an accumulated dose of 1 mg.[1] Specific IgE levels to house dust mite (Dermatophagoides pteronyssinus), cat, timothy grass and Cladosporium herbarum were measured with the Pharmacia CAP system (Pharmacia Diagnostics, Uppsala, Sweden). Atopy was defined as sensitization (specific IgE levels >0.35 kU/L) to any allergen.

In order to increase accuracy in the diagnosis, we considered subjects as cases for any of the three traits if they reported them in both the baseline survey (ECRHS I) and in the follow-up (ECRHS II). Subjects with atopy, BHR or those who reported ever having had asthma in either of the two stages of ECRHS were excluded from controls. In addition, subjects recruited in the symptomatic sample (and who did not qualify as

cases) were also excluded from the control set. Centres from Belgium and Estonia where excluded from the analysis because they did not perform the BHR assessment in both surveys. Figure 1 describes the subject selection process of the ECRHS study and show numbers available for this analysis.

SNP selection and genotype characterization

In order to reduce SNP redundancy and to incorporate all putative functional SNPs in genotyping design without loss of power, we selected tagSNPs combined with binned functional variants (most likely to influence gene expression or function) when possible. SNPs on candidate genes were selected in the gene region and 10kb upstream from 5'UTR and 10Kb downstream from 3' UTR derived from the HapMap Project database (http://www.hapmap.org , phase I, release #16) and Perlegen database (http://genome.perlegen.com/). Those SNPs described in caucasian populations with a minor allele frequency (MAF) >5% were retained. In addition all described SNPs from public databases were analysed (http://www.ncbi.nlm.nih.gov/projects/SNP). The *Ensemble* search engine ENSMART (http://www.ensembl.org/index.html) was used to filter by frequency and validation status all described SNPs in a gene region. Selection of tagSNPs was derived from the disequilibria patterns described from the HapMap project or from Perlegen. Selection of tagSNPs was carried out with the algorithm *Tagger* implemented in Haploview[5] using a threshold of r^2> 0.80 and a multimarker approach (pairwise and two-marker tagSNPs) to define correlated SNPs (binset) in selected region. SNP selection criteria were changed for genes *NPSR1* and *DPP10* because of the high number of inferred tagSNPs. In these cases more relaxed criteria (r^2 threshold ≥ 0.60 and a MAF > 20%). In the case of *DPP10* we focused in the candidate region, defined by an area of 50 kbp around the microsatellite marker D2S308 which have been reported to be linked to asthma previously.[6] The list of SNPs examined can be found in Supplementary Table 1.

The ECRHS DNA bank is maintained at Helmholtz Zentrum München in Germany and genotyping was performed at the Centre for Genomic Regulation (CRG) in the Barcelona node from the "Centro Nacional de Genotipado" (CeGen) in Spain

(http://www.cegen.org). SNPs were genotyped using the SNPlex™ platform (Applied Biosystems, Foster City, CA) according to manufacturer instructions and analyzed on an Applied Biosystems 3730/3730xl DNA Analyzer. Allele-calling was done by clustering analysis using Genemapper software (Genemapper v.4.0). The genotype call rate was >98%. Genotyping quality was controlled in two ways. First, internal positive and negative controls provided by ABI's manufacturer were included in the reaction plates. Second, six duplicated samples of two HapMap reference trios were incorporated in the genotyping process. Both genotype concordance and correct Mendelian inheritance were verified. Genotype concordance was tested using SNPator, a web-based tool for genotyping management and SNP analysis developed.[7]

Statistical analysis

We tested for genotype deviations from Hardy-Weinberg equilibrium (HWE) [8] in the control set of subjects (Supplementary Table 1). All analyses were adjusted for country, body mass index (BMI), smoking status and sex. Analysis of single marker effect was performed assuming an additive genetic model considering the most frequent allele as reference category and using logistic regression implemented in the SNPassoc package (version 1.5-1)[9] from R statistical software (version 2.6.1).[10] To avoid false-positive results due to multiple testing, we applied Bonferroni correction for the 51 independent loci genotyped (significant p value = 0.001).

Haplotypes were estimated using haplo.em function from haplo.stats package (version 1.3.8).[11] D', R^2 and χ^2 p-values for marker independence were estimated to determine linkage disequilibrium between genetic markers. In order to detect regions associated with high risk, we used a sliding windows approach to construct haplotypes in windows up to 4 adjacent SNPs within each gene. The sliding windows analysis was performed using the haplo.score.slide command from HaploStats package in R.[11] In order to avoid false positives we restricted the haplotype analysis to those genes with significant associations in the single-marker analysis.

Population attributable risk (PAR) for haplotypes was calculated from the odds ratios (OR) using the formula PAR= 100*(P*[OR-1]/ [P*(OR-1)+1]) , where P is the prevalence of the haplotype in the population.

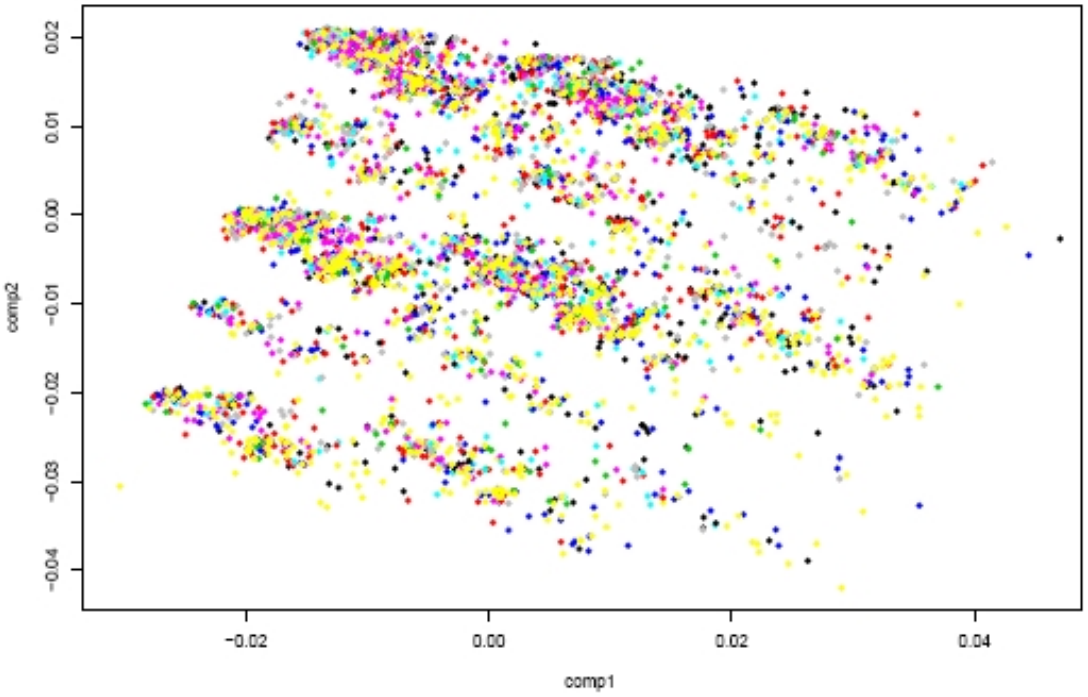
In order to test the hypothesis of association between genetic polymorphisms and age at onset of asthma we identified the most appropriate cut-off for age at onset following a modification of the method described by Bouzigon.[12] For each specific age from 1 to 40 years, the association between genetic polymorphisms and asthma was assessed by multinomial regression analysis stratifying cases using the specific age at onset as a cut-off (0 control; 1 onset of asthma less or equal to specific age; 2 onset of asthma before specific age). By using this model, we compare each group of cases with the whole set of non-asthmatics individuals. Homozygosity for the more common allele among controls was set as the reference class. Tests for linear trend of odds ratios (OR) were calculated using the categorised variable as quantitative 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 polymorphism and age at onset was maximum. Interaction was assessed using likelihood ratio test, by comparing the models with and without the interaction term.

Population stratification analysis in ECRHS was previously reported.[3] Stratification was assessed using a set of ancestry-informative markers (AIM)[13] in addition to oculocutaneous albinism II (*OCA2)* and lactase (*LCT*) markers which have been found to be strongly associated with the south-north gradient in Europeans.[14,15] Genomic control fail to evidence a population stratification[16] (inflation factor (λ) = 1.06) as well as principal component analysis using EIGENSTRAT (Supplementary Figure 1).[17]

Supplementary Table 1. Characteristics of SNPs selected for Genomic control and EIGENSTRAT

SNP	Chromosome	Position	Gene	% Genotype rate
rs1490413	1	4277696	-	99,4
rs10495407	1	234765349	-	99,2
rs876724	2	104974	-	98,9
rs4988235	2	136442378	Lct	99,0
rs309125	2	136477287	Lct	99,1
rs907100	2	239345579	-	98,6
rs1357617	3	936782	-	99,5
rs1979255	4	190693229	-	99,4
rs717302	5	2932395	-	99,0
rs1029047	6	1080939	-	99,5
rs917118	7	4230244	-	98,3
rs2056277	8	139468298	-	99,7
rs1015250	9	1813774	-	99,8
rs2076848	11	134172756	-	98,8
rs2111980	12	104830721	-	99,6
rs1335873	13	19799724	-	99,3
rs873196	14	97915284	-	86,5
rs1900758	15	25903692	OCA2	91,0
rs1800404	15	25909368	OCA2	99,4
rs729172	16	5546198	-	99,55
rs740910	17	5647347	-	98,1
rs1024116	18	73561374	-	86,41
rs719366	19	33155177	-	98,94
rs1005533	20	38920524	-	99,67
rs722098	21	15607469	-	99,37
rs2040411	22	46156931	-	98,69

Supplementary Figure 1. The figure shows the first two axes of genotypic variance for each of the nine countries, using EIGENSTRAT procedure as described in Price et al, 2006. EIGENSTRAT, uses principal components analysis to explicitly model ancestry differences between control and cases (current asthma). Each point represents each one of the 5,065 individuals who were genotyped. Colours represent the different countries included in the study. The plot indicates no subdivision within the population.



Supplementary Table 2. SNP summary

Gene	SNP	Chromosomal position	Gene location	Alleles	Major allele frequency (%)	P-value HWE	Missing (%)
ADAM33	rs6084432	3591652	3' near gene	G/A	83.6	0,24	0.6
	rs512625	3596377	3' near gene	G/A	69.4	0,50	0.5
	rs3918395	3601148	intron 13	G/T	86.7	0,31	1
	rs2787095	3603942	intron 3	G/C	59.4	0,08	0.9
	rs2853215	3614254	5' near gene	G/A	73.5	0,23	0.3
NPSR1	rs714588	34466465	5' upstream	A/G	55.7	0,75	0.6
	rs1023555	34469618	5' upstream	T/A	76.4	0,72	0.6
	rs898070	34472104	intron 1	G/A	62.9	0,87	0.7
	rs963218	34485008	intron 1	C/T	53.7	0,49	0.4
	rs1419835	34497159	intron 1	C/T	78.2	0,65	0.6
	rs765023	34515290	intron 2	T/C	65.6	0,01	6.2
	rs1345267	34521298	intron 2	A/G	62.3	0,11	0.1
	rs324381	34541350	intron 2	G/A	65.5	0,76	10.2
	hopo546333	34546333	intron 2	G/A	93.2	0,23	0.4
	rs184448	34546349	intron 2	T/G	54.4	0,20	1.7
	rs324396	34563362	intron 2	C/T	69.1	0,61	0.2
	rs324957	34574611	intron 2	G/A	55.3	0,13	0.4
	rs324960	34575630	intron 2	C/T	67.2	0,24	1.1
	rs10486657	34575966	intron 2	C/T	82.2	0,60	3.9
	rs324981	34591352	exon 3 N170I	A/T	54.3	0,34	0.2
	rs1419780	34593540	intron 3	C/G	81.6	0,45	0.3
	rs325462	34623085	intron 3	A/T	50.1	0,88	0.5
	rs727162	34647277	exon 6 S241R	G/C	78.5	0,47	0.3
	rs10250709	34660064	intron 7	G/A	66.2	0,18	0
	rs6958905	34662791	3' UTR	T/C	65.6	0,33	0.4
	rs10238983	34672812	3' downstream	T/C	76.5	0,22	0.5
PHF11	rs4941643	48966387	5' near gene	A/G	55	0,78	6.4
	rs3794381	48971563	intron 1	C/G	72.8	0,64	6.3
	rs2031532	48978847	exon 2	G/A	65.5	0,45	0
	rs2247119	48985142	intron 2	T/C	70.8	0,95	0.8
	rs8000149	48988025	intron 3	T/C	63.8	0,40	0.3
	rs2274276	48993953	intron 5	G/C	58.1	1,00	0.7
	rs7332573	48996376	intron 8	G/T	91.9	0,72	1.2
	rs3829366	49001411	3' near gene	T/A	52.9	0,75	1
DPP10	rs4490198	114880688	5' near gene	A/G	58.2	0,07	0.6
	rs4849332	114891859	5' near gene	G/T	61.9	0,44	0.2
	rs1367179	114894785	5' near gene	G/C	81.7	1,00	0.9
	rs11123242	114897278	5' near gene	C/T	81.9	0,86	0.4
	rs13014858	114909119	5' near gene	G/A	58.2	0,67	0.3
	rs1430094	114912983	5' near gene	G/A	66.6	0,19	0.3
	rs1430093	114920665	5' near gene	C/A	66.1	0,68	3.7
	rs746710	114922262	5' near gene	G/C	51	0,96	0.1
	rs1430090	114926871	5' near gene	T/G	69.6	0,12	1.9
	rs6737251	114929904	5' near gene	C/T	69.2	0,81	0.4
	rs11685217	114938917	5' near gene	C/T	80.4	0,006	3.8
	rs1430097	114956909	5' near gene	C/A	65.7	0,95	0.9
	rs10496465	114974292	5' near gene	A/G	85.1	1,00	0.6
SPINK5	rs3756688	147422971	5' near gene	T/C	63.1	0,57	1
	rs2303063	147460219	exon 13	A/G	51.9	0,79	1.3
	rs1422993	147484012	intron 27	G/T	74.9	0,23	0.1
	rs2400478	147491058	exon 31	G/A	62.2	0,40	0.9

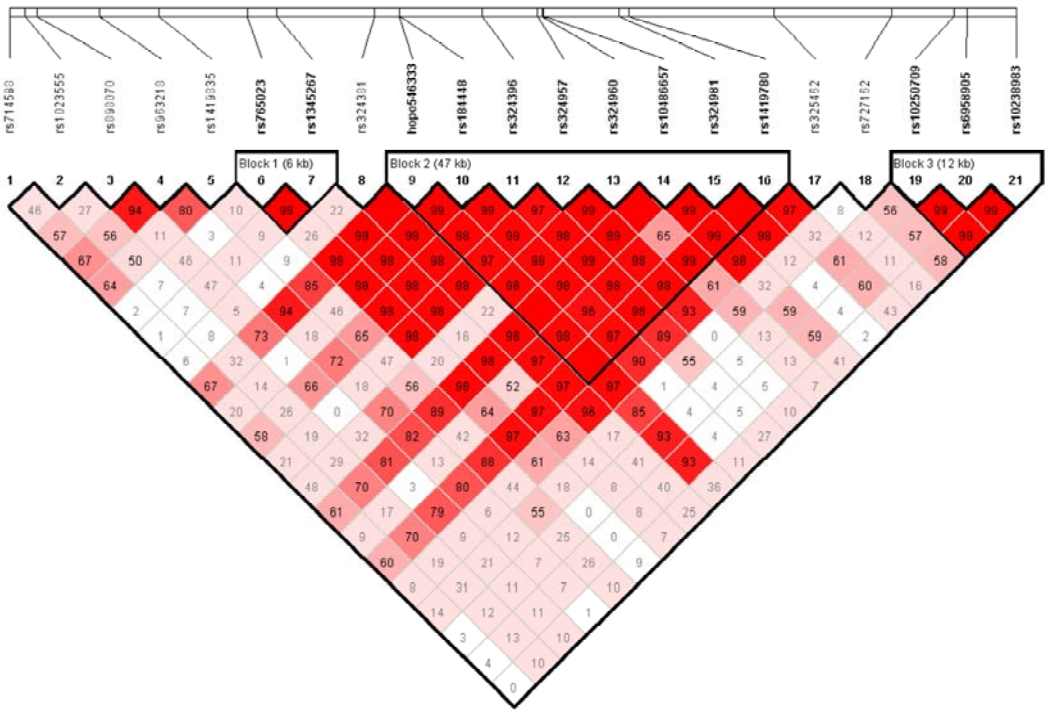
Supplementary Table 3. Odds ratio and p-value of the association of selected SNPs with persistent asthma and atopy under additive genetic model. Model adjusted by country, body mass index, age, sex and smoking status.

Gene	SNP	Persistent Asthma		Persistent Atopy		Persistent BHR	
		OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value
ADAM33	rs6084432	1.27(0.99-1.64)	0.0643	1.13(0.96-1.33)	0.1402	1.03(0.79-1.34)	0.8500
ADAM33	rs512625	0.93(0.75-1.15)	0.4923	1.03(0.9-1.17)	0.6909	1(0.81-1.24)	0.9725
ADAM33	rs3918395	1.08(0.81-1.44)	0.6101	1.08(0.9-1.29)	0.4133	1.11(0.84-1.49)	0.4616
ADAM33	rs2787095	1.06(0.87-1.29)	0.5403	1.12(0.99-1.27)	0.0626	1.23(1.01-1.5)	0.0382
ADAM33	rs2853215	1.07(0.86-1.34)	0.5194	0.93(0.81-1.07)	0.3223	0.9(0.71-1.12)	0.3394
NPSR1	rs714588	1.04(0.86-1.27)	0.6709	0.87(0.77-0.99)	0.0285	0.9(0.74-1.11)	0.3245
NPSR1	rs1023555	1.05(0.83-1.32)	0.6919	1.03(0.89-1.19)	0.6828	1(0.79-1.26)	0.9874
NPSR1	rs898070	1.07(0.87-1.31)	0.5251	0.91(0.8-1.04)	0.1715	0.94(0.76-1.16)	0.5551
NPSR1	rs963218	1.03(0.85-1.25)	0.7463	0.89(0.79-1.01)	0.0748	0.94(0.77-1.15)	0.5635
NPSR1	rs1419835	1.07(0.84-1.35)	0.5948	0.91(0.78-1.06)	0.2476	0.94(0.74-1.19)	0.5842
NPSR1	rs765023	0.81(0.65-1)	0.0533	0.9(0.78-1.03)	0.1205	1.02(0.82-1.26)	0.8572
NPSR1	rs1345267	0.81(0.66-1)	0.0437	0.91(0.8-1.03)	0.1326	0.94(0.76-1.16)	0.5622
NPSR1	rs324381	0.81(0.64-1.01)	0.0636	0.88(0.77-1.02)	0.0887	1(0.79-1.26)	0.9963
NPSR1	hopo546333	0.74(0.49-1.13)	0.1552	0.8(0.62-1.04)	0.0958	0.66(0.42-1.03)	0.0557
NPSR1	rs184448	1.48(1.2-1.82)	0.0002	1.2(1.06-1.36)	0.0049	1.14(0.93-1.4)	0.2111
NPSR1	rs324396	1.21(0.97-1.51)	0.0868	1.25(1.09-1.43)	0.0010	1.16(0.94-1.45)	0.1721
NPSR1	rs324957	1.41(1.15-1.74)	0.0010	1.2(1.05-1.36)	0.0051	1.14(0.93-1.4)	0.1999
NPSR1	rs324960	0.74(0.6-0.93)	0.0076	0.85(0.74-0.97)	0.0140	0.93(0.75-1.16)	0.5270
NPSR1	rs10486657	0.83(0.64-1.07)	0.1480	0.92(0.79-1.09)	0.3502	0.98(0.76-1.27)	0.8822
NPSR1	rs324981	0.69(0.56-0.85)	0.0004	0.84(0.74-0.95)	0.0064	0.87(0.71-1.06)	0.1591
NPSR1	rs1419780	0.86(0.67-1.11)	0.2508	0.98(0.84-1.14)	0.7800	1(0.78-1.28)	0.9879
NPSR1	rs325462	0.76(0.62-0.93)	0.0085	0.88(0.78-0.99)	0.0367	0.86(0.71-1.05)	0.1356
NPSR1	rs727162	1.16(0.92-1.47)	0.2055	1(0.86-1.16)	0.9903	1.08(0.85-1.36)	0.5397
NPSR1	rs10250709	0.99(0.8-1.22)	0.9173	0.92(0.81-1.05)	0.2317	1.01(0.81-1.24)	0.9600
NPSR1	rs6958905	0.98(0.8-1.21)	0.8683	0.92(0.8-1.05)	0.1963	1.01(0.82-1.25)	0.8927
NPSR1	rs10238983	1.13(0.9-1.42)	0.3122	1.01(0.87-1.17)	0.9164	1.1(0.88-1.39)	0.3998
PHF11	rs4941643	1.06(0.87-1.29)	0.5892	0.95(0.84-1.08)	0.4497	1.02(0.84-1.24)	0.8488
PHF11	rs3794381	0.97(0.77-1.21)	0.7659	0.89(0.77-1.02)	0.1032	0.83(0.66-1.04)	0.1044
PHF11	rs2031532	1.07(0.87-1.31)	0.5444	0.91(0.8-1.04)	0.1721	0.94(0.76-1.15)	0.5402
PHF11	rs2247119	1(0.81-1.23)	0.9888	1.05(0.92-1.2)	0.4722	1.02(0.82-1.26)	0.8859
PHF11	rs8000149	1.07(0.87-1.31)	0.5395	0.91(0.8-1.03)	0.1393	0.95(0.77-1.16)	0.6026
PHF11	rs2274276	0.97(0.8-1.18)	0.7719	0.9(0.79-1.02)	0.0876	0.92(0.75-1.12)	0.3977
PHF11	rs7332573	1.11(0.78-1.56)	0.5674	1.05(0.84-1.32)	0.6522	1.36(0.97-1.9)	0.0781
PHF11	rs3829366	1(0.83-1.22)	0.9772	0.89(0.79-1)	0.0590	1.02(0.84-1.23)	0.8758
DPP10	rs4490198	1.04(0.86-1.27)	0.6841	1.04(0.92-1.18)	0.5245	0.97(0.8-1.19)	0.7903
DPP10	rs4849332	1.01(0.83-1.24)	0.8870	0.98(0.87-1.12)	0.7961	0.89(0.73-1.09)	0.2718
DPP10	rs1367179	1.13(0.88-1.45)	0.3309	0.9(0.77-1.07)	0.2298	1.1(0.86-1.41)	0.4345
DPP10	rs11123242	1.13(0.88-1.45)	0.3442	0.91(0.78-1.08)	0.2756	1.09(0.85-1.4)	0.5015
DPP10	rs13014858	1.06(0.87-1.28)	0.5822	1.05(0.93-1.19)	0.4301	0.98(0.81-1.2)	0.8732
DPP10	rs1430094	1.04(0.85-1.28)	0.7140	1.12(0.98-1.27)	0.0847	1.03(0.84-1.27)	0.7835
DPP10	rs1430093	1.03(0.84-1.27)	0.7640	1.12(0.98-1.28)	0.0916	1.04(0.84-1.29)	0.7176
DPP10	rs746710	1.01(0.83-1.23)	0.8970	1.07(0.95-1.22)	0.2542	0.96(0.79-1.18)	0.7195
DPP10	rs1430090	0.99(0.8-1.22)	0.9266	0.99(0.87-1.13)	0.9081	1.09(0.88-1.35)	0.4214
DPP10	rs6737251	1.01(0.82-1.24)	0.9558	1.06(0.93-1.2)	0.4108	0.86(0.69-1.07)	0.1614
DPP10	rs11685217	1.11(0.88-1.41)	0.3727	1.11(0.96-1.3)	0.1556	0.82(0.63-1.06)	0.1227
DPP10	rs1430097	0.99(0.81-1.22)	0.9403	1.06(0.93-1.2)	0.3986	0.83(0.67-1.02)	0.0769
DPP10	rs10496465	1.08(0.81-1.44)	0.5831	1.06(0.89-1.27)	0.5194	0.97(0.73-1.3)	0.8329
SPINK5	rs3756688	0.97(0.79-1.18)	0.7304	0.95(0.83-1.07)	0.3907	0.95(0.77-1.16)	0.5992
SPINK5	rs2303063	0.9(0.74-1.09)	0.2836	0.96(0.85-1.08)	0.4904	0.9(0.74-1.1)	0.3083
SPINK5	rs1422993	1.2(0.97-1.49)	0.1002	1.07(0.93-1.23)	0.3472	1.09(0.88-1.36)	0.4347
SPINK5	rs2400478	1.02(0.84-1.25)	0.8229	1.05(0.93-1.19)	0.4280	0.97(0.79-1.19)	0.7653

Supplementary Table 4. Odds ratios and p-values for highly statistically significant (p-value<0.001) *NPSR1* polymorphisms for asthma or atopy, stratified by atopic and asthmatic status. Model adjusted by country, body mass index, age, sex and smoking status.

		ALL		Atopy:ALL		Atopy:No	
***NSPR1* SNP**	**Asthma**	**OR (95%CI)**	**P-VALUE**	**OR (95%CI)**	**P-VALUE**	**OR (95%CI)**	**P-VALUE**
rs184448	All	-	-	1.2(1.06-1.36)	0.0049	-	-
	Yes	1.48(1.2-1.82)	0,0002	1.54(1.19-1.99)	0,0009	1.09(0.72-1.66)	0,0002
	No			1.15(1.01-1.31)	0.0364	Reference	
rs324396	All			1.25(1.09-1.43)	0.0010		
	Yes	1.21(0.97-1.51)	0.0868	1.23(0.94-1.6)	0.1263	1.1(0.7-1.74)	0.0868
	No			1.26(1.09-1.45)	0.0013	Reference	
rs324957	All			1.2(1.05-1.36)	0.0051		
	Yes	1.41(1.15-1.74)	0.0010	1.5(1.16-1.92)	0.0017	1.09(0.71-1.66)	0.0010
	No			1.15(1.01-1.31)	0.0358	Reference	
rs324960	All			0.85(0.74-0.97)	0.0140		
	Yes	0.74(0.6-0.93)	0.0076	0.75(0.58-0.98)	0.0305	0.77(0.49-1.2)	0.0076
	No			0.86(0.75-0.99)	0.0335	Reference	
rs324981	All			0.84(0.74-0.95)	0.0064		
	Yes	0.69(0.56-0.85)	0.0004	0.67(0.53-0.87)	0.0018	0.75(0.5-1.14)	0.0004
	No			0.88(0.77-1)	0.0489	Reference	
rs325462	All			0.88(0.78-0.99)	0.0367		
	Yes	0.76(0.62-0.93)	0.0085	0.72(0.56-0.92)	0.0076	0.9(0.6-1.34)	0.0085
	No			0.91(0.8-1.04)	0.1678	Reference	

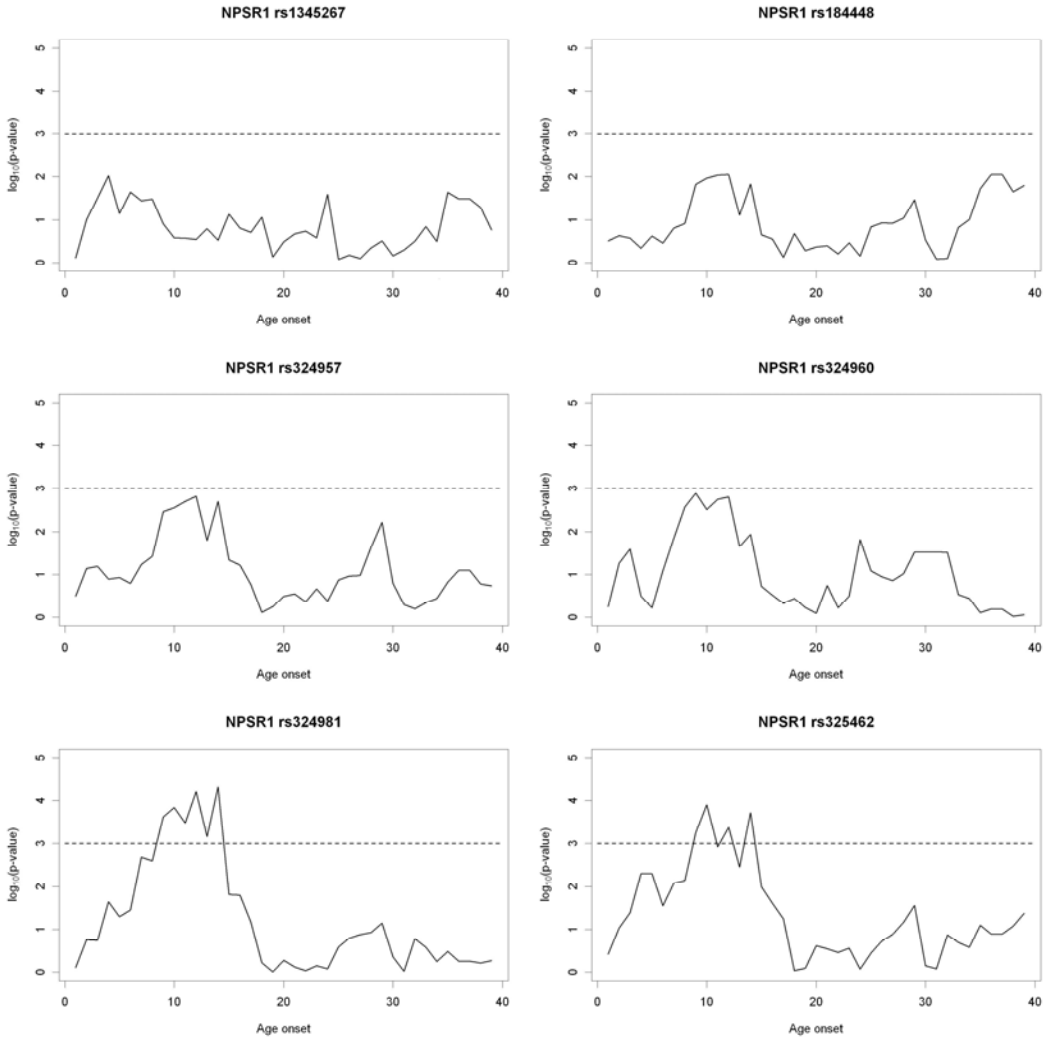
Supplementary Figure 2. Linkage Disequilibrium pattern in *NPSR1* gene.



Supplementary Table 5. Odds ratio and p-value of the association of selected SNPs with asthma and atopy either in baseline or follow-up evaluations and physician diagnosed asthma. Logistic regression performed under additive genetic model. Model adjusted by country, body mass index, age, sex and smoking status.

Gene	SNP	Asthma either in baseline or follow-up		Atopy either in baseline or follow-up		Persistent physician diagnosed asthma	
		OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value
NPSR1	rs714588	0.95(0.82-1.11)	0.5130	0.89(0.8-0.98)	0.0364	0.89(0.75-1.04)	0.1468
NPSR1	rs1023555	1.02(0.86-1.22)	0.7876	1.04(0.92-1.17)	0.2586	0.97(0.8-1.17)	0.7645
NPSR1	rs898070	0.97(0.83-1.13)	0.7091	0.92(0.83-1.03)	0.2356	0.85(0.72-1)	0.0539
NPSR1	rs963218	0.93(0.8-1.08)	0.312	0.91(0.83-1.01)	0.1041	0.84(0.71-0.98)	0.0296
NPSR1	rs1419835	0.92(0.76-1.1)	0.3627	0.94(0.83-1.06)	0.3108	0.99(0.81-1.2)	0.8964
NPSR1	rs765023	0.88(0.75-1.04)	0.1296	0.92(0.82-1.02)	0.1102	0.85(0.71-1.02)	0.0835
NPSR1	rs1345267	0.89(0.76-1.04)	0.1317	0.9(0.81-1)	0.0537	0.91(0.77-1.08)	0.2909
NPSR1	rs324381	0.85(0.72-1.01)	0.0676	0.93(0.83-1.05)	0.1423	0.86(0.72-1.04)	0.1153
NPSR1	hopo546333	0.9(0.66-1.22)	0.4881	0.93(0.76-1.14)	0.2067	0.78(0.56-1.1)	0.1553
NPSR1	rs184448	1.25(1.07-1.46)	0.0052	1.16(1.05-1.29)	0.0023	1.28(1.08-1.51)	0.0039
NPSR1	rs324396	1.22(1.03-1.44)	0.0200	1.17(1.04-1.3)	0.0013	1.27(1.07-1.51)	0.0075
NPSR1	rs324957	1.22(1.05-1.43)	0.0108	1.16(1.05-1.29)	0.0020	1.22(1.04-1.44)	0.0176
NPSR1	rs324960	0.86(0.73-1.01)	0.0641	0.89(0.79-0.99)	0.0223	0.77(0.64-0.91)	0.0029
NPSR1	rs10486657	0.82(0.67-1)	0.0537	0.94(0.83-1.08)	0.2933	0.95(0.77-1.17)	0.6307
NPSR1	rs324981	0.8(0.68-0.93)	0.0040	0.89(0.8-0.98)	0.0074	0.8(0.68-0.94)	0.0067
NPSR1	rs1419780	0.87(0.72-1.06)	0.1706	0.97(0.85-1.1)	0.6169	0.98(0.8-1.19)	0.8096
NPSR1	rs325462	0.88(0.76-1.02)	0.0967	0.91(0.82-1)	0.0247	0.9(0.76-1.05)	0.1777
NPSR1	rs727162	1.08(0.9-1.3)	0.3855	1.07(0.95-1.21)	0.2104	1.14(0.94-1.38)	0.1819
NPSR1	rs10250709	1.06(0.9-1.24)	0.5005	0.97(0.87-1.08)	0.2125	1(0.84-1.2)	0.9701
NPSR1	rs6958905	1.05(0.9-1.24)	0.5124	0.97(0.87-1.08)	0.1468	0.99(0.83-1.17)	0.8678
NPSR1	rs10238983	1.15(0.97-1.37)	0.1132	1.01(0.89-1.14)	0.8015	1.12(0.93-1.36)	0.2312

Supplementary Figure 3. P values for interaction from the multinomial logistic regression for each cut-off of age at onset for the 6 significant SNPs from *NPSR1*. Dotted line indicates the nominal p-value (0.05). Model adjusted by country, BMI, age, sex and smoking status.



Supplementary Table 6. Multinomial analysis of age at onset asthma and selected SNPs under the additive genetic model. Model adjusted by country, body mass index, age, sex and smoking status.

Gene	SNP	All (n=320)	Early onset (n=141)	Late onset (n=146)	P interaction	OR (95%CI) young	OR (95%CI) adult
ADAM33	rs6084432	0.0643	0.0358	0.3893	0.1179	1.46(1.03-2.08)	1.16(0.83-1.63)
ADAM33	rs512625	0.4923	0.1029	0.7146	0.1476	0.77(0.57-1.05)	1.05(0.8-1.39)
ADAM33	rs3918395	0.6101	0.7425	0.5638	0.4820	1.07(0.71-1.62)	1.12(0.77-1.63)
ADAM33	rs2787095	0.5403	0.6163	0.3478	0.7577	1.07(0.81-1.42)	1.13(0.87-1.47)
ADAM33	rs2853215	0.5194	0.2613	0.7181	0.5959	1.19(0.88-1.63)	1.06(0.79-1.41)
NPSR1	rs714588	0.6709	0.9704	0.4011	0.3358	1.01(0.76-1.33)	1.12(0.86-1.45)
NPSR1	rs1023555	0.6919	0.6322	0.2803	0.0738	0.92(0.65-1.3)	1.18(0.88-1.59)
NPSR1	rs898070	0.5251	0.5850	0.5193	0.9548	1.08(0.81-1.45)	1.09(0.84-1.43)
NPSR1	rs963218	0.7463	0.8617	0.4615	0.2642	0.98(0.74-1.29)	1.1(0.85-1.42)
NPSR1	rs1419835	0.5948	0.6106	0.4764	0.2294	0.91(0.64-1.3)	1.12(0.82-1.52)
NPSR1	rs765023	0.0533	0.0908	0.2495	0.6819	0.77(0.56-1.04)	0.84(0.63-1.13)
NPSR1	rs1345267	0.0437	0.2163	0.0655	0.5927	0.83(0.62-1.12)	0.77(0.58-1.02)
NPSR1	rs324381	0.0636	0.0167	0.8274	0.0465	0.66(0.47-0.93)	0.97(0.72-1.3)
NPSR1	hopo546333	0.1552	0.5170	0.0570	0.5711	0.83(0.47-1.46)	0.54(0.29-1.02)
NPSR1	rs184448	0.0002	0.0005	0.0281	0.1604	1.71(1.26-2.32)	1.36(1.03-1.78)
NPSR1	rs324396	0.0868	0.1340	0.3644	0.4345	1.27(0.93-1.74)	1.14(0.86-1.53)
NPSR1	rs324957	0.0010	0.0003	0.0850	0.0674	1.75(1.29-2.35)	1.27(0.97-1.67)
NPSR1	rs324960	0.0076	0.0067	0.1070	0.1431	0.64(0.46-0.88)	0.79(0.59-1.05)
NPSR1	rs10486657	0.1480	0.1300	0.7217	0.4827	0.75(0.51-1.09)	0.94(0.67-1.31)
NPSR1	rs324981	0.0004	0.0001	0.1331	0.0134	0.54(0.4-0.74)	0.81(0.62-1.06)
NPSR1	rs1419780	0.2508	0.2027	0.8431	0.5454	0.79(0.55-1.14)	0.97(0.7-1.34)
NPSR1	rs325462	0.0085	0.0007	0.2786	0.0243	0.6(0.44-0.81)	0.86(0.66-1.13)
NPSR1	rs727162	0.2055	0.4983	0.1303	0.8080	1.12(0.8-1.57)	1.26(0.93-1.71)
NPSR1	rs10250709	0.9173	0.1339	0.4532	0.1696	1.26(0.93-1.7)	0.9(0.67-1.19)
NPSR1	rs6958905	0.8683	0.1869	0.5290	0.2685	1.22(0.91-1.65)	0.91(0.69-1.21)
NPSR1	rs10238983	0.3122	0.1088	0.5028	0.3845	1.3(0.94-1.79)	1.11(0.82-1.51)
PHF11	rs4941643	0.5892	0.8979	0.5509	0.6020	1.02(0.77-1.35)	1.08(0.83-1.41)
PHF11	rs3794381	0.7659	0.8669	0.6443	0.8222	1.03(0.75-1.42)	0.93(0.69-1.26)
PHF11	rs2031532	0.5444	0.7365	0.6974	0.8267	1.05(0.78-1.42)	1.06(0.8-1.39)
PHF11	rs2247119	0.9888	0.9336	0.2890	0.3679	0.99(0.73-1.34)	0.85(0.64-1.14)
PHF11	rs8000149	0.5395	0.7714	0.6694	0.7938	1.04(0.78-1.4)	1.06(0.81-1.39)
PHF11	rs2274276	0.7719	0.9597	0.7194	0.9283	0.99(0.75-1.32)	0.95(0.73-1.24)
PHF11	rs7332573	0.5674	0.3589	0.5496	0.7510	1.25(0.78-2.01)	1.15(0.73-1.81)
PHF11	rs3829366	0.9772	0.9729	0.9163	0.7635	1(0.76-1.33)	1.01(0.78-1.31)
DPP10	rs4490198	0.6841	0.5135	0.5782	0.7004	1.1(0.83-1.45)	1.08(0.83-1.4)
DPP10	rs4849332	0.8870	0.9558	0.7912	0.9576	1.01(0.76-1.34)	1.04(0.79-1.35)
DPP10	rs1367179	0.3309	0.3149	0.7387	0.4404	1.2(0.84-1.7)	1.06(0.76-1.47)
DPP10	rs11123242	0.3442	0.2838	0.8479	0.3203	1.21(0.85-1.73)	1.03(0.74-1.45)
DPP10	rs13014858	0.5822	0.8610	0.2389	0.2990	0.98(0.74-1.29)	1.17(0.9-1.51)
DPP10	rs1430094	0.7140	0.5733	0.5544	0.8858	1.09(0.81-1.45)	1.09(0.83-1.42)
DPP10	rs1430093	0.7640	0.6349	0.5989	0.8794	1.07(0.8-1.44)	1.08(0.82-1.42)
DPP10	rs746710	0.8970	0.5098	0.7932	0.8234	1.1(0.83-1.45)	1.04(0.8-1.34)
DPP10	rs1430090	0.9266	0.1833	0.2678	0.0414	0.81(0.59-1.11)	1.17(0.89-1.54)
DPP10	rs6737251	0.9558	0.9323	0.7942	0.8518	1.01(0.75-1.37)	1.04(0.79-1.37)
DPP10	rs11685217	0.3727	0.6712	0.2603	0.7845	1.08(0.76-1.52)	1.19(0.88-1.62)
DPP10	rs1430097	0.9403	0.9734	0.9915	0.6705	1.01(0.75-1.35)	1(0.76-1.32)
DPP10	rs10496465	0.5831	0.4742	0.0934	0.0283	0.85(0.55-1.32)	1.36(0.95-1.94)
SPINK5	rs3756688	0.7304	0.7354	0.3126	0.2801	1.05(0.79-1.4)	0.87(0.67-1.14)
SPINK5	rs2303063	0.2836	0.5458	0.1315	0.5833	0.92(0.69-1.21)	0.82(0.63-1.06)
SPINK5	rs1422993	0.1002	0.0716	0.6142	0.2360	1.33(0.98-1.81)	1.08(0.81-1.44)
SPINK5	rs2400478	0.8229	0.4386	0.3703	0.2066	1.12(0.84-1.48)	0.89(0.68-1.16)

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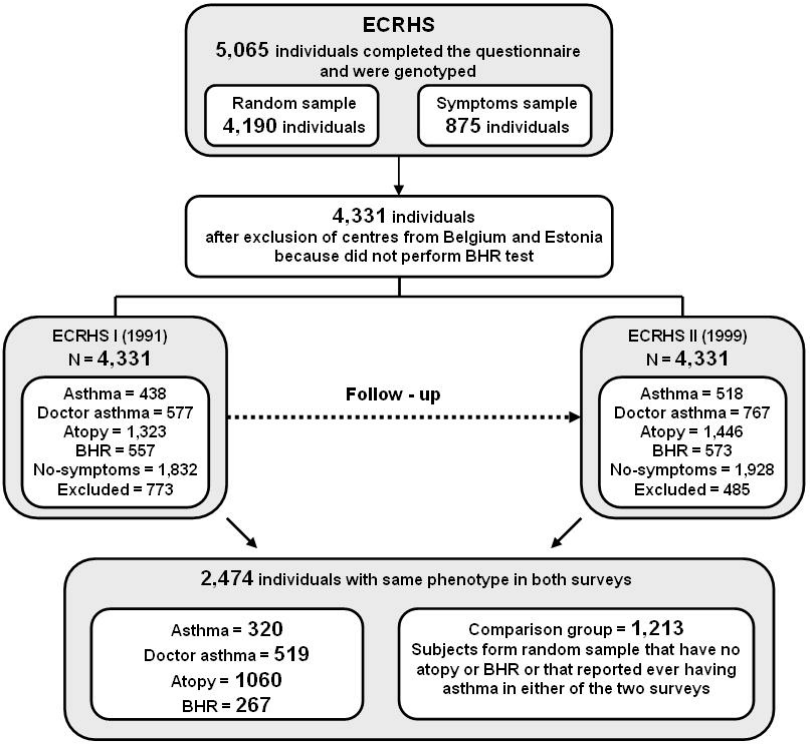
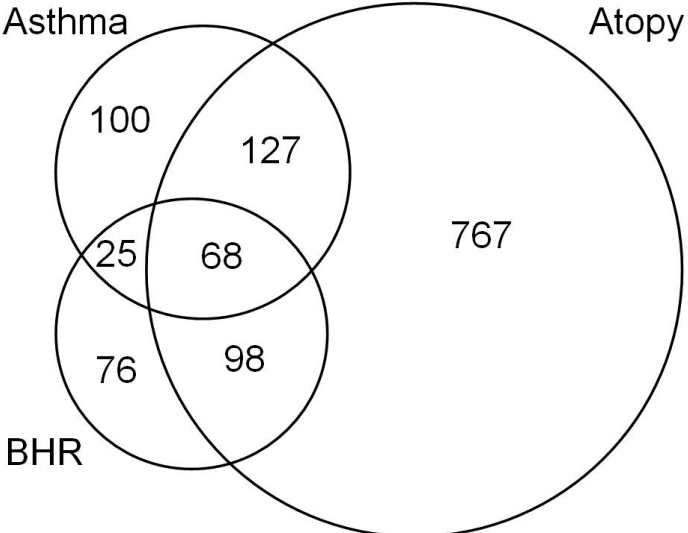
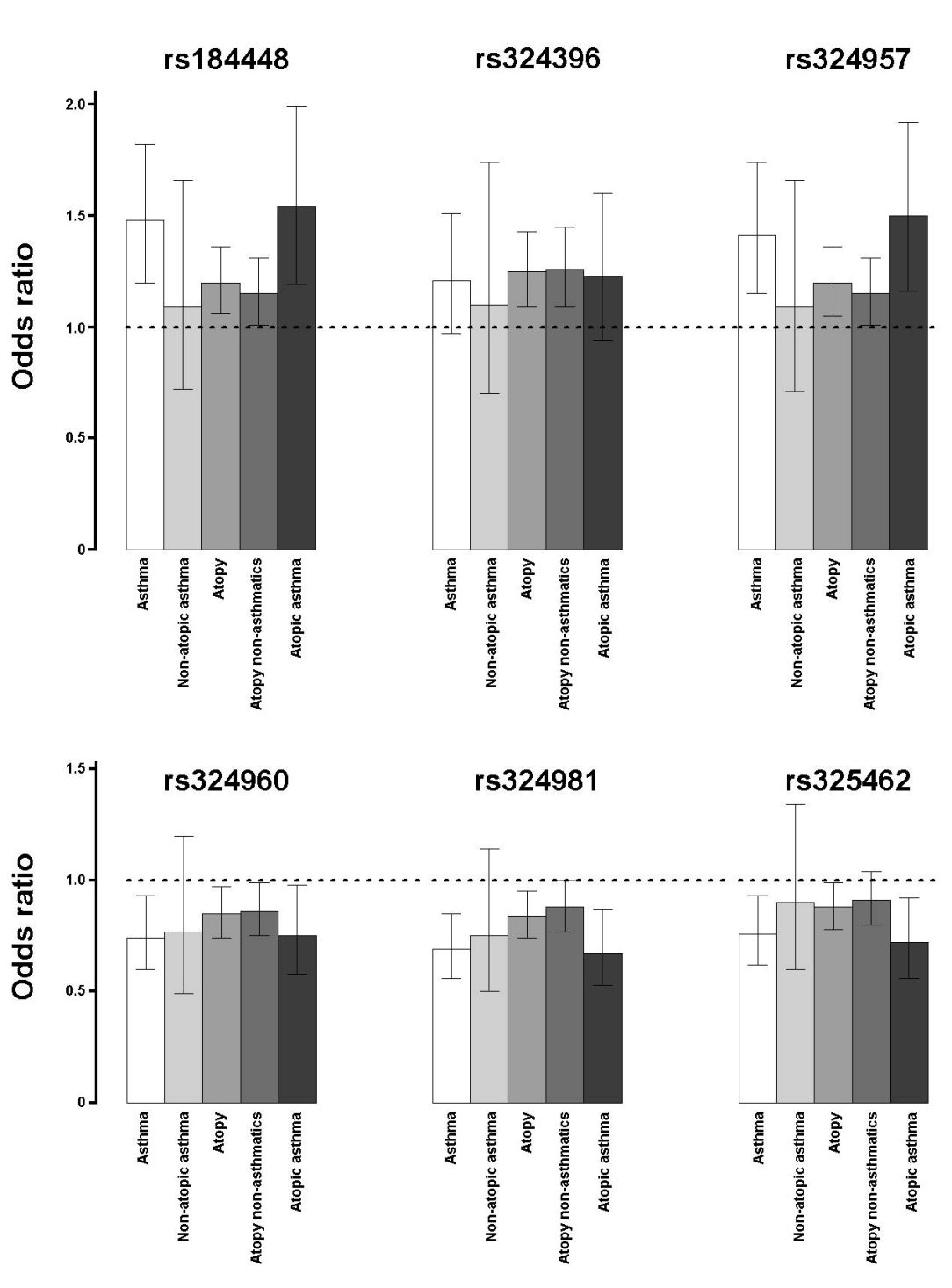
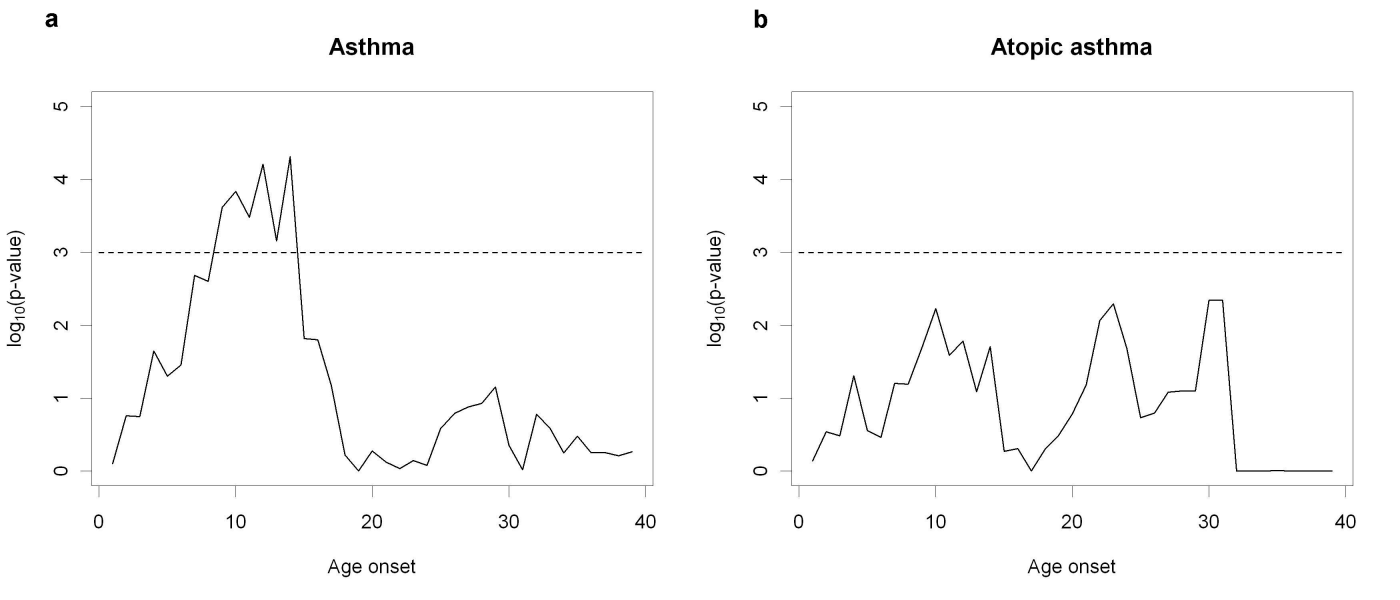

ECRHS
5,065 individuals completed the questionnaire and were genotyped
Random sample
4,190 individuals
Symptoms sample
875 individuals
4,331 individuals
after exclusion of centres from Belgium and Estonia because did not perform BHR test
ECRHS I (1991)
N = 4,331
Asthma = 438
Doctor asthma = 577
Atopy = 1,323
BHR = 557
No-symptoms = 1,832
Excluded = 773
Follow - up
ECRHS II (1999)
N = 4,331
Asthma = 518
Doctor asthma = 767
Atopy = 1,446
BHR = 573
No-symptoms = 1,928
Excluded = 485
2,474 individuals with same phenotype in both surveys
Asthma = 320
Doctor asthma = 519
Atopy = 1060
BHR = 267
Comparison group = 1,213
Subjects form random sample that have no atopy or BHR or that reported ever having asthma in either of the two surveys


Asthma
Atopy
100
127
25
68
767
76
98
BHR


rs184448
rs324396
rs324957
Odds ratio
2.0
1.5
1.0
0.5
0
Asthma
Non-atopic asthma
Atopy
Atopy non-asthmatics
Atopic asthma
rs324960
rs324981
rs325462
Odds ratio
1.5
1.0
0.5
0
Asthma
Non-atopic asthma
Atopy
Atopy non-asthmatics
Atopic asthma


a
Asthma
log10(p-value)
0
1
2
3
4
5
0
10
20
30
40
Age onset
b
Atopic asthma
log10(p-value)
0
1
2
3
4
5
0
10
20
30
40
Age onset