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Genetic variants in RORA are associated with asthma and allergy markers in an admixed population

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ABSTRACT

Asthma and allergy affect hundreds of millions of people from childhood to old age. In most of them, the inflammatory process of respiratory allergies involves the participation of type 2 cytokines, derived from T helper-2 (Th2)-cell, and Group 2 Innate Lymphoid (ILC2) Cells. An efficient memory Th2 cell response is dependent on IL-13 produced by ILC2s, causing allergic lung inflammation and elevated serum levels of immunoglobulin E. ILC2 cells are derived from common lymphoid progenitors and their growing depends on the transcription factor RORA. The aim of this work was to identify genetic variants in RORA associated with asthma phenotypes and allergy markers. Genomic DNA samples of 1246 individuals participating from Social Changes Asthma and Allergy in Latin America Program (SCAALA) have been genotyped using Illumina Human 2.5 Omni Beadchip. Logistics regressions have been performed to analyze the association among RORA variants and asthma, skin prick tests (SPT), specific IgE and type 2 cytokine production. Twelve single nucleotide variants (SNVs) were significantly associated with atopy ($P < 0.01$), in which four of them, rs10162630, rs17191519, rs17270243, and rs55796775 and their haplotypes were strongly and positively associated ($P < 0.001$). Furthermore, these variants increased the RORA gene expression in silico analysis. Other SNVs in RORA were associated with allergy markers, atopic and non-atopic asthma. Therefore, it is believed that variants in RORA gene may influence immunologic features of asthma and allergies and could be possible targets for future treatment of allergic diseases.

1. Introduction

The major chronic respiratory diseases include asthma and allergic rhinitis, which affect hundreds of millions individuals worldwide. The frequency of these diseases has caused prevalence rates to increase in both developed and developing countries [1]. Clinical asthma features include wheezing, mucus production, airflow limitation and bronchial hyperreactivity to environmental stimuli [2]. Among several asthma phenotypes, two are very well characterized: (1) atopic asthma, defined by positive serum specific Immunoglobulin E (IgE) antibodies and/or skin prick test (SPT) positive to any tested allergen; (2) non-atopic asthma, with absence of allergen-specific serum IgE and/or SPT and is associated with air pollution, including ozone, cigarette smoke and diesel particulates, and higher frequency in adult women [3].

The inflammatory process of respiratory allergies involves interactions and cooperation of many cell types, causing a type 2 immune response [4,5] mediated by eosinophils, mast cells, basophils, Th2 cells, Group 2 Innate Lymphoid (ILC2) cells and IgE-producing B cells. Th2 cells are sources of type 2 cytokines, such as IL-4, IL-5, IL-9 and IL-13. These cytokines are responsible for unleashing a cascade of events, such as IgE-triggered hypersensitivity to aeroallergens, activation and migration of eosinophils, differentiation for a Th2 immune response, bronchial hyperreactivity and metaplasia of globet cells with mucus production in the epithelium and mucosal subepithelium of the airways. However, for some time asthma is no longer considered as pathology of only Th2 profile, other cell types participate in the

Abbreviations: *B. germanica*, *Blattella germanica*; *B. tropicalis*, *Blomia tropicalis*; *D. pteronyssinus*, *Dermatophagoides pteronyssinus*; GWAS, Genome-Wide Association Studies; ILC2, Group 2 Innate Lymphoid; LD, linkage disequilibrium; *Periplaneta americana*, *P. americana*; sIgE, specific Immunoglobulin E; SPT, skin prick tests; SNV, single nucleotide variant

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pathophysiology of asthma and allergic diseases such as ILC2 cells [6]. There is also the participation of Th17 cells, responsible for the neutrophilic inflammatory profile of asthma in some patients. ILC2 cells do not have specific antigenic receptors, but like Th2 cells, they respond to the stimulus by triggering IL-25, IL-33, and Thymic Stromal Lymphopoietin (TSLP), producing IL-5, IL -9 and IL-13. ILC2-derived IL-13 mounts an efficient memory Th2 cell response to allergens, causing allergic lung inflammation and elevated serum levels of IgE [7,8]. ILC2 cells are derived from lymphoid progenitors common in response to IL-7 and IL-33 and their growing depend on the transcription factors Zinc Finger 3 (GATA-3) and Retinoic acid-dependent Orphan Receptor Alpha (RORA) [9–11].

Retinoic acid-dependent Orphan Receptors (RORs) are members of the Nuclear Receptor protein superfamily and comprise three members: ROR-alpha, ROR-beta and ROR-gamma. RORA has four variants, called RORA-1, RORA-2, RORA-3 and RORA-4, which differ in the amino-terminal region, as well as gene expression in different tissues [12,13]. In addition, RORA, as well as the other nuclear receptors, shares a common modular structure composed of several domains: the amino-terminal domain, the DNA Binding Domain (DBD), the Ligand Binding Domain (LBD) and a flexible domain that connects the DBD and LBD. In RORA, the DNA binding domain is named RORE [9,14]. RORA regulates gene transcription through monomer binding to the RORE response element. When it interacts with coactivators and corepressors, RORA can induce or suppress, respectively, the transcription of target genes [15]. The main known functions of RORA are: circadian rhythm regulator, participation in the metabolism and development of the immune system [5,16]. Experimental models of airway inflammation have shown that mice deficient in the *RORA* gene have attenuated immune system cell infiltration in the lung, decreased pulmonary mucosal hyperplasia, reduced Th2 cells, ILC2 cells, IL-4, IL-5 and IL-13 cytokines (Fig. 1). Other papers suggest a role for *RORA* in the regulation of Th2 inflammation occurring in the lung, as well as a risk factor for asthma in European Americans [13,17].

Asthma is a complex disease and genetically heterogeneous; the interaction between environmental and genetic factors leads to the occurrence of allergic respiratory diseases [18,19]. Genome-Wide Association Studies (GWAS) have demonstrated association between genetic variants in *RORA* and asthma. Moreover, other GWAS have described genes, such as *IL33*, *IL13* and *STAT6* which, like *RORA*, are molecular components of the pathophysiology of asthma [20–23]. Acevedo et al. [24] have demonstrated that genetic variants in *RORA* are risk factors for childhood asthma and have epistasis with polymorphisms in *NPSR1*. The NPS/NPSR1 pathway has biological interactions with *RORA* and other circadian clock genes that may have effects on the rhythmic occurrence of asthma symptoms.

Studies suggest that orphan nuclear receptors are an active field of research because of its potential for binding to ligands, which can be used to modulate these receptors in order to develop targeted therapies for various diseases [9,13]. It is known that RORA is constitutively active, presenting greater transcriptional activity when binding to a coactivator. However, there is the possibility of RORA binding to an inverse agonist, may allow suppression of transcription. Studying genetic variants in *RORA* can contribute to the identification of the pathogenic asthma mechanisms that are not still well elucidated and thus, discover new therapeutic options. In this context, the aim of this work was to identify genetic variants in *RORA* associated with asthma phenotypes and atopy markers in an admixed population of individuals from Salvador, Bahia, Brazil.

2. Material and methods

2.1. Study population and data collection

Data collected from 1246 children, included in the SCAALA (Social Changes Asthma and Allergy in Latin America) cohort, were analyzed. SCAALA was created to study the effect of a sanitation program on the children's health (age 4 to 11 years) in the city of Salvador, located in the Northeast of Brazil, from 1996 to 2004. They were again evaluated in 2005, with the application of new questionnaires for asthma diagnosis. The methods applied in this study were reported in previous studies [25–27].

Ethical approval was obtained by the National Research Ethics Committee 120.616. The written, free and informed consent was appropriately obtained from parents or legal guardian of each child.

2.2. Definition of asthma and atopy

Using the ISAAC questionnaire phase II (International Study of Asthma and Allergies in Childhood), children were classified as having asthma if their parents or guardians reported wheezing in the last 12 months and at least one of the following: clinical diagnosis of asthma, wheezing with exercise in the last 12 months, four or more episodes of wheezing in the last 12 months and waking up at night because of wheezing in the last 12 months [26].

Atopy was defined as the presence of at least one anti-allergen IgE test with serum levels equal to or greater than 0.70 kU/L, regardless of results of SPT [25].

The skin prick test was performed on the right forearm of each child using standardized extracts (ALK-Abelló, São Paulo, Brazil) for four

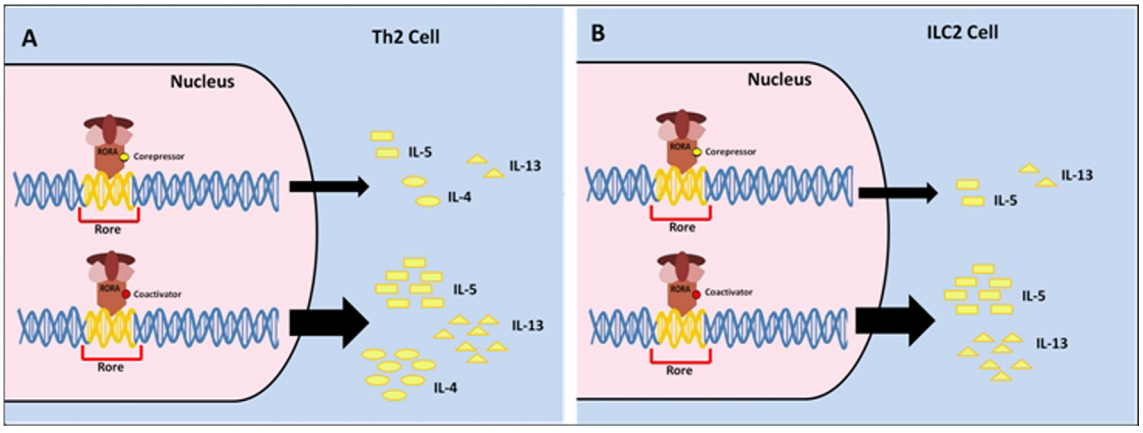

Fig. 1. Interaction of RORA with: Th2 (A) and ILC2 cells (B). The action of RORA on Th2 and ILC2 cells depends on their binding to RORE and binding of coactivators to LBD, leading to increased expression of cytokines in Th2 and ILC2 cells. On the other hand, the binding of corepressor molecules to LBD inhibits cytokine expression of the type 2 immune response in Th2 and ILC2 cells. Th2 (T helper type 2); Group 2 Innate Lymphoid (ILC2); RORE (DNA Binding Domain of RORA); LBD (Ligand Binding Domain).

allergens: *Blattella germanica, Blomia tropicalis, Dermatophagoides pteronyssinus* and *Periplaneta americana*. These tests were previously described by Barreto et al. [26]. Saline solution was used for negative control and 10 mg/mL of histamine for positive control. The reaction to each allergen was read after 15 min and considered positive if the papule diameter was at least 3 mm larger when compared to the negative control.

Each child's blood was collected in a heparinized tube and the serum kept frozen until use. The Pharmacia Immuno CAP System IgE FEIA (Pharmacia, Uppsala, Sweden) was used to measure the serum levels of IgE anti-mite (*Dermatophagoides pteronyssinus* and *Blomia. tropicalis*) and anti-cockroach (*Periplaneta americana* and *Blatella germanica*) in the subjects' blood, according to the manufacturer's instructions. Atopic condition was considered when serum IgE levels were greater than or equal to 0.70 kU/L for at least one tested allergen.

Whole blood was cultured in a 5% CO_2 atmosphere during 5 days for the detection of IL-13 and IL-5. These cytokines were detected in culture supernatant by the capture ELISA method, according to the manufacturer's instructions (Pharmigen, San Diego, CA, USA) [25].

2.3. DNA extraction and genotyping

DNA extraction was performed from the peripheral blood samples of 1246 SCAALA subjects, according to the protocols of QIAGEN kit (Gentra Puregene Blood Kit, Hilden, Germany). All samples to be genotyped were standardized at a concentration of 50 ng/μL and identified in bar code tubes. The samples were genotyped using the Illumina Human Omni 2.5–8 Bead Chip Kit, which consists of a large platform with approximately 2.5 million markers of the human genome (www.ilummina.com). For this study, the RORA genetic information was extracted between positions 60781040 and 61517218 on chromosome 15.

2.4. Statistical analysis

Using Plink program (version 1.07), the single nucleotide variants (SNVs) were filtered according to the following criteria: Hardy-Weinberg equilibrium test (P-value < 0.001); minor allele frequency (> 0.1) and percentage of *loci* lost (MIND > 0.1 and GENO > 0.1). The association analyses were adjusted for sex, age, helminth infection and major components (PC1 and PC2) to correct confounding factors from the population structure [20,28].

The analyses were performed under an additive model and the Odds Ratio (OR) was estimated, as well as 1000 permutations procedures was used to estimate the statistical significance of multiple correlation tests in the genetic association analysis [29,30]. Results obtained from this analysis having a confidence interval of 99.0% and a P-value less than 0.01 were considered statistically significant.

The haplotype analysis was performed in the SNPStats version 1.22.0 program (www.rdocumentation.org).

The Mann-Whitney test was used to compare IL-5 and IL-13 production levels among different rs10162630, rs17191519, rs17270243 and rs55796775 genotypes. Statistical significance was considered with an alpha error of 5%. These statistical analyses were performed using Prism software version 5 (GraphPad Inc., San Diego, CA).

The linkage disequilibrium (LD) analysis was carried out for each selected SNV, which refers to the association of alleles on the same chromosome but at different *loci*. Haploview 4.2 software was used to calculate the degree of confidence in the Ď value [31].

2.5. In silico analysis

The possible role of each SNV was obtained through information available from the National Biotechnology Information Center (NCBI) (www.ncbi.nlm.nih.gov).

Each SNV was also scored according to the scale of the RegulomeDB project (regulomedb.org), which consists of a database for the interpretation of regulatory variants in the human genome. RegulomeDB identifies probable functions of functional genetic variants and regulatory mechanisms through computational predictions and manual annotations. Data sources are combined in a tool that scores functional variants and provides testable hypotheses regarding their function [32]. The authors of the RegulomeDB project have developed a heuristic scoring system based on the functional reliability of a variant. The scoring system represents, on a growing scale of confidence, the functional location of a variant and its probable functional consequence. Scores from 1a to 1f indicate that the variant is likely to affect binding and is linked to the expression of a target gene, and the scores from 2a to 2c only indicate that the variant is likely to affect binding. While scores from 3a and 3b indicate that variants are less likely to affect binding, and scores of 4, 5, and 6 indicate minimal binding evidence or no data are available [32].

All SNVs were subjected to an analysis on the GTEx portal (www.gtexportal.org), from a consortium (Consortium, 2015), created by the National Institutes of Health Common Fund (Genotype-Tissue Expression Project). This consortium provides resources that enable the study of human gene expression and regulation and its relation to genetic variation and other molecular phenotypes in various human tissue types. The project authors analyzed the expression of global RNA in individual tissues and the levels of gene expression were treated as quantitative traits. Therefore, variations in gene expression that are highly correlated with genetic variation can be identified as expression of quantitative trait *loci*, named eQTLs.

3. Results

3.1. Study population

Table 1 summarizes the characteristics of the study population. It

Table 1
Characteristics of the SCAALA population according to asthma status and the variables included in the study.

Variables	Subject group (1246)				
	Nonasthmatic	%	Asthmatic	%	P-value
	942*	75.9%	274*	22%	
Age					
≤5	314	33.3%	132	48.2%	
6–7	337	35.8%	88	32.1%	0.000
≥8	291	30.9%	54	19.7%	
Sex					
Male	506	53.7%	151	55.1%	0.684
Female	436	46.3%	123	44.9%	
Specific IgE for					
Blomia tropicalis	288	30.6%	128	46.7%	0.000
Dermatophagoides pteronyssinus	179	19%	91	33.2%	0.000
Periplaneta americana	81	8.6%	31	11.3%	0.171
Blatella germanica	117	12.4%	47	17.2%	0.044
Skin Prick test for					
Blomia tropicalis	192	20.4%	76	27.7%	0.010
Dermatophagoides pteronyssinus	131	13.9%	62	22.6%	0.001
Periplaneta americana	120	12.7%	49	17.9%	0.030
Blatella germanica	70	7.4%	31	11.3%	0.040
IL-13 production by *Dermatophagoides. pteronyssinus stimulus*[a]	174	18.5%	52	19%	0.907

Data were analyzed using the chi-squared test.

* 30 subjects presented data miss and were excluded to analysis.

[a] Response of asthmatic and nonasthmatic individuals from the lowest detection point for each cytokine in cells stimulated cultures.

Table 2
Genetics information of main SNVs associated with asthma and allergy markers in this study.

SNV	Location Allele[a]	MAF[b]	HWE[c]	Function[d]	RegulomeDB score
rs10162630	A/G	0.45	0.73	Intron variant	5
rs11071584	A/C	0.31	0.64	Intron variant	5
rs12903172	C/T	0.25	0.26	Intron variant	5
rs17191519	G/A	0.10	0.65	Intron variant	3a
rs17270243	G/A	0.17	0.28	Intron variant	6
rs2414681	A/C	0.31	0.59	intron variant, nc transcript variant	6
rs34720147	T/C	0.26	0.30	Intron variant	-7
rs4775301	C/T	0.29	0.44	Intron variant	4
rs55796775	T/G	0.40	0.52	Intron variant	6
rs7169281	C/T	0.31	0.55	Intron variant	5
rs726914	A/G	0.19	0.45	Intron variant	5
rs8024133	T/C	0.30	0.95	Intron variant	4

[a] First is alternative allele and the second is reference allele (1/2).
[b] MAF: Minor Allele Frequency.
[c] HWE: Hardy–Weinberg Equilibrium.
[d] NCBI (National Center for Biotechnology Information).

was observed different proportions of asthmatic subjects across all age groups ($P < 0.01$). Differences in markers of allergy, such as SPT and specific IgE levels were statistically significant ($P < 0.05$) between non-asthmatic and asthmatic groups, being all of them greater among asthmatics.

3.2. Description of variants in RORA gene

For the accomplishment of this study, 824 SNVs from the Illumina genotyping chip belonging to the *RORA* were extracted. 178 SNVs were excluded by the frequency test (MAF < 0.10). No SNV was excluded because of poor genotyping (MIND > 0.1), missingness (GENO > 0.1) or by the Hardy-Weinberg Equilibrium (HWE) test ($P \leq 0.001$). Thus, 646 SNVs were included in the association analysis.

Table 2 contains information on the top-12 SNVs, in accordance with the statistical criteria established in Section 2.4 and which were associated with two or more outcomes. It also shows a MAF ranging between 0.10 and 0.45. The complete list of all SNVs associated with asthma and allergy markers with a P value less than 0.01 and MAF > 0.05 can be found in the Supplementary Table 1.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.07.004.

Supplementary Table 2 provides a list of independent SNVs in RORA gene.

3.3. Association of asthma and SPT with variants in RORA gene

The allele A of rs2414681 and the allele C of rs7169281 were negatively associated with asthma (OR 0.70 and OR = 0.69, respectively; $P < 0.01$), the latter being also associated with non-atopic asthma (Table 3). Both SNVs were also positively associated with SPT for *Blomia tropicalis* ($P < 0.01$) (Table 5).

Table 3
Association between SNVs in *RORA* gene and asthma by logistic regression analysis adjusted for sex, age, helminth infections and ancestry.

SNV	OR	99%CI	P value	Perm
Asthma				
rs2414681	0.70	0.56–0.88	0.0020	0.0015
rs7169281	0.69	0.55–0.86	0.0011	0.0009
Non-atopic asthma vs control				
rs7169281	0.66	048–0.90	0.0010	0.0009

SNV: single nucleotide variant; OR: odds ratio; 9% CI: 99% confidence interval; Perm: permutational P value.

Table 4
Significant association between *RORA* SNVs and specific IgE by logistic regression adjusted for sex, age, helminth infections and ancestry.

SNV	OR	99% CI	P Value	Perm
Specific IgE for *B. tropicalis*				
rs17191519	1.65	1.25–2.16	0.0003	0.0002
rs17270243	1.44	1.15–1.80	0.0017	0.0016
rs726914	1.38	1.11–1.72	0.0035	0.0021
Specific IgE for *D. pteronyssinus*				
rs10162630	1.43	1.17–1.73	0.0003	0.0004
rs17191519	1.68	1.25–2.26	0.0005	0.0004
rs17270243	1.55	1.21–1.98	0.0005	0.0007
rs55796775	1.38	1.13–1.68	0.0012	0.0013
Specific IgE for *P. Americana*				
rs11071584	0.59	0.42–0.83	0.0022	0.0016
Specific IgE for *B. germanica*				
rs34720147	1.56	1.21–2.01	0.0005	0.0005
rs4775301	1.44	1.12–1.84	0.0045	0.0068
rs726914	1.70	1.28–2.24	0.0002	0.0002

SNV: single nucleotide variant; OR: odds ratio; 9% CI: 99% confidence interval; Perm: permutational P value.

Table 5
Significant associations between *RORA* SNVs and skin prick test (SPT) by logistic regression adjusted for sex, age, helminth infections and ancestry.

SNV	OR	99% CI	P-value	Perm
Skin Prick Test for *Blomia tropicalis*				
rs10162630	1.35	1.11–1.64	0.0026	0.0030
rs17191519	1.57	1.17–2.12	0.0028	0.0023
rs17270243	1.47	1.14–1.89	0.0026	0.0028
rs2414681	1.41	1.15–1.74	0.0011	0.0012
rs4775301	1.41	1.14–1.74	0.0013	0.0016
rs55796775	1.32	1.08–1.60	0.0060	0.0068
rs7169281	1.39	1.13–1.71	0.0017	0.0020
rs8024133	1.33	1.07–1.64	0.0087	0.0107
Skin Prick Test for *Dermatophagoides pteronyssinus*				
rs17191519	1.74	1.25–2.41	0.0009	0.0007
rs4775301	1.42	1.12–1.80	0.0038	0.0029
rs55796775	1.36	1.09–1.70	0.0068	0.0058
rs8024133	1.40	1.10–1.78	0.0058	0.0049
Skin Prick Test for *Periplaneta Americana*				
rs10162630	1.35	1.07–1.71	0.0106	0.0122
rs11071584	0.63	0.48–0.83	0.0010	0.0010
rs12903172	1.53	1.18–1.99	0.0013	0.0012
rs34720147	1.40	1.09–1.80	0.0087	0.0132
rs726914	1.51	1.14–1.99	0.0037	0.0038
Skin Prick Test for *Blatella germanica*				
rs12903172	1.74	1.26–2.40	0.0007	0.0004
rs726914	1.59	1.13–2.24	0.0084	0.0085

SNV: single nucleotide variant; OR: odds ratio; 99% CI: 99% confidence interval; Perm: permutational P value.

3.4. Association of specific IgE levels for aeroallergens with variants in RORA gene

The polymorphisms rs10162630, rs17191519, rs17270243 and rs55796775 were positively associated with sIgE positivity for *D. pteronyssinus* (A allele, OR 1.43; G allele, OR 1.68; G allele, OR 1.55 and T allele, OR 1.38, respectively; $P < 0.01$, two of which rs17191519 (G allele, OR 1.65, $P = 0.0003$) rs17270243 (G allele, OR 1.44, $P = 0.0017$) were also positively associated with sIgE for *B. tropicalis*. Moreover, the allele A rs726914 was positively associated with sIgE for *B. tropicalis* (OR 1.38, $P = 0.0035$) and sIgE for *B. germanica* (OR 1.70, $P = 0.0002$). Two other variants were positively associated with sIgE

for *B. germanica* and only the variant rs11071584 (A allele) had a negative association for *P. americana* (OR 0.59 and P = 0.0017) (Table 4).

3.5. Association of skin prick test with variants in RORA gene

Table 5 shows the association between variants in *RORA* and skin prick test. It was observed a statistically positive association for eight SNVs in the *RORA* gene with SPT for *B. tropicalis* (P < 0.01). Of these eight, four SNVs were also positively associated with SPT for *D. pteronyssinus* (P < 0.01). It has been demonstrated a positive association between variants rs12903172 and rs726914 with SPT for cockroaches. Similarly, the association with *P. americana* specific IgE rs11071584 was negatively associated with SPT for this same aeroallergen (OR 0.63; P = 0.001).

3.6. Association between SNVs in RORA gene and type 2 cytokine production

The T allele of rs55796775 was positively associated with IL-13 production upon *D. pteronyssinus* stimulation in peripheral blood cells (OR 1.33, CI 1.08–1.64; P = 0.0072). It was not possible to obtain the adjusted analysis for IL-5 production due to the small number of individuals with polymorphic alleles for SNVs in RORA gene.

It is noteworthy that rs10162630, rs17191519, rs17270243 and rs55796775 were associated with SPT and sIgE for *D. pteronyssinus* and/or *B. tropicalis,* then it was evaluated the effect of presence one or two polymorphic alleles on the production of IL-5 and IL-13 stimulated by these mites in atopic individuals. The A alele of rs10162630 was associated with high levels of IL-13 production by *Blomia tropicalis* stimulus (P = 0.046, Fig. 2A). Atopic individuals with rs17191519 AG/GG genotypes exhibited higher IL-5 production upon stimulation with *D. pteronyssinus*, when compared to individuals with rs17191519 AA genotype (P = 0.045, Fig. 2B). Regardless of other genotypes, no significant differences were observed in IL-5 and IL-13 production under stimulation with *B. tropicalis* and/or *D. pteronyssinus* among the study groups (data not shown).

3.7. Haplotype analysis

To perform the haplotype analysis, SNVs in high linkage disequilibrium and associated with at least four different variables. In Table 6 we present the haplotype analysis for SNVs in *RORA* obtained our study. The SNVs rs10162630 (A allele), rs17191519 (G allele), rs17270243 (G allele) and rs55796775 (T allele) have statistically significant positive associations with two different outcomes (Tables 4 and 5). In the haplotype analysis, the positive association was observed between the haplotype AGGT and SPT and between the haplotype AGGT and sIgE, for house dust mites (P = 0.001). Other haplotypes of these SNVs were also associated with the SPT and sIgE allergy markers.

3.8. In silico analysis

Analysis from the regulomeDB showed that only the variant rs17191519 had a value equals to 3a (Table 2). For the other SNVs of this study, the values of the regulomeDB were above the 3a value.

Using the Haploview program, the linkage disequilibrium (LD) analysis, was performed, in which three blocks were found (Fig. 3).

The differential expression of RORA in cutaneous tissue was obtained using the GTEx platform. In skin not exposed to sunlight it was found polymorphisms rs10162630 and rs55796775 significantly associated with expression of the RORA gene (P < 0.05). While in sun-exposed skin, it was found that rs17191519 and rs17270243 were also associated with the expression of RORA (P < 0.01) (Fig. 4A–D). Only variant rs17270243 was significantly associated with expression of the RORA gene in lung tissue (Fig. 4E).

4. Discussion

Many advances have been made to unveil, at the molecular level, the complexity of asthma and allergic diseases [33]. The RORA transcription factor has been investigated and it has been found that it participates in lung development during the fetal life and is associated with pulmonary allergic inflammation [24,34]. Studies of candidate genes and GWAS have identified the *RORA* gene region as involved in susceptibility to various diseases, including asthma [20,22,24]. Herein it is reported for the first time that genetic variants in *RORA* are significantly associated with asthma and allergy markers in an admixed population, and since atopic sensitization still remains the strongest risk factor for asthma on the western part of the planet [35], it is speculated that these variants may play a role in asthma occurrence.

In our study, four SNVs in *RORA* (rs10162630, rs17191519, rs17270243 and rs55796775) and their haplotypes, especially the

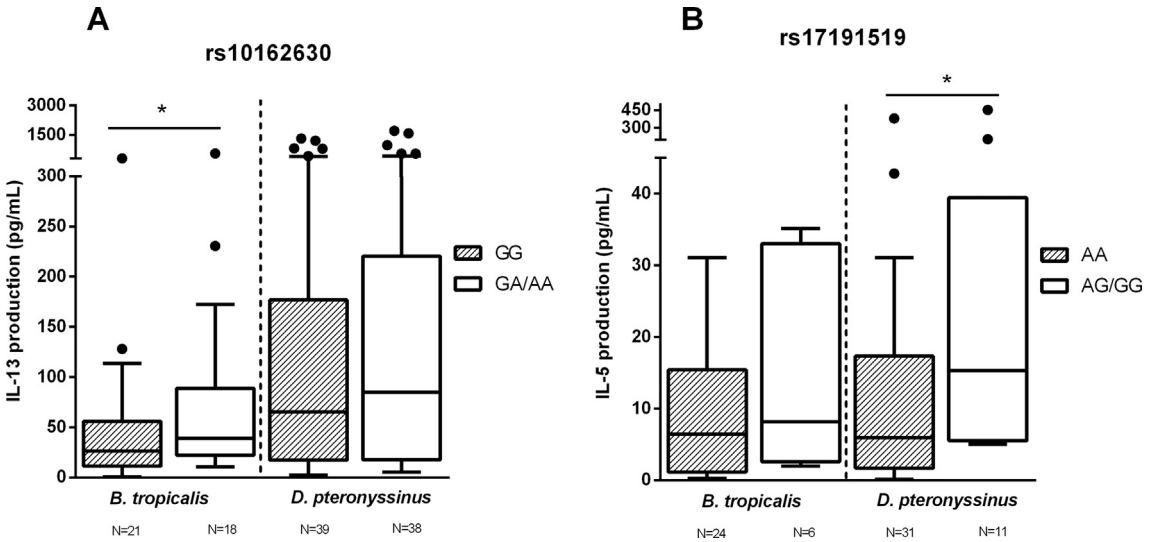

Fig. 2. IL-5 and IL-13 production by atopic individuals according to SNVs RORA genotype. (A) IL-13 production by *B. tropicalis* (GG = 26,56 [11.65–55.99] pg/ml; AG/AA = 40.79 [23.50–89.94] pg/ml) and *D. pteronyssinus* stimulus (GG = 66.67 [17.45–184.70] pg/ml; AG/AA = 87.36 [17.73–231.30] pg/ml). (B) IL-5 production by *Blomia tropicalis* (AA = 6.46 [1.14–15.42] pg/ml; AG/GG = 8.14 [2.58–33.01]) and *D. pteronyssinus* stimulus (AA = 5.92 [1.67–17.32] pg/ml; AG/GG = 14.18 [5.46–81.15]).

Table 6
RORA haplotypes associated with skin prick test and specific IgE for house dust mites.

Haplotype	rs10162630	rs17191519	rs17270243	rs55796775	Frequencies		OR[a] (95% CI)	P-value
					Cases	Controls		
Reference	G	A	A	G	0.46	0.37	1.00	
Haplotype association with SPT for *B. tropicalis*								
1SPT.B	A	A	A	T	0.37	0.32	1.44 (1.12–1.84)	0.004
2SPT.B	G	A	G	G	0.06	0.05	1.64 (0.96–2.81)	0.071
3SPT.B	G	G	G	G	0.05	0.04	1.72 (0.96–3.07)	0.067
4SPT.B	A	G	G	T	0.07	0.04	2.38 (1.45–3.89)	0.0006
5SPT.B	A	A	A	G	0.04	0.04	1.33 (0.78–2.27)	0.03
6SPT.B	A	A	G	T	0.01	0.01	1.06 (0.31–3.60)	0.93
Haplotype association with IgE specific for *B. tropicalis*								
1IgE.B	A	A	A	T	0.33	0.33	1.11 (0.90–1.38)	0.32
2IgE.B	G	A	G	G	0.06	0.05	1.47 (0.91–2.38)	0.12
3IgE.B	G	G	G	G	0.05	0.04	1.53 (0.92–2.55)	0.1
4IgE.B	A	G	G	T	0.06	0.04	2.01 (1.26–3.21)	0.004
5IgE.B	A	A	A	G	0.04	0.04	1.14 (0.71–1.83)	0.58
6IgE.B	A	A	G	T	0.01	0.02	0.69 (0.23–2.06)	0.51
Haplotype association with SPT for *D. pteronyssinus*								
1SPT.D	A	A	A	T	0.39	0.32	1.52 (1.15–2.00)	0.003
2SPT.D	G	A	G	G	0.06	0.05	1.61 (0.90–2.86)	0.11
3SPT.D	G	G	G	G	0.06	0.04	2.17 (1.19–3.95)	0.011
4SPT.D	A	A	A	G	0.04	0.04	1.11 (0.58–2.10)	0.75
5SPT.D	A	G	G	T	0.07	0.04	2.41 (1.42–4.10)	0.001
6SPT.D	A	A	G	T	0.005	0.02	0.39 (0.05–2.89)	0.36
Haplotype association with IgE specific for *D. pteronyssinus*								
1IgE.D	A	A	A	T	0.38	0.32	1.55 (1.21–1.98)	0.0005
2IgE.D	G	A	G	G	0.06	0.05	1.84 (1.09–3.11)	0.023
3IgE.D	G	G	G	G	0.06	0.04	2.01 (1.16–3.51)	0.014
4IgE.D	A	G	G	T	0.07	0.04	2.73 (1.68–4.44	0.0001
5IgE.D	A	A	A	G	0.05	0.04	1.59 (0.95–2.66)	0.08
6IgE.D	A	A	G	T	0.01	0.02	1.04 (0.30–3.58)	0.95

Adjusted by gender. age. helminth infection. ancestry markers. SPT.B = Skin Prick Test for Blomia tropicalis; IgE.B = Specif IgE for Blomia tropicalis; SPT.D = Skin Prick Test for Dermatophagoides pteronyssinus; IgE.D = Specific IgE for Dermatophagoides pteronyssinus.

AGGT haplotype, were associated with increasing risk for atopy markers production. *In silico* analysis GTEx revealed that these SNVs have increased *RORA* expression for the polymorphic allele. In addition, rs55796775 and rs10162630 were associated with increased production of IL-13 in dust mite-stimulated cell cultures, while atopic individuals with genotype GG/AG of rs17191519 presented high levels of IL-5 production when stimulated with *D. pteronyssinus*. The transcription factor RORA is essential for ILC2 cells development that are rapid and potent producers of the type 2 cytokines Il-5 and IL-13 [11,17]. Recently, Halim et al. have proved the importance of ILC2-derived IL-13 in the efficient recruitment of memory Th2 cells to the allergen-challenged lung through collaboration with lung dendritic cells [8,36]. Therefore, as the presence of *RORA* is related to increased allergic lung inflammation mainly through an immune response mediated by ILC2 cells, the genetic variants previously cited may play a role in the pathogenesis of asthma and allergic diseases [6,37]. However, to our knowledge, none of these variants in *RORA* have been associated with asthma and/or allergies in other populations so far. Probably this result is related to the type of population evaluated in this study, composed of African descendants, whereas other studies involving the *RORA* gene and asthma were carried out in Europeans and/or their descendants.

Two SNVs, rs2414681 and rs7169281, were associated with

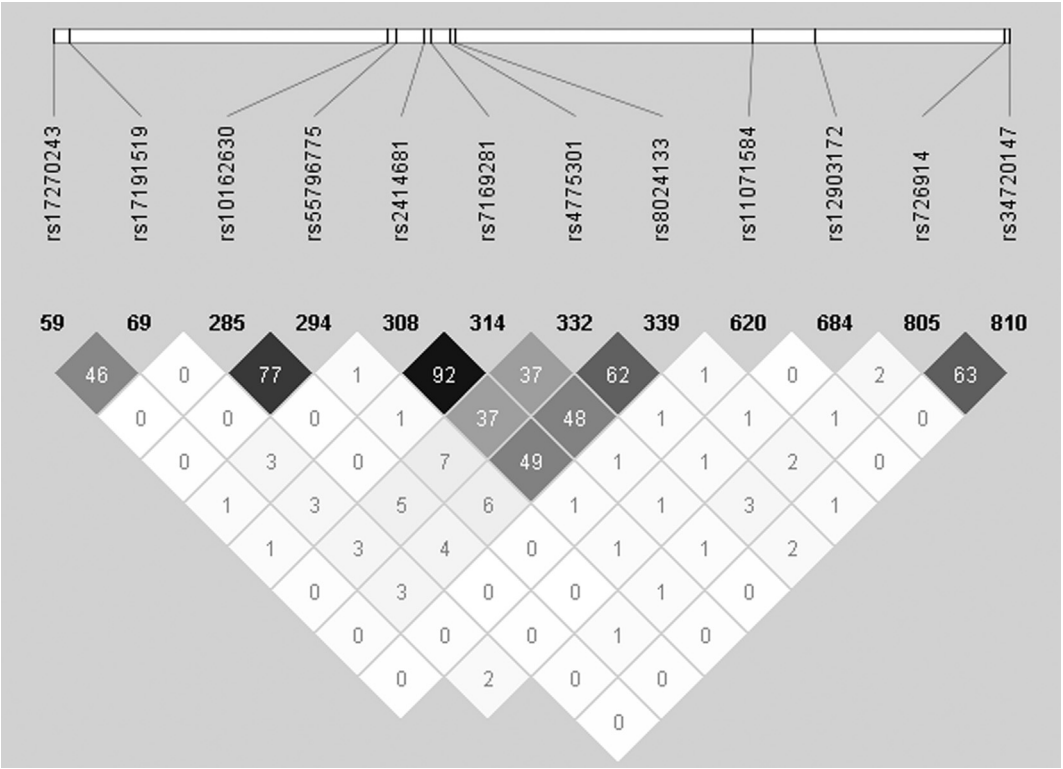

Fig. 3. Pairwise LD within Haploview using the r squared statistic for the RORA gene. Intensity of shading indicates the degree of confidence in the r squared value.

decreased risk for asthma and rs7169281 (allele C) was negatively associated with non-atopic asthma, suggesting that these variants could provide protection against the development of this disease phenotype. According to literature data, non-atopic asthma has independent pathogenic mechanisms of a type 2 immune response and often progresses to severe asthma with participation of Th17 and Th1 cytokines, but not type 2 cytokines [7]. On the other hand, these SNVs can trigger atopy as they were associated with SPT positivity for *B. tropicalis*, which is a risk factor for the development of allergies, such as atopic asthma [35,37].

Alleles C of rs12903172, T of rs34720147 and A of rs726914 were

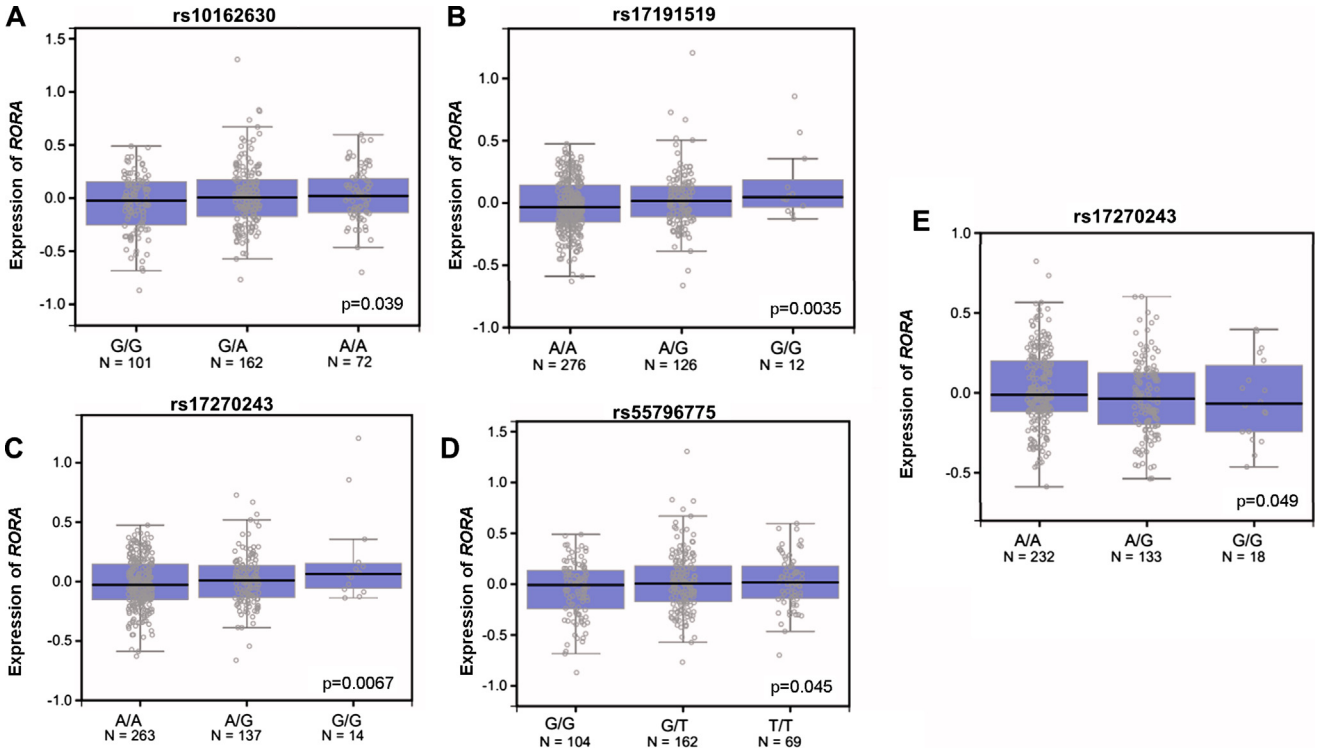

Fig. 4. GTEx-Gene expression level of RORA grouped by genotypes of (A) 10162630 from skin non-exposed to sunlight; (B) rs17191519 from sun-exposed skin; (C) rs17270243 from sun-exposed skin; (D) rs55796775 from skin non-exposed to sunlight; (E) rs17270243 from lung tissue.

positively associated with SPT positivity and serum IgE production for cockroach allergens. Other SNVs (Allele C of rs4775301 and allele T of rs8024133) also showed association with increased allergy markers in this study. Therefore, exposure to these aeroallergens in those individuals with such polymorphisms may trigger an immune response with production of sIgE and type 2 cytokines, leading to the emergence of allergic diseases [6]. In this work, only the rs11071584 polymorphism showed negative association with reactivity for SPT and sIgE tests, suggesting that this may be a protective marker for allergic diseases.

The LD graph shows that six of the SNVs analyzed here form three blocks in strong LD. They were associated with allergy markers indicating that the 15q22 region, in which *RORA* is found, can be responsible for occurrence of allergic diseases. All the variants presented here are in the intronic region of the *RORA*-1 isoform for this transcription factor, which is considered the active isoform in humans. SNVs located in regulatory parts of the intronic region may alter gene transcoding and alternative splicing, and consequently the RORA production [38]. On the other hand, the values of regulomeDB score did not present high values for the SNVs in this study and this fact is credited to the absence of known ligands for *RORA*.

Although some GWAS have shown association of the *RORA* gene region with asthma and allergic diseases [20,22,39], only one candidate gene study was found in which variants in the *RORA* gene were significantly associated with asthma [24]. However, these variants were different from those observed in our study. In addition, unlike our findings, the same author [24] has not found association of RORA gene with atopic sensitization. It is hypothesized that these differences can be attributed to the differences in the genotyping panels used to study allelic frequencies and the different ethnicities of the studied populations. In fact, the studies carried out to date on the *RORA* gene involve populations of European origin, whereas in our study we dealt with admixed populations of African descendants. Literature data report notable disparities in the frequencies of susceptible alleles for asthma in African descendants [40,41].

In conclusion, individuals in our population with genetic variants in *RORA* have a higher risk of developing asthma and atopy and protection for non-atopic asthma. To our knowledge, this is the first work associating SNVs in the *RORA* gene with asthma and allergic sensitization in a population of African descendants. Studies in diverse populations are important to reveal new etiological mechanisms, involving possible gene-environment differences, as well as to examine the consistency of established associations. Future work will be necessary to understand how the *loci* described in this study control the disease and whether this gene could be used as a target for the treatment of allergic diseases.

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