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Polymorphisms in *DENND1B* gene are associated with asthma and atopy phenotypes in Brazilian children

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ABSTRACT

Asthma is a heterogeneous disease associated with a complex basis involving environmental factors and individual variabilities. The DENN Domain Containing 1B (*DENND1B*) gene has an important role on T cell receptor (TCR) down-regulation on Th2 cells and studies have shown that mutations or loss of this factor can be associated with increased Th2 responses and asthma. The aim of this work is to evaluate the association of polymorphisms in the *DENND1B* with asthma and allergy markers phenotypes in Brazilian children. Genotyping was performed using a commercial panel from Illumina (2.5 Human Omni bead chip) in 1309 participants of SCAALA (Social Change, Asthma, Allergy in Latin American) program. Logistic regressions for asthma and atopy markers were performed using PLINK software 1.9. The analyzes were adjusted for sex, age, helminth infections and ancestry markers. The *DENND1B* gene was associated with different phenotypes such as severe asthma and atopic markers (specific IgE production, skin prick test and IL-13 production). Among the 166 SNPs analyzed, 72 were associated with asthma and/or allergy markers. In conclusion, polymorphisms in the *DENND1B* are significantly associated with development of asthma and atopy and these polymorphisms can influence *DENND1B* expression and consequently, asthma.

1. Introduction

Asthma is a complex and heterogeneous disease characterized by chronic airway inflammation, bronchial hyper responsiveness, remodeling and airflow obstruction spontaneously reversible or with treatment, it is manifested by recurrent episodes of wheezing, dyspnea, chest tightness and cough (Barreto et al., 2014; Troy et al., 2016). About 334 million people in the world suffer from asthma (Asher and Pearce, 2014) and an estimated 100 million asthma cases are estimated to increase by 2025 worldwide (Barreto et al., 2014). The prevalence of asthma has been growing steadily throughout the world, affecting mainly children (GINA, 2016).

There are several factors suggested to explain the increase in the prevalence of asthma, including environmental, nutritional, economic and psychosocial aspects (Barreto et al., 2014). The classic risk factors are: family history of allergic diseases, sensitization to environmental allergens (including aeroallergens), endotoxins exposure, fungi and viral respiratory infections in early life; however, such factors do not fully explain the establishment and maintenance of the disease, and other factors have been associated with this disease such as being male, low birth weight, maternal or domestic smoking, secondary exposure to pollution in large cities, climatic changes, exposure to chemical irritants, physical exercise and psychosocial factors (Coelho et al., 2016).

Th2 cells are the most studied cell phenotype in asthma, however evidence shows that other helper T cell lines, such as Th1, Th17 and Tregs, act in different ways in the immunopathology of asthma, because of that it has been suggested that asthmatics can be divided into different subgroups (Malmhäll et al., 2012), being typically classified as atopic and non-atopic asthma. Atopic asthma is characterized by Th2-type TCD4+ cells, which secrete cytokines such as IL-4, IL-5 and IL-13, and granulocyte–macrophage colony-stimulating factor (GM-CSF), which in turn leads to the IgE, mast cell and eosinophilic responses that

Abbreviations: BDCA, markers for distinct subsets of *dendritic cells* in human peripheral blood; CI, confidence interval; DENN, differentially expressed in normal and neoplastic cells domain; DENND1B, DENN domain containing 1B; ELISA, enzyme-linked immunosorbent assay; eQTLs, expression quantitative trait *loci*; GEF, guanine nucleotide exchange factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; GTEx, The Genotype-Tissue Expression; GTP, guanosine triphosphate; GWAS, genome-wide association studies; HWE, Hardy-Weinberg equilibrium; IgE, immunoglobulin E; IL, interleukin; LD, linkage disequilibrium; MAF, frequency of minor allele; OR, odds ratio; Rab, Ras-related protein; SCAALA, Social Change, Asthma, Allergy in Latin American; SNP, single nucleotide polymorphism; TCR, T cell receptor; Th cells, T helper cells; TF, transcription factor; Tregs, regulatory T cell

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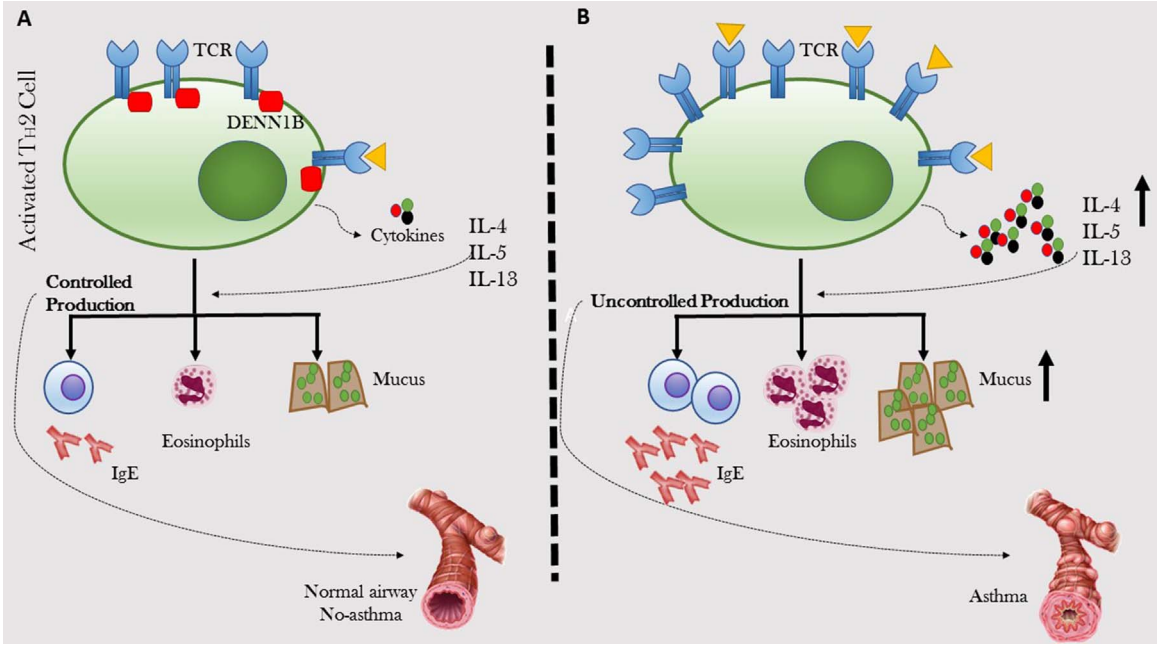

Fig. 1. DENND1B mechanism. (A) Presence of *DENN1DB* in the cell controls the internalization of the TCR and, consequently, the production of Th2 cytokines**. (B)** Cell without *DENN1B* has exacerbated production of cytokines, leading to Th2 asthma.

are characteristic of allergic asthma (Holgate et al., 2015). Asthmatic patients that do not manifest atopy (non-atopic asthma) are called intrinsic asthmatics and they normally show negative skin tests and there is no clinical or family history of allergy. Additionally, lack of specific IgE antibodies directed against common allergens (Humbert et al., 1999).

The heterogeneous condition of asthma arises from different causes associated with a complex genetic basis involving environmental factors and individual variabilities (Kabesch et al., 2007). A number of studies on candidate genes have been conducted in recent years in an attempt to understand the genetic mechanisms of asthma (Costa et al., 2015). Several candidate genes, at different levels, have been associated with different asthma phenotypes, fifty-three genes have been identified and replicated in more than one study, and 20 genes have been replicated in more than 10 separated investigations (Vercelli, 2008).

Genetic polymorphisms, along with environmental factors, play a significant role in asthma pathogenesis, and therefore interest in asthma genetics has grown over the last two decades (Bazzi et al., 2011). The main type of genetic variation observed in individuals is single nucleotide polymorphism (SNP), which can alter the structure, function, levels and expression of proteins (Hawkins et al., 2006).

The differentially expressed in normal and neoplastic cells domain (DENN) interacts with members of the Rab family of small GTPases and has enzymatic function as a guanine nucleotide exchange factor (GEF) for Rab, being able to activate monomeric GTPases by stimulating the release of guanosine diphosphate (GDP) and allowing binding to guanosine triphosphate (GTP) (Yang et al., 2016). In addition, it controls proteins that have been implicated in various cellular functions, including the recycling receptor, synaptic vesicle endocytosis and autophagy (Cherfils and Zeghouf, 2013; Yang et al., 2016).

There are 18 human proteins containing the DENN domain and their alternative splicing variants. Mutations (coding for a different amino acid), chromosomal translocations and altered levels of expression have been described in association with neurological disorders, ocular disorders and cancers (Yang et al., 2016).

The *DENND1B* gene is located on chromosome 1, region 1q31.3 and is expressed in a subgroup of dendritic cells (BDCA3+ and BDCA4+) and natural killer cells and also in activated T cells (Sleiman et al., 2010). In addition, it is GEF for Rab35, a key endocytic recycling regulator and important for negative modulation of the T cell receptor (TCR) in Th2 cells (Yang et al., 2016).

In 2016 Yang et al. proposed the following mechanism for DENND1B in mice: in normal conditions, that is, a cell with *DENND1B* expressing, this gene will control cytokine production of Th2 cells by modulating the duration of T cell receptor (TCR), when stimulated by an antigen, with a continuous process of endocytosis and recycling via the endosomes. After exposure to a certain antigen, TCRs molecules in activated T cells are targeted to the immunological synapse but are quickly, within 5–10 min, targeted to endosomes for degradation or recycling (Fig. 1A). In DENND1B-deficient T cells, TCR internalization is significantly slowed down, leading to prolonged TCR signaling that could increase production of effector cytokines (IL-4, IL-5, IL-13) involved in the pathology of atopic asthma (Godar and Lambrecht, 2016) (Fig. 1B). In this way, mutations or loss of this factor may be associated with increased Th2 responses and asthma (Yang et al., 2016).

The first study about *DENND1B* was published by Sleiman et al. (2010), a genome-wide association study (GWAS), which identified a locus containing *DENND1B* on chromosome 1q31.3 significantly associated with susceptibility to asthma in patients of European ancestry.

The present project is a candidate gene, replicated in some populations of the world, but not yet explored in the Brazilian population. Impact of knowing this susceptibility can increase knowledge about the pathophysiology of asthma and the possibility of personalizing the treatment of patients based on genetic information.

No study with polymorphism in the *DENND1B* gene has been conducted so far in Latin America. In this way, our aim was to evaluate the association of polymorphisms in the gene *DENND1B* with atopy and asthma phenotypes in Brazilian children.

2. Material and methods

2.1. Study population

The present study was conducted with 1309 children who had participated in the SCAALA (Social Change, Asthma, Allergy in Latin American) program and lived in the city of Salvador with a population of approximately 2.9 million habitants (IBGE, 2016). The prevalence of wheezing in the last 12 months in school children aged 12–13 years in

Salvador is approximately 27.1% (Rodrigues et al., 2008). This population has already been described in other works of the group (Barreto et al., 2006; Rodrigues et al., 2008). The children were recruited in childhood to participate in a prospective study that aimed to measure the impact of a sanitation program in the city of Salvador on child morbidity (Barreto et al., 2007). Data were collected from children born between 1994 and 2001, who lived in sentinel neighborhoods of the city. Standardized questionnaires were applied to the legal guardian of each child between 1997 and 2003 to collect data on demographic and social variables, as well as on the domestic environment (Figueiredo et al., 2010). The children were interviewed again in 2005 and the same demographic and social variables were collected to obtain stool and blood samples for the laboratory tests and isolation of the genetic material. This work has the approval of the National Research Ethics Committee 120.616 and free informed consent was appropriately obtained from the legal guardian of each child.

2.2. Variables and markers used in the association study

Asthma symptoms were defined using a Portuguese adapted phase II International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire and the children were classified as asthmatic when the parents reported wheezing in the previous 12 months and at least one of the following situations: diagnosis of asthma by a doctor at any time in their lives; wheezing with exercise in the last 12 months; four or more episodes of wheezing in the last 12 months; and waking up at night due to wheezing episodes in the last 12 months (Costa et al., 2015; Figueiredo et al., 2013). We defined wheeze phenotypes (atopic and nonatopic by a positive IgE result (> 0.70 kU/L) or skin prick test above 3 mm of diameter.

Severe asthma was considered cases that, besides mentioning wheezing in the last 12 months, had one or more affirmative answers to the following questions: inability to speak at least two complete words during a wheezing crisis, number of attacks greater than 12 in the last year, number of nocturnal awakenings as a result of asthma (> 1 night per week) (Simões et al., 2010).

Determination of specific IgE serum concentrations was performed for mite (*Dermatophagoides pteronyssinus* and *Blomia tropicalis)* and for cockroach (*Blatella germanica*, and *Periplaneta americana*) by using the ImmunoCAPassay (Phadia Diagnostics AB, Uppsala Sweden). Children with 0.70 kU/L or greater of specific IgE for any allergen tested were considered to have positive results (Figueiredo et al., 2013).

All individuals were submitted to skin puncture tests for seven common aeroallergens: D*ermatophagoides pteronyssinus*, *Blomia tropicalis*, *Periplaneta Americana*, *Blatela germanica*, fungi (*Aspergillus fumigatus* and *Penicillium notatum*), dog and cat epithelium. Saline and 10 mg/mL histamine solution were used as negative and positive controls, respectively. After 15 min, the diameter wheal was measured and a mean wheal of at least 3 mm greater than the negative control was considered positive (Figueiredo et al., 2013).

Venous blood was collected into heparinized tubes and cultured at a dilution of 1:4 in RPMI (Gibco, Auckland, New Zealand) containing 10 mmol/L glutamine (Sigma-Aldrich, St Louis, Mo) and 100 μg/mL gentamicin (Sigma-Aldrich). The cells were cultured within 6 h of collection in the presence or not of 10 μg/mL endotoxin-free and were maintained in a humidified environment of 5% CO2 at 37 °C for 5 for detection of IL-13 in the presence of pokeweed mitogen (Sigma-Aldrich). We determined the optimal time course of cytokine accumulation in whole- blood culture via a standardization process in our own laboratory (Figueiredo et al., 2010).

The IL-13 concentration was measured in whole-blood culture supernatants by using commercially available antibody pairs and recombinant cytokine standards (BD Pharmingen, San Diego, CA), by means of sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Cytokine concentrations were determined by interpolation of standard curves. The detection limits (low/high) for the cytokine was 62.5/4000 pg/mL. Responders were defined as those children with cytokine concentrations above the lower detection limits.

2.3. Genomic DNA extraction and genotyping

DNA extraction was performed from blood samples according to the protocol of the Gentra® Puregene® Blood Kit (Quiagen). All samples to be genotyped were standardized at a concentration of 5 ng/μL and stored at −30 °C until use.

The genotyping was performed using standardized commercial panel 2.5 HumanOmni Beadchip and currently available from Illumina (www.illumina.com). The *DENND1B* genetic information were extracted from 197473878 to 197744623 position (NC_000001.10) at the chromosome 1. Most of these SNPs were selected from the 3.1 million genotyped SNPs in the HapMap International Project (http://www.hapmap.org/), which represents the largest international initiative for mapping genomic variability and the pattern of imbalance in the human genome (The International HapMap Consortium, 2007).

2.4. Data analysis

For genetic association analysis, each SNPs were subjected to quality control tests that include parameters such as genotype frequency, Hardy-Weinberg equilibrium (HWE > 0.001), population stratification, genotypic allocation, frequency of minor allele (MAF > 0.05), and *p*-value of less than 0.05 were consider to associations statically significant. These parameters were evaluated using the software PLINK (version 1.9) (Purcell et al., 2007). Logistic regressions for asthma and allergy markers (skin tests and IgE production) were performed using the same software, adjusted for sex, age, helminth infections and ancestry markers (Principal components (PC)), were used as covariates to control confounding by population structure (Costa et al., 2015). Empirical p values were generated through a permutational approach for correction for multiple tests and the OR value (odds ratio) was estimated. The analyses were performed using the additive genetic model. Pairwise LD was created with Haploview.

2.5. In silico analysis

Information about the function of each SNP was obtained in the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov).

RegulomeDB (http://www.regulomedb.org/) is a database that integrates a collection of SNPs with known regulatory information in the intergenic regions of the human genome, thus allowing functional allocation of regulatory information in any set of variants. It uses a scoring system representing the presence of a variant in a functional location that is likely to result in a functional consequence (Boyle et al., 2012). This score ranges from 1 to 6 and the lower this value, greater the probability of this variant being located in a functional region. The 1a to 1f scores are likely to affect binding and linked to expression of a gene target, the 2a to 2c scores are likely to affect binding, the 3a to 3b scores are less likely to affect binding, the 4–6 scores are minimal binding evidence (Boyle et al., 2012) and 7 no information (Supplementary Material − Table A1).

The Genotype-Tissue Expression (GTEx) project of the National Institutes of Health Common Fund aims to establish a resource database and associated tissue bank in which to study the relationship between genetic variation and gene expression and other molecular phenotypes in multiple reference tissues (Lonsdale et al., 2013). This project collected and analyzed multiple human tissues from donors who are also densely genotyped, to assess genetic variation within their genomes. By analyzing global RNA expression within individual tissues and treating the expression levels of genes as quantitative traits, variations in gene expression that are highly correlated with genetic variation can be identified as expression quantitative trait loci, or eQTLs (Lonsdale et al., 2013).

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

Variables	Non-asthmatic	Asthmatic	p-value
	N (%)	N (%)	
Total	949	360	
Sex (1198)			
Male	594 (64.5)	99 (35.7)	< 0.0001[a]
Female	327 (35.5)	178 (64.3)	< 0.0001[a]
Age group (1201)			
≤5 years	341 (36.6)	96 (35.7)	0.488
6–7 years	320 (34.3)	85 (31.6)	0.491
≥8 years	271 (29.1)	88 (32.7)	0.419
[a]Positive specific IgE production (1223)			
At least one allergen	327(51.6)	134(48.4)	< 0.0001[a]
D. pteronyssinus	179(18.9)	91 (32.9)	< 0.0001[a]
B. tropicalis	289(30.5)	129(46.6)	< 0.0001[a]
B. germanica	118(12.5)	47(17.5)	0.054
P. americana	81(8,6)	31(11.2)	0.182
[b]Positive skin prick test response (1222)			
At least one allergen	273 (28.9)	100 (36.1)	0.022
D. pteronyssinus	132(14.0)	62(22.4)	0.001
B. tropicalis	193(20.4)	76(27.4)	0.013
B. germanica	71(7,5)	31(11.2)	0.037
P. americana	120(12.7)	49(17.7)	0.034

[a] > 0.70 kU/L.
[b] > 3 mm diameter.

3. Results

3.1. Study population and data collection

Table 1 summarizes the characteristics of the study population and demonstrate that there is statistical difference between both genders and specific IgE production for at least on allergen, *D. pteronyssinus* and *B. tropicalis*; existing more males in the non-asthmatic group than females, on the other hand, there are more females than males in the asthmatic group.

3.2. Description of the DENND1B polymorphisms

In this study, 166 SNPs were extracted from Illumina 2.5 HumanOmni Beadchip. Among these markers, only one was excluded by of Hardy-Weinberg Equilibrium (HWE) test ($p \leq 10^{-3}$) and 93 SNPs were excluded due to very low minor allele frequency (MAF < 0.05). Thus from 166 SNPs typed, 72 were included in the association analysis. Table 2 presents information from 23 SNPs discussed in the present study, that had SNPs with at least two associations with asthma and/or atopy phenotypes and the other 49 with only one association are showed in the supplementary material (Tables A2 and A3).

3.3. Association of DENND1B polymorphisms with asthma

The C allele of rs6691216 (OR 0.76, CI 0.60–0.96) was negatively associated with asthma (Table 3). The A allele of rs1421396 (OR 1.45, CI 1.04–2.00) and the allele C of rs1421389 (OR 1.44, CI 1.04–1.99) were positively associated with asthma (Table 3) and they are in perfect linkage disequilibrium (Fig. 2).

3.4. Association of DENND1B polymorphisms with atopic and non-atopic asthma

The allele T of rs16841893 (OR 2.37, CI 1.43–3.94), allele C of rs6694441 (OR 2.37, CI 1.43–3.94), allele G of rs73077640 (OR 2.39, CI 1.51–3.79), allele A of rs57589685 (OR 2.00, CI 1.31–3.05) and allele A of rs73073636 (OR 1.72, CI 1.05–2.83), respectively, were positively associated with non-atopic asthma and the allele C of rs6685897 (OR 1.62, CI 1.04–2.52) was positively associated with atopic asthma when compared with control (Table 4).

Table 2
Base pairs, allele, Minor allele frequency, Hardy–Weinberg equilibrium, function and RegulomeDB score of *DENND1B* SNPs.

SNP	Location allele[a]	MAF	HWE	Function[b]	RegulomeDB Score
rs73077640	G/A	0.05	0.57	intron variant	5
rs6694441	C/T	0.05	0.57	intron variant	7
rs17641842	G/T	0.06	0.02	intron variant	5
rs57589685	A/G	0.06	0.63	intron variant	6
rs10801621	G/A	0.07	0.14	intron variant	7
rs6685897	C/T	0.07	0.08	intron variant	5
rs73073636	A/G	0.07	0.70	nc transcript variant,utr variant 3 prime	5
rs981520	A/G	0.08	0.72	intron variant	5
rs2147771	A/G	0.08	0.73	intron variant	7
rs4427436	G/A	0.08	0.73	intron variant,utr variant 5 prime	6
rs3902061	G/T	0.08	0.73	intron variant	5
rs1421396	A/G	0.09	0.74	intron variant	4
rs1421389	C/A	0.09	0.74	nc transcript variant,utr variant 3 prime	5
rs12354257	C/T	0.19	0.92	intron variant	7
rs2884629	G/T	0.20	0.44	intron variant	7
rs1998598	G/A	0.20	0.50	intron variant	6
rs6691216	C/T	0.25	0.41	intron variant	3a
rs11799915	G/A	0.27	0.88	intron variant	6
rs1573100	C/T	0.30	0.43	intron variant	5
rs4915551	A/G	0.46	0.26	intron variant	7
rs12066841	T/C	0.46	1	intron variant	5
rs16841893	T/C	0.06	0.81	intron variant	7
rs17641524	T/C	0.08	0.48	intron variant,synonymous codon,utr variant 3 prime	3a

[a] First is the alternative allele and the second is the reference allele (A1/A2).
[b] NCBI National Center for Biotechnology Information. MAF: Minor allele frequency. HWE: Hardy–Weinberg equilibrium.

Table 3
Association between SNPs in *DENND1B* gene and asthma by logistic regression adjusted for sex, age, helminth infections and ancestry.

SNP	OR	95% CI	P value	Perm
rs6691216 (C/T)	0.76	0.60–0.96	0.024	0.019
rs1421396 (A/G)	1.45	1.04–2.00	0.025	0.021
rs1421389 (C/A)	1.44	1.04–1.99	0.027	0.023

SNP: single nucleotide polymorphism; OR: odds ratio; 95% CI: 95% confidence interval; Perm: permutational P value for correction for multiple tests.

3.5. Association of DENND1B polymorphisms with asthma severity

The allele A of rs57589685 (OR 1.92, CI 1.24–2.98), allele A of rs73073636 (OR 1.90, CI 1.28–2.83), allele T of rs16841893 (OR 1.71, CI 1.08–2.73), allele C of rs6694441 (OR 1.67, CI 1.02–2.73) and allele G of rs73077640 (OR 1.67, CI 1.02–2.73) were positively associated with asthma severity and the allele A rs4915551 (OR 0.68, CI 0.53–0.87) and allele C of rs6691216 (OR 0.62, CI 0.45–0.86) were negatively associated with asthma severity (Table 5).

3.6. Association of DENND1B polymorphisms with specific IgE serum levels

The results of the analysis between the *DENND1B* SNPs and specific IgE serum levels are showed in Table 6. The allele A of rs57589685 (OR

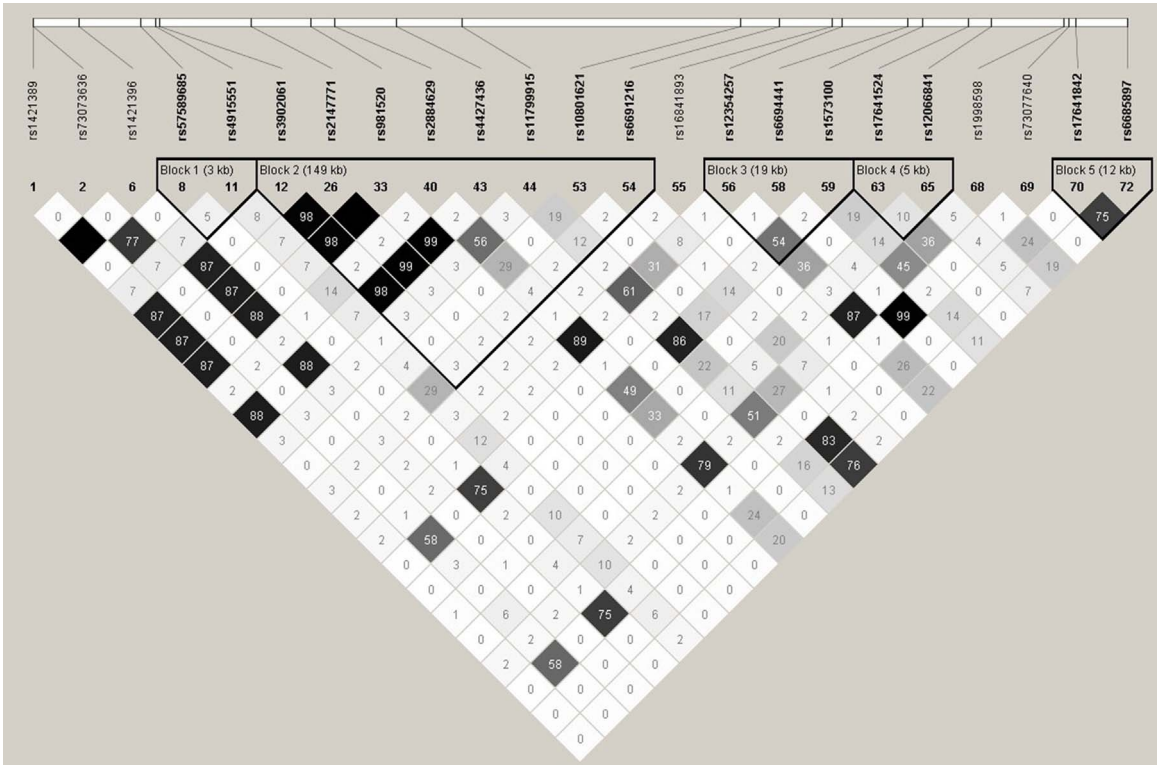

Fig. 2. Pairwise LD within Haploview by using the R‘ squared statistic for the *DENND1B* gene. Intensity of shading indicates the degree of confidence in the R‘ value.

Table 4
Association between SNPs in *DENND1B* gene and atopic and non-atopic asthma by logistic regression adjusted for sex, age, helminth infections and ancestry.

SNP	OR	95% CI	P value	Perm
Non-atopic Asthma vs Control				
rs6694441 (C/T)	2.37	1.43–3.94	0.0007	0.0005
rs73077640 (G/A)	2.37	1.43–3.94	0.0007	0.0005
rs57589685 (A/G)	2.39	1.51–3.79	0.0002	0.0001
rs73073636 (A/G)	2.00	1.31–3.05	0.0011	0.0010
rs16841893 (T/C)	1.72	1.05–2.83	0.0312	0.0302
Atopic Asthma vs Control				
rs6685897 (C/T)	1.62	1.04–2.52	0.0308	0.0460

SNP: single nucleotide polymorphism; OR: odds ratio; 95% CI: 95% confidence interval; Perm: permutational P value for correction for multiple tests.

Table 5
Association between SNPs in DENND1B gene and asthma severity by logistic regression adjusted for sex, age, helminth infections and ancestry.

SNP	OR	95% CI	P value	Perm
rs4915551 (A/G)	0.68	0.53–0.87	0.003	0.002
rs6691216 (C/T)	0.62	0.45–0.86	0.003	0.003
rs57589685 (A/G)	1.92	1.24–2.98	0.003	0.003
rs73073636 (A/G)	1.90	1.28–2.83	0.001	0.001
rs16841893 (T/C)	1.71	1.08–2.73	0.023	0.024
rs6694441 (C/T)	1.67	1.02–2.73	0.040	0.030
rs73077640 (G/A)	1.67	1.02–2.73	0.040	0.030

SNP: single nucleotide polymorphism; OR: odds ratio; 95% CI: 95% confidence interval; Perm: permutational P value for correction for multiple tests.

0.68, CI 0.47–0.98) and the allele A of rs73073636 (OR 0.71, CI 0.51–0.98) were negatively associated with specific IgE serum levels for *Blomia tropicalis*. The allele A of rs4915551 (OR 1.23, CI 1.01–1.48) was positively associated with specific IgE serum levels for *Dermatophagoides pteronyssinus*.

The allele C of rs6685897 was positively associated with specific IgE levels for all the allergens tested (*D. pteronyssinus* (OR 1.46, CI 1.05–2.02), *B. tropicalis* (OR 1.38, CI 1.02–1.86), *B. gemanica* (OR 1.50, CI 1.02–2.20) and *P. americana* (OR 1.69, CI 1.10–2.61)). The rs10801621 was positively associated with specific IgE serum levels for *B. gemanica* (OR 1.53, CI 1.03–2.28) and *P. americana* (OR 1.72, CI 1.10–2.70). The allele G of rs17641842 (OR 1.74, CI 1.09–2.77) and the allele G rs3902061 (OR 0.52, CI 0.28–0.98) were associated with specific IgE serum levels for *P. americana*, positively and negatively, respectively.

Table 6
Association between SNPs in DENND1B gene and specific IgE levels by logistic regression adjusted for sex, age, helminth infections and ancestry.

SNP	OR	95% CI	P value	Perm
Specific IgE for *Dermatophagoides pteronyssinus*				
rs6685897 (C/T)	1.46	1.05–2.02	0.022	0.027
rs4915551 (A/G)	1.23	1.01–1.48	0.030	0.045
Specific IgE for *Blomia tropicalis*				
rs6685897 (C/T)	1.38	1.02–1.86	0.033	0.041
rs57589685 (A/G)	0.68	0.47–0.98	0.039	0.022
rs73073636 (A/G)	0.71	0.51–0.98	0.042	0.035
Specific IgE for *Blatela gemanica*				
rs10801621 (G/A)	1.53	1.03–2.28	0.034	0.045
rs6685897 (C/T)	1.50	1.02–2.20	0.035	0.043
Specific IgE for *Periplaneta americana*				
rs6685897 (C/T)	1.69	1.10–2.61	0.016	0.011
rs10801621 (G/A)	1.72	1.10–2.70	0.016	0.013
rs17641842 (G/T)	1.74	1.09–2.77	0.019	0.016
rs3902061 (G/T)	0.52	0.28–0.98	0.045	0.031

SNP: single nucleotide polymorphism; OR: odds ratio; 95% CI: 95% confidence interval; Perm: permutational P value for correction for multiple tests.

3.7. Association of DENND1B polymorphisms with skin prick test

The results of the analysis between the *DENND1B* SNPs and skin prick test are shown in Table 7. The allele C of rs6694441 and the allele G of rs73077640 were negatively associated with skin test for at least

Table 7
Association between SNPs in DENND1B gene and skin prick test by logistic regression adjusted for sex, age, helminth infections and ancestry.

SNP	OR	95% CI	P value	Perm
Skin prick test for at least one allergen tested				
rs6685897 (C/T)	1.64	1.21–2.2	0.001	0.001
rs10801621 (G/A)	1.62	1.18–2.22	0.002	0.002
rs12354257 (C/T)	1.39	1.12–1.72	0.002	0.003
rs11799915 (G/A)	1.31	1.08–1.59	0.005	0.007
rs17641842 (G/T)	1.59	1.13–2.23	0.006	0.006
rs1998598 (G/A)	1.32	1.07–1.62	0.009	0.009
rs1573100 (C/T)	1.25	1.04–1.51	0.016	0.028
rs2884629 (G/T)	1.29	1.04–1.59	0.017	0.024
rs4915551 (A/G)	1.21	1.02–1.44	0.026	0.029
rs6694441 (C/T)	0.63	0.41–0.96	0.034	0.031
rs73077640 (G/A)	0.63	0.41–0.96	0.034	0.031
Skin prick test for *Dermatophagoides pteronyssinus*				
rs6694441 (C/T)	0.43	0.22–0.82	0.010	0.009
rs73077640 (G/A)	0.43	0.22–0.82	0.010	0.009
rs1998598 (G/A)	1.36	1.05–1.77	0.017	0.019
rs6685897 (C/T)	1.55	1.08–2.23	0.017	0.014
rs12354257 (C/T)	1.34	1.03–1.75	0.029	0.026
rs17641524 (T/C)	1.47	1.03–2.10	0.033	0.041
Skin prick test for *Blomia tropicalis*				
rs6685897 (C/T)	1.83	1.33–2.53	0.0001	0.0001
rs12354257 (C/T)	1.48	1.17–1.88	0.0009	0.0008
rs1998598 (G/A)	1.42	1.13–1.79	0.002	0.002
rs11799915 (G/A)	1.38	1.12–1.71	0.002	0.002
rs10801621 (G/A)	1.65	1.17–2.31	0.003	0.003
rs17641842 (G/T)	1.63	1.14–2.34	0.007	0.007
rs1573100 (C/T)	1.31	1.06–1.60	0.010	0.009
rs2884629 (G/T)	1.35	1.07–1.70	0.010	0.017
rs17641524 (G/T)	1.48	1.07–2.04	0.015	0.017
rs4915551 (A/G)	1.25	1.03–1.51	0.019	0.028
rs57589685 (A/G)	0.58	0.37–0.92	0.020	0.029
rs6694441 (C/T)	0.57	0.35–0.95	0.031	0.029
rs73077640 (G/A)	0.57	0.35–0.95	0.031	0.029
Skin prick test for *Blatela gemanica*				
rs6685897 (C/T)	1.80	1.15–2.82	0.009	0.007
rs10801621 (G/A)	1.83	1.15–2.93	0.010	0.007
rs12066841 (T/C)	0.55	0.40–0.76	0.0002	0.0001
Skin prick test for *Periplaneta americana*				
rs6694441 (C/T)	0.46	0.24–0.90	0.024	0.030
rs73077640 (G/A)	0.46	0.24–0.90	0.024	0.030
rs6685897 (C/T)	1.66	1.15–2.40	0.006	0.005
rs4915551 (A/G)	1.26	1.01–1.59	0.039	0.045
rs12354257 (C/T)	1.46	1.11–1.92	0.006	0.007
rs1998598 (G/A)	1.36	1.04–1.78	0.021	0.016
rs10801621 (G/A)	1.62	1.10–2.38	0.013	0.011
rs11799915 (G/A)	1.30	1.01–1.67	0.037	0.032
rs2884629 (G/T)	1.39	1.06–1.82	0.015	0.014
rs17641842 (G/T)	1.55	1.03–2.34	0.034	0.035
Skin prick test for cat epithelium				
rs12066841 (T/C)	3.49	1.24–9.78	0.017	0.012
Skin prick test for fungi				
rs3902061 (G/T)	8.48	2.05–35.13	0.003	0.004
rs981520 (A/G)	5.73	1.59–20.62	0.007	0.011
rs4427436 (G/A)	5.71	1.58–20.6	0.007	0.011
rs2147771 (A/G)	5.70	1.58–20.54	0.007	0.011
rs1421396 (A/G)	6.27	1.62–24.13	0.007	0.009
rs1421389 (C/A)	6.24	1.62–24.05	0.007	0.009

SNP: single nucleotide polymorphism; OR: odds ratio; 95% CI: 95% confidence interval; Perm: permutational P value for correction for multiple tests.

one allergen tested (OR 0.63, CI 0.41–0.96), *Dermatophagoides pteronyssinus* (OR 0.43, CI 0.22–0.82), *Blomia tropicalis* (OR 0.57, CI 0.35–0.95) and *Periplaneta americana* (OR 0.46, CI 0.24–0.90) and the rs1998598 (OR 1.32, CI 1.07–1.62; OR 1.36, CI 1.05–1.77; OR 1.42, CI 1.13–1.79; OR 1.36, CI 1.04–1.78; respectively) and the allele C of rs12354257 (OR 1.39, CI 1.12–1.72; OR 1.34, CI 1.03–1.75; OR 1.48, CI 1.17–1.88; OR 1.46, CI 1.11–1.92; respectively) presented the inverse result, that is, positively associated with these phenotypes.

The allele A of rs4915551, allele G of rs11799915, allele G of rs17641842 and allele G of rs2884629 were positively associated with skin test for at least one allergen tested (OR 1.21, CI 1.02–1.44; OR 1.31, CI 1.08–1.59; OR 1.59, CI 1.13–2.23; OR 1.29, CI 1.04–1.59; respectively), *B. tropicalis* (OR 1.25, CI 1.03–1.51; OR 1.38, CI 1.12–1.71; OR 1.63, CI 1.14–2.34; OR 1.35, CI 1.07–1.70) and *P. americana* (OR 1.26, CI 1.01–1.59; OR 1.30, CI 1.01–1.67; OR 1.55, CI 1.03–2.34; OR 1.39, CI 1.06–1.82). The allele C of rs6685897 was positively associated with skin test for at least one allergen tested (OR 1.64, CI 1.21–2.2), *D. pteronyssinus* (OR1.55, CI 1.08–2.23), *B. tropicalis* (OR 1.83, CI 1.33–2.53), *B. gemanica* (OR 1.80, CI 1.15–2.82) and *P. americana* (OR 1.66, CI 0.15–2.40).

The allele T of rs17641524 was positively associated with skin test for *D. pteronyssinus* (OR 1.47, CI 1.03–2.10) and *B. tropicalis* (OR 1.48, CI 1.07–2.04). The allele G of rs10801621 positively associated with skin test for at least one allergen tested (OR 1.62, CI 1.18–2.22), *B. tropicalis* (OR 1.65, CI 1.17–2.31), *B. gemanica* (OR 1.83, CI 1.15–2.93) and *P. americana* (OR 1.62, CI 1.10–2.38).

The allele C of rs1573100 was positively associated with skin test for at least one allergen tested (OR 1.25, CI 1.04–1.51) and *B. tropicalis* (OR 1.31, CI 1.06–1.60). The allele T of rs12066841 was positively associated with skin test for cat epithelium (OR 3.49, CI 1.24–9.78) and negatively associated with skin test for *B. gemanica* (OR 0.55, CI 0.40–0.76).

The allele A of rs57589685 (OR 0.58, CI 0.37–0.92) was associated negatively with skin test for *B. tropicalis*. The allele G of rs3902061, allele A of rs981520, allele G of rs4427436, allele A of rs2147771, allele A of rs1421396 and allele C of rs1421389 were positively associated with skin test for fungi (OR 8.48, CI 2.05–35.13; OR 5.73, CI 1.59–20.62; OR 5.71, CI 1.58–20.6; OR 5.70, CI 1.58–20.54; OR 6.27, CI 1.62–24.13; OR 6.24, CI 1.62–24.05; respectively).

3.8. Association of DENND1B polymorphisms with IL-13 levels concentration on blood cell culture supernatant

All the SNPs that presents statistical significance were positively associated with production of IL-13 by blood cell culture supernatant stimulated with mitogen (Table 8) and they are in higher linkage disequilibrium (Fig. 3B).

3.9. GTEX in silico analysis

The eQLT was examined to evaluate the expression of the gene depending on the genotype in tissues. GTex data that were statistically significant showed that the polymorphic allele of rs6691216 (C) (Fig. 4A) decreases the expression and the rs1998598 (G) (Fig. 3B), rs12354257 (C) (Fig. 4C), rs1573100 (C) (Fig. 4D) and rs17641524 (T) (Fig. 4E) increases the expression of *DENND1B* in the whole blood (Fig. 4A–E). No significant P-value was found for the eQTL analyzis for the other SNPs described in the study. (Lonsdale et al., 2013).

Table 8
Association between SNPs in *DENND1B* gene and IL-13 production stimulated with mitogen by logistic regression adjusted for sex, age, helminth infections and ancestry.

SNP	OR	95% CI	P value	Perm
rs3902061 (G/T)	1.78	1.17–2.71	0.006	0.006
rs4427436 (G/A)	1.66	1.10–2.51	0.014	0.015
rs981520 (A/G)	1.65	1.09–2.48	0.016	0.019
rs2147771 (A/G)	1.64	1.09–2.48	0.017	0.019
rs10801621 (G/A)	1.65	1.05–2.59	0.027	0.034
rs1421396 (A/G)	1.56	1.04–2.32	0.028	0.026
rs1421389 (C/A)	1.56	1.04–2.32	0.029	0.027

SNP: single nucleotide polymorphism; OR: odds ratio; 95% CI: 95% confidence interval; Perm: permutational P value for correction for multiple tests

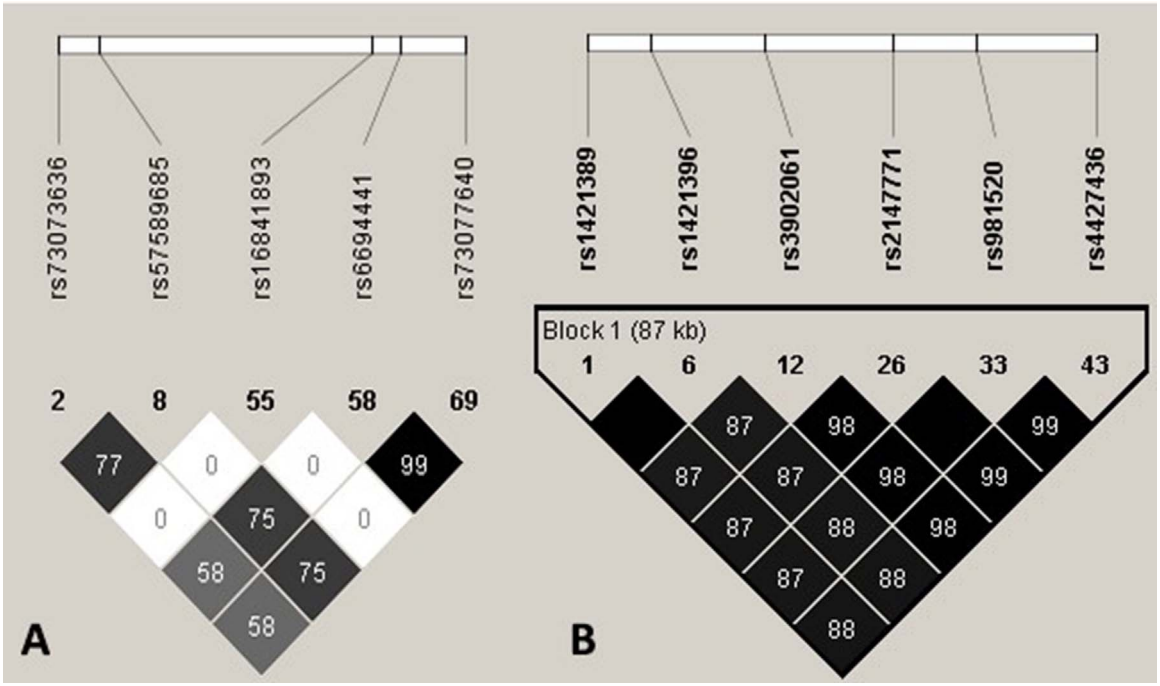

Fig. 3. Pairwise LD within Haploview by using the R‘ squared statistic for the *DENND1B* gene. **A.** Positively association with non-atopic asthma and severity. **B.** Positively association with skin test for fungi and IL13 production stimulated with mitogen.

4. Discussion

The present study is the first to show the influence of genetic variants in *DENND1B* gene on asthma and atopy in a Brazilian population. We find associations of SNPs in *DENND1B* with asthma and atopy markers and also, for the first time, with non-atopic asthma.

In our study the rs6691216 (C) was related to asthma, acting as a protective factor for asthma, non-atopic asthma and asthma severity due to its negatively association and its score from RegulomeDB is 3a which means that this polymorphism can influence the binding of the transcription factor (TF), some motif sequences (indicates sequence-specific binding sites for proteins such as TF) and in the DNase peak, a technique to verify the influence of the SNP. The rs1421396 (A) and rs1421389 (C) were risk factors for asthma, skin test for fungi and IL-13 production stimulated with mitogen. This finding is corroborating with the Sleiman et al. (2010) in a genome wide-association study (GWAS), single nucleotide polymorphisms (SNPs) in *DENND1B* was related to asthma in young children. Probably these polymorphisms are interfering in the expression of *DENND1B*. Four SNPs reported by Sleiman et al. were found in our study. Both SNPs (rs10737692, rs12127378, rs10442656 and rs1775454) were negatively associated with skin prick test for *Blatela germanica* and in Sleiman’s et al. study they were associated with asthma, not atopy.

Risk factors by decreasing the expression of *DENND1B*, are presumably influencing the modulation of TCR, increasing, and consequently leading to a higher production of effector cytokines leading to Th2 asthma (atopic asthma). While the protective factors act in reverse, with a controlled production of effector cytokines. Data from the GTex showed that the rs6691216 decrease the expression of *DENND1B* in the whole blood (Fig. 3C), in contrast with our associations results. Two subsequent studies, in multiple countries, with older children and adults did not corroborating with this finds (Ferreira et al., 2011; Moffatt, 2010). These differences may reflect the different ages of the asthmatic populations studied since genetic variants of *DENND1B* have recently been associated with high levels of exhaled nitric oxide in healthy newborns, a phenotype associated with early-onset wheezing and a family history of allergy or allergic disorders (Chawes et al., 2015; Godar and Lambrecht, 2016) and our population is composed of children from 4 to 11 years old.

The rs1998598 (G), rs12354257 (C), rs17641524 (T), rs11799915 (G), rs10801621 (G), rs17641842 (G), rs1573100 (C) and rs2884629 (G) are risk factor for some allergen sensitizations measured by skin prick tests, indicating that these polymorphisms are more likely associated with atopy, due to the positively association with allergies markers. The rs1998598, rs12354257, rs1573100 and rs17641524 are associated positively with some allergen skin prick tests, so they are risk factors that will probably decrease the DENND*1B* expression but GTex data shows that these polymorphisms have higher expression of *DENND1B* in whole blood cell (Fig. 4B–E).

The rs6694441 (C), rs73077640 (G), rs57589685 (A), rs73073636 (A) and rs16841893 (T) were risk factors for non-atopic asthma and asthma severity and four of them were negatively associated with allergy markers, which confirms the association with non-atopic asthma and severity once these phenotype are associated with neutrophilic inflammation, are usually triggered by the presence of pathogens and pollutants and, because of that, have been shown to contribute to severe asthma and relative corticosteroid insensitivity (Brook et al., 2015). But severity is not exclusive for this asthma phenotype, since that atopic asthma also can be severe due to some allergens that causes cell damaged. The rs73073636 and rs57589685 are in linkage disequilibrium such as rs57589685 and rs6694441, and rs57589685 and rs73077640. The rs6694441 and rs73077640 are in higher linkage disequilibrium (Fig. 2A). Thus, we hypothesize that the associations of *DENND1B* with non-atopic asthma suggest that this gene can also be related with Th1/Th17 cytokine production and not only specifically Th2 cytokine production as previously described for Yang et al. (2016), since this phenotype of asthma is usually triggered by Th1/Th17 immune response reaction to pathogens and pollutants.

The rs4915551 (A) was positively associated with specific IgE levels for *Dermatophagoides pteronyssinu*, skin test for at least one allergen tested, *Blomia tropicalis* and *Periplaneta americana* indicates that this variant is a risk factor for allergies markers and is also negatively associated with severity, corroborating the association with atopy.

The rs10801621 (G), rs17641842 (G) and rs6685897 (C) were positively associated with some skin prick test, specific serum IgE, IL-13 production by blood cell cultures stimulated with mitogen (only rs17641842)

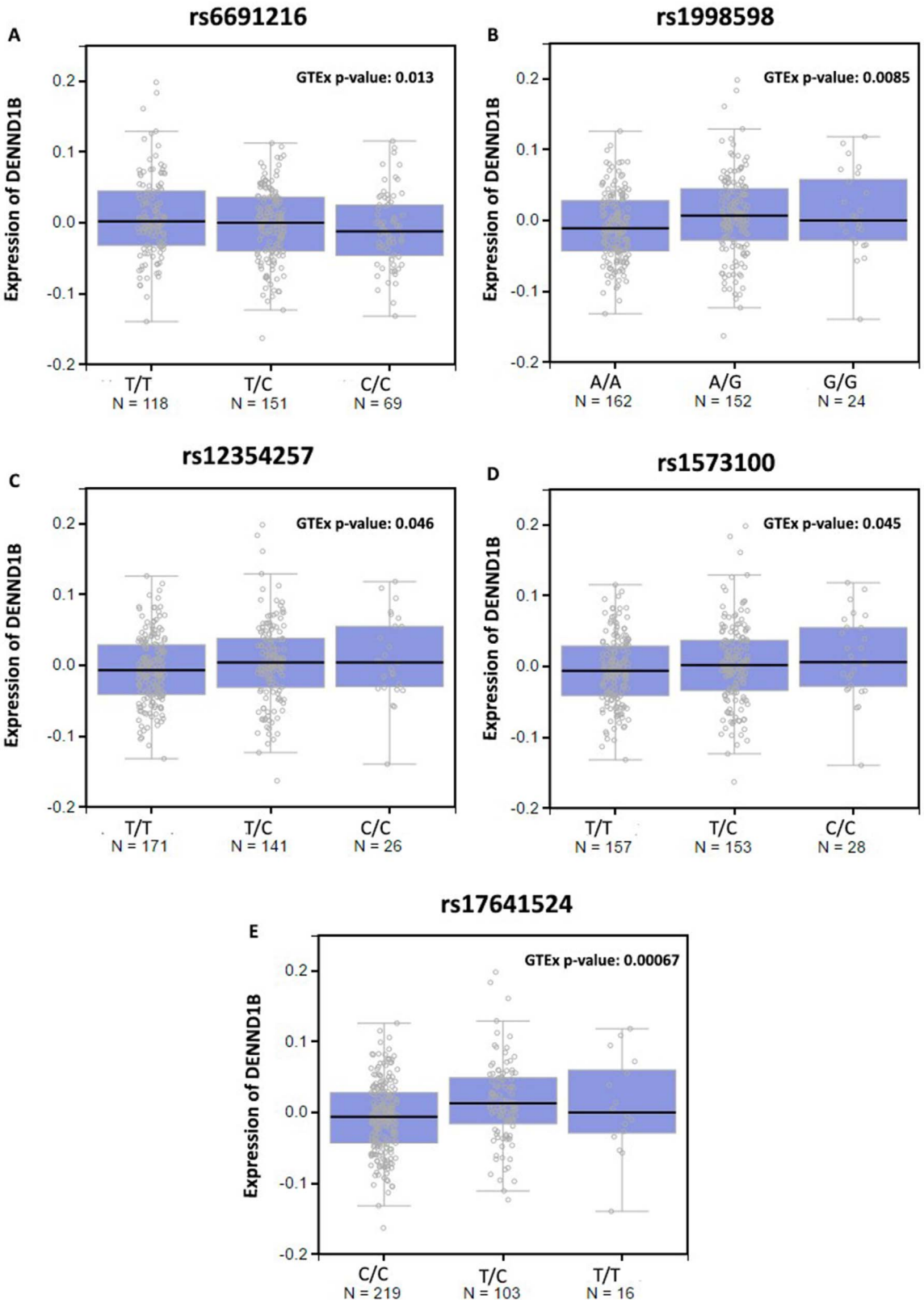

Fig. 4. GTEx-Gene expression level of *DENND1B* from whole-blood grouped by genotypes of A. rs6691216 (*p*-value 0.013), B. rs1998598 (*p*-value 0.0085), C. rs12354257 (*p*-value 0.046), D. rs1573100 (*p*-value 0.045) and E. rs17641524 (*p*-value 0.00067).

and atopic asthma (only rs6685897). With this result, we can infer that they are risk factors for atopy and can increase the production of Th2 cytokines and consequently of IgE. The presence of IgE induces the mast cells degranulation and releases their cytoplasmatic granules, leading to papule formation and positivity for skin testing and also leads to an atopic asthma. In the same way, the rs3902061 (G), rs981520 (A), rs4427436 (G) and rs2147771 (A) were positively associated with skin prick test for fungi and IL-13 production by blood cell cultures stimulate with mitogen. All the SNPs associated with skin prick test for fungi and IL-13 production are in higher or total linkage disequilibrium (Fig. 2B).

The rs6691216, rs1998598, rs12354257, rs1573100 and rs17641524 showed opposing results in GTex. This can be explained by the fact that the project uses several populations that are different from those used in this study. Thus, ethnic components will influence the protective or risk function of SNPs, showing that is necessary to replicate this study in others populations, because the same SNP can have different outcomes depending on the population. Moreover, the majority of the polymorphisms found in this are intronic variants that can affect the mechanisms of alternative splicing and gene transcription when located in consensus sequences or sequences accentuating/inhibiting these mechanisms. That can explain the effect of the SNPs in the expression of *DENND1B*.

Although the study has reached its aims, there were some unavoidable limitations. First, due to limit time, it was not possible to observe the functional impact of the gene. Second, the lack of studies showing the influence of *DENND1B* in Th1/Th17 cells makes it impossible to discuss more the association of *DENND1B* with non-atopic asthma and the gene impact on this phenotype.

In conclusion, the polymorphisms in *DENND1B* gene may influence the asthma severity and the status of atopy. This was the first study to demonstrate association of the gene with non-atopic asthma, thus showing the need to identify why *DENND1B* specifically regulates the production of Th2 effector cytokines but not the production of Th1 or Th17 effector cytokines. Also, more studies are needed to elucidate the potential role of *DENND1B* gene on asthma and atopy, and how it could be used as a strategy to control the disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2017.06.030.

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