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RESEARCH ARTICLE

HAPTOGLOBIN POLYMORPHISM AND BRONCHIAL ASTHMA

Margarida Cortez[*1,2], Andreia Matos[2,3], Joana Ferreira[2,3] and Manuel Bicho[2,3]

[1]ImmunoAllergy Department-CHLN-HSM, Lisbon Portugal
[2]Genetics Laboratory and Environmental Health Institute, Faculty of Medicine, University of Lisbon, Portugal
[3]Instituto de Investigação Científica Bento Rocha Cabral, Lisboa, Portugal

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A B S T R A C T

Asthma is considered a heterogeneous disease, characterized most of the times by a Th2 inflammatory response. Haptoglobin (Hp), is an alfa2-sialoglycoprotein known to bind free hemoglobin (Hb) and has been implicated in modulation of Th1/Th2 response, Intervening in innate and adaptive immune response. The Hp locus is situated at 16q22 chromosome, being in humans, polymorphic for the α chain. The α chain of Hp has 2 major co-dominant alleles Hp*1 and Hp*2, with 3 genotype variants, Hp1-1, Hp2-1, Hp2-2. The aim of the study is to establish a relation between Hp genotypes and Hp levels (intermediate phenotype), and the pathophysiology of asthma when compared with a control group of healthy blood donors. In a group of 114 asthmatic patients and 50 controls we studied the Hp levels that were determined by nephelometry and genotypes by polyacrylamide gel electrophoresis (PAGE). Statistical analysis was performed with statistical software PASW version 18, having established a level of significance of $p< 0.05$.

We found that Allelic (Hp*1 e Hp*2) and Hp genotypes (Hp 1-1, Hp 2-1, Hp 2-2) distribution in asthmatics, are not statistical different from control group ($p> 0.05$). There is no statistical differences in the asthmatics between, gender, age-group, atopics and non-atopics, controlled and non-controlled asthma (p>0, 05). The different genotypes seem not to be related with an increased risk of having asthma when compared with the control group (p>0, 05). In control group there is no statistical differences in Hp levels by genotype and age- group (p>0, 05). When we compare asthmatics with control group we verified that in asthma , the levels of Hp are always lower than in the control group (125,13±50,95vs137,86±51,39mg/dL) and there was a statistical difference in Hp2-2 genotype (95,60±41,43 vs 128, 40±51,48mg/dL) (p<0, 05). In asthmatics Hp levels, are statistical different between ages >30 years and <15 years (135.6±50.05 vs 87.45± 38.89 mg/dL) (p<0.05). In asthmatics Hp levels, present statistical differences by genotype (p=0,000). Those who express Hp 2-2 had the lower levels of the circulating protein when compared with Hp 2-1 and Hp 1-1(95,6 ± 41,93 vs 137,37±49,58 vs 146,09±47,37mg/dL) ,and it is statistical different (p=0.000).In those asthmatics with age ≥15 years Hp levels are different by genotype (p<0.05): 1-1 and 2-1 differ from 2-2. Those patients with age <15 years, Hp levels were no different between genotypes (p>0, 05). In a pos-Hoc analysis Hp 2-2 is an independent factor, as age <15 years, associated with lower levels of Hp.

Although no statistical differences were find between Hp genotype and allelic distribution in the group of asthmatics when compared to control group we verified that asthmatics had lower levels of the circulating Hp when compared to the control- group and that this difference is associated with Hp 2-2 genotype. In asthmatics, Hp levels are different between genotypes (with age ≥15 years) because Hp levels are lower in the Hp2-2 genotype when compared with the other genotypes.In the future, studies done with Hp should be controlled by age , because the Hp levels are lower in the pediatric group. These data point to differences among groups that could be related to Hp genotypes, and possibly with different immunological profiles.

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INTRODUCTION

Haptoglobin (Hp), an alpha 2-sialoglycoprotein (acute phase protein), that is known by its ability to bind free hemoglobin (Hb) and to form an Hp-Hb complex that allows the recycling of globin and heme [1-4]. The Hp-Hb complex can be cleared from plasma by two different pathways, one in the hepatocyte (90%) and another in monocytes- macrophages by the CD163 receptor [5-7]. Hp consists of two different polypeptide chains, the α and β-chain. The Hp α gene, located in 16q22 chromosome, is highly polymorphic in humans, presenting 2 major co-dominant alleles: Hp*1 and Hp*2 that originate 3

different genotypes: Hp1-1, 2-1 and 2-2. The Hp gene has two major alleles: Hp*1, (with five exons) and Hp*2, (with seven exons) which probably arose from a duplication event involving exons 3 and 4, producing a 61 kDa protein. In its ancestral form, Hp is a dimer, however, the Hp 1–2 encoded protein exists as linear polymers containing 2–8 monomers, while the Hp 2–2 encoded protein exists as circular polymers of 3–10 Hp monomers [8].

These genetic variants present different affinities to bind hemoglobin [9] and so can modulate the toxicity and inflammatory nature of free iron, namely its capacity to consume nitric oxide [10,11] and to serve as a Fenton reagent [9]. Besides its antioxidant role, Hp also plays an immunoregulatory function, through the CD163 receptor in macrophages, modulating the cytokine profile released after endocytosis of the Hp-Hb complex [9].

One of the consequences of the allergic reaction is the increase of free hemoglobin. Hp*1 has greater affinity for free Hb presenting higher antioxidant capacity; on the other hand, Hp*2 has less affinity for Hb, being associated with more susceptibility to oxidative stress damage [9, 12-15]. As a response to tissue injury or infection, the target cells segregated IL-1β and TNFα that activate endothelial cells and neutrophils. The activated neutrophils, the first line of defense in immune response, help in the recruitment of other inflammatory cells, which promote reactive oxygen species (ROS) generation [16]. Hp is synthesized during neutrophils differentiation and is stored to be released when these are activated. Hp synthesis occurs mainly at hepatocyte level, and also in the alveolar macrophages and lung eosinophils with active inflammation, but not in the healthy lung [17]. The Hp binds to Apo-A1, protecting from free radical attack and preventing HDL to form complexes with other lipoproteins. The Hp has the ability to inhibit lipoxygenase and cicloxygenase activity, modulating the synthesis of prostaglandins and leukotrienes [13]. Hp is also an excellent suppressor of T cell proliferation [13]. The macrophages activated by the complex Hp2-2: Hb through the CD163 receptor deviates T helper response to a Th1 profile, while macrophages activated by complex Hp1-1: Hb phagocytosis produces Th2 cytokines. The balance between these T cell responses is particularly important in the extravascular space, since a localized expression of Hp minimizes tissue injury [9].

In extravascular space, dendritic cells respond to alert signals, like oxidative stress, and interact with antigens/allergens. These dendritic cells differentiate to mature cells and migrate to lymphatic nodes, where they interact with naïve T cells. Being the Hp a ligand to monocytes and macrophages, this protein may play an important role in the activation of these immune cells [9].

Allergic asthma is an inflammatory disease where predominates a Th2 response in the bronchial airways. Th2 response might be related to a more ancestral immune response. Asthma appears as a result from a failure in the immunoregulatory mechanisms of the respiratory epithelium. According to the "hygiene hypothesis", a lack of Th1 response stimulation upon the adaptive immunity leads to a prevalence of Th2 response [19, 20]. Nowadays, there has been an increase prevalence of autoimmune diseases (Th1 diseases) in the western societies, namely type I Diabetes and multiple sclerosis, suggesting that both diseases (Th1 and Th2) can coexist in the same patient. These data seem to oppose the bipolarization of an immunological environment Th1 or Th2, and points to a deficient regulation in both diseases (role of the Treg cells). Nevertheless, the defenders of this hypothesis assert that the absence of necessary stimulation could deprive the immune system from the necessary signals to the development of regulatory pathways able to control Th1 and Th2 response. Advances in the immunology field, came to focus the attention in the central role of innate immunity, as the "orchestrator" of immune response and maintenance of tolerance [21].

The activation of innate immunity through the antigen presenting cells (APCs), where are involved the Hp and its receptor, CD163 (marker of M2 macrophages), might be an additional factor in Th2 polarization of allergic response, similarly to the allergens [19]. Identically to the Th1/Th2 nomenclature, the polarized macrophages are reported as M1 and M2 [22, 23]. The M1, also designed as activated by classic pathway, can be induced by IFNγ, LPS, TNF-α and GM-CSF. These macrophages act in the initial phase of the inflammation and produce great amounts of pro-inflammatory cytokines (IL1-β, TNFα, IL-6), oxygen free radicals and nitrogen compounds, and participate as effectors in the polarized reactions Th1. The resolution phase is characterized by macrophages that produce anti-inflammatory cytokines with high phagocytic capacity and overexpression of the mannose receptor, CD206 and the Hb-Hp receptor, CD163. M2 macrophages are activated by the alternate pathway and can be induced by IL-4, IL-13, immune complexes, IL-10, glucocorticoids, activin-A (a member of the TGF-β family) and IL- 21. In general, M1 macrophages are IL-12 high, IL-23 high and IL-10 low [22]. Despite participating in Th1 response, they are also responsible for the resistance against intracellular parasites and activity against the tumoral cells [22]. In contrast, M2 macrophages are IL-12 low, IL-23 low, IL-10 high, and have a variable capacity to produce inflammatory cytokines with a Th2 profile [22]. They are associated with atopic and allergic asthma, being able to promote the proliferation and tumor metastization.

The aim of this study is to establish a relation between Hp genotype and asthma susceptibility and to correlate the Hp genotype with plasma Hp levels (intermediate phenotype-endotype) establishing a possible, relationship between the modulation of Th1/Th2 immune response by Hp polymorphism and asthma pathophysiology.

MATERIAL AND METHODS

To identify if there were differences in the two groups, we did a case-control study with a group of 114 asthmatic patients, (Immuno Allergy Department-CHLN/HSM-Director: Prof. Manuel Barbosa), 70 females and 44 males, mean age 41±18 years; in the control group were 50 healthy blood donors, 45 females and 5 males, mean age 50±13 years. Asthmatic patients were classified according to severity in intermittent and persistent (mild/moderate/severe) asthma as stated by GINA classification [24] (Global Initiative for Asthma) and according to levels of asthma control (controlled, partly controlled and uncontrolled) in compliance with ACQ7 (Asthma Control Questionnaire, Portuguese Version by Juniper) and PAQLQ (Pediatric Asthma Quality of Life Questionnaire- Portuguese Version by Juniper adjusted for

patients between 7-17 years with at least 6 months of therapeutics for asthma) [25].

The sample consisted in 98 atopics and 16 non atopics (according to the definition of atopy by the WAO/EAACI [26]); 45 with uncontrolled asthma (evaluated by validated instrument ACQ7 (cutpoint: 0.75) and PAQLQ: global score is the average of all the answers, < 4 imply uncontrolled asthma) and 69 with controlled asthma. The exclusion criteria were non adhesion to the anti-asthmatic therapy; existence of other co- morbidities that could interfere with the severity of the respiratory disease; existence of a diagnosis of chronic obstructive pulmonary disease or another pulmonary disease; smoking habits and other co-infections, namely HIV, parasitosis or other type of infection , anemia or chronic liver disease. All participants gave their written informed consent for the study. Written informed consent was obtained from all participating individuals. The genetic study was done under the standards of the Bioethics Commission for research at Lisbon Medical School.

Patients were diagnosed by physicians for asthma according to the guidelines of GINA, and as having atopy or not according to WAO/EAACI guidelines, they were examined for a selfreported history of breathlessness, wheezing, atopic dermatitis and family history, atopic individuals have a positive skin prick test (SPT) for at least one of the common environmental allergens or the presence of specific IgE ,associated with high serum IgE levels estimated using enzyme-linked immunosorbent assay and suffered from asthma.

Blood samples were collected after an overnight fast. The determination of the haptoglobin polymorphism was done from plasma samples (Hb-supplemented plasma) using a phenotyping method for Hp, based on polyacrylamide gel (4.7% in TRIS/HCl 0.504M, pH 8.9) electrophoresis (PAGE) followed by o-dianisidine staining, and assigned corresponding genotype. The Hb-supplemented samples for application into the gel (10µL) were previously prepared using a mixture of 40% sacarose (w/v), Hb 282 mg/mL and plasma in the 3:2:4 proportion for a final volume of 45µL. For identification of the Hp migratory bands we used a method of coloration by contact with o-dianisidine 16mM in 50% acetic acid and, subsequently, with 0.6% hydrogen peroxide (Fig.1). Samples underwent electrophoresis for 180 minutes (180 V, 0.05 A; Cleaver). Plasma haptoglobin concentration was measured by nephelometric method on a BN ProSpec (Siemens Helthcare Diagnostics) [27].

Statistical analysis was performed with Statistical software, and the continuous variables were summarized as means (standard deviation) or as medians according to their homogeneity. Categorical variables (allele and genotypes frequencies) were compared with the c2 test. Continuous variables among patients and controls were compared with Student t test. To compare the Hp levels in the different groups defined for genotype and age group, an ANOVA test was used, after verified the normality and the homogeneity of the variances, and pos-Hoc tests. Associations are given as odds ratios with a confidence interval established at 95%. All statistical analysis was done using PASW version18. A two-sided probability value of $p < 0.05$ was considered significant.

RESULTS

There were no statistically significant differences regarding the distribution of Hp genotypes between asthmatics and controls (Hp1-1: 19.3 vs 10.0, Hp 2-1: 47.4 vs 58.0, Hp 2-2: 33.3 vs 32.0, p> 0.05), as well as in their allele frequencies (Hp 1*: 0.43 vs 0.39, Hp 2*:0.57 vs 0.61, p> 0.05) (Table 1). Our results concerning the frequency of allele 1 in the control group are consistent with those described by Carter and Worwood relatively to the European population (Fig.2) [28]. The Hp genotype frequencies among the asthmatic patients showed no statistically significant differences between males and females, uncontrolled and controlled asthma, atopic or non-atopic, age- group (p> 0.05).

Table 1 Haptoglobin genotype and allele frequencies in asthma and control group

	ASTHMA		CONTROL		
GENOTYPE	N	Frequency (%)	N	Frequency (%)	p- value
Hp 1-1	22	0.193	5	0.10	
Hp 2-1	54	0.474	29	0.58	>0.05
Hp 2-2	38	0.333	16	0.32	
Hp* 1		0.39		0.43	>0.05
Hp* 2		0.61		0.57	

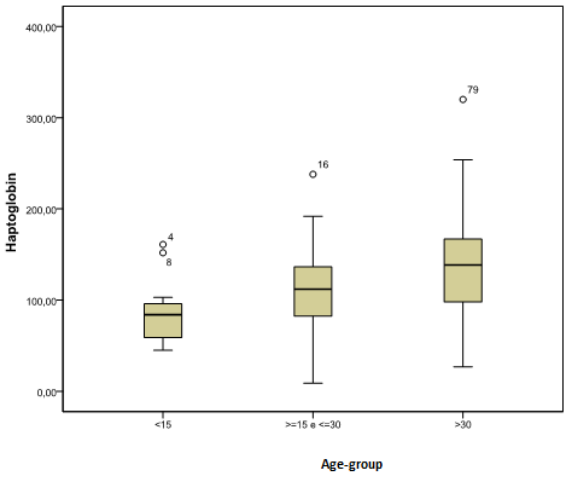

Figure 1 Plasma Hp levels (mg/dL) in asthmatics stratified by Age-group

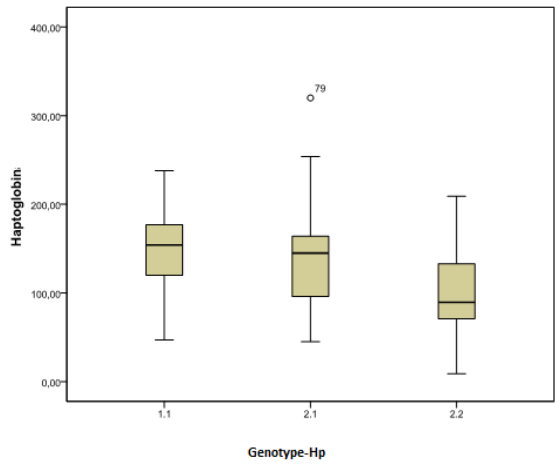

Figure 2 Plasma Hp levels stratified by Hp genotypes (genotype-phenotype association) in asthmatics.

In patients with asthma (Table 2), the plasma concentration of Hp (intermediate phenotype) did not differ significantly between controlled and uncontrolled asthma, atopic and non atopic and between males and females, there is, however, a

significant variation with age, having the patients with age > 30 years (135.60 ± 50.05 mg/dL); 15- 30 years (111.00 ± 48.43 mg/dL) and <15 years (87.45 ± 38.89 mg/dL)(ANOVA p=0,008 between > 30 years and <15 years: pos- hoc test) (Fig. 3). The asthmatics with Hp 2-2 genotype presented lower concentrations of circulating protein when compared with patients Hp 2-1 and Hp 1-1 (95.60 ± 41.93 vs 137.37 ± 49.58 vs 146.09 ± 47.36 mg/dL), being this difference statistically significant (p = 0.000, ANOVA) (Fig. 4); this pattern was not observed among individuals in the control group. These differences in Hp concentration was significant only in asthmatics aged ≥ 15, with no differences in patients <15 years (ANOVA) (Fig. 5). When analyzing the levels of circulating Hp between asthmatics and controls (Fig. 6), it was found that, overall, no statistically significant differences between mean values of this protein was found among both groups (125.13±50.95 vs 137.86 ± 51.39 mg/dL, p> 0.05), despite the Hp 2-2 individuals (95.6 ± 41.9 vs 128.4 ± 51.5 mg/dL) presented significantly differences in mean Hp values between asthma and control group (p=0,018) (Fig. 6). Relatively to Hp 1-1 and Hp 2-1 genotypes, no significant differences were observed between asthmatics and controls (146.09±47.36 vs 175.8±15.78 ;p> 0.05; 137.37 ± 49.58 vs 136.55 ± 53.46; p> 0.05) (Fig. 7). Thus, although not always significant, the asthma group had lower mean values of circulating Hp.

Table 2 Plasma concentrations of Hp in asthmatics, stratified by: age; gender; clinical characteristics; and by Hp genotype

		N	Hp SD (mg/dL)	p-value
AGE	<15 years	11	87,45±37.8	0,008 among <15 and >30
	15-29 years	27	111.00±48,43	
	≥30 years	76	135,60±50,05	
GENDER	Female	70	129.17 ±53.36	
	Male	46	118.70±46.73	0,288
ASTHMA	Controlled	69	120.81±46.79	
	Uncontrolled	47	131,75±56.64	0.264
ATOPY	YES	98	126.81±51.83	
	NO	18	114.87±45.27	0.388
GENOTYPE	Hp 1-1	22	146,09±47,36	
	Hp 2-1	54	137,37±49,58	0.000
	Hp 2-2	38	95,60±41,93	

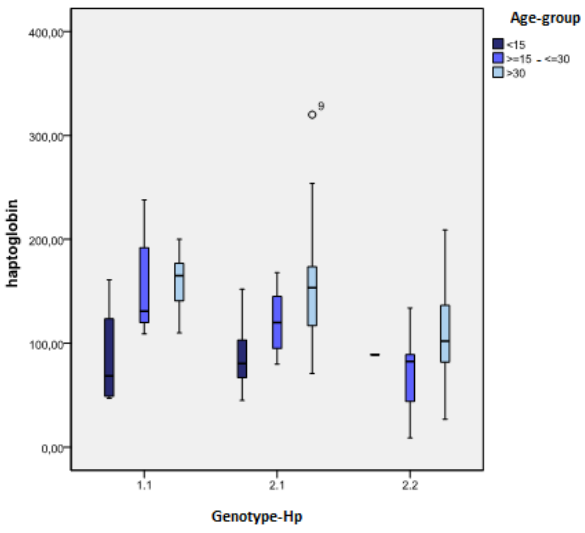

Figure 3 Plasma Hp concentrations in asthmatics patients, stratified by Hp genotype and age-groups.

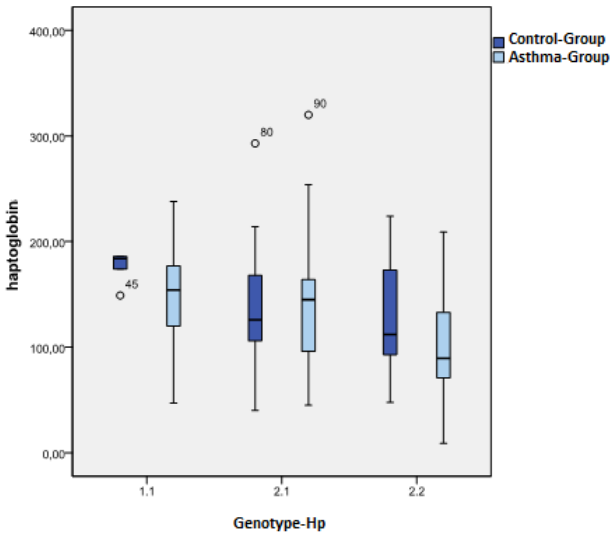

Figure 4 Plasma Hp levels in asthmatics and controls, stratified by the haptoglobin genotypes.

In the control group there were no differences in the levels of Hp between individuals with age > 30 years (138.99 ± 53.67 mg/dL) and 15- 30 years (127.76 ± 23.08 mg/dL) ;(p=0.648), the same with their distribution by genotype (Hp 1-1: 175.8 ± 15.78 mg/dL vs Hp 2-1: 136.55 ± 53.46mg/dL vs Hp2-2: 128.40 ± 51.48mg/dL, p = 0.196 (ANOVA). The control group followed the Hardy-Weinberg equilibrium.

DISCUSSION

With this study it was possible to put in evidence that the genetic polymorphism for the α chain of Hp may be associated with differences in haptoglobin levels and that these differences are more pronounced in the asthmatics with longer disease evolution (the differences in relation to the Hp genotype are observed only in patients aged > 15 years). These observations could be derived from differences in the activation and polarization of macrophages (M1 and M2) in the innate immunity system, and therefore different immune response, Th1 (pro-inflammatory) or Th2 (anti-inflammatory) [22]. The genetic polymorphism of haptoglobin has a major role, by conditioning the nature and intensity of the response of macrophages to extravascular and extracorpuscular hemoglobin, and potentially interfering with modulation of immunity that accompanies the allergic response and bronchial asthma [13, 18]. Haptoglobin also plays an important role by interacting with CD22 and the integrin CD11b/CD18, as recipients of the haptoglobin in the cells of the immunological system [29,30].

The toxicity and inflammatory nature of free hemoglobin is due to its ability to consume nitric oxide and to act as an oxidant, producing highly reactive radicals such as anion superoxide and hydroxyl. Haptoglobin binds to hemoglobin, inhibiting the ability of Hb to act as an oxidant and promoting its removal, despite the Hp-Hb complex is not completely inert and can also catalyze the formation of oxygen radicals. The removal of the Hp-Hb complex is done through hepatocytes and the CD163 receptor of monocytes/ macrophages. In the extravascular space there is only one way to remove the Hp-Hb, the CD163 receptor on macrophages [5, 9]. The allele 1 seems to generate a complex Hp1-Hb redox-inactive that binds to the CD163 receptor of the

macrophage, inducing the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β. By contrast, the allele 2 potentiate the generation of a complex redox active Hp2-Hb, with release of pro-inflammatory cytokines, and consequent vascular injury and inflammation [9, 13]. Atopy and allergic asthma may be due to an M2 polarization of innate immunity (with an important role for the Hp-CD163 receptor on macrophages) and consequent Th2 polarization in detriment of the adaptive immunity, Th1 [20, 22]. This polarization of the immune response may be related to genetic polymorphism of haptoglobin.

The highest values of circulating haptoglobin were observed in asthmatics with longer time of disease evolution (>15 years of age) , and this increase was dependent on Hp genotype (Hp1-1> Hp 2-1 > Hp 2-2). This fact might be related to the chronicity of the disease, reflecting a stress response and consequent induction of the release of glucocorticoids and catecholamines, which stimulate the production of haptoglobin and other acute phase proteins [31]. The age-group under 15 years has lower levels of haptoglobin when compared with patients aged > 30 years ($p< 0.05$). The difference in the Hp levels between genotypes occurs particularly in the age- group over 30 years ($p< 0.05$). The asthmatics with genotype Hp 2-2 have lower levels of circulating haptoglobin when compared with Hp1-1 and 2-1 ($p<0.05$). Many studies point to the fact that the Hp1-1 genotype is associated with higher levels of Hp and a Th2 profile, whereas the Hp2-2 genotype was associated with lower levels of Hp and a Th1 profile [13, 28]. It is also described that asthma and respiratory allergy, are associated with the lowest levels of Hp [-32], and that this protein could act as a natural antagonist of the activation of the immune system when related to a series of stimulus. On the other hand, it was observed a lower expression of the CD163 receptor on macrophages of individuals who express Hp2-2 genotype, and that Hp1-1:Hb complex increases the activity of casein kinase II (CK II) associated with CD163 phosphorylation , leading to a distinct mechanism of activation as well as a different profile of cytokines after endocytosis of Hp1-1 complex (with increase of IL-10) versus Hp2-2 [33,34].

CONCLUSIONS

Considering the results obtained, and the population samples studied, we believe it is essential to increase the number of patients studied in each subgroup, in order to increase the statistical power, leading to a better characterization of these subgroups, particularly, that under 15 years.We also think that future studies must be controlled by age-group, and that different polymorphisms could lead to different genotype-specific response to treatment and different asthma endotypes/phenotypes among patients. Our results suggest an important role of haptoglobin polymorphism in bronchial asthma, possibly associated with the polarization of the immune response, disease severity and response to anti-asthmatic therapy.

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Statement of Contribution of Each Author

All authors contributed equally.

Conflict of Interest

The authors declare that they have no conflict of interests.

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