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Impact of *FOXP3* gene polymorphisms and gene-environment interactions in asthma and atopy in a Brazilian population

Cintia Rodrigues Marques [a,*], Bianca Sampaio Dotto Fiuza [b], Thiago Magalhães da Silva [c], Tamires Cana Brasil Carneiro [b], Ryan Santos Costa [b], Monica Francisca de Assis Silva [d], Wagma Lauane Luz Viana [d], Valdirene Leão Carneiro [d], Neuza Maria Alcantara-Neves [b], Maurício Lima Barreto [e], Camila Alexandrina Figueiredo [b]

[a] *Multidisciplinary Health Institute, Federal University of Bahia, Vitória da Conquisra, Brazil*
[b] *Institute of Health Sciences, Federal University of Bahia, Salvador, Brazil*
[c] *Department of Biological Sciences, State University of Southwestern Bahia, Jequié, Brazil*
[d] *Department of Life Sciences, State University of Bahia, Salvador, Brazil*
[e] *Center for Data and Knowledge Integration for Health, Fiocruz, Salvador, Brazil*

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ABSTRACT

Background: Polymorphisms in genes related to the activation and development of regulatory T cells (Tregs), such as *FOXP3*, may be associated with asthma and atopy development. Additionally, environmental factors such as exposure to infections can modify the effect of these associations. This study evaluated the impact of polymorphisms in the *FOXP3* on the risk of asthma and atopy as also gene-environment interactions in these outcomes.

Methods: This study included 1,246 children from the SCAALA program, between 4 and 11 years of age. DNA was extracted from peripheral blood and eight SNPs (rs2280883, rs11465476, rs11465472, rs2232368, rs3761549, rs3761548, rs2232365 and rs2294021) were genotyped using the 2.5 HumanOmni Beadchip from Illumina (San Diego, California, USA) or TaqMan qRT-PCR.

Results: The rs2232368 (Allele T) was positively associated with asthma symptoms (OR = 1.95, CI = 1.04 to 3.66, p = 0.040) and skin prick test (SPT) reactivity to aeroallergens (OR = 2.31, CI = 1.16 to 4.59, p = 0.017). The rs3761549 (Allele T) was positively associated with SPT reactivity (OR = 1.44, CI = 1.03 to 2.02, p = 0.034). The rs2280883 (Allele C) was negatively associated with specific IgE to aeroallergens (OR = 0.83, CI = 0.70 to 0.99, p = 0.040). Furthermore, the rs2280883 played a protective role in the development of atopy only in individuals seropositive to Epstein-Barr virus (EBV) infection (OR = 0.74, CI = 0.60 to 0.92, p = 0.003 and OR = 0.74; 95% CI = 0.61–0.91, p = 0.007 for SPT and sIgE respectively), but not in individuals without EBV infection.

Conclusion: Polymorphisms in the *FOXP3* gene were associated with the risk of atopy and asthma development in our population. In addition, EBV infection had an effect modifier of the observed association for rs2280883 variant.

1. Introduction

Asthma is a complex disease determined by interactions between host genetic factors and environmental exposure (Upton et al., 2000). It is estimated that 339 million people have asthma worldwide (Childhood, 2014) and approximately 250 thousands annual deaths occur due to the disease (WHO, 2007). The pathogenesis of allergic diseases is related to the involvement of regulatory T lymphocytes $CD4^{+}CD25^{+}$ (Treg) which play a central role as regulators of the immune response (Nakashima et al., 2014). Regulatory mechanisms are

Abbreviations: CI, confidence interval; eQTL, Expression Quantitative Trait Locus; FOXP3, forkhead box protein 3; CACNA1F, calcium voltage-gated channel subunit alpha1 F GTEx, Genotype-Tissue Expression; LD, linkage disequilibrium; MAF, minor allele frequency; OR, odds ratio; PC, principal component; SCAALA, Social Change, Asthma and Allergy in Latin America; SNP, Single Nucleotide Polymorphism.

* Corresponding author.
E-mail address: cintiabiomedica@yahoo.com.br (C.R. Marques).

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important to maintain peripheral tolerance by the immune system. Tregs inhibit Th2 cell activation (by suppressing of IL-4, IL-5, IL-9 and IL-13), blocking the migration of T cells to inflamed tissue, suppressing IgE production, inducing IgG production in B cells and limiting inflammation induced by Th17 (Stelmaszczyk-Emmel et al., 2012). Treg cells express the protein forkhead box protein 3 (FOXP3), a transcription factor that controls their development and function (Zhang et al., 2009). The human *FOXP3* gene is located on the X chromosome and consists of 11 exons encoding a protein with 431 amino acids (Takenaka et al., 2013). Several polymorphisms in *FOXP3* have been described and some studies have evaluated the association of polymorphisms in this gene with the development of allergic conditions such as asthma and atopy (Cintia et al., 2015). In addition, it has been suggested that gene-environment interaction in asthma development may result in genetic associations only under specific conditions (Kraft et al., 2007). Thus, environmental factors such as exposure to infections, especially virus infections, may modify the effect of these associations. There is evidence that viral pathogens such as hepatitis A (HAV), herpes simplex, herpes zoster, and Epstein-Barr virus can modulate allergy development (Alcantara-Neves et al., 2012). Additionally, an association between genetic polymorphisms and asthma and/or atopy may only occur in individuals previously exposed to infection (McIntire et al., 2004). Thus, in the present study, we evaluated the association between polymorphisms in the *FOXP3* gene and asthma and atopy in children living in Salvador - BA, Brazil. Furthermore, we analyzed the influence of exposure to viral infections on the impact of these genetic factors on the risk of asthma and allergies.

2. Methods

2.1. Study design and participant characteristics

This study included 1,246 unrelated children living in Salvador, Bahia, Brazil, who participated in the SCAALA project (Social Changes, Asthma and Allergy in Latin America). Briefly, the children were previously studied to evaluate the impact of a sanitation program and enrolled when they were 0–3 years old in the period 1996 to 2003 (Barreto et al., 2006). Standardized questionnaires were administered to the children's guardians between 1997 and 2003 (baseline) and data was collected on housing, sanitation, and socioeconomic conditions. Data collection was repeated in 2005 when children were aged 4 to 11 years, at which time blood samples were collected and serological tests were performed. This work has ethical approval from the National Commission of Ethics in Research and consent was obtained from the legal guardian of each child.

2.2. Skin prick tests

Skin Prick tests (SPTs) were performed using house dust mite, *Blomia tropicalis*, cat and dog epithelium, fungi, *Blatella germanica* and *Periplaneta americana* using ALK-ABELLO antigens (São Paulo, Brazil).

2.3. Detection of IgE-Antiallergens

The presence and levels of specific IgE (sIgE) to aeroallergens from house dust mite, *Blomia tropicalis, Blatella germanica* and *Periplaneta americana* were determined using available commercially available kits (Immuno CAP System, Phadia AB, Uppsala, Sweden). Children with 0.70 kU/L or greater of specific IgE for any allergen tested were considered to have positive results.

2.4. Asthma definition

The definition of asthma symptoms was done using the phase II International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire according to previous publications (Figueiredo et al., 2013). Asthma cases were defined by the presence of wheezing in the last 12 months and one of the following conditions in the last 12 months: diagnosis of asthma, wheezing while exercising, four or more episodes of wheezing or waking at night due to wheezing. Atopic and non-atopic asthma phenotypes were defined by the presence of asthma symptoms, as defined above, in the presence or absence, respectively, of a positive SPT result and/or specific IgE for at least one of the aeroallergens tested.

2.5. Serological tests for pathogens

Specific IgG levels were measured in serum by available commercial kits (Diamedix, Miami, Florida, USA) for herpes simplex virus (HSV), varicella zoster virus (VZV) and Epstein-Barr virus (EBV). The exposure to the hepatitis A virus (HAV) was determined by the presence of the IgG anti-HAV antibody using ADALTIS kits (Toronto, Canada).

2.6. Genotyping

DNA was extracted from peripheral blood cells from 1,246 children using a commercial kit (Gentra® Puregene® Blood Kit (Qiagen). Polymorphisms included and analyzed in this work were obtained by two different methodologies. SNPs rs2280883, rs11465476, rs11465472, rs2232368 and rs3761549 were selected from the genotyping results for the 2.5 HumanOmni Beadchip of Illumina. Otherwise, the SNPs rs3761548, rs2232365, rs2294021 and rs2280883 were genotyped by RT-PCR platform, TaqMan probe-based 5′-nuclease (Applied Biosystems, Foster City, CA, USA) on QuantStudio 12 k Flex equipment.

2.7. Statistical analysis

Associations of *FOXP3* SNPs with asthma, asthma phenotypes (atopic and non-atopic asthma) SPT and sIgE were analyzed considering the additive genetic model and the X chromosome inactivation process (Konig et al., 2014). To measure associations between polymorphisms of *FOXP3* and different outcomes investigated, the o*dds ratio* (OR) was calculated with confidence intervals of 95% by logistic regression models adjusted for sex, age and the proportions of individual ancestry, estimated as previously described (Lima-Costa et al., 2015). For binary outcomes (IgE, SPT and asthma), analyses were performed on Plink 1.9. To test for differences in effect size according to sex we performed tests of heterogeneity using the METAL program (Willer et al., 2010). Associations between *FOXP3* SNPs and asthma phenotypes were analyzed by polytomous regression models using SPSS 20.0. All analyses adopted a significance level of 5% ($\alpha = 0.05$).

2.8. In silico analysis

To assess the potential role of *FOXP3* polymorphisms as expression quantitative trait locus (eQTL) on lung tissue and whole blood cells, we used data available from the Genotype-Tissue Expression (GTEx) Portal (https://www.gtexportal.org/home/) (Battle et al., 2017).

3. Results

The general characteristics of the study population are described in Supplementary Table 1. Most of the sample consisted of males and aged between 6 and 7 years.

Analysis of the association between *FOXP3* SNPs and asthma symptoms is shown in Table 1. The T allele of rs2232368 was positively associated with asthma in the global sample (OR = 1.95, 95% CI = 1.04–3.36, p = 0.040) and among males (OR = 2.33, 95% CI = 1.16–4.67, p = 0.017), but not among females (OR = 0.58, 95% CI = 0.07–5.01, p = 0.62). No other *FOXP3* SNPs evaluated was significantly associated with asthma symptoms in either the global sample or in the sex-stratified analyses.

Table 1
Association between polymorphisms in the FOXP3 gene and asthma symptoms.

SNP	Reference/ Variant*	Global		Males		Females		P-heterogeneity
		OR (CI 95%)	p value*	OR (CI 95%)	p value*	OR (CI 95%)	p value*	
rs2280883	T/C	0.99 (0.81–1.20)	0.90	0.94 (0.74–1.19)	0.63	1.07 (0.73–1.57)	0.71	0.530
rs11465476	T/C	1.31 (0.68–2.50)	0.42	1.39 (0.66–2.94)	0.38	1.12 (0.30–4.18)	0.87	0.791
rs11465472	G/A	1.27 (0.81–2.00)	0.29	1.18 (0.69–2.02)	0.55	1.68 (0.70–4.04)	0.25	0.475
rs2232368	**C/T**	**1.95 (1.04–3.66)**	**0.04**	**2.33 (1.16–4.67)**	**0.017**	0.58 (0.07–5.01)	0.62	0.229
rs3761549	C/T	1.26 (0.87–1.83)	0.23	1.47 (0.98–2.22)	0.06	0.66 (0.27–1.65)	0.38	0.125
rs3761548	G/T	0.95 (0.76–1.18)	0.66	0.88 (0.68–1.15)	0.35	1.17 (0.77–1.77)	0.45	0.350
rs2232365	C/T	0.97 (0.80–1.18)	0.82	0.92 (0.72–1.17)	0.49	1.05 (0.75–1.48)	0.77	0.133
rs2294021	T/C	0.95 (0.80–1.14)	0.62	1.05 (0.85–1.29)	0.64	0.79 (0.56–1.09)	0.16	0.090

* p value for logistic regression adjusted for sex, age and individual genetic ancestry.

The results of the associations between *FOXP3* variants and atopy are shown in Table 2. In the general population, two SNPs were positively associated with SPT reactivity, the T allele of rs2232368 (OR = 2.313; 95% CI 1.16 – 4.59p = 0.017) and the T allele of rs3761549 (OR = 1.44; p = 0.034). A negative association was observed between the C allele of rs2280883 and specific IgE to aeroallergens (OR = 0.836; p = 0.040). No other SNP had statistically significant association with these outcomes. In the sex-stratified analyses, positive associations for rs2232368 and rs3761549 with SPT reactivity were observed only among the females (OR = 6.4, 95% CI = 1.21–33.49, p = 0.028 and OR = 2.61, 95% CI = 1.33–5.09, p = 0.005 for the T allele of rs2232368 and the T allele of rs3761549, respectively). Similarly, the negative association between rs2280883 and sIgE to aeroallergens was significant only among females (OR = 0.68, 95% CI = 0.47–0.97, p = 0.033). Furthermore, the SNPs rs2232368 and rs3761549 were positively associated with sIgE to aeroallergens only among the females (OR = 5.49, 95% CI = 1.05–28.79, p = 0.044 and OR = 2.10, 95% CI = 1.08–4.08, p = 0.029, respectively).

Despite the sex-specific associations observed for some of the *FOXP3* SNPs evaluated here, the only statistically significant difference in the effect according to sex was observed for rs3761549 in the SPT reactivity outcome (P-heterogeneity = 0.042).

The associations between *FOXP3* polymorphisms and asthma phenotypes are shown in Table 3. None of the SNPs evaluated were significantly associated with atopic asthma or with non-atopic asthma in our population.

Regarding the interaction of *FOXP3* polymorphism with different viral infections in the investigated outcomes, no significant result was observed for asthma symptoms (data not shown). On the other hand, for atopy, a significant interaction with EBV infection was observed (Fig. 1).

Table 3
Association between polymorphisms in the FOXP3 gene and asthma in atopic and non-atopic children.

	Atopic asthmatic × No-asthmatic atopic		No-atopic asthmatic × No-asthmatic no-atopic	
	OR (CI 95%)	p value	OR (CI 95%))	p value
rs2280883	0,94 (0,70–1,26)	0,679	1,09 (0,83–1,43)	0,523
rs11465476	1,24 (0,30–5,13)	0,765	1,51 (0,72–3,16)	0,276
rs11465472	1,20 (0,63–2,27)	0,575	1,33 (0,71–2,52)	0,365
rs2232368	1,51 (0,76–2,97)	0,232	3,19 (0,60–16,85)	0,171
rs3761549	1,28 (0,81–2,01)	0,283	1,02 (0,51–2,03)	0,958
rs3761548	0,86 (0,62–1,21)	0,394	1,09 (0,81–1,48)	0,541
rs2232365	1,00 (0,76–1,32)	0,977	0,92 (0,68–1,22)	0,570
rs2294021	0,98 (0,76–1,27)	0,907	0,93 (0,72–1,20)	0,606

* p value for polytomous logistic regression adjusted for sex, age and individual genetic ancestry.

The C allele of rs2280883 was negatively associated with SPT reactivity (OR = 0.74; 95% CI = 0.60–0.92, p = 0.003) and sIgE to aeroallergens (OR = 0.74; 95% CI = 0.61–0.91, p = 0.007) in individuals seropositive for EBV but not in those seronegative (OR = 1,45; 95% CI = 0.87–2.39, p = 0.145 and OR = 1.35; 95% CI = 0.84–2.19, p = 0.215 for SPT and sIgE, respectively). These results were later corroborated in gene-environment interaction analyzes conducted in the MDR software (Moore, 2004) and are presented in Supplementary Table S2.

The linkage disequilibrium pattern for the *FOXP3* SNPs is shown in Fig. 2. Although the SNPs significantly associated with asthma and atopy are not in the same haplotypic block, pairwise LD analyzes reveal a perfect linkage disequilibrium among the same (D '= 1.0), except for the

Table 2
Association between polymorphisms in the FOXP3 gene and SPT and IgE for aeroallergens.

SNP	Global		Males		Females		P-heterogeneity
	OR (CI 95%)	p value*	OR (CI 95%)	p value*	OR (CI 95%)	p value*	
SPT							
rs2280883	0.87 (0.73–1.05)	0.159	0.95 (0.77–1.16)	0.613	0.72 (0.49–1.03)	0.076	0.205
rs11465476	0.71 (0.34–1.49)	0.372	0.59 (0.20–1.70)	0.330	1.07 (0.33–3.47)	0.913	0.465
rs11465472	1.18 (0.77–1.81)	0.433	1.02 (0.61–1.70)	0.938	1.77 (0.78–4.02)	0.168	0.257
rs2232368	**2.31 (1.16–4.59)**	**0.017**	1.84 (0.90–3.78)	0.095	**6.4 (1.21–33.49)**	**0.028**	0.174
rs3761549	**1.44 (1.03–2.02)**	**0.034**	1.17 (0.79–1.74)	0.425	**2.61 (1.33–5.09)**	**0.005**	**0.042**
rs3761548	1.01 (0.82–1.23)	0.954	1.08 (0.86–1.36)	0.494	0.82 (0.55–1.22)	0.326	0.134
rs2232365	0.92 (0.77–1.10)	0.381	0.92 (0.74–1.14)	0.444	0.91 (0.65–1.25)	0.555	0.753
rs2294021	1.07 (0.91–1.26)	0.394	1.02 (0.84–1.24)	0.807	1.23 (0.91–1.67)	0.184	0.525
sIgE							
rs2280883	**0.83 (0.70–0.99)**	**0.040**	0.90 (0.74–1.10)	0.305	**0.68 (0.47–0.97)**	**0.033**	0.174
rs11465476	0.49 (0.22–1.08)	0.079	0.46 (0.16–1.31)	0.145	0.61 (0.17–2.22)	0.455	0.733
rs11465472	0.89 (0.58–1.37)	0.608	0.80 (0.48–1.32)	0.384	1.18 (0.52–2.72)	0.685	0.428
rs2232368	1.94 (0.98–2.85)	0.057	1.54 (0.75–3.14)	0.233	**5.49 (1.05–28.79)**	**0.044**	0.165
rs3761549	1.30 (0.93–1.82)	0.121	1.10 (0.75–1.62)	0.625	**2.10 (1.08–4.08)**	**0.029**	0.099
rs3761548	0.89 (0.74–1.08)	0.263	0.94 (0.75–1.18)	0.597	0.77 (0.52–1.14)	0.193	0.343
rs2232365	0.98 (0.82–1.16)	0.805	1.07 (0.87–1.33)	0.511	0.83 (0.60–1.14)	0.246	0.196
rs2294021	1.06 (0.91–1.24)	0.426	1.04 (0.86–1.26)	0.271	1.17 (0.87–1.58)	0.297	0.556

* p value for logistic regression adjusted for sex, age and individual genetic ancestry.

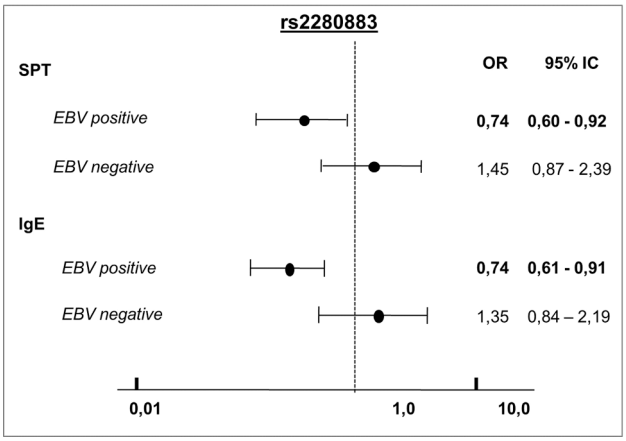

Fig. 1. Association between polymorphisms in the FOXP3 gene and SPT and IgE for aeroallergens stratified by EBV serology.

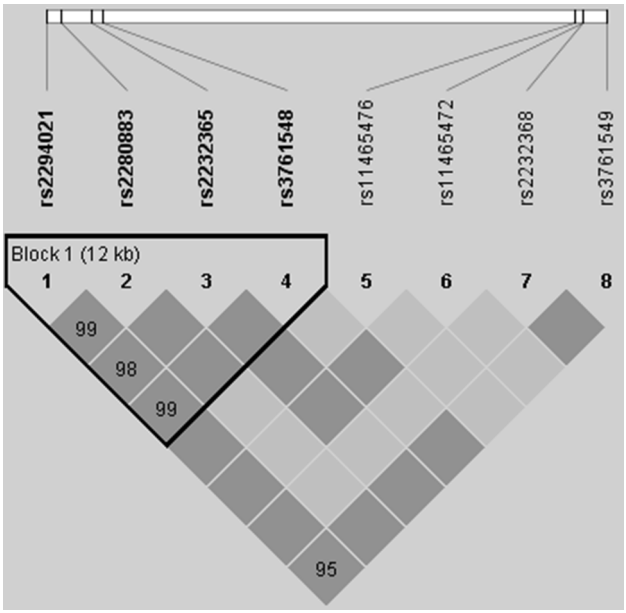

Fig. 2. Linkage disequilibrium (LD) plot of SNPs within *FOXP3* gene in our study population.

pair rs2280883 and rs2232368. As shown in Table 4, the haplotype formed by the combination of the highest risk alleles for asthma or atopy in our population (T alleles of rs2280883, rs2232368 and rs3761549) was also a risk factor for asthma symptoms (OR = 1.94; 95% CI = 1.03–3.65, p = 0.039) and SPT reactivity (OR = 2.31; 95% CI = 1.16–4.59, p = 0.016). The same trend for this haplotype was observed in relation to sIgE to aeroallergens, although it was not statistically significant (OR = 1.95; 95% CI = 0.98–3.86, p = 0.056). In addition, the haplotype formed by the combination of alleles associated with a lower risk of asthma and atopy in our population (C alleles of rs2280883, rs2232368 and rs3761549) was a protective factor for sIgE to aeroallergens (OR = 0.83; 95% CI = 0.70–0.99, p = 0.041).

Analyzes conducted through the GTEx project portal indicated that rs2280883 was the most significantly associated as eQTL, influencing *CACNA1F* gene expression in lung ($p = 2.7 \times 10^{-13}$) and whole blood cells ($p = 1.5 \times 10^{-7}$). In both tissues, the minor allele of rs2280883 (allele C) was negatively associated with *CACNA1F* expression (Supplementary Fig. 1).

4. Discussion

In the present study, we reported significant associations of *FOXP3* polymorphisms with asthma and atopy in children of a large Latin American urban center. Furthermore, EBV infection was an effect modifier of the association of these genetic variants with the risk of atopy in our population. The rs2232368 was associated as a risk factor for asthma symptoms. This SNP is located in the intronic region and there are few studies with this marker. Association between this SNP and infertility has been previously reported (Andre et al., 2011). Thus, to the best of our knowledge, this is the first study to analyze the impact of this polymorphism in asthma and atopy. Regarding the other *FOXP3* SNPs evaluated, none of them were significantly associated with asthma symptoms, although, couple of them (rs3761548, rs2294021, rs3761549) have been previously reported to be associated with asthma, atopy or allergic rhinitis (Zhang et al., 2009; Bottema et al., 2010a; Bottema et al., 2010b; Fodor et al., 2011; Zhang et al., 2012). Furthermore, no *FOXP3* polymorphisms was significantly associated with atopic or non-atopic asthma in our population.

In relation to the atopy status, two SNPs (rs2232368 and rs3762549) were associated with positive SPT to aeroallergens and rs2280883 was negatively associated with specific IgE to aeroallergens. Another study also reported an association of this SNP with specific IgE to egg (food allergy) in children at the earliest age (Bottema et al., 2010b).

Many studies have suggested that viral infections can influence atopic disorders, depending on the virus type, the degree of infection and genetic interaction. Interestingly, different studies have reported an association between seropositivity for Epstein Barr virus and protection for atopy (Nilsson et al., 2005; Veiga et al., 2011; Alcantara-Neves et al., 2012). However, interactions of genetic polymorphisms with infections can modulate the effect of such associations. For instance, a negative association of HAV infection and protection for atopy only in individuals with a polymorphism in the TIM-1 gene (cell surface receptor used by HAV to infect cells) has been previously demonstrated (McIntire et al., 2004). Herein, we reported that the C allele of rs2280883 was negatively associated with SPT reactivity only in EBV seropositive individuals. Polymorphisms in the *FOXP3* gene may influence the expression of this transcriptional factor and therefore the development of regulatory T cells and modulate the allergies. Otherwise, EBV has several escape mechanisms that can directly modulate immune responses. Interestingly, one study has shown a homology region of the EBV genome with the human IL10 gene, suggesting that the virus could regulate the expression of IL-10 levels (Moore et al., 1990). Furthermore, other studies indicate that EBV is capable of stimulating the production of LMP1 (anti-apoptotic protein) decreasing IFN-ɣ levels and may lead to IgE decrease (Marshall et al., 2003). In this context, we can suppose that in our population, EBV could influence Treg development and lead to a protection against atopy especially in individuals with the rs2280883 (C

Table 4
Haplotypes formed by the combination of the highest risk alleles for asthma or atopy.

Haplotypes	Asthma Symptoms	pValue	SPT+	pValue	sIgE > 0.70	pValue
rs2280883/rs2232368/rs3761549	OR (95% CI)		OR (95% CI)		OR (95% CI)	
TTT	1.94 (1.03–3.65)	**0.039**	2.31 (1.16–4.59)	**0.016**	1.95 (0.98–3.86)	0.056
TCT	1.09 (0.70–1.71)	0.687	1.23 (0.83–1.82)	0.297	1.16 (0.78–1.70)	0.450
CCC	0.98 (0.80–1.20)	0.883	0.87 (0.73–1.05)	0.154	0.83 (0.70–0.99)	**0.041**
TCC	1.05 (0.87–1.26)	0.576	0.98 (0.83–1.15)	0.809	0.91 (0.78–1.07)	0.274

allele). However, other studies should be conducted to better understand the impact of gene-environment interactions on the development of asthma and atopy.

Our results also suggest the potential involvement of *FOXP3* gene polymorphisms as eQTLs influencing the expression of genes important for T cell development, survival and function, such as *CACNA1F* (Omilusik et al., 2011). This gene encodes an α-1 subunit (Cav1.4) of the voltage-dependent Ca^{2+} channel (Cav1), which is expressed in different cells of the immune system, such as mast cells, CD4 + T cells, TH1 cells, and TH17 cells (McRory et al., 2004; Robert et al., 2014). A previous study using a murine asthma model demonstrated that knocking down of Cav1 Ca^{2+} channels prevented the development of airway inflammation and reduced the total serum IgE concentration compared to the control group (Cabral et al., 2010). Interestingly, the rs2280883 of *FOXP3*, which was negatively associated with *CACNA1F* expression in lung and whole blood cells in GTEx samples, was a protective factor for atopy measured by sIgE in the present study. Noteworthy, the rs2280883 was also negatively associated with serum total IgE levels in our population assuming an additive genetic model adjusted for sex, age, individual genetic ancestry and helminth infections ($\beta = -0.20$; $p = 0.023$).

5. Conclusion

Polymorphisms in the *FOXP3* gene were associated with the development of asthma and atopy in our population. Importantly, the impact of these polymorphisms on such outcomes can be influenced by exposure to EBV. Studies with other populations and functional analyses can help better understand the impact of these polymorphisms on asthma and atopy.

Contributors

CRM and CAF conceived the study. MLB is the cohort coordinator, providing samples and data. TMS coordinated the genetic analyses. CRM and TMS wrote the manuscript. All the authors contributed with discussion on the results and on the manuscript. The authors BSDF, TCBC, RSC, MFAS, WLLV, VLC and NMA-N contributed with data, laboratorial analyses or statistical analyses.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2022.146706.

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