

ORIGINAL ARTICLE

SPINK5 and ADRB2 haplotypes are risk factors for asthma in Mexican pediatric patientsN. E. Martínez-Aguilar, MD, PhD¹, B. E. Del Río-Navarro, MD, MSc², E. Navarro-Olivos, MD, MSc³, H. García-Ortíz, PhD⁴, L. Orozco, MD, PhD⁴, and S. Jiménez-Morales, PhD⁴¹Escuela Superior de Medicina, Instituto Politécnico Nacional, México City, Mexico, ²Allergy and Immunology Department, Hospital Infantil de México, México City, México, ³Dirección de Enseñanza e Investigación, Secretaría de Salud de Guanajuato, Guanajuato, Gto. México, and ⁴Laboratory of Immunogenomics and Metabolic Diseases, Instituto Nacional de Medicina Genómica, SS, México City, Mexico**Abstract**

Background: Asthma is one of the most common respiratory diseases worldwide, and the complexity of its etiology has been widely documented. Chromosome 5q31-33 is one of the main loci implicated in asthma and asthma-related traits. *IL13*, *CD14* and *ADRB2*, which are located in this risk locus, are among the genes most strongly associated with asthma susceptibility. **Objectives:** This study evaluated whether single-nucleotide polymorphisms or haplotypes at 5q31-33 conferred risk for asthma in Mexican-Mestizo pediatric patients. **Methods:** We performed a case-controlled study including 851 individuals, 421 of them affected with childhood-onset asthma and 430 ethnically matched unaffected subjects. We used the TaqMan Allelic Discrimination Assay to genotype 20 single-nucleotide polymorphisms within *IL5*, *RAD50*, *IL13*, *IL4*, *CD14*, *SPINK5*, *HTR4*, *ADRB2* and *IL12B*. **Results:** Although no association was detected for any risk allele, three *SPINK5* haplotypes (GGCT: $p = 6 \times 10^{-6}$; AATC: $p = 0.0001$; AGTT: $p = 0.0001$) and five *ADRB2* haplotypes (AGGACC: $p = 0.0014$; AGGAAG: $p = 0.0002$; TGAGAG: $p = 0.0001$; AGGAAC: $p = 0.0002$; AAGGAG: $p = 0.003$) were associated with asthma. Notably, the AGTT *SPINK5* haplotype exhibited a male gender-dependent association ($p = 7.6 \times 10^{-5}$). **Conclusion:** Our results suggest that *SPINK5* and *ADRB2* haplotypes might play a role in the susceptibility to childhood-onset asthma.

Keywords5q31-33 locus, *ADRB2*, asthma, case-controlled study, pediatric patients, *SPINK5***History**Received 14 May 2014
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Published online 8 October 2014**Introduction**

Asthma is a chronic respiratory disease that has a high prevalence worldwide. It is characterized by recurrent wheezing, shortness of breath, chest tightness and chronic cough. This pathology involves a complex etiology, but many reports have demonstrated the influence of a genetic component for the risk for developing asthma [1–4]. Chromosome 5q31-33 is one of the main regions implicated in susceptibility to asthma and asthma-related traits [1,5–8]. This locus harbors multiple genes that may influence atopic responses, inflammation and drug responses. The genes identified in this region include Th2 profile interleukins such as *IL4*, *IL5*, *IL9* and *IL13*; cluster differentiation 14 (*CD14*); a DNA repair protein (*RAD50*); protocadherin-1 (*PCDH1*); serine protease inhibitor kazal type 5 (*SPINK5*); and genes involved in the therapeutic response to asthma, such as β 2-adrenergic receptor (*ADRB2*), glucocorticosteroid receptor (*GCR*), and

hydroxytryptamine receptor 4 (*HTR4*) [2–4,8–10]. Polymorphisms in *IL4*, *IL13* and *CD14* are some of the variants most robustly associated with susceptibility to asthma and bronchial hyperresponsiveness (BHR), total serum IgE levels, and the clinical course of the disease [2,4,8,10]. The *IL13* Arg130Gln single-nucleotide polymorphism (SNP), which modulates a set of genes relevant to asthma pathogenesis including *STAT6* and *CD23*, is one of the most robustly associated [11]. SNPs in *ADRB2*, mainly the Arg16Gly and Gln27Glu variants, have been related to the clinical course of the disease and to drug response [9,12–14].

Use of a transgenic mouse model led to the identification of a Th2 locus control region at 5q21-35, suggesting a potential contribution to asthma. Recently, a genome-wide association study provided evidence for the role of multiple SNPs in the *RAD50-IL13* region in susceptibility to asthma and asthma-related quantitative traits [15–17].

Despite the wide replication of results suggesting associations between asthma and *IL4* and *IL13*, previous studies that included adult Mexican patients detected no distribution differences in *IL4* C590T and *IL13* Arg130Gln between asthmatic cases and controls [18,19]. The lack of association of highly reproduced polymorphisms in many ethnic groups highlights the genetic heterogeneity underlying asthma and

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emphasizes the need for investigation of candidate genes in each population. The aim of this study was to evaluate whether 5q31-33 SNPs or haplotypes confer risk to asthma in Mexican-Mestizo pediatric patients.

Methods

Study population

Four hundred and twenty-one asthmatic patients recruited from four tertiary-level institutions located in Mexico City were enrolled in the current study (Table 1). A physician diagnosed asthma based on the presence of a history of shortness of breath, wheezing due to chest tightness, spirometry test and positive skin prick test (criteria from the American Thoracic Society and the Global Initiative for Asthma). All patients were younger than 17 years of age and the mean ($\pm$ standard deviation) age at onset of asthma was 10.8 ± 2.9 years and the gender distribution were 263 males (62%) and 158 females (38%). Most children had intermittent asthma (55%) whereas mild and moderate disease was present in 17% and 27%, respectively. Severe asthma represented the lowest percentage ($>1\%$). A battery of 25 aeroallergens (*Aspergillus fumigatus*, *Alternaria* sp., *Mucor* sp., *Blattella germanica*, *Periplaneta americana*, *Penicillium* sp., cat, dog, horse, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Ambrosia* sp., *Artemisia ludoviciana*, *Cynodon dactylon*, *Chenopodium album*, *Quercus robur*, *Fraxinus* sp., *Helianthus annuus*, *Ligustrum vulgare*, *Lolium perenne*, *Plantago lanceolata*, *Rumex crispus*, *Schinus molle*, *Salsola* sp. and *Phleum pratense*) common in Mexico City was used for skin prick testing as described previously [20,21]. Skin prick test data were available in 322 cases, and 266 (83%) of cases had a positive skin test to at least one aeroallergen.

We included 430 adults unrelated ethnically and sex-matched healthy individuals subjects as a control group. All of them were enrolled from a blood bank in Mexico City, identified themselves without symptoms or a past history of asthma, atopy, autoimmune or other pulmonary diseases and no first-degree relatives with a history of asthma. The gender distribution in the healthy controls was: 254 (59%) males and 176 (41%) females.

Table 1. Clinical features of subjects included in the present study.

Clinical characteristics	Mean (SD) or n (%)
Age at the study (years)	421 10.8 ± 2.9
Gender	
Male	263 (62)
Female	158 (38)
Atopy ^a	268 (83)
N = 322	
Asthma severity ^a	
N = 341	
Intermittent	189 (55)
Mild persistent	57 (17)
Moderate persistent	93 (27)
Severe persistent	2 (<1)

SD: standard deviation.

^aData are missing from several cases, atopy: positive skin test to at least one aeroallergen.

All patients and controls were descended from parents and grandparents born in Mexico and all of them were recruited from public hospitals located in Mexico City. Furthermore, the cases and controls had previously been ethnically matched using 10 ancestry-informative markers that mainly identify the two primary parental populations of the Mexican-Mestizo population (Amerindian and European) [22]. This study was conducted with the approval of the ethics and research committees from all participating institutions. Written informed consent was obtained from each individual, parents provided consent for the child's participation with the child's consent.

Genotyping analysis

Genomic DNA was extracted from blood samples using the standard salting out technique (Qiagen, Valencia, CA). Twenty SNPs in the 5q31-33 locus were included considering two key selection criteria: (a) their potential function (coding SNP, miRNA binding site and transcription factor binding site) [http://snpinform.nih.gov/snpfunc.htm], and (b) previous association data with asthma and its related phenotypes in several populations (Table 2) [4,8,11,13,16,17]. Genotyping of 20 polymorphisms at the 5q31-33 loci (rs56753728, rs2244012, rs1800925, rs20541, rs4986964, rs2569190, rs6892205, rs2303067, rs777134, rs4349706, rs6580550, rs17778257, rs2053044, rs1042713, rs1042717, rs1042718, rs1042719, rs6870828, rs3213096 and rs3181217) was performed using the TaqMan Allelic Discrimination Assay (Applied Biosystems, Foster City, CA). Polymerase chain reaction was carried out using the ABI PRISM 7900 system following established conditions [22].

The overall genotype call rate by TaqMan was 99.9%, and genotyping reproducibility was 100% in 180 randomized samples genotyped in duplicate. To confirm the SNP genotypes, three random samples were sequenced for each genotype (heterozygote and both homozygotes). Polymerase chain-reaction products were sequenced directly with the DNA Sequencing Kit with the BigDye Terminator on an automated ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA). The forward and reverse primers were designed using primer3 version 4.0 [23].

Statistical analysis

Testing for Hardy–Weinberg equilibrium in cases and controls was performed using the FINETTI software (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). Association between all the SNPs and asthma was analyzed with chi-squared tests on 2×2 and 2×3 contingency tables for allele and genotype frequencies, respectively. Statistical analysis and odds ratios (ORs) with 95% confidence intervals (95% CIs) were performed with StatCalc (Epi Info 2005 V.3.2; Centers for Disease Control and Prevention, Atlanta, GA). Haplotype frequencies and linkage disequilibrium (LD; r^2) were evaluated with Haploview [24]. We performed multiple tests for association to avoid spurious results. p Values were corrected by applying a permutation test based on 100 000 permutations; since we analyzed 20 SNPs, $p < 0.0025$ was set as the significance threshold for an α level of 0.05. Statistical power was estimated using QUANTO version 1.2 (<http://hydra.usc.edu/>)

GxE) and considering the childhood-onset asthma prevalence in Mexico (8%) [25] and both the minor allele frequencies and ORs (under the additive model) identified in the present study.

Although the pairwise r^2 values between some of the SNPs were low, all of the informative SNP studied were incorporated in the haplotype analysis.

Results

With the exception of rs4986964, which was not polymorphic in our population, all SNP genotype distributions were in Hardy–Weinberg equilibrium among cases and controls (data not shown). Comparative analysis of all SNPs did not show significant differences in allele or genotype frequencies between asthma patients and healthy controls (Table 3). After gender stratification, only the heterozygote genotype of *IL5* rs56753728 showed significantly decreased risk of asthma (CC versus CT: OR = 0.73, 95% CI 0.54–0.98, $p = 0.03$) in the female population.

Haplotyping analysis revealed that an *IL12β* CGA haplotype, harboring two minor (rs6870828C and rs3181217A) alleles and one major (rs3213096G) alleles, occurred more frequently in asthma cases than in controls (2.8% versus 1.1%, respectively; OR = 2.57, 95% CI 1.16–5.81, $p = 0.009$). However, these differences were not statistically significant after multiple comparison correction ($p = 0.09$).

Interestingly, two LD blocks involving *SPINK5* and *ADRB2* variants showed statistically significant differences in distribution between cases and controls. The *SPINK5* block (rs6892205 A/G, rs2303067 A/G, rs3777134 C/T, and rs4349706 C/T) exhibited eight haplotypes with frequencies >0.01 (GGTT, AACC, AATT, GGCT, AATC, AGTT, GACC and GGCC). The GGCT, AATC and AGTT haplotypes were significantly associated with asthma (GGCT: OR = 3.99, 95% CI 2.02–8.02, $p = 6 \times 10^{-6}$; AATC: OR = 3.58, 95% CI

1.69–7.78, $p = 0.0001$; AGTT: OR = 0.25, 95% CI 0.10–0.57, $p = 0.0001$). Statistical significance persisted for the three haplotypes after multiple comparison tests (Table 4), although the AGTT haplotype was only significant in males (OR = 0.13, 95% CI 0.04–0.4, $p = 2.3 \times 10^{-5}$).

Although the *ADRB2* SNPs showed low LD with each other, 5/9 haplotypes significantly differed between cases and controls. The haplotype AGGAAC that consists of three major and three minor alleles was associated with decreased risk for asthma (OR = 0.68, 95% CI 0.55–0.83, $p = 0.0014$), while the other four haplotypes were associated with susceptibility to asthma (AGGACC: OR = 1.80, 95% CI 1.2–2.71, $p = 0.002$; TGAGAG: OR = 2.65, 95% CI 1.52–4.65, $p = 0.0001$; AGGAAG: OR = 3.74, 95% CI 1.7–8.46, $p = 0.0002$; AAGGAG: OR = 4.41, 95% CI 1.39–15.54, $p = 0.003$). However, the gender stratification analysis displayed a female gender-dependent association between four *ADRB2* haplotypes (AGGACC: $p = 0.0003$; AGGACC: $p = 9.3 \times 10^{-6}$; TGAAG: $p = 0.0011$; AGGAAG: $p = 0.0007$) and asthma.

Unexpectedly, the haplotype analysis involving only the Arg175Arg (rs1042718A/C) and Gly351Gly (rs1042719C/G) previously associated with *ADRB2* translational efficiency, exhibit an association between haplotypes AC (OR = 0.72, 95% CI 0.59–0.89, $p = 0.002$) and AG (OR = 3.03, 95% CI 2.3–4.53, $p = 3.4 \times 10^{-8}$) and asthma. Actually, the AG haplotype showed an even stronger association than any haplotype containing all six SNPs. Significant associations between these haplotypes and asthma remained after multiple comparisons.

The association analysis between patients with mild asthma (intermittent and mild persistent) and severe asthma (moderate persistent and severe) did not show statistical significance in allele or haplotype frequencies. However, the comparative analysis including healthy subjects as a control group, showed statistical differences in the distribution of the AATC ($p = 4.12 \times 10^{-5}$) and AGTT ($p = 9.0 \times 10^{-6}$) *SPINK5* haplotypes

Table 2. Genes and polymorphisms included in the present study.

Location ^a	Gen	SNP	Alleles	Position into the gene	Associated phenotype or potential function
Chr5:131877406	<i>IL5</i>	rs56753728	C/T	3'UTR	miRNA binding site ^b
Chr5:131901225	<i>RAD50</i>	rs2244012	A/G	Intron	Asthma
Chr5:131992809	<i>IL13</i>	rs1800925	C/T	5' near gene	Asthma, rhinitis, atopy, TFBS ^b
Chr5:131995964		rs20541	A/G	Pro144Arg	Asthma
Chr5:132009821	<i>IL4</i>	rs4986964	C/T	Arg27Cys	Asthma/Breakage of a disulphide bond ^c
Chr5:140012916	<i>CD14</i>	rs2569190	A/G	Intron	Asthma, asthma severity, eczema/TFBS ^b
Chr5:147475386	<i>SPINK5</i>	rs6892205	A/G	Arg267Gln	Dermatitis
Chr5:147480955		rs2303067	A/G	Glu420Lys	Eczema
Chr5:147478212		rs3777134	C/T	Arg711Gln	Splicing, protein structure ^c
Chr5:147516598		rs4349706	C/T	3'UTR	miRNA binding site ^b
Chr5:147856232	<i>HTR4</i>	rs6580550	C/T	Intron	miRNA binding site ^b
Chr5:148204577	<i>ADRB2</i>	rs17778257	A/T	5' near gene	TFBS ^b
Chr5:148205372		rs2053044	A/G	5' near gene	TBS ^b
Chr5:148206440		rs1042713	A/G	Gly16Arg	Asthma, asthma severity,
Chr5:148206646		rs1042717	A/G	Leu84Leu	Asthma
Chr5:148206917		rs1042718	A/C	Arg175Arg	Protein translation
Chr5:148207447		rs1042719	C/G	Gly351Gly	Protein translation
Chr5:158738512	<i>IL12B</i>	rs6870828	C/T	5' near gene	–
Chr5:158750329		rs3213096	A/G	Ile33Val	ESE/ESS ^b
Chr5:158752270		rs3181217	A/G	Intron	–

ESE: Exonic splicing enhancer; ESS: exonic splicing silencer; TFBS: transcription factor binding sites.

^aPositions are from NCBI dbSNP, Short Genetic Variations. Created/Update in build: 129/137 for Human; ^b<http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm> or ^c<http://www.snps3d.org/>.

Table 3. Genotype and allele frequencies of the 5q31-33 SNPs in asthma affected and unaffected Mestizo-Mexican subjects.

Gene	Polymorphism	Allele (1/2)	Population	Genotype frequencies			Allele frequencies		<i>p</i>	
				11	12	22	1	2	Genotype	Allele
<i>IL5</i>	rs56753728	C/T	Control	0.39	0.50	0.11	0.64	0.36	0.047 ^a	0.69
			Case	0.45	0.41	0.14	0.65	0.35		
<i>RAD50</i>	rs2244012	A/G	Control	0.98	0.02	0.0	0.98	0.02	0.92	0.85
			Case	0.98	0.01	0.0	0.99	0.01		
<i>IL13</i>	rs1800925	C/T	Control	0.43	0.44	0.13	0.65	0.36	0.23	0.15
			Case	0.48	0.42	0.10	0.69	0.31		
	rs20541	A/G	Control	0.41	0.44	0.15	0.63	0.37	0.55	0.39
			Case	0.37	0.48	0.15	0.65	0.35		
<i>CD14</i>	rs2569190	A/G	Control	0.27	0.46	0.27	0.5	0.5	0.36	0.23
			Case	0.23	0.49	0.28	0.47	0.53		
<i>SPINK5</i>	rs6892205	G/A	Control	0.57	0.36	0.07	0.75	0.25	0.47	0.3
			Case	0.61	0.32	0.07	0.78	0.22		
	rs2303067	G/A	Control	0.59	0.35	0.06	0.76	0.24	0.86	0.84
			Case	0.59	0.34	0.07	0.68	0.32		
	rs3777134	T/C	Control	0.65	0.29	0.06	0.78	0.22	0.28	0.70
			Case	0.63	0.28	0.09	0.77	0.23		
	rs4349706	T/C	Control	0.66	0.29	0.06	0.80	0.20	0.87	0.83
			Case	0.64	0.30	0.06	0.79	0.21		
<i>HTR4</i>	rs6580550	C/T	Control	0.66	0.28	0.06	0.80	0.20	0.17	0.14
			Case	0.59	0.34	0.07	0.76	0.24		
<i>ADRB2</i>	rs17778257	A/T	Control	0.38	0.47	0.15	0.61	0.39	0.33	0.21
			Case	0.35	0.46	0.19	0.58	0.42		
	rs2053044	G/A	Control	0.72	0.25	0.03	0.84	0.16	0.58	0.78
			Case	0.69	0.28	0.03	0.83	0.17		
	rs1042713	G/A	Control	0.33	0.49	0.18	0.58	0.42	0.29	0.43
			Case	0.36	0.44	0.22	0.56	0.44		
	rs1042717	G/A	Control	0.33	0.49	0.18	0.58	0.42	0.9	0.67
			Case	0.34	0.44	0.22	0.56	0.44		
	rs1042718	C/A	Control	0.32	0.52	0.16	0.58	0.42	0.7	0.93
			Case	0.34	0.48	0.18	0.58	0.42		
	rs1042719	G/C	Control	0.26	0.50	0.24	0.51	0.49	0.1	0.09
			Case	0.33	0.46	0.21	0.56	0.43		
<i>IL12B</i>	rs6870828	T/C	Control	0.43	0.47	0.10	0.66	0.34	0.91	0.75
			Case	0.43	0.46	0.11	0.66	0.34		
	rs3213096	G/A	Control	0.99	0.01	0	0.99	0.01	0.3	0.14
			Case	0.99	0.01	0	0.99	0.01		
	rs3181217	G/A	Control	0.30	0.51	0.19	0.55	0.45	0.73	0.61
			Case	0.30	0.53	0.18	0.56	0.44		

^aNo statistical significance remained after Bonferroni correction test.

between mild asthma and controls subjects. Meanwhile, the *AGGAAC* ($p=0.001$), *AGGACC* ($p=0.004$), *TGAGAG* ($p=0.005$) *ADRB2* haplotypes were associated with mild asthma and the *AGGAAG* ($p=6.67^{-9}$) with the more severe forms of this respiratory disease.

Discussion

One of the most important loci for asthma susceptibility is located at 5q31-33, which harbors the genes that are most robustly associated with this disease and its related phenotypes (allergy, atopy BHR, etc.). Furthermore, several studies in animal models have provided evidence supporting the participation of this locus in asthma-related phenotypes. In a mouse model, McIntire et al. [26] detected a deletion at 5q23-35 related to BHR development and low production of the IL4 and IL13 cytokines; these mice displayed a drastic reduction in lung inflammation, eosinophil infiltration and mucosa production after induction of allergic asthma. To evaluate whether DNA variations at 5q31-33 confer risk to asthma in Mexican-Mestizo pediatric patients, we performed an association study in ethnically matched cases and controls. When we compared cases and controls, no statistically significant

differences were observed in the frequencies of 20 SNPs located at 5q31-33. A few Mexican studies have investigated genes located at 5q31-33; studies including Mexican adults or children were not able to replicate the association of *IL13* and *IL4* SNPs with asthma [18,19,27]. These findings, which are in contrast to results from investigations of other populations, may be due to the complex genetic background of the Mexican population, which stems from the mixture of races during conquest, but also could be due to differential interaction between genotype and environmental background in these populations [2,28]. In addition, we cannot exclude the possibility that there are other asthma-susceptibility SNPs in the 5q31-33 region, since polymorphism screening did not cover the entire locus [29–32].

One gene-asthma interaction observed in this study involved *IL5*, a cytokine that places key roles in allergy and eosinophilic inflammation [33]. We noted that the *TC* genotype of *IL5* rs56753728 confers protection against asthma. Functional-effect evaluation predicted that this SNP is located in the hsa-miR-516b binding sequence on the *IL5* 3' untranslated region [34]. It is possible that this SNP modifies miRNA-*IL5* binding and diminishes *IL5* expression via degradation of its mRNA [35].

Table 4. Haplotype frequencies of *SPINK5* and *ADRB2* SNPs in case and control groups.

Block	Haplotype	Frequency		OR[95% CI]	<i>p</i>
		Healthy controls <i>n</i> (%)	Asthmatic cases <i>n</i> (%)		
<i>SPINK5</i> ^a					
1: rs6892205 G/A, rs2303067 G/A, rs3777134 T/C, rs4349706 T/C	<i>GGTT</i>	607 (0.71)	580 (0.69)	0.92 [0.75–1.14]	0.44
	<i>AACC</i>	135 (0.16)	117 (0.14)	0.87 [0.66–1.14]	0.29
	<i>AATT</i>	31 (0.04)	25 (0.03)	0.82 [0.46–1.44]	0.46
	<i>GGCT</i>	12 (0.01)	45 (0.06)	3.99 [2.02–8.02]	6 × 10^{−6}*
	<i>AATC</i>	10 (0.01)	34 (0.04)	3.58 [1.69–7.78]	0.0001*
	<i>AGTT</i>	32 (0.04)	8 (0.01)	0.25 [0.10–0.57]	0.0001*
	<i>GACC</i>	17 (0.02)	9 (0.01)	0.54 [0.22–1.28]	0.18
	<i>GGCC</i>	10 (0.01)	9 (0.01)	0.92 [0.34–2.45]	0.96
<i>ADRB2</i> ^a					
2: rs17778257 A/T, rs2053044 G/A, rs1042713 G/A, rs1042717 G/A, rs1042718 C/A, rs1042719 G/C	<i>AGGAAC</i>	326 (0.38)	246 (0.29)	0.68 [0.55–0.83]	0.0014*
	<i>TGAGCG</i>	274 (0.32)	242 (0.29)	0.86 [0.7–1.07]	0.16
	<i>AAGGCG</i>	112 (0.13)	83 (0.10)	0.73 [0.53–1.00]	0.04
	<i>AGGACC</i>	43 (0.05)	73 (0.09)	1.80 [1.2–2.71]	0.002*
	<i>TGAGCC</i>	42 (0.05)	41 (0.05)	1.0 [0.63–1.58]	0.72
	<i>TGAGAG</i>	20 (0.02)	50 (0.06)	2.65 [1.52–4.65]	0.0001*
	<i>AGGAAG</i>	9 (0.01)	32 (0.04)	3.74 [1.7–8.46]	0.0002*
	<i>AAAGCG</i>	14 (0.02)	14 (0.02)	1.01 [0.46–2.28]	0.95
	<i>AAGGAG</i>	4 (0.004)	17 (0.02)	4.41 [1.39–15.54]	0.003*

^ahaplotypes >1% are showed,
*p Values remained statistically significant after 100 000 permutations (words in bold).

Likewise, this study revealed that *SPINK5* and *ADRB2* haplotypes, rather than single SNPs, may be involved in susceptibility to asthma, contributing to its pathogenesis. On the other hand, SNPs in *SPINK5* have been consistently associated with atopic dermatitis and allergic rhinitis, but their association with asthma remains controversial. We did not observe allelic or genotype association with asthma, consistent with results from Chinese, Dutch and German populations [36–38]. However, we identified an association between *SPINK5* haplotypes and childhood-onset asthma in Mexicans. Furthermore, the association of the *AGAT* haplotype was male gender-dependent. Since the LD patterns of the *SPINK5* SNPs in our study were very similar to those published elsewhere [6,39], haplotype association should be carried out in populations in which isolated SNPs in *SPINK5* are not associated with asthma. Despite the genetic studies, which have shown that variants in *SPINK5* may be associated with atopic diseases and its related phenotypes, the functional role of *SPINK5* in asthma still remain unclear. Recently, it was reported that *SPINK5* is involved in the production of pro-inflammatory molecules, including IL-6, IL-8 and RANTES in epithelial cells, suggesting that *SPINK5* could play an important role in asthma pathogenesis [40]. In this regard, all SNPs comprising the *SPINK5* haplotypes have a potential function, three non-synonymous and one in the 3'UTR. In fact, the Arg711 residue affected by rs3777134 (Arg711Gln) is highly conserved in mammals and prediction function tools report that the Gln variant is likely to be not tolerable. Moreover, the rs4349706 (3'UTR) *SPINK5* variant is predicted to be a potential binding site for several members of the pro-inflammatory *hsa-let-7* family gene, which has also an important role in the asthma development [41].

Otherwise, the gender-dependent associations documented in the present study are common in case-control studies of immune-mediated diseases, including asthma [22,42,43]. For example, we previously reported an association between *TNFα* and *MMP9* SNPs and asthma in females [22,44]. Ours

and other reports of gender association suggest that the interaction between genotype and sexual hormones plays a role during the development of asthma.

On the other hand, although *ADRB2* has been extensively studied for its pharmacogenetic effects on β2-AR agonist responses, the contributions of single *ADRB2* SNPs to asthma risk have not been clarified. Many studies of asthmatic patients have focused on the role of the *ADRB2* SNPs, especially those affecting codons 16 and 27, in lung function, BHR, and asthma management, but controversial results have been reported [45–49]. Recently, an *ADRB2* haplotype has been reported to possibly contribute to increased asthma risk in a Chinese population [49]. In addition, in Caucasians the Arg16Gly/Gln27Gln (rs1042713/rs1042714) haplotype was identified as a risk factor to severe asthma exacerbations [50].

It has been reported that *ADRB2* polymorphisms are associated with bronchodilator response in Puerto Ricans but not in Mexican children [13]. As well, the heterozygous genotype Arg16Gly (rs1042713) has been associated with severe asthma in children [51,52], however, in this study we analyzed six *ADRB2* variants, including the rs1042713 SNP, and no significant differences of genotype and allele frequencies were observed between asthmatic patients and healthy controls, even stratifying by severity. This discrepancy could be due to ethnic differences, but we cannot discard that the small sample size of moderate/severe cases included in the present study may be a confounding factor.

Nevertheless, we identified five *ADRB2* haplotypes associated with asthma. Interestingly, the haplotypes harboring the AC minor alleles of Arg175Arg (rs1042718) and Gly351Gly (rs1042719) conferred protection (OR = 0.72) against asthma in our patients, while the AGGACC, TGAGAG, AGGAAG and AAGGAG haplotypes were associated with asthma risk. Similarly, protective and risk-conferring *ADRB2* haplotypes have also been associated with asthma and lung function in Asian and Caucasian populations [51,52]. Moreover, Zhao et al. [53] reported that

the haplotype AC, containing minor synonymous alleles of rs1042718 and rs1042719, markedly reduced *ADRB2* translational efficiency compared with wild-type haplotype CG in transfected HEK293 cells. There is evidence indicating that certain synonymous SNPs involving frequent-to-rare codon substitutions may result in ribosome stalling, due to a lower concentration of cognate tRNAs or to alteration of the RNA structure [54]. However, it is still not clear how reduced translation of *ADRB2* caused by these genetic variants could confer a low risk for asthma development.

In the present study, the age of the case patients (<17 years old) and healthy controls (>18 years) were significantly different; however, given that we found similar frequencies of the risk alleles between children (cases) and adults (controls), age was not considered as a confounding factor. Moreover, in a study testing the relationship between common *ADRB2* variants and human longevity in Chinese healthy people, the synonymous rs1042718 and rs1042719 SNPs and their haplotypes tended to be associated with longevity [53]. Those results would suggest that we might expect to find a higher frequency of these alleles in our controls in comparison with cases contrary to what we actually observed.

Various *ADRB2* haplotypes have been described, and data from other reports have suggested that ethnicity may modify the association between *ADRB2* haplotypes and human diseases and that rare haplotypes are also associated with genetic predisposition to those entities, including asthma [55–57]. Numerous evidences show that non-synonymous SNPs could drive differences in the transcriptional activation, protein expression and cellular localization of the mutant protein and have a potential pathological effect [58,59]. For instance, Panebra et al. 2007 demonstrated that non-synonymous SNPs showing varying levels of LD with *ADRB2* 5'UTR SNPs (far upstream position) have a negative effect in obstructive lung phenotypes through altered transcription factor binding [59]. So, specific coding variants contained in a haplotype might have an additive biological effect on the secondary structure of the mRNA, which, in addition with variants (3'UTR) that potentially can result in gene dysregulation at either transcriptional or post-transcriptional level, could impact the number of peptides with the appropriate structure and activity. We performed a correction by permutation testing using multiple comparisons, and our study, particularly for haplotype analysis, is still underpowered; so, we interpret with caution our haplotype asthma-associated data. Replication in future studies with independent cohorts or populations with the same background and age-matched could confirm these findings.

In conclusion, extensive haplotype analysis in the asthmatic population could benefit the identification of ethnic-specific haplotypes and might provide a better understanding of the ethnic and individual differences in asthma risk and drug responses.

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Declaration of interest

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