ARQUIVOS DE ASMA, ALERGIA E IMUNOLOGIA

Original Article

Study of variants in the mTOR gene with asthma and therapeutic control in a population of Salvador/BA

Estudo de variantes no gene mTOR com asma e controle terapêutico em uma população de Salvador/BA

Ítalo Santos Uzêda[1], Raisa Coelho[1], Ryan Santos Costa[1], Camila Figueiredo[1]

ABSTRACT

Introduction: Asthma is an inflammatory airway disease that is influenced by several factors. An evolutionarily conserved serine/threonine kinase named mTOR plays a key role in the integration of environmental signals in the form of growth factors, amino acids, and energy. In the immune system, mTOR is a critical regulator. The mTOR pathway exerts central control over processes in the immune response and in T-cell proliferation, multiplication, and differentiation. Variations in the gene responsible for mTOR complexes have been associated with different critical levels of cytokines, increased likelihood of developing asthma, and increased prevalence of atopy. **Objective and method:** This study aimed to investigate the association of mTOR gene variants with asthma, asthma severity, and atopy, as well as to perform a cytokine analysis. **Result and conclusion:** The findings reinforce the importance of mTOR gene variants in the development of asthma.

Keywords: Asthma, TOR serine-threonine kinases, single nucleotide polymorphism.

RESUMO

Introdução: A asma é uma doença inflamatória das vias aéreas, com diversos fatores influenciando essa condição inflamatória. A mTOR, uma serina/treonina quinase evolutivamente conservada, desempenha um papel central na integração de sinais ambientais na forma de fatores de crescimento, aminoácidos e energia. No sistema imunológico, a mTOR se apresenta como um regulador crítico. A via mTOR se destaca pelo controle central na resposta do sistema imunológico, bem como na proliferação, multiplição e diferenciação das células T. Variações no gene responsável pelos complexos mTOR têm sido associadas a diferentes níveis críticos de citocinas, aumento da probabilidade de desenvolver asma e aumento da prevalência de atopia. **Objetivo e método:** O objetivo do presente estudo foi investigar a associação entre as variantes do gene mTOR com asma e sua gravidade, atopia, além da análise de citocinas. **Resultado e conclusão:** Os achados reafirmam a importância das variantes do gene mTOR no desenvolvimento da asma.

Descritores: Asma, serina-treonina quinases TOR, polimorfismo de nucleotídeo único.

Introduction

Asthma is an inflammatory disease of the airways of heterogeneous and chronic origin whose varied symptoms are cough, shortness of breath, wheezing and chest pain with variation in time and intensity in the air flow.[1] Because it is a complex disease, it is the result of many factors, whether genetic, environmental (dust, mites, animal hair or cigarette smoke), viral infections or use of drugs that culminate in the characteristic symptoms of asthma.[2] About 339 million people worldwide are affected by this disease, and the trend is for the prevalence to increase.[1]

Atopic asthma is characterized by the presence of specific IgE for these aeroallergens and by the action of cytokines and molecules that make up the

1. Universidade Federal da Bahia, Programa de Pós-Graduação em Imunologia - ppGim - Salvador, BA, Brazil.

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profile of T helper 2 or Th2 lymphocytes, this being a subtype of effector CD4 + T cells, which lead to an inflammatory cascade.[3] The identification of allergens by Th2 cells in atopic asthma induces the interleukins (IL) of this profile, such as IL-4, IL-5 and IL-13, cytokines involved in the isotype change of the B cell heavy chain, which results in the release of Immunoglobulin E (IgE) by plasmocytes, in eosinophil chemotaxis, in airway hyperreactivity and mucus secretion. Non-atopic asthma, in turn, points to a distinct pattern of T cell activation, producing IL-5, IL-2 and IFN-γ.[4] The mTOR, or target of rapamycin in mammals, is a serine / threonine kinase whose function in the central regulation of cell metabolism, growth, proliferation and survival has already been reiterated.[5] In the activation cascade by extrinsic factors PI3K (p-PI3K) phosphorylates Akt, which activates mTOR and its effective ribosomal protein to S6 kinase 1 (S6K1). Phosphorylated S6K1 (p-p70S6k) promotes protein translation and cell growth. Aberrant mTOR signaling is involved in many diseases, including cancer, cardiovascular disease and diabetes.[6] The mTOR, which also regulates the cellular immunity of lymphocytes, stimulates the release of cytokines from inflammatory cells.[7] In addition, systemic lupus erythematosus was suppressed when patients were treated with the mTOR inhibitor rapamycin.

The mTOR pathway regulates the differentiation and activation of subsets of CD4 + T cells. Therefore, it is believed that the mTOR signaling pathway is strongly associated with the loss of balance between Th1 and Th2 cytokines and between Th17 and Treg cells in immunological diseases, as well as asthma, whose phenotypic profile is variable.[8] In this perspective, the objective of the study was to analyze the association of variants in the MTOR gene with asthma, atopy and therapeutic control of the disease in a population in Salvador, Ba.

Methods

Population

The present study was conducted in 1178 patients from ProAR (Program for the Control of Asthma and Allergic Rhinitis in Bahia) and these were divided into the following groups:

a) Patients with severe asthma: 401 patients with severe asthma, of both genders and age over 18 years, living in Salvador, were included. The cases were recruited consecutively among patients followed by ProAR for at least one year, with a confirmed diagnosis of severe asthma according to the classification of the Global Initiative against Asthma[9] by an audit carried out by two experts.

b) Controls (patients with mild asthma): 413 control patients with mild or intermittent persistent asthma[9], also resident in Salvador and matched to cases by gender, age and socioeconomic status, were evaluated.

c) Controls (patients without asthma): 364 individuals with no history of asthma, also resident in Salvador and matched to cases by gender, age, socioeconomic status and place of residence, were evaluated, undergoing a medical consultation to assess their condition, health supplemented by basic blood, feces and urine tests, to all procedures for obtaining environmental information and blood samples for DNA analysis and genetic study.

The diagnosis of asthma and the definition of severity, carried out by the doctor, followed the classification of the Global Initiative for Asthma.[1] Combined history, physical examination, spirometry, daytime airway variation and response to treatment lead to the diagnosis of the disease. The main associated symptoms included wheezing, chronic cough, chest tightness, dyspnoea, chest discomfort, at specific times and under certain circumstances: exposure to cold, post-exercise, respiratory infection, exposure to inhalers, respiratory irritants and / or exposure to allergens. Severity was based on reports of daily symptoms, exacerbations or frequent nocturnal symptoms, limitation in physical activities, reduced lung function (FEV_1 or peak expiratory flow ≤ 60%) or variability in FEV_1 or peak expiratory flow > 30%.

The individuals underwent a medical consultation to assess their general health condition, with basic tests (blood, feces and urine), a questionnaire to collect information on the home environment and blood samples for DNA analysis and genetic study.

Atopy was defined based on the dosage of allergen-specific IgE (asIgE) in the individuals' serum, combined with the results of skin tests. All cases and controls were subjected to skin prick tests (SPT) for the most common inhalable allergens in our region (mites – *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Blomia tropicalis*; cockroach - *American periplaneta* and *Blatella germanica*; fungi - *Aspergillus fumigatus* and *Penicilliumnotatum*; animals - dog

and cat epithelium) (ALKAbello, Denmark), on the foreleg. The diameter of the papules was measured after 15 minutes. The test was considered positive if the average of the largest perpendicular diameters (excluding pseudopods) was at least 3 mm greater than the negative control.

Assessment of response to treatment

Asthma control is assessed considering the week prior to the date of the clinical evaluation and reflects the response to the treatment the patient is using. Asthma is considered uncontrolled if the patient has three or more of the following characteristics: symptoms more than twice a week that trigger limitations in his activities, nocturnal symptoms, FEV_1 or Peak Expiratory Flow < 80%. The patient is also considered uncontrolled if asthma exacerbation occurred in the week of the assessment[10].

The evaluation of asthma control was performed using the ACQ7 questionnaire. The ACQ, in its full version, consists of seven questions. Five questions refer to asthma symptoms (nighttime symptoms, morning symptoms, limitations in daily activities, dyspnea and wheezing), one question refers to the use of rescue β2-agonist medication and the seventh question takes into account a gauge measure of the airways: the percentage value of the forced expiratory volume in the first second (FEV_1) in relation to the predicted.

The final score of the questionnaire is the average score of the answers chosen by the patient, ranging from 0 (fully controlled) to 6 (uncontrolled) points. When validated in English, the ACQ presented two cutoff points to discriminate between controlled and uncontrolled asthma: the score of 0.75 is used in clinical practice, with a negative predictive value of 0.85 (meaning that if the score is < 0.75, there is an 85% chance of asthma being well controlled), and the score of 1.50 is used in clinical studies, with a positive predictive value of 0.88 (meaning that if the score is > 1.50, there is an 88% chance that asthma is not well controlled).

DNA Extraction and genotyping

DNA extraction was performed from blood samples according to the Gentra® Puregene® Blood Kit (Quiagen) protocol. All samples were standardized at a concentration of 5 ng/μL and stored at -30 °C until genotyping. 1 11166592 11333554

The genotyping was performed using the Illumina Infinium Multi-Ethnic AMR/AFR-8 kit. The data of the genetic variants to be studied were extracted considering the genetic location of the MTOR according to NCBI data (www.ncbi.nlm.nih.gov) [Chromosome 1, NC_000005.10 (11166592 - 11333554)].

After the tests were carried out, the results of the genotyped SNVs were stored in a database of phenotypes of the patients monitored and submitted to a quality control process. In this analysis, only individuals and SNVs with a genotyping rate "call rates" of at least 90% and presenting $p > 0.05$ in the Hardy – Weinberg balance analysis using healthy individuals of the population, as well as variants whose frequency of polymorphic allele (AMF) was greater than 0.5% in the population. As controls, wells without DNA were used to evaluate non-specific amplification and a family triad (mother, father and son) to evaluate inconsistencies in genotyping.

Dosage of cytokines and chemokine

The samples were tested for a panel of 11 cytokines and chemokines (IL1β, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17A, IFN-γ, TNF and CCL11) using okill MEXIPEX® (Merck, Darmstadt, Germany) and following the manufacturer's instructions. The assay was performed using the Luminex MAGPIX® system (LifeTechnology, USA), based on the measurement of fluorescent signals released by a suspension of microspheres with specific cytokine antibodies, in 96-well plates. The combination of the fluorimetric signal of the microspheres with that released by the secondary antibody allows the measurement of signals related to the concentration of cytokines converted by a processor. For this, a standard curve of eight points was used for each cytokine. The data were analyzed using the Analyst 5.1 MILLIPLEX® software (Merck, Darmstadt, Germany).

Analyzes in silica

The link disequilibrium (LD) analysis of the observed SNPs was also performed. LD measures the non-random association of alleles at different loci[11]. The observed associations can be affected by mutation, recombination, gene conversion, selection, genetic drift or demographic factors, such as inbreeding, migration and population structure.[12] Thus, LD patterns are used to infer genetic parameters of a population[13].

SNPs were also researched on the RegulomeDB and Ensembl platforms. RegulomeDB is a database for the interpretation of regulatory variants in the human genome. The platform identifies, through computational forecasts and manual annotations, the regulatory potential for productivity and functional variants. The score ranges from 1 to 6, where lower scores indicate increased evidence. Thus, a score of 1 indicates a likely effect on binding and gene expression, while scores 2 do not affect gene expression. Scores greater than 3 indicate a low probability of affecting the connection[14].

Statistical analysis

The phenotypes chosen in 3 genetic models (additive, dominant and recessive) for each SNP were analyzed. Empirical p-values were generated through a permutational approach for correction for multiple tests using the PLINK program. A series of current studies show that analyzing the 3 genetic models (additive, dominant and recessive) inserts more statistical power since the significance is determined by permutation. For adjusted association tests, we used logistic regression corrected for age, sex, BMI and main components of ancestry. For the analysis of quantitative traits (for example, specific IgE level as an outcome), the association tests were performed using a linear regression approach.

In addition, it was necessary to check the Hardy-Weinberg (H-W) balance. The H-W balance assumes that the genotype and allele frequencies are maintained randomly for generations and that there is a relationship between the allele and the gene frequency, their deviations or errors can be caused by genotyping errors. The tests are two-tailed and the statistical significance was established for the 95% confidence interval. The genetic associations followed analysis in the PLINK 1.9 program and the graphics produced in STATA 8.2 (StataCorp LP, CollegeStation, TX, USA).

Genotypic comparisons were made between the variants in the mTOR gene, in the three models (recessive, additive and dominant) and the dosed cytokines (IL-1β, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17A, IFN-γ, TNF and CCL11). For data with normal distribution, we used the One-Way analysis of variance (ANOVA) and Tukey's test or Green House-Geisser correction as Post-test; for data with non-normal distribution, Kruskall Walis and Dunn's Post-test (multiple comparisons) or Mann-Whitney (comparisons between two groups), adopting a value of $P < 0.05$ to determine statistical significance between groups, using the Graph Program Pad v6 (Graph Pad Software Inc., San Diego, CA, USA).

Ethical considerations

This is a subproject of the project entitled "Risk factors, biomarkers and endemic phenotypes of severe asthma" coordinated by Prof. Álvaro Cruz, Faculty of Medicine, Federal University of Bahia in which he proposes to investigate the genetic mechanisms linked to asthma. This project was approved by the Research Ethics Committee of Maternidade Climério de Oliveira (MCO/UFBA), opinion No. 095/2012. The cell culture stage is a subproject of the project entitled "Assessment of Biological Pathway Markers in Endophenotypes and in the Therapeutic Response of Asthma and Allergy" coordinated by Prof[a] Camila Alexandrina Viana de Figueiredo Fontana, Institute of Health Sciences, Federal University of Bahia. This project was approved by the Research Ethics Committee of the Faculty of Medicine of Bahia, Federal University of Bahia, opinion No. 2,549,881/2018.

Results

The study population, PROAR, was analyzed according to asthma phenotypes, asthma severity and atopy. The data collected, in turn, in a system were analyzed for the prevalence of characteristics that could describe in an analytical way the different effects of the variables in the subsequent biostatistical analysis (Table 1).

Description of variants in the mTOR gene

74 variants were observed in the MTOR gene. Of the initial total of variants, 62 were excluded because they had FAM < 0.05 and 03 by the Hardy Weinberg (HWE) balance test. The low genotyping criteria (Mind > 0.1) did not exclude any variant. After the quality control steps, the study included 09 SNPs in the mTOR (Table 2).

Association of variants in mTOR with asthma

The analysis was performed using non-asthmatic individuals as a control group and all asthmatic individuals as a case group. In this sense, of the variants of the mTOR gene included in the study,

Table 1
Characteristics of the Proar population according to the asthma, gravity and atopy phenotypes

	Individuals (n)					Individuals with Asthma (n)				
Variables	**Not asthmatic (n=342)**	**%**	**Asthmatics (n=754)**	**%**	**p value**	**Light (n=385)**	**%**	**Serious (n=369)**	**%**	**p value**
Age										
Average±DP	43.84±12.9	–	43.36±15.1	–	0.271	36.27±12.8	–	50.75±13.63	–	0.000*
Sex										
Woman	294	26.8	598	54.6		298	39.5	300	39.8	
Man	48	4.4	156	14.2	0.09	87	11.5	69	9.2	0.187
Body mass index										
Average±DP	26.9±5.7	–	28.0±5.81	–	0.001	26.9±5.77	–	29.1±5.8	–	0.000*
Smoking index										
Yes	127	11.8	239	22.2		106	14.4	133	32.6	
No	216	20.1	495	46.0	0.150	260	35.4	235	67.4	0.033
Positive skin test (atopy)										
For at least one of the main allergens tested	80	9.0	404	45.7	0.000*	233	37.5	171	27.5	0.008

Table 2
Description of variants in the mTOR gene

Chromosome	SNP	Position	Alleles	MAF	HWE	Occupation	Score DB regulation
1	rs12139042	11167146	A/G	0.081	0.147	Intronic variant	5
	rs17036350	11171226	A/G	0.160	0.567	Intronic variant	5
	rs12122483	11193408	A/G	0.095	0.339	Intronic variant	3a
	rs1057079	11205058	A/G	0.392	0.072	Intronic variant	4
	rs12122605	11248020	A/G	0.152	0.552	Intronic variant	5
	rs28990992	11249789	C/G	0.056	0.380	Intronic losses variant	4
	rs61773703	11281952	A/G	0.072	1	Intronic variant	7
	rs2788570	11289466	A/G	0.113	0.304	Intronic variant	5
	rs7525957	11318236	A/G	0.485	0.825	Intronic variant	5

only two showed positive associations compatible with asthma, one identified by the additive model (rs1057079) and the other by the recessive model (rs7525957). The other variants (rs12139042, rs17036350, rs12122483, rs12122605, rs28990992, rs61773703, rs2788570) were not associated with the outcome in this population. Table 3 below shows such associations adjusted for sex, age, BMI, and main component 1.

Association of variants in mTOR with asthma control

Table 4 shows the significant association of the rs7525957 variant with severe asthma control. In this case, the analysis was done restricting the group of asthmatic individuals with a severe asthma profile, having as control the group whose asthma was controlled after treatment and the case the group whose control was not possible. In this sense, individuals with severe asthma who have the AA genotype are twice as likely to have the disease uncontrolled when compared with the other genotypes (OR 2.05; 95% CI 1.23-3.42). The other variants (rs12139042, rs17036350, rs12122483, rs12122605, rs28990992, rs61773703, rs1057079, rs2788570) were not associated with the outcome in this population.

Association of variants in mTOR with atopy

This immune response is promoted by the production of an antibody called immunoglobulin

Table 3
Significant association between SNPs in mTOR and asthma by logistic regression adjusted for sex, age, BMI and main component 1

SNP	Model	GENO	Control	Case	OR	95% CI	P value	P perm
rs1057079	ADD	GG	151 (45%)	272 (36.2%)	1.22	1.00-1.48	0.046	0.043
		GA	136 (40.5%)	336 (44.8%)				
		AA	48 (14%)	141 (18%)				
	DOM	GG	151 (45%)	272 (36.2%)	1.31	0.98-1.73	0.0062	0.069
		GA+AA	184 (54.5%)	477 (0.63%)				
	REC	GG+GA	287 (0.86%)	608 (0.81%)	1.28	0.19-1.88	0.190	0.184
		AA	48 (14%)	141 (18%)				
rs7525957	ADD	GG	102 (30.4%)	199 (26.5%)	1.18	0.98-1.42	0.087	0.100
		GA	168 (50.1%)	345 (46%)				
		AA	65 (19%)	205 (27%)				
	DOM	GG	102 (30.4%)	199 (26.5%)	1.084	0.81-1.45	0.592	0.555
		GA+AA	233 (69.1%)	550 (73%)				
	REC	GG+GA	270 (80.5%)	544 (72.5%)	1.45	1.05-2.01	0.002	0.026
		AA	65 (19%)	205 (27%)				

Table 4
Association between SNPs in MTOR and asthma control by logistic regression adjusted for sex, age, BMI and main component 1

Gene	SNP	Model	GENO	Control	Case	OR	95% CI	P value	P perm
			GG	71 (28.7%)	35 (30.4%)				
		ADD	AG	121 (48.9%)	39 (33.9%)	1.278	0.929-1.760	0.132	0.114
			AA	55 (22.0%)	41 (35.0%)				
mTOR	rs7525957	DOM	GG	71 (28.7%)	35 (30.4%)	0.745	0.557-1.554	0.783	0.857
			AA+AG	176 (70.9%)	80 (68.9%)				
		REC	GG+AG	192 (77.6%)	74 (64.3%)	2.05	0.261-3.421	0.006	0.009
			AA	55 (22.0%)	41 (35.0%)				

E (IgE), and some people are born with a genetic predisposition to show reactions due to the increase in this antibody. Of asthmatic patients, approximately half of them are atopic or allergic, the first symptoms occur in childhood and tend to regress in adolescence.

In this sense, the analysis is essential and was performed using non-asthmatic individuals as a control group and all asthmatic individuals as a case group. In this sense, three of the variants presented a significant association with the outcome atopy (rs1057079, rs7525957, rs12122483). As demais variantes (rs12139042, rs17036350, rs12122605, rs28990992, rs61773703, rs2788570) were not associated with the outcome in this population (Table 5).

The rs1057079 variant in the additive model and the recessive model, the rs7525957 variant in the additive and recessive model and the rs12122483 variant in the recessive model.

Eotaxin production among mTOR genotypes

Eotaxin production in patients with asthma was compared between mTOR genotypes. The rs1057079 variant was related to the serum increase in eotaxin. In this sense, the occurrence of allele A was significantly associated in the additive model, showing greater circulation of this cytokine when compared to polymorphic homozygosis and wild homozygosity as well as, in heterozygosis with p respectively 0.006 and 0.029 (Figure 1A).

There was also a difference in Eotaxin production between the rs7525957 genotypes, with the presence of the A allele, in heterozygote and wild homozygote different from the wild homozygote (p value 0.044 and 0.041, respectively) (Figure 1B).

The rs12122483 genotype showed a difference in Eotaxin production, with the wild homozygote ($p = 0.022$) having less expression compared to the heterozygote (Figure 1C).

Finally, the AA genotype of rs17036350 showed a reduction in eotaxin production when compared with the AG ($p < 0.01$) and GG ($p = 0.001$) genotypes (Figure 1D).

Cytokine production among MTOR genotypes

Individuals with asthma presenting the rs17036350 variant had lower IL-17 cytokine production between AA and heterozygous genotype ($p < 0.05$) and

between AA and GG genotypes (p < 0.05). As well as lower expression of IL-13 between genotype AA and AG (p < 0.01), between AA and GG (p < 0.01); IL-1B between genotype AA and AG (p < 0.05) and between AA and GG (p < 0.05); IL-8 between genotype AA and AG (p < 0.05); IL-6 between genotype AA and AG (p < 0.05), between AA and GG (p < 0.01); IL-5 between genotypes AA and GG (p < 0.05);

Table 5
Significant association between SNPs in mTOR and Atopy by logistic regression adjusted for sex, age, BMI and main component 1

SNP	Model	GENO	Control	Case	OR	95% CI	P value	P perm
rs1057079	ADD	GG	168 (43.2%)	163 (34.6%)	1.27	1.04-1.56	0.021	0.013
		GA	163 (42.0%)	210 (44.5%)				
		AA	57 (14%)	98 (20.0%)				
	DOM	GG	168 (43.2%)	163 (34.6%)	1.31	0.96-1.76	0.785	0.074
		GA+AA	220 (56%)	308 (65.5%)				
	REC	GG+AG	331 (85.3%)	373 (79.1%)	1.48	1.02-2.16	0.039	0.044
		AA	77 (20%)	98 (20%)				
rs7525957	ADD	GG	122 (31.4%)	117 (24.8%)	1.34	1.10-1.63	0.004	0.003
		GA	189 (49.3%)	207 (43.9%)				
		AA	77 (20.0%)	147 (31.0%)				
	DOM	GG	122 (31.4%)	117 (24.8%)	1.24	0.90-1.70	0.183	0.158
		GA+AA	266 (69.3%)	354 (54.9%)				
	REC	GG+GA	311 (80.7%)	324 (68.7%)	1.79	1.28-2.49	0.001	0.001
		AA	77 (20%)	147 (31%)				
rs12122483	ADD	GG	321 (82.7%)	387 (82.1%)	1.15	0.83-1.58	0.411	0.523
		GA	65 (16.7%)	73 (15.4%)				
		AA	2 (0.5%)	11 (2.3%)				
	DOM	GG	321 (82.7%)	387 (82.1%)	1.03	0.72-1.48	0.875	0.875
		GA+AA	67 (17.2%)	84 (17.7%)				
	REC	GG+AG	386 (99.4%)	460 (97.5%)	5.37	1.62-24.79	0.031	0.013
		AA	2 (0.5%)	11 (2.3%)				

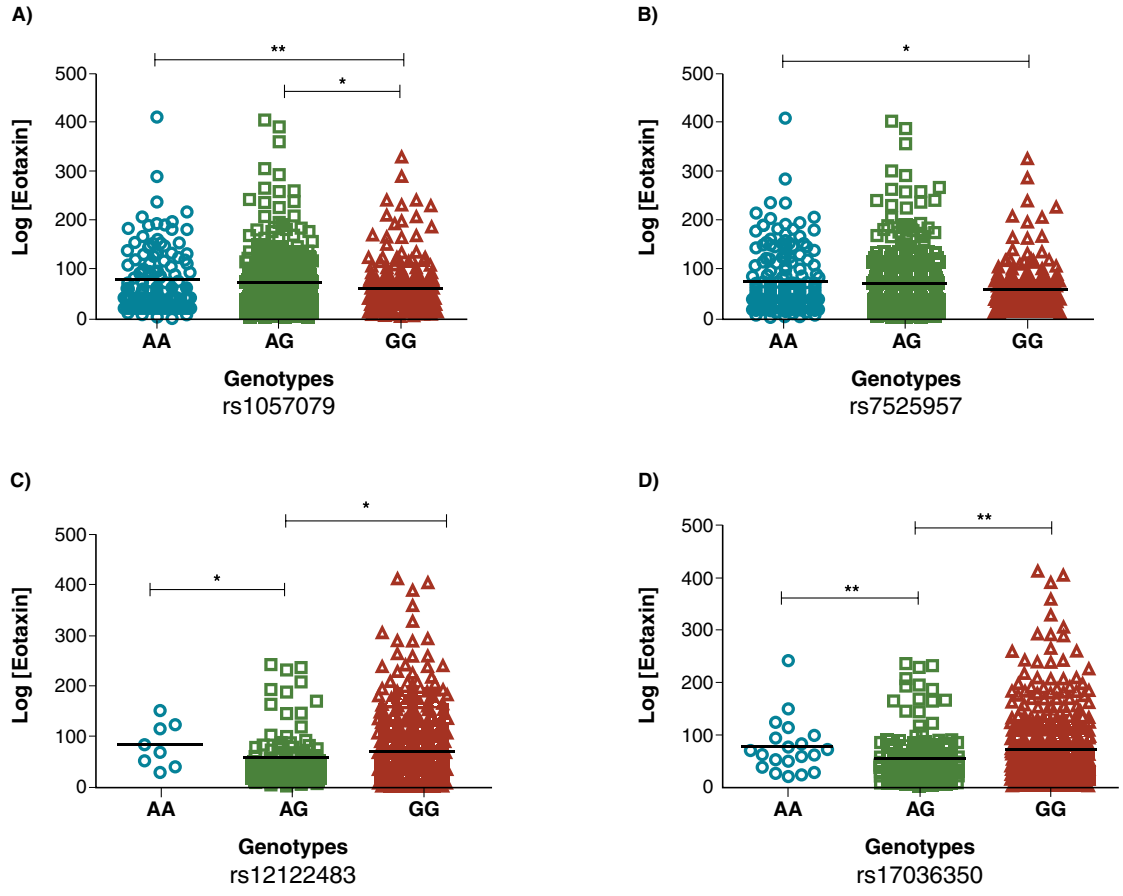

Figure 1
Average production of Eotaxin in individuals with asthma separated by rs1057079 (A), rs7525957 (B), rs12122483 (C) and rs17036350 (D), all with increased production in the polymorphic genotype. Tests used: Shapiro-Wilk; Kruskal-Wallis with Dunn and Mann-Whitney post-test

IL-12 between genotypes AA and GG ($p < 0.05$); IFN, between genotypes AG and GG ($p < 0.05$) and cytokine IL 10 between genotypes AA and GG ($p < 0.05$) (Figure 2A-I).

Additionally, the expression of Interleukin 8 (IL-8) is significantly reduced in the polymorphic genotype (AA) of the rs12122483 variant when compared to the heterozygous group ($p = 0.00061$) and when compared to the wild homozygote ($p = 0.0015$) (Figure 3).

Between the rs1057079 and rs7525957 genotypes there was no significant difference in the levels of the analyzed cytokines.

Tissue expression of mTOR among the studied genotypes

The in silico analysis by the gTex platform of the variants shows, in a global analysis, the expression of the variants in different tissues and their relevance is analyzed as to the significance value. Through this analysis it was observed that the expression of the variants in the blood and pulmonary tissues were significant in individuals with the AA genotype of the rs7525957 variant ($p = 1.2e\text{-}4$) presenting less expression of the mTOR gene in the blood. Also individuals with the AA genotype of the rs1057079 variant ($p = 5.1e\text{-}16$) showed lower expression of

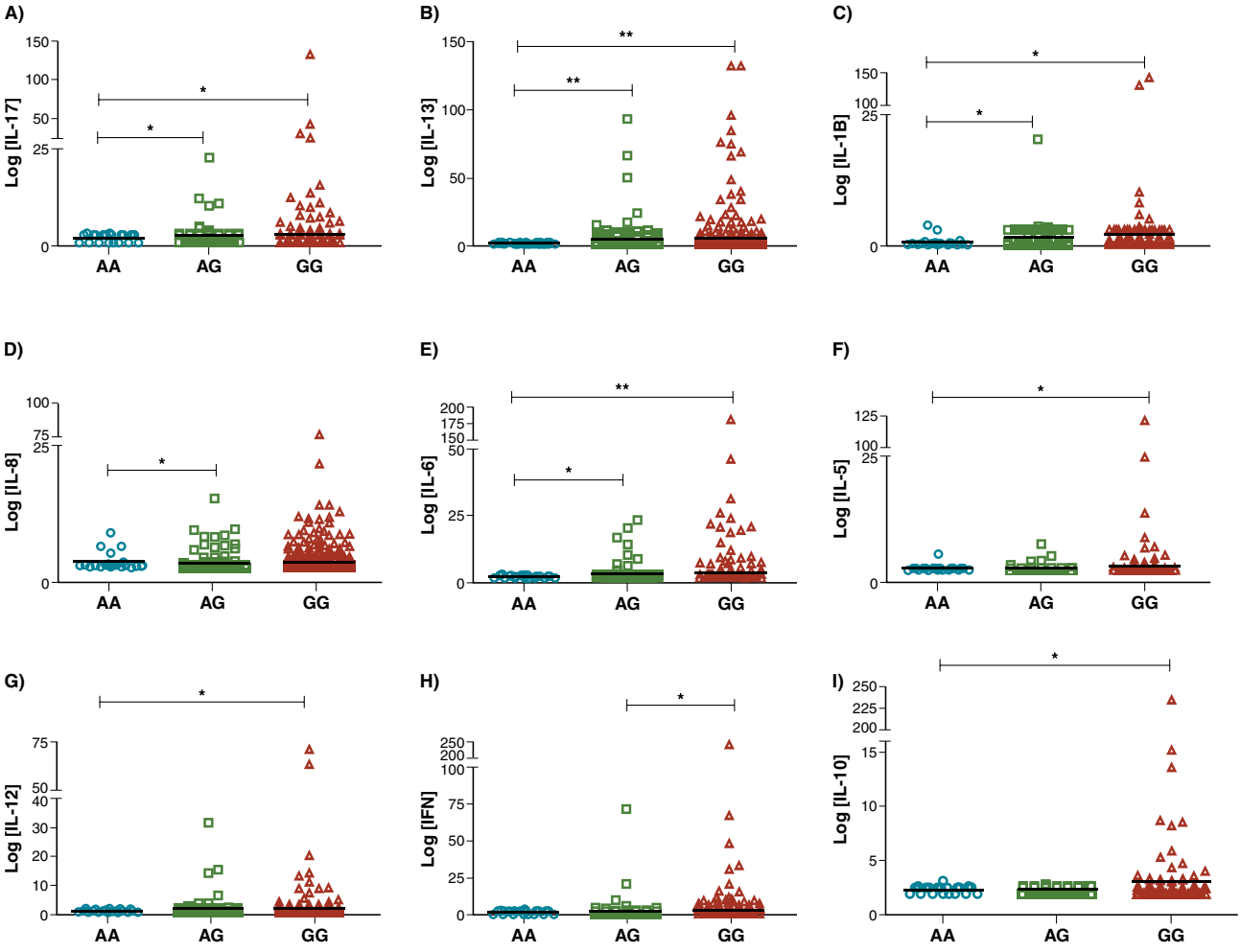

Figure 2

Average production of cytokines IL-17, IL-13, IL-1B, IL-8, IL-6, IL-5, IL-12, IFN (AH), IL-10 in individuals with asthma separated by genotype of rs17036350. All cytokines analyzed showed lower levels in the wild genotype group. Tests used: Shapiro-Wilk; Kruskal-Wallis with Dunn and Mann-Whitney post-test

mTOR in whole blood (p = 5.1e-16) and in lung tissue (p = 1.7e-6) when compared with the others genotypes (Figure 4).

Connection imbalance

Using Haploview, linkage imbalance analyzes were performed, which clarifies the non-random association of alleles in two or more loci. LD reflects historical events of natural selection, gene conversion, mutation and other evolutionary forces. In this scenario, it implies a joint heritability of the rs 1057079, rs7525057 variants of 60% in contrast to the rs 17036350 variant whose association with the aforementioned ones was less than 15% (Figure 5).

Discussion

Asthma is a chronic inflammatory disease with airway remodeling as one of the main symptoms. The PI3K/Akt/mTOR signaling pathway plays a central role in a broad spectrum of cellular activities, including cell proliferation, survival and differentiation[15]. Zhang et al.[16] demonstrated that the remodeling of the airways in mice was strongly associated with high levels of mTOR expression. MTORC1 can selectively inhibit the myeloid precursor to differentiate into

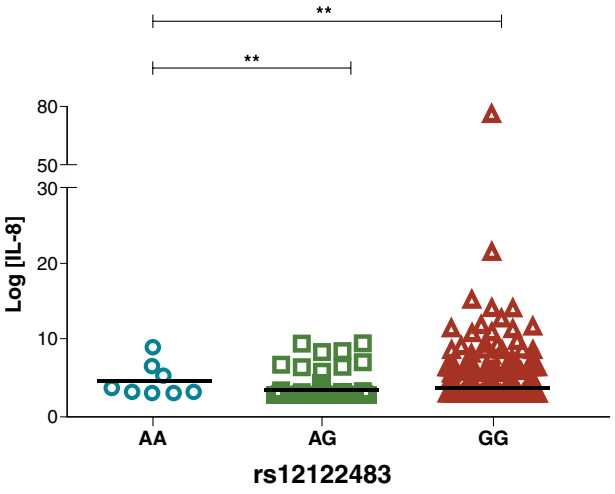

Figure 3
Average production of IL-8 in individuals with asthma separated by rs12122483 genotype. The group with the polymorphic genotype showed a higher level of cytokine ($p < 0.001$). Tests used: Shapiro-Wilk; Kruskal-Wallis with Dunn and Mann-Whitney post-test

eosinophil lineage, while promoting this differentiation in eosinophils. Activation of mTOR appears to be indispensable in controlling the excessive development of eosinophils, which can be a potential therapeutic target in the treatment of asthma.[17] On the other hand, Zhu et al.[18] demonstrated that inhibition of mTOR, either by gene deletion or by molecular antagonism, potentiated eosinophilia in a murine model of asthma, evidencing a dual role of mTORC1 and mTORC2 in the orchestration of the inflammatory process. In view of the complex role of mTOR in the immunological context, the present study evaluated variants in the mTOR gene in a population of patients with asthma.

In the analyzed population, the variant rs1057079 was significantly associated as a risk factor for asthma. This same variant was also associated with the risk of atopy, suggesting an impact on a common biological pathway for both outcomes. Similarly, the polymorphic homozygosis of the A allele of rs7525957 indicated a greater risk for asthma and atopy. Asthmatic patients carrying the polymorphic genotype presented airway obstruction determined by a spirometric test. Both variants are intronic, however, to date, they have no evidence of clinical significance in the literature related to their functional impact in asthmatic patients. On the other hand, single nucleotide varianters7525957 has been suggested as a marker of advanced esophageal tumor.[19] The link value and imbalance showed a 60% probability of heritability of these variants in the same individual, and the information may converge for both when observed in one of these, due to the chances of culminating in the association.

The in silico analysis of gene expression revealed that individuals who have rs7525957 or rs1057079 present a reduced tissue expression of mTOR, which suggests a negative regulatory role of these variants in the formation of mTORC1 and mTORC2. The inhibition of these complexes has already been simulated using substances such as Rapamycin, which specifically blocks mTORC1, while the use of Torina-1, which blocks both complexes, preventing the formation of the two complexes.[20] It has been shown that selective blocking of mTORC1 results in inhibition of eosinophilic differentiation. However, the blocking

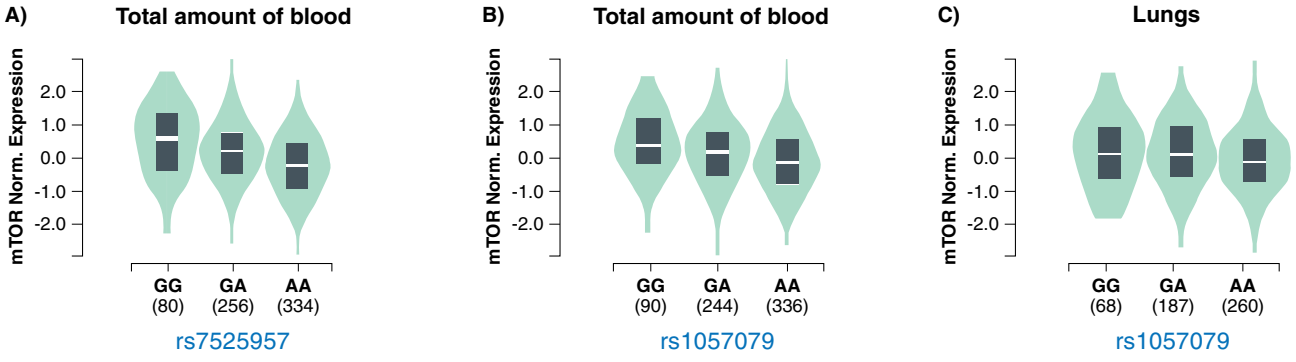

Figure 4
Image of the Violin Plot with the expression of the MTOR gene according to the genotypes of rs7525957 in whole blood (A), rs1057079 in whole blood (B) and rs1057079 in the lung (C). The polymorphic genotype showed reduced gene expression compared to the other genotypes ($p < 0.001$)

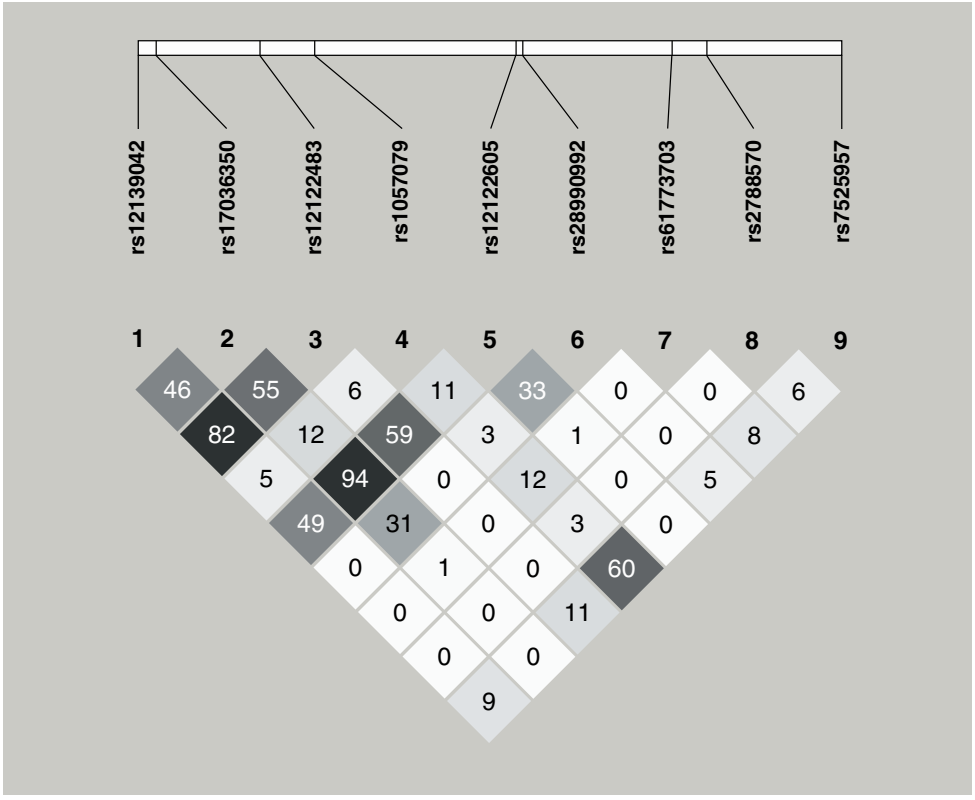

Figure 5
Graph of LD generated by Haploview using the R-squared statistic for SNPs in the mTOR gene

of both complexes has the consequence of increased eosinophilia, as demonstrated by Zhu et al.[18] Thus, it is believed that the variants rs7525957 and rs1057079, by reducing the expression of mTOR, contribute to the inflammatory process, increasing the susceptibility of individuals carrying the polymorphic alleles for the development of asthma and atopy.

The contribution of the rs7525957 and rs1057079 variants to the eosinophilic inflammatory process can be characterized, at least in part, by the level of production of Eotaxin, an eosinophilic chemotactic protein. In the population whose research was carried out, the variants were related to a higher level of Eotaxin in patients with asthma carrying the polymorphic allele, which may be associated with increased migration of eosinophils that potentiate the atopic process. Allergic diseases, such as asthma, allergic rhinitis and atopic dermatitis, are characterized by an increase in the number of eosinophils in the circulating blood and degranulation in the tissue.[21] The action of some cellular and molecular signals, including eotaxin, drives the exacerbated action of eosinophils. In this sense, eotaxin-1 binds with high affinity to the chemokine CC 3 receptor, which is expressed by a variety of inflammatory cells.[21]

In addition to the positive relationship with atopy whose influence of greater eotaxin expression has been previously reported, rs7525957 represented a twice as high risk for the lack of therapeutic control in patients with severe asthma. The uncontrolled asthma condition is thus defined when the use of inhaled corticosteroids only influences the reduction of exacerbations, but not the reduction of symptoms or the control of.[22] What is observed, particularly in this variant, is that its presence is attributed to the increased risk of asthma, atopy, eotaxin expression and possible resistance to inhaled anti-inflammatory drugs. The activity of mTORC1 has already been associated with insensitivity to corticosteroids[23] suggesting a greater expression of mTORC1.

The AA allele of rs12122483, also in homozygosis, was associated with a five times greater risk for atopy. In addition, it also presents higher levels of Eotaxin production, which leads us to think that the AA genotype of these variants has an impact on the expression of mTOR, similar to previous SNVs.

The aforementioned variant is also related to a higher level of production of Interleukin-8 (IL-8), being more produced in patients with AA genotype when compared with the other genotypes. The chemotactic cytokine IL-8 activates inflammatory cells by recruiting neutrophils, mononuclear phagocytes, mast cells and T cells.[24] Secreted by immune cells, bronchial epithelial cells, smooth muscle cells and macrophages, IL-8 is involved in the beginning of the acute and chronic inflammatory process.[25] This cytokine is associated with Th17 cells, as belonging to its secretion profile, which in turn, have been positively associated with difficult-to-control asthma in African-American children.[26] In a study of the mTOR pathway, the overexpression of these complexes was reversed by treatment with IL-8, demonstrating their regulatory role under this pathway.[27]

The rs17036350 variant was not associated with any of the study phenotypes, however there was an impact on the production of the tested cytokines. This variant, however, is not in imbalance of connection with the variants discussed earlier, rs1057079, rs7525957 and rs12122483, indicating reduced possibility of being inherited at the same time. Patients with asthma who have the AA genotype of this variant have a lower level of IL-17, IL-6, IL-13, IL5, IL-1B, IL-12 and IL-10 when compared to the wild genotype, which demonstrates its immunomodulatory impact. As for the cytokine IL-8, there was an average increase in its expression in asthmatic individuals, suggesting a possible activation of a feedback mechanism modulating the expression of mTOR in these individuals through the expression of cytokines, although this hypothesis was not tested in the study.

Conclusion

This study demonstrated for the first time that variants in the MTOR gene suggest risk factors for asthma, atopy and can influence the therapeutic control of asthma through the immunological regulation observed by the expression of cytokines. The variants have a direct influence on the immunogenic control that directly influences the responsiveness to asthma, mainly atopic, due to the strong relationship with the external environment. Further studies are needed to understand the functional impact of the variants associated here.

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Corresponding author:
Ítalo Santos Uzêda
E-mail: italozeda@gmail.com