Elisabete Cristina Peres Resende

IL4R, *IL13*, *IL17A* and *GSTP1* polymorphisms and susceptibility to asthma and rhinitis

Dissertation for Master Degree in Biomedical Research
presented to the Faculty of Medicine of the University of Coimbra

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Dissertation presented to the Faculty of Medicine of the University of Coimbra for the fulfillment of the requirements for a Master degree in Biomedical Research, area of Infection and Immunity.

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1 Coríntios 13:2

Abstract

BACKGROUND: Asthma and rhinitis have a complex etiology, depending on multiple genetic and environmental risk factors. An increasing number of susceptibility genes are currently being identified, but the majority of reported associations have not been consistently replicated across populations of different genetic backgrounds.

AIMS: To evaluate whether polymorphisms of *IL4R* (rs1805015), *IL13* (rs20541), *IL17A* (rs2275913) and *GSTP1* (rs1695) genes are associated with rhinitis and/or asthma in adults of Portuguese ancestry and to compare genetic profiles of these two related phenotypes.

METHODS: 192 unrelated healthy individuals and 232 patients, 83 with rhinitis and 149 with asthma, were studied. All polymorphisms were detected by real-time polymerase chain reaction (PCR) using TaqMan assays.

RESULTS: Compared to controls, a significant association with asthma was observed for *GSTP1* rs1695 AA genotype (Odds Ratio (OR) – 1.96; 95% CI - 1.18 to 3.25; *p*= 0.010). The association sustains for allergic asthma (OR – 2.17; 95% CI - 1.23 to 3.80; *p*= 0.007). *IL13* rs20541 GG genotype was associated with less susceptibility to asthma (OR – 0.55, 95% CI - 0.33 to 0.94, *p*= 0.028). Comparing both phenotypes, *IL17A* rs2275913 AA genotype was less associated with asthma than with rhinitis (OR – 0.20; 95% CI of 0.07 to 0.56; *p*= 0.002). A similar association was found for *IL13* rs20541 GG genotype (OR – 0.48; 95% CI of 0.25 to 0.93; *p*= 0.031). There were no significant differences in the distribution of allelic and genotypic frequencies between patients and controls for the *IL4R* polymorphism' analysed. Hardy-Weinberg equilibrium was verified for all SNPs.

CONCLUSION: These results support the existence of a significant association between *GSTP1* rs1695 and *IL13* rs20541 SNPs, with susceptibility to asthma, in the population studied. Different genotype profiles of *IL17A* and *IL13* genes seem to influence the clinical pattern of disease expression mainly confined to the upper airways, as rhinitis, or including the lower airways, as asthma.

Key words: asthma, rhinitis, allergy, *IL4R*, *IL13*, *IL17A*, *GSTP1*, genetic polymorphism, genetic profile.

Resumo

INTRODUÇÃO: A asma e a rinite têm uma etiologia complexa, dependendo de vários fatores de risco genéticos e ambientais. Um crescente número de genes de suscetibilidade tem sido identificado, mas os resultados não têm sido consistentemente reproduzidos em estudos de populações de diferentes origens genéticas.

OBJETIVOS:. Avaliar se os polimorfismos *IL4R* (rs1805015), *IL13* (rs20541), *IL17A* (rs2275913) e *GSTP1* (rs1695) estão associados a suscetibilidade para rinite e/ou com a asma em indivíduos adultos de ascendência Portuguesa e comparar os perfis genotípicos dos dois fenótipos.

MÉTODOS: Os polimorfismos foram estudados em 210 doentes, 83 com rinite e 149 com asma, e em 192 indivíduos controlo, não consanguíneos. A genotipagem foi realizada pela técnica de reação de polimerização em cadeia (PCR) em tempo real com sondas TaqMan.

RESULTADOS: Comparativamente ao grupo controlo, foi observada uma associação significativa com a asma para o genótipo AA do *GSTP1* rs1695 (Odds Ratio (OR) - 1,96; IC 95% - 1,18-3,25; p = 0,010). Esta associação mantém-se para a asma alérgica (OR - 2,17; IC de 95% - 1,23-3,80; p = 0,007). O genótipo GG do *IL13* rs20541 foi associado a uma menor suscetibilidade para a asma (OR - 0,55, 95% CI - 0,33 a 0,94, p = 0,028). Comparando os dois grupos de doentes, o genótipo AA do *IL17A* rs2275913 está menos associado com asma do que com rinite (OR - 0,20; IC 95% de 0,07-0,56; p = 0,002). Um resultado semelhante foi encontrado para o genótipo GG de *IL13* rs20541 (OR - 0,48; IC 95% entre 0,25 e 0,93; p = 0,031). Não foram encontradas diferenças significativas na distribuição das frequências alélicas e genotípicas, entre os grupos estudados, para o polimorfismo do *IL4R* analisado. O equilíbrio de Hardy Weinberg foi verificado para todos os SNPs.

CONCLUSÃO: Estes resultados sugerem, para a população estudada, a existência de uma associação significativa entre os SNPs rs1695 do *GSTP1* e rs20541 da *IL13* e a suscetibilidade para a asma. Diferentes perfis dos genes *IL17A* e *IL13* parecem influenciar a localização preferencial das manifestações da doença, com predomínio nas vias respiratórias superiores, como na rinite, ou nas vias aéreas inferiores, no caso da asma.

Palavras-chave: asma, rinite, alergia, *IL4R*, *IL13, IL17A*, *GSTP1*, polimorfismo genético, perfil genético

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List of Abbreviations

A	Adenine
ADAM33	ADAM Metallopeptidase Domain 33
AdjOR	Adjusted
ADRB2	Adrenoceptor beta 2
ARIA	Allergic Rhinitis and its Impact on Asthma
C	Cytosine
CI	Confidence Interval
C11orf30	chromosome 11 open reading frame 30
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CNV	Copy number variation
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EP3OS	European Position Paper on Rhinosinusitis and Nasal Polyps
FEV1	Forced expiratory flow in one second
FVC	Forced vital capacity
G	Guanine
GA^{2}LEN	Global Allergy and Asthma European Network
GINA	Global Initiate for Asthma
GSTP1	Glutathione S-transferase pi 1
GWAS	Genome-wide association studies
HLA	Major histocompatibility complex
HWE	Hardy-Weinberg Equilibrium
IFNγ	Interferon gamma
IgE	Immunoglobulin E
IL	Interleukin
IL1RL1	Interleukin 1 receptor like 1
IL4	Interleukin 4

IL4R	IL4 receptor
IL13	Interleukin 13
IL17A	Interleukin 17A
ILC2	Innate lymphoid cells
MGB	Minor groove binder
NaCl	Sodium chloride
NARES	Nonallergic rhinitis with eosinophilia syndrome
NCBI	National Center for Biotechnology Information
NOD1/CARD4	Nucleotide binding oligomerization domain containing 1
NFAT	nuclear factor activated T
ORMDL3	ORMDL sphingolipid biosynthesis regulator 3
OX40L	OX40 ligand
OR	Odds Ratio
PCR	Polymerase chain reaction
PEF	Peak expiratory flow
SD	Standard deviation
SNP	Single nucleotide polymorphism
SPINK5	Serine peptidase inhibitor Kazal-type 5
SPT	Skin prick test
STAT6	Signal transducer and activator of transcription 6
STIM1	Stromal interaction molecule–1
STRs	Short tandem repeats
T	Thymine
TGF	Transforming growth factor
Th	T helper
TNF-α	Tumor necrosis factor alpha
Treg	regulatory T
TSLP	Thymic stromal lymphopoietin
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late Antigen-4

I. INTRODUCTION

1. Phenotypes: Asthma and rhinitis

Asthma and rhinitis are a serious global health problem affecting all age groups and representing a substantial proportion of primary care practice. Their prevalence is increasing in many countries, with consequent rising in treatment costs, reduced work productivity, and lost school days.

The World Health Organization has estimated that 400 million people in the world suffer from allergic rhinitis, and 235 million from asthma [1]. With global prevalence ranging from 1% to 21% in adults, it is estimated that asthma causes 346,000 deaths worldwide every year [2]. Rhinitis is rarely a cause of death but affects between 10% and 30% of all adults and as many as 40% of children [3].

According to studies conducted in Portugal, asthma has a prevalence of 5.5 to 10% and rhinitis of 22.1% to 26.1% [4]. Pegas et al. find a prevalence of allergic rhinitis of 43% among primary school children in Lisbon [5].

1.1. Asthma

The Global Initiative for Asthma (GINA 2014) defines asthma as “a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation” [6]. These symptoms are often triggered by factors such as exercise, allergen or irritant exposure, change in weather, or viral respiratory infections. Characteristically, symptoms and airway obstruction are least partially reversible.

Although airway inflammation and hyperresponsiveness has been found to be essential in the pathogenesis of asthma, these are not necessary or sufficient to make the diagnosis of asthma. According to GINA guidelines [7], the diagnosis should be based on the history of a characteristic symptom pattern and evidence of variable airflow limitation (Supplemental material 1). If possible, the evidence for the diagnosis of asthma should be documented before starting treatment, as it is often more difficult to confirm the diagnosis afterward.

In recent years, asthma heterogeneity has been recognized, and clusters of demographic, clinical and/or pathophysiological characteristics, often called 'asthma phenotypes', have been identified [8,9]. GINA 2016 [7] suggest five different phenotypes (Table 1): allergic asthma, nonallergic asthma, late-onset asthma, asthma with fixed airflow limitation and asthma with obesity. However, to date, we still don´t have strong, objective, markers to identify these specific phenotypes.

Table 1. Phenotypes of asthma according to GINA 2016 [7]

Phenotype	Characteristics
Allergic asthma	Often initiates in childhood; associated with allergic symptoms such as eczema, allergic rhinitis, or food or drug allergy. Total and allergen-specific immunoglobulin E is often high. Frequently, there is a T-helper type 2 dominant inflammatory process and both airway and blood eosinophilia.
Non-allergic asthma	Frequent in adults. The cellular profile of the sputum of these patients may be neutrophilic, eosinophilic or contain only a few inflammatory cells. Can be associated with sinusitis, nasal polyps and is sometimes aspirin-exacerbated. Th2-type inflammation is also associated with this phenotype
Late-onset asthma	Some adults, particularly women, present asthma for the first time in adult life. These patients tend to be non-allergic, and often require higher doses of Inhaled corticosteroid or are relatively refractory to treatment
Asthma with fixed airflow limitation	Some patients with long-standing asthma develop fixed airflow limitation that is thought to be due to airway wall remodeling
Asthma with obesity	In obese patients; little eosinophilic airway inflammation

Identifying the different phenotypes of asthma may significantly affect the choice of diagnostic tests and long-term prognosis, and most importantly, predict responsiveness to specific pharmacotherapies. For research, improvement in the definition of asthma phenotypes will enhance interpretation of data, promote appropriate comparisons among studies and facilitate genetics research in which phenotype is correlated with genotype.

Being an inflammatory disorder of the airways, asthma involves multiple inflammatory cells and mediators that contribute to characteristic clinical and pathophysiological changes. Although the clinical spectrum of asthma is highly variable, the presence of airway inflammation is a common feature. Airway inflammation is persistent, affecting all airways in special medium-sized bronchi [8]. This pattern appears to be similar in all clinical forms of asthma, whether allergic or nonallergic, and is characterized by the presence of activated mast cells, eosinophils, natural killer T cells and T helper 2 lymphocytes (Th2), which release chemokines and cytokines that contribute to symptoms[10] (Figure 1). Neutrophils seem also to participate, particularly when T-helper 17 (Th17) lymphocytes are involved [8].

In the immunopathogenesis of allergic asthma, the pivotal role of Th2 cells – mediated immune response against environmental allergens is an established fact (Figure 1). Th2 polarization is associated with the early production of interleukin 4 (IL4) during the primary response [11]. Cytokines and chemokines produced by Th2 cells, including GM-colony stimulatory factors, IL4, IL5, IL9, IL13, and those produced by other cell types account for most pathophysiologic aspects of allergic disorders, including production of immunoglobulin E (IgE) antibodies [10]. Activated tissue innate immunoinflammatory cells will release bronchoconstrictor mediators, including cysteinyl leukotrienes and prostaglandin D2 [10] (Figure 1). The coexistence of a defect in regulatory T (Treg) cells may favors further Th2-cell proliferation[10].

Airflow limitation in asthma is recurrent and caused by a variety of changes that lead to a reduction of airway diameter, like contraction of smooth muscles, vascular congestion, edema of the bronchial wall and production of thick secretions [12]. In some patients, airflow limitation may be only partially reversible because permanent structural changes can occur, often described as airway remodeling. These alterations can include subepithelial fibrosis, extracellular matrix deposition, smooth muscle hypertrophy, and goblet cell hyperplasia in the airways [12].

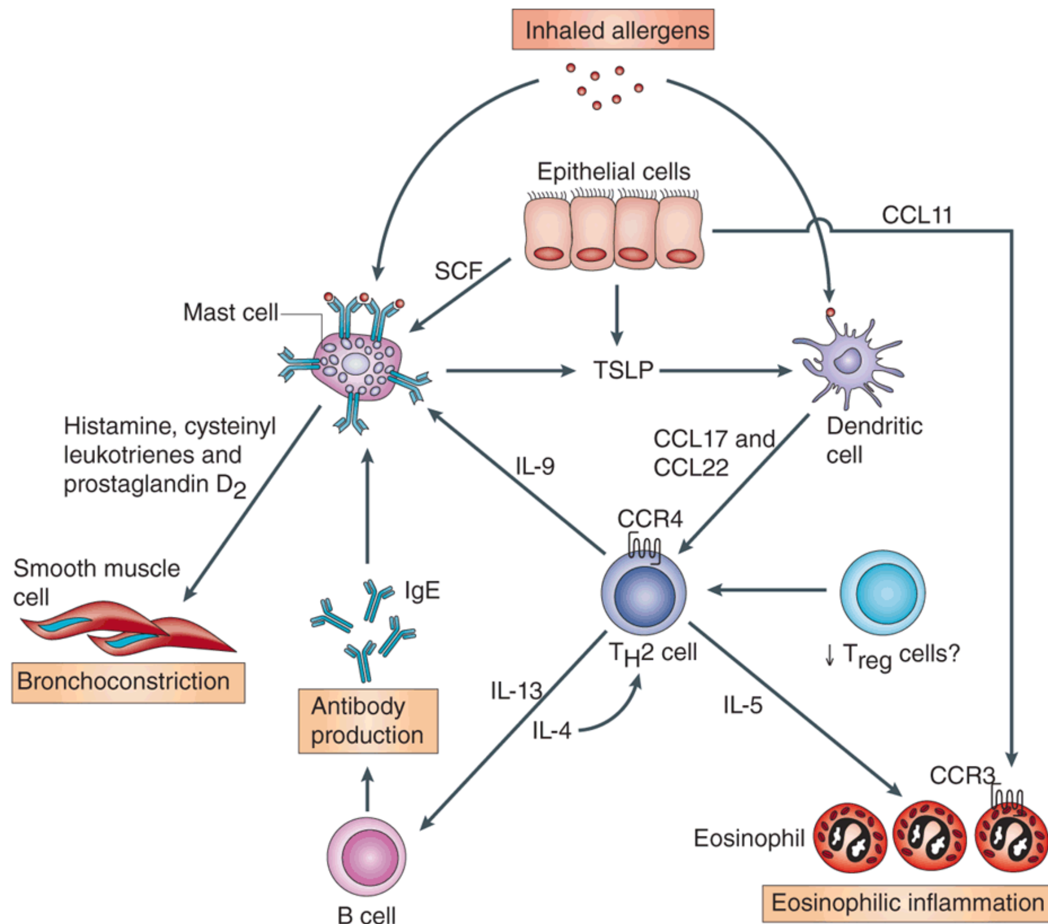

Figure 1. Immunoinflammatory mechanisms involved in allergic asthma. Inhaled allergens activate sensitized mast cells by crosslinking surface-bound IgE molecules to release several bronchoconstrictor mediators. Epithelial cells release stem-cell factor (SCF), which is important for maintaining mucosal mast cells at the airway surface. Allergens are processed by myeloid dendritic cells, which release the chemokines that attract Th2 cells. Th2 cells have a central role in orchestrating the inflammatory response in allergy through the release of interleukin-4 (IL-4), IL-13 (which stimulate B cells to produce IgE), IL-5 (which is necessary for eosinophilic inflammation) and IL-9 (which stimulates mast-cell proliferation). TSLP: thymic stromal lymphopoietin; CCL11, CCL17 and CCL22 are chemokines; CCR4 and CCR3 are: chemokine receptors. Adapted from Barnes, 2008 [10].

1.2. Rhinitis

According to the global guidelines on Allergic Rhinitis and its Impact on Asthma (ARIA), rhinitis is defined as "an inflammation of the lining of the nose that is characterized by nasal symptoms including anterior or posterior rhinorrhea, sneezing, nasal blockage and/or itching of the nose" [13]. These symptoms occur during two or more consecutive days for more than one hour on most days"[13]. Rhinitis is a recognized risk factor for asthma and is commonly associated with rhinosinusitis [14].

The diagnosis of rhinitis should be based on the existence of 2 suggestive nasal symptoms and confirmation of nasal inflammation by clinical examination. Inhalant allergic triggers are identified by skin prick tests.

Rhinitis is an even more heterogeneous disease than asthma, including several different phenotypes, each of which is defined by distinct clinical, functional and pathobiological patterns (Table 2). Classically, patients are grouped in three major clinical phenotypes: allergic rhinitis, infectious rhinitis, and nonallergic noninfectious rhinitis, with the possibility of a combined (mixed) presentation in some patients [3] (Table 2). Most cases of rhinitis are allergic (only 23% have non-allergic rhinitis) [15].

Table 2. Phenotypes of rhinitis [3]

Phenotypes
I. Allergic (nonoccupational)
II. Infectious: Acute and chronic a. Viral b. Bacterial **c.** Fungal
III. Nonallergic, noninfectious rhinitis a. Idiopathic Rhinitis (vasomotor rhinitis) b. Nonallergic rhinitis with eosinophilia syndrome (NARES) c. Hormonal rhinitis (pregnancy and menstrual cycle-associated) d. Gustatory rhinitis (induced by spicy food) e. Drug-induced rhinitis (local alpha-adrenergic agonist, vasodilators) f. Rhinitis of the elderly g. Atrophic rhinitis (commonly, by bacterial colonization) h. Occupational rhinitis (irritant-induced rhinitis or corrosive rhinitis) **i.** Associated with systemic conditions (vasculitis, granulomatous diseases)

As in allergic asthma, in allergic rhinitis, the process of allergen sensitization involves the involvement of antigen-presenting cells, T lymphocytes, and B lymphocytes and depends on environmental allergen exposure [15] (Figure 2). Sensitization results in the generation of allergen-specific IgE that circulates in the peripheral blood and attaches itself to the surface of all mast cells and basophils including those that home to the nasal mucosa.

Subsequent nasal exposure to allergen activates these cells and, through the release of the classic mediators of the allergic reaction, produces acute nasal symptoms. In a later-phase, this allergic response is characterized by Inflammatory and immune cell infiltrate, including eosinophils, basophils, neutrophils, T lymphocytes, and monocytes. As the mucosal inflammation develops, the nose becomes primed to allergen, reacting more vigorously to subsequent allergen exposure, and also becomes hyperresponsive to irritants and to changes in atmospheric conditions [16].

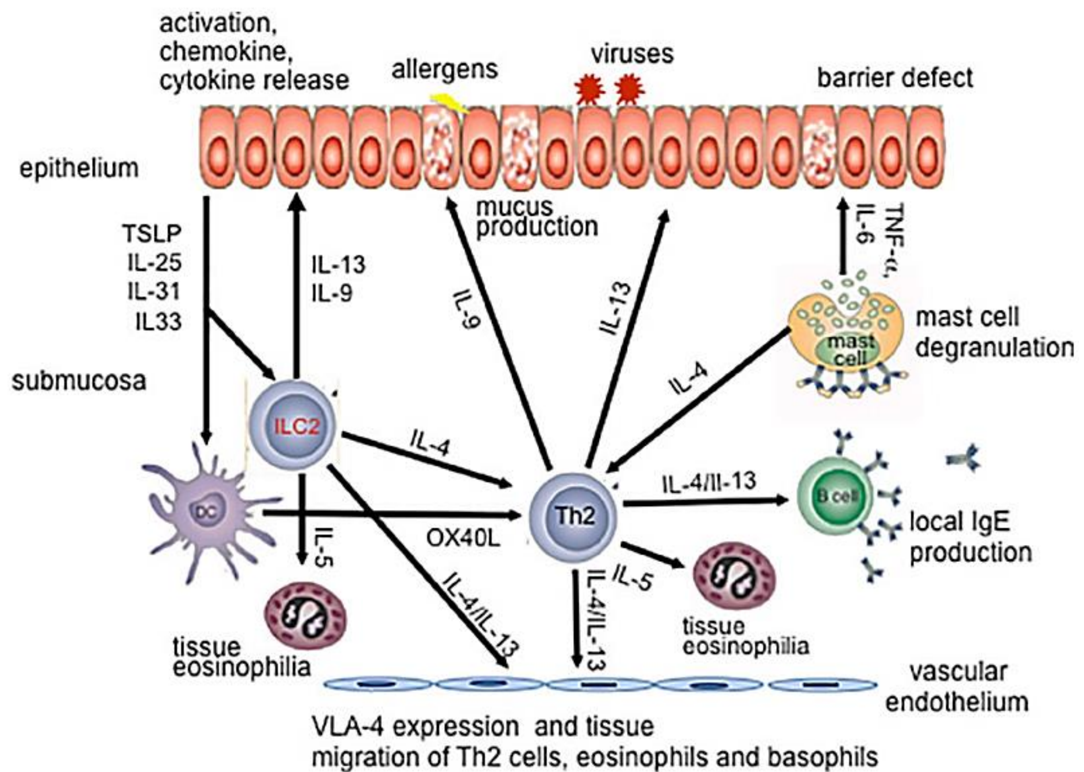

Figure 2. Type 2 inflammation and cytokine network in allergic rhinitis. Nose inflammation develops as a combination of innate and adaptive immune response and involvement of resident tissue cells. IL: interleukin; DC: Dendritic cells; ILC2: Innate lymphoid cells; TNF-α: Tumor necrosis factor alpha; TLSP: Thymic stromal lymphopoietin; OX40L: OX40 ligand; IFNγ: Interferon gamma; VLA-4: Very late Antigen-4. Adapted from Agache, 2016 [17].

In nonallergic rhinitis, several profiles have been identified. These include a group of patients with apparent hyperresponsiveness of the C-fiber sensory nerves with no inflammatory changes in the nasal mucosa and a group with mucosal eosinophilia who may have allergic sensitization to common aeroallergens circumscribed to the nasal mucosa (local allergic rhinitis) [16].

2. Asthma and rhinitis comorbidity

Asthma and rhinitis are two strongly associated diseases, caused by inflammation of the airways, often triggered by common allergens and other risk factors. ARIA 2008 update clearly support the links between upper and lower airways manifestations [15].

Several studies have demonstrated that nonallergic and mostly allergic upper airway disease are important risk factors for asthma development and severity [15,18]. There is some evidence that uncontrolled rhinitis can increase bronchial hyperresponsiveness and asthma severity, primarily via inflammatory mediators and activated cells that reach the lung through circulation [18]. Most patients with asthma (both allergic and nonallergic) also have rhinitis, whereas 10% to 40% of patients with allergic rhinitis have asthma [15]. Contrary to childhood, in adults, asthma development in individuals with rhinitis is often independent of allergy [19]. The frequency of the association between asthma and rhinitis without sinusitis found by Sa-Sousa et al. [20] in Portuguese population (Odds Ratio 3.67, 95% CI 2.79-4.82) is similar to other European studies [15].

Nonetheless, the explanation for the disease comorbidity remains unclear: is one disease causal for the other one, are there common environmental and genetic risk factors, or more likely, a combination of both reasons is at work?

3. Host and environmental risk factors to asthma and rhinitis

Many host and environmental factors are known to influence susceptibility and the clinical course of asthma and rhinitis.

Gender affects the development of asthma depending on the age of individuals. Prior to the age of 14, the prevalence of asthma is nearly twice as great in boys than in girls [21], but by adulthood, the prevalence of asthma is higher in women than in men. The reasons for this gender-related difference are not clear, one potential contributor may be differences in lung and airway size, which are smaller in males than in females in infancy[21]. For rhinitis, in childhood affected boys also outnumber girls, in adult studies results are controversial, some describing a female preponderance, especially for allergic rhinitis [22,23], and others no gender differences' [24].

Obesity has recently been identified as a major risk factor for the development of asthma, particularly for women. Asthma in obese individuals tends to be more severe and has a poorly response to treatment [25]. The association is not well understood. Potential contributing factors include changes in airway function due to the effects of obesity on lung mechanics, the development of a pro-inflammatory state, an increased prevalence of comorbidities and genetic, development, hormonal or neurogenic influences [25].

Family history of asthma is a strong determinant of asthma. Parental asthma, especially maternal, not only contributes to a greater risk but also to severity and age of onset of disease [26]. A family history of atopy or allergic diseases, like atopic dermatitis, is also associated with increased risk of developing asthma and rhinitis[23].

Atopy is a personal and/or familial tendency to become sensitized and produce IgE antibodies in response to common allergens, especially inhaled allergens and food allergens [27]. Is identified by positive skin prick tests results or by increased levels of specific IgE in serum, and increases the probability of an individual to develop allergic diseases such as asthma, rhinitis, atopic dermatitis, food allergy or hay fever [15].

Prenatal maternal **smoking habits** have been consistently associated with early childhood wheezing and an increased risk of rhinitis [23]. Postnatal exposure to parental tobacco smoke has also been consistently associated with respiratory symptoms [28]. In children and adults, passive and active exposure to tobacco exacerbate asthma symptoms, accelerate declining of lung function and decrease response to treatment [28].

Besides tobacco, allergic airway diseases are related to exposure to other **air pollutants** [24]. Children raised in a polluted environment have diminished lung function and exposure to outdoor air pollutants has been associated with significant effects on asthma morbidity in children and adults [29].

Other factors have been associated with development and course of asthma and rhinitis like stress, diet, viral infections, host microbiome composition or exposure to drugs like acetaminophen [19].

4. Genetic susceptibility to asthma and rhinitis

The etiology of complex diseases, like rhinitis and asthma involves low penetrance variants in numerous genes, environmental factors, and their interactions. Twin and family studies suggest a considerable genetic contribution for asthma and rhinitis, with an estimated heritability of 60–70% [30,31].

When exploring genetic susceptibility to human complex traits, genetic approaches had to evolve from family-based linkage studies, which traditionally mapped Mendelian disorders, to population-based association studies.

In linkage analysis, hundreds to thousands highly variable markers, usually short tandem repeats (STRs), are typed in families with multiple affected relatives. Markers that segregate with the trait in relatives, localize the disease locus. This approach has been successfully applied to find causal genes for many single-gene disorders. However, linkage analysis has been less successful for polygenic, multifactorial traits, mainly because of the small effect (low penetrance) of each genetic variant and the lower frequency of traits in families [32].

Association studies look for markers that are correlated with the phenotype across a population, rather than within families, and have much greater power to detect the effects of common, low-penetrance, genetic variants [33]. In the last decade, following the International HapMap Project, the development of high-throughput genotyping platforms has led to large-scale genome-wide association studies (GWAS), which are now frequently used to determine the genetic basis for the complex human diseases. Essentially, association studies compare the frequency of alleles and/or genotypes between individuals with the phenotype and controls. A variant with significantly higher frequency in cases than in controls is considered to be a risk variant [32]. Single nucleotide polymorphisms (SNPs) are the most frequently used genetic variant, both on a small scale (the candidate-gene approach) and large scale (GWAS) association studies. SNPs have several advantages: they are the DNA polymorphism with a higher density in the genome, they are represented in both coding and no coding regions, they are the main responsible for individual phenotype variability and they are easily genotyped. Also, the use of tagSNPs allows to improve genome coverage, and haplotype analysis increases the power of associations.

GWAS have the advantage of interrogating SNPs across the whole genome to identify novel disease susceptibility genes unrestrained by prior knowledge [33]. To reach statistical power, even when identifying only common variants, this approach imposes the study of large population samples. In a typical GWAS, only one or two of the thousands of SNPs analysed will reach statistical significance, and frequently, they will localize to a no coding region and will be of unknown biological function [34].

Candidate-gene approach has some benefits compared to GWAS. The issue with multiple testing is less pronounced since the numbers of SNPs studied are much smaller. It is also possible to choose functionally relevant SNPs in genes which prior knowledge suggests a role in the pathogenesis of the disease [35]. These studies tend to be inexpensive and sufficiently powered to detect common variants of modest effects, and results are usually easier to interpret [33]. However, candidate-gene studies are restricted to the effects of one (or a few) genetic risk factors selected and their potential for discovery relies on previous knowledge [33]. Also, frequently, SNPs selected have an ambiguous functional impact in gene expression, mostly because these are the ones with an allelic frequency above 5% in the population.

Other problem with case-control association studies derives from the potential population stratification, which could result in differences in allelic frequencies between cases and controls not due to disease. This requires strict sample selection methods and imposes the use of specific statistical analysis [33].

Either by GWA, gene-candidate or, less frequently, by linkage studies, many susceptibility loci have been identified for asthma and rhinitis. Many of these SNPs are localized in or near by genes encoding proteins involved in innate immunity and immunoregulation, genes associated with Th2-cell differentiation and effector functions, genes associated with epithelial biology and mucosal immunity, and genes associated with lung function, airway remodelling and disease severity [36].

From rare linkage studies, one of the most well-replicated regions is in chromosome 5q31-33, and includes 14 genes, such as interleukin 4 (*IL4*), interleukin 13 (*IL13)*, adrenoceptor beta 2 (*ADRB2*), and serine peptidase inhibitor Kazal-type 5 (*SPINK5*) genes. These loci have been associated with asthma, atopy or related intermediate phenotypes [36].

Results of GWAS investigating asthma phenotypes have frequently been replicated [37,38]. The *ARDB2* gene, for example, was identified more than 40 times, and association of genes like *IL4*, interleukin 4 receptor (*IL4R*), *IL13*, interleukin 10 (*IL10*), ADAM Metallopeptidase Domain 33 (*ADAM33*), ORMDL sphingolipid biosynthesis regulator 3 (*ORMDL3*), signal transducer and activator of transcription 6 (STAT6) and glutathione S-transferase pi 1 (*GSTP1*) have been replicated more than ten times [39,40].

GWAS also revealed that different allergic diseases with common immunological physiopathology, share susceptibility loci [38,41]. For instance, the interleukin 1 receptor like 1 (*IL1RL1*), *IL13*, chromosome 11 open reading frame 30 (*C11orf30*) and major histocompatibility complex (*HLA*) regions are associated with atopic dermatitis, asthma and allergic rhinitis [38,42].

Although more than 100 SNPs have been described, the genetic basis of allergic rhinitis is less understood. Genes like thymic stromal lymphopoietin (*TSLP*), Toll-like receptor 6 (*TLR6*) and nucleotide binding oligomerization domain containing 1 (*NOD1/CARD4*) were identified by candidate-gene approaches [43].

Despite all efforts and high costs, the huge quantity of data collected to date only explains a fraction of the heritability of these and other complex diseases.

The recent introduction of parallel sequencing, promising genomic screening of all variants, from SNPs to copy number variations (CNVs), raises a new hope for the discovery of the “missing heritability” of complex traits.

4.1. Interleukin 4 receptor

IL4 induces differentiation of Th2 cells. Activated Th2 cells subsequently produce additional IL4 in a positive feedback loop (Figure 1). IL4 mediates important pro-inflammatory functions in allergic phenotypes, being the major responsible for the IgE isotype switch by B lymphocytes. IL4 also induces vascular cell adhesion molecule 1(VCAM-1) on vascular endothelium and thus directs the migration of T lymphocytes, monocytes, basophils and eosinophils to the inflammation site.[11] In asthma, IL4 contributes both to

inflammation and to airway obstruction through the induction of mucin gene expression and the hypersecretion of mucus [11].

The IL4 action is mediated through activation of its receptor (IL4R), a cell-surface heterodimeric complex, located in the airway epithelium and in activated T lymphocytes. This receptor has the ability to bind both IL4 and IL13, which explains the similarity of their biological functions in the development of allergic responses [11]. IL4 signals through the type I (IL4Rα/common γ-chain) and the type II (IL4Rα/-13Rα1) IL4 receptors, whereas IL13 utilizes only the type II receptor [11].

A number of *IL4R* gene polymorphisms were described in the coding region, many of which producing amino acid substitutions (missense variants). Several of these variants have been associated with susceptibility to atopy, allergic rhinitis, sinusitis, asthma and eczema [44,45]. For example, the missense SNP rs1805015 (**T**CC > **C**CC), that results in the replacement at codon 503 of a Serine by a Proline (Ser503Pro), was associated with an increased response to IL4 in asthma and rhinitis, especially in the presence of atopy [46,47]. Several studies associate *IL4R* rs1805015 with asthma, frequently, with opposing and statistically not significant results (Table 3).

Table 3. Association studies of *IL4R* rs1805015 polymorphism and asthma in child and adult populations

Study, year	Country, ethnicity	Age group	Case (n)	Controls (n)	OR (CI) CC+CT vs. TT
Lee et al., 2004 [48]	Korea, Asian	Child	193 (At)	98	0.86 (0.43-1.71)
			59 (NAt)	98	1.13 (0.47-2.71)
Battle et al., 2007 [49]	USA, African American	Adults	263	171	0.88 (0.59-1.31)
Zhang et al., 2007 [47]	China, Asian	Mixed	352	114	**4.48 (1.75-11.45)***
Wang et al., 2009 [50]	China, Asian	Child	446	508	**0.71 (0.49-1.03)***
Bottema et al., 2010 [45]	Netherlands, Caucasian	Adults	118	102	0.81 (0.46-1.43)

Adapted from Zhu, 2013 [46]. This table also includes child studies as there are very few studies for adults. Only gene candidate studies with sample size >100 patients were included.

* $p < 0.05$; At - Atopic asthma; NAt- Non-atopic asthma; OR-odd ratio; CI-Confidence interval

4.2. Interleukin 13

The type 2 cytokine IL13 is a key mediator of allergic airway diseases and is up-regulated in response to many asthma-promoting exposures. It is involved in several stages of B lymphocytes maturation and differentiation and like IL4 promotes IgE isotype switching of these cells. IL13 production in the airway promotes the survival and migration of eosinophils, activation of macrophages, increased permeability and mucus production by airway epithelial cells, and stimulates airways hyperresponsiveness [51]. IL13 also modulates Ca2+ responses in vitro in human airway smooth muscles [52]. Sputum IL13 concentration and the number of IL13 positive cells in the bronchial submucosa and airway smooth muscles bundle were shown to be increased in severe asthmatics [52]. Although IL13 has a critical role in the pathogenesis of allergen-induced asthma, it can also operate through mechanisms independent of IgE and eosinophils [51].

IL13 is one of the most studied candidate genes for allergic diseases. Between the replicated SNPs is rs20541, that was significantly correlated with higher total IgE levels, allergic rhinitis (Table 4) and asthma (Table 5) [45,53-55].

Table 4. Association studies of *IL13* rs20541 polymorphism and allergic rhinitis in adult population

Study, year	Country, ethnicity	Cases (n)	Controls (n)	OR (CI) A vs. G
Wang et al., 2003 [56]	China, Asian	188	87	1.43 (0.95-2.16)
Nieters et al., 2004 [57]	Germany, Caucasian	318	321	1.14 (0.87-1.49)
Cheng et al., 2006 [58]	Japan, Asian	95	94	0.78 (0.50-1.22)
Kim et al., 2007 [59]	Korea, Asian	307	268	**1.30 (1.02-1.65)***
Llanes et al., 2009 [60]	Spain, Caucasian	37	50	**3.33 (1.45-7.65)***
Black et al., 2009 [61]	UK, Caucasian	651	2072	1.11 (0.95-1.31)
Lu etal., 2011 [62]	China, Asian	264	273	1.22 (0.94-1.58)
Shazia et al., 2013 [63]	Pakistan, Asian	106	120	**1.46 (0.99-2.15)***
Andiappan et al., 2013 [64]	Singapore, Asian	349	260	**1.21 (1.02-1.43)***

Adapted from Ying, 2013 [55]. Only gene candidate studies with sample size close or >100 subjects were included.

G- Guanine; A- Adenine., * p< 0.05. OR-odd ratio; CI-Confidence interval

Table 5. Association studies of *IL13* rs20541 (+2044A/G) polymorphism and asthma in adult population

Study, year	Country, ethnicity	Case (n)	Controls (n)	OR (CI) (AA+AG vs GG)
Howard et al., 2001 [65]	Holland, Caucasian	152	120	0.89 (0.55-1.45)
Donfack et al., 2005 [66]	USA, Caucasian	127	205	0.99 (0.63-1.56)
Battle et al., 2007 [49]	USA, African American	261	174	1.08 (0.72-1.62)
Black et al., 2009 [61]	UK, Caucasian	275	2462	**1.55 (1.20-2.00)***
Daley et al., 2009[67]	Australia, Caucasian	644	750	1.16 (0.92-1.45)
Llanes et al., 2009 [60]	Spain, Caucasian	108	145	0.88 (0.53-1.47)
Wang et al., 2009 [68]	China, Asian	446	505	0.87 (0.67-1.12)
Bottema et al., 2010 [45]	Netherlands, Caucasian	114	89	**2.30 (1.28-4.11)***
Dmitrieva-Zdorova et al., 2010 [69]	Russia, Caucasian	283	227	1.18 (0.83-1.68)
Palikhe et al., 2010 [70]	Korea, Asian	463	430	1.14 (0.87-1.48)
Undarmaa et al., 2010 [39]	Japan, Asian	367	676	1.10 (0.85-1.42)
Shazia et al., 2013[63]	Pakistan, Asian	108	120	1.00 (0.68-1.48)

Adapted from Ying, 2013 [55]. Only gene candidate studies with sample size with >100 patients were included.
* $p < 0.05$. OR-odd ratio; CI-Confidence interval

IL13 rs20541 (CAG>CGG; p.Gln144Arg) results in the non-conservative replacement of a neutral glutamine for a positively charged arginine in the IL13 α helix of D domain, the region that is thought to interact with IL4Rα/IL13Rα1 heterodimers [71]. The A allele has been associated with decreased affinity of IL13 for its receptor and increased expression of IL13 in patients with asthma [72,73]. Huang et al. [74] found that asthma patients, submitted to allergen challenge, had a significant enhancement of *IL13* gene expression at mRNA and protein levels in bronchoalveolar lavage samples, compared with the saline-challenged control sites.

4.3. Interleukin 17A

Interleukin 17 (IL17) family is a group of cytokines produced by activated T cells. There are six family members (IL17A-F) that bind to five receptors (IL17RA-RD and SEF). Among all IL17 family members, interleukin 17A (IL17A) is one of the most important and it may play a role in autoimmune and chronic inflammatory diseases, such as asthma and rhinitis [75].

IL17A can increase the expression level of proinflammatory cytokines and induce the release of chemokines to promote neutrophil infiltration into the airways. This ability suggests that it is involved in severe asthma, in which accumulation of neutrophils in the airways is a major defining hallmark [76]. Moreover, IL17A is increased in the blood, sputum and bronchoalveolar lavage fluid of asthma patients, and positively correlates with asthma severity [77]. IL17A could also modulate the activation and proliferation of B cells, thus enhancing IgE production [78].

Some authors showed that IL17A expression in the nasal mucosa is associated with the pathophysiology of allergic rhinitis, including disease severity and nasal eosinophilia[79,80].

Although several *IL17A* gene polymorphisms were identified, their influence in the immune and inflammatory response of these diseases is unclear. One of the polymorphisms studied, *IL17A* rs2275913, is located in 5' regulatory region within a binding motif for the nuclear factor activated T cells (NFAT), which is a critical regulator of the *IL17A* gene promoter, supporting its functional role [81]. Comparing to G allele, the A allele was associated with the higher production of IL17A, higher promoter activity, and increased affinity to transcriptional factor NFAT [82]. In the last years, the association of this polymorphism with asthma has been analysed by several authors (Table 6), but until now, with inconsistent results [83]. With regard to rhinitis, no study for this polymorphism was found.

Table 6. Association studies of *IL17A* rs2275913 polymorphism and asthma

Study, year	Country, ethnicity	Age group	Case (n)	Controls (n)	OR (CI) G vs A
Wang et al., 2009 [84]	China, Asian	Child	481	546	1.00 (0.84-1.20)
Chen et al., 2010 [85]	China, Asian	Child	168	205	**0.72 (0.54-0.97)***
Schieck et al., 2014 [86]	German, Caucasian	Child	651	652	0.88 (0.75-1.04)
Maalmi et al., 2014 [76]	Tunisia, African	Child	171	171	**1.89 (1.21-2.97)***
Du et al., 2016 [87]	China, Asian	Adult	125	132	**0.61 (0.40-0.95)***

Adapted from Zhu, 2016 [83]. This table includes child studies as there are very few studies for adults.
G- Guanine; A- Adenine., * $p< 0.05$. OR-odd ratio; CI-Confidence interval

4.4. Glutathione S-transferase P1

Glutathione S-transferases (GSTs) belong to a superfamily of phase II detoxification enzymes. GSTs play an important role in protecting cells from damage caused by numerous environmental toxins and endogenous reactive oxygen species (ROS). They conjugate reactive intermediates with glutathione to produce less reactive water-soluble compounds. In mammals, based on sequence homology and substrate specificity, eight classes of GSTs have been identified: alpha (GSTA), mu (GSTM), theta (GSTT), Pi (GSTP), zeta (GSTZ), sigma (GSTS), kappa (GSTK), and omega (GSTO) [88].

Reactive oxygen and nitrogen species, originated from air pollutants or released by inflammatory cells and stressed bronchial epithelia, are major contributors to asthma and asthma-related phenotypes [89]. Several studies have highlighted that oxidative stress damages pulmonary function and might act as a key player in the worsening of asthma symptoms [89].

Glutathione S-transferase P1 (GSTP1) is the predominant cytosolic GST expressed in lung epithelium. [88]. *GSTP1* gene has received considerable interest given the existence of common functional variants in the general population. *GSTP1* rs1695 polymorphism is one of the most studied in adult populations (Table 7), but results remain controversial [40,90]. *GSTP1* rs1695 (**A**TC>**G**TC) is a missense SNP that results in isoleucine substitution to valine in codon 105 of exon 5 (Ile105Val). This genetic variant (allele G) significantly lowers GST

enzyme activity [88], and depending on studied population, and has been reported to be both protective [91,92] and a risk factor [93] for asthma.

For rhinitis few studies are available, and the majority could not find any significant association for this *GSTP1* rs1695 polymorphism [94,95].

Table 7. Association studies of *GSTP1* rs1695 polymorphism and asthma in adult population

Study, year	Country, ethnicity	Cases (n)	Controls (n)	OR (CI) AG+GG vs AA	OR (CI) GG vs AG+AA
Fryer et al., 2000 [96]	UK, European	127	44	**0.40 (0.18-0.88)**	**0.26 (0.09-0.77)**
Aynacioglu et al., 2004 [91]	Turkey, Asian	210	265	0.95 (0.66-1.36)	**0.29 (0.13-0.64)***
Tamer et al., 2004 [93]	Turkey, Asian	101	103	**1.87 (1.06-3.30)***	**3.50 (1.48-8.26)***
Oh et al, 2005 [97]	Korea, Asian	197	119	0.80 (0.50-1.26)	1.09 (0.37-3.20)
Plutecka et al., 2006 [98]	Poland, European	399	210	0.80 (0.57-1.12)	0.84 (0.47-1.43)
Mak et al., 2007 [99]	China, Asian	312	314	1.09 (0.78-1.52)	1.24 (0.51-3.03)
Castro-Ginger et al., 2009 [100]	European comunity, European	327	2250	0.89 (0.70-1.14)	0.85 (0.54-1.35)
Undarmaa et al., 2010 [39]	Japan, Asian	359	624	0.96 (0.71-1.30)	0.80 (0.30-2.12)
Piacentini et al., 2012 [101]	Italy, European	199	200	1.32 (0.89-1.96)	1.07 (0.54-2.14)

Adapted from Piancentini et al.2013. [90]. Only gene candidate studies with sample size with >100 patients were included.

* p< 0.05. OR-odd ratio; CI-Confidence interval

II. AIMS

For asthma and rhinitis, an increasing number of susceptibility genes have been identified in the last decades but the majority of reported associations have not been consistently replicated across populations of different genetic backgrounds. This work had as main objectives:

1. To study the allelic and genotypic frequencies of polymorphisms of *IL4R* rs1805015, *IL13* rs20541, *IL17A* rs2275913 and *GSTP1* rs1695 in an adult population of Portuguese ancestry.

2. To analyse the association of these polymorphisms with susceptibility to asthma and rhinitis.

3. To compare genetic profiles of susceptibility to asthma and rhinitis

III. MATERIALS AND METHODS

1. Population – sample selection

This study was conducted under the project GA²LEN (Global Allergy and Asthma European Network) in which the Allergology group of the University Hospitals of Coimbra participated. Between 2009 and 2010, 2200 individuals aged between 18 and 74 years, randomly selected, as recommended by GA²LEN protocol, received a short questionnaire to be filled out and sent by mail (three attempts to get a response were made). The questionnaire collected information on age, gender, smoking and the presence of symptoms of asthma, rhinitis and chronic rhinosinusitis. Questions about characteristic signs and symptoms of chronic bronchitis were also included.

A random subsample of 275 symptomatic individuals, corresponding to 12.5% of the total sample was selected respecting a balanced distribution between the several groups. Patients willing to participate attended a clinical consultation, answered to clinical inquiry and performed pulmonary functional studies and skin prick tests (SPT). SPT is considered a reliable method to diagnose IgE-mediated allergic disease in patients with rhinitis and asthma and a positive result in a symptomatic individual was used as criteria of allergy.

For genetic analysis, 232 nonconsanguineous patients with rhinitis and/or asthma, were selected. The patient population was divided into three groups, according to the clinical diagnosis, using ARIA 2008[15], GINA 2008[102] and EP3OS (European Position Paper on Rhinosinusitis and Nasal Polyps 2007)[103] criteria, as recommended by GA²LEN: group 1 included patients with persistent asthma and no rhinitis; group 2 included patients with asthma and concurrent rhinitis, and group 3 included patients with rhinitis but not asthma.

A population of 192 individuals without any symptoms related to respiratory system and no history of allergic disease or other known inflammatory or chronic condition, including cancer, was selected from the whole sample for a control group.

All participants signed a written informed consent and stringent standards were followed to protect their privacy (respecting deliberation 227/2007 of the National Committee for Data Protection). The study was approved by the by the Ethics Committee of the University Hospitals of Coimbra (HUC - 32-08).

2. Skin Prick tests

SPTs were performed with *a standard panel* of 18 aeroallergens (Supplemental material 2) following GA^2LEN recommendations [104]. Allergen extracts were placed on the anterior side of the forearms and the sting was carried out with metallic lancets of Morrow-Brown type. Results were read after 20 min. The test was considered positive when wheal diameter was ≥ than 3 mm and controls showed adequate reactions [104].

3. DNA extraction

DNA was extracted from 5ml of frozen peripheral blood collected in tubes containing ethylenediaminetetraacetic acid disodium salt (Na 2 EDTA) as an anticoagulant and stored at -20°C until further use.

DNA was extracted by salting-out method, adapted from by Miller et al. [105]. After defrosting, the blood sample was treated with 40 ml of an erythrocyte lysis buffer consisting of 1x PBS with 1% IGEPAL. This solution promotes the lysis of erythrocytes and hemoglobin release. Samples were subsequently placed on an automatic shaker for 10 min and then the suspension was centrifuged for 20min at 2500rpm. Supernatant was aspirated and wasted and the pellet suspended again in 40 ml of the same erythrocyte lysing solution. The procedure was repeated until a clear supernatant with complete removal of hemoglobin was obtained.

The resulting pellet was resuspended in 1 to 6 ml of a membrane lysis buffer consisting of 300 mM NaCl, 10mM EDTA, 10mM Tris-HCl, pH 7.4 and urea 7 M, until complete homogenization. 10% SDS (sodium dodecyl sulfate) was added in a volume corresponding to 1/5 of the volume of the homogenate. This mixture was allowed to incubate for 8 hours at 37°C with constant shaking at 100 rpm on a shaker (Orbital Shaker shape, Thermo).

For protein precipitation, 6mM of NaCl solution at a ratio of 1 ml per 3.5 ml of sample was added. After vigorously shacked the homogenate was centrifuged for 25 min at 3500 rpm at room temperature. The supernatant was carefully collected and DNA was precipitated by adding 2.5 volumes of 100% ethanol. DNA was removed with a pipette and

washed with 70% ethanol. After ethanol evaporation, the DNA was dissolved in 200 µl bidistilled water and incubated at 65° for 30 min to inactivate RNases and DNases. The samples were stored at -20° until use.

4. DNA quantification

DNA concentration and purity was assessed using spectrophotometry (Nanodrop™2000) by measuring the absorbance at wavelengths of 230, 260 and 280 nm.

5. Genotyping

The genetic variants selected in the present study were functional SNPs of genes that have been reported to have a role in rhinitis and/or asthma pathogenesis (Table 8).

Table 8. SNPs evaluated

Gene	Variation	dbSNP rs ref (NCBI)	Chrom;Gene location	Alleles	TaqMan Assay ID
IL4R	p.Ser503Pro	**rs1805015**	16p; exon 11	T > C	C__8903092_20
IL13	p.Gln144Arg	**rs20541**	5q; exon 4	A > G	C__2259921_20
IL17A	c.-197G>A (regulatory region)	**rs2275913**	6p; 5'UTR	G > A	C__15879983_10
GSTP1	p.Ile105Val	**rs1695**	11q; exon 5	A > G	C__3237198_20

5.1. Sequencing

Although genotyping of selected genes was achieved by real-time polymerase chain reaction (PCR) using the TaqMan® assays (Applied Biosystems), some samples were previously sequenced to confirm TaqMan assay specificity and to later use as positive controls.

For the initial PCR, primers were designed using the Beacon Design 2.0 software (Biosoft international) (Table 9). For each amplification, 200 ng of DNA, 1.5 mM MgCl2, 200 uM dNTPs, 12.25 uM primers, 1 x buffer and 1 unit of Taq polymerase were used in a final volume of 25µl. After an initial denaturation of 5 min at 95°C, 35 cycles of 30 seconds at 95°C, 30 seconds at annealing temperature (Table 9) and 30 seconds extension at 72°C were performed; finishing with 5 min extension at 72°C. A thermal cycler "My cycler" from Biorad was used.

Table 9. Primers and annealing temperatures

Gene	Primer Sequence*	Annealing temperature
IL4R	F 5' ATGCCTTCTTCCACCTTC R 5' CATCTCGGGTTCTACTTCC	58ºC
IL13	F 5' CCGTGAGGACTGAATGAG R 5' CACAGGCTGAGGTCTAAG	55ºC
IL17A	F 5' ATGACACCAGAAGACCTAC R 5' TGTGCCTGCTATGAGATG	55ºC
GSTP1	F 5' TGTGGCAGTCTCTCATCC R 5' GCAGGTTGTGTCTTGTCC	58ºC

The quality of amplification (absence of contamination, specificity and signal intensity) was checked by electrophoresis of the PCR products on a 2 % agarose gel.

Before sequencing, PCR products were purified using Jetquick kit (Genomed). For each 20 µl of PCR product, 80 µl H1 solution were added, the mixture was applied to the column previously seated in a 1.5 ml tube and centrifuged at 20.000 x *g* for 1 minute. Then,

500 µl of H2 solution were added and centrifugation was repeated. The column was transferred to a new 1.5 ml microtube, 30 µl of sterilized water were added and the last centrifugation was performed for 2 minutes at 20.000 x *g*.

Sequencing was performed using ABI PRISM BigDye v1.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), the same primers as in PCR and an ABI PRISM 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). For sequencing reactions, 14µL H2O, 2µL buffer, 2 µL terminators, 1µL of PCR product and 1µL of primer (sense or antisense) were used. After an initial denaturation of 1 min at 96°C, 25 cycles of 10 seconds at 96°C, 5 seconds at 50 ºC and 4 minutes at 60°C were performed; finishing with 5 min extension at 72°C. The same thermal cycler "My cycler" from Biorad was used.

Samples were then purified in Sephadex™ G-50 Fine DNA Grade (GE Healthcare, Sweeden) column by centrifugation at 6000 x *g* for 1minute.

To 4 µl of purified sample, 12 µl de formamide were added and the mixture was applied in the genetic analyser. Results were analysed with sequencing analysis software v 5.2 (Applied Biosystems, Foster City, CA, USA).

5.2. TaqMan® SNP Genotyping Assay

Using this technique for detection of SNPs increases the specificity of the PCR assays and eliminates the need for any post-PCR procedures.

Each TaqMan® SNP genotyping assay contains a pair of sequence-specific forward and reverse primers as well as two minor groove binder (MGB) probes around the SNP of interest with identical sequences except in the SNP site. Each allele-specific probe consists of an oligonucleotide labeled with VIC® or 6-FAM ™ dye, attached covalently to the 5' end, to differentiate between the two SNP alleles. Near the 3 'end, there is a non-fluorescent quencher (NFQ) that, while the probe is intact, by proximity, greatly reduces the fluorescence emitted by the reporter dye. The MGB stabilizes the double-stranded structure formed between the target and the probe (Figure 3).

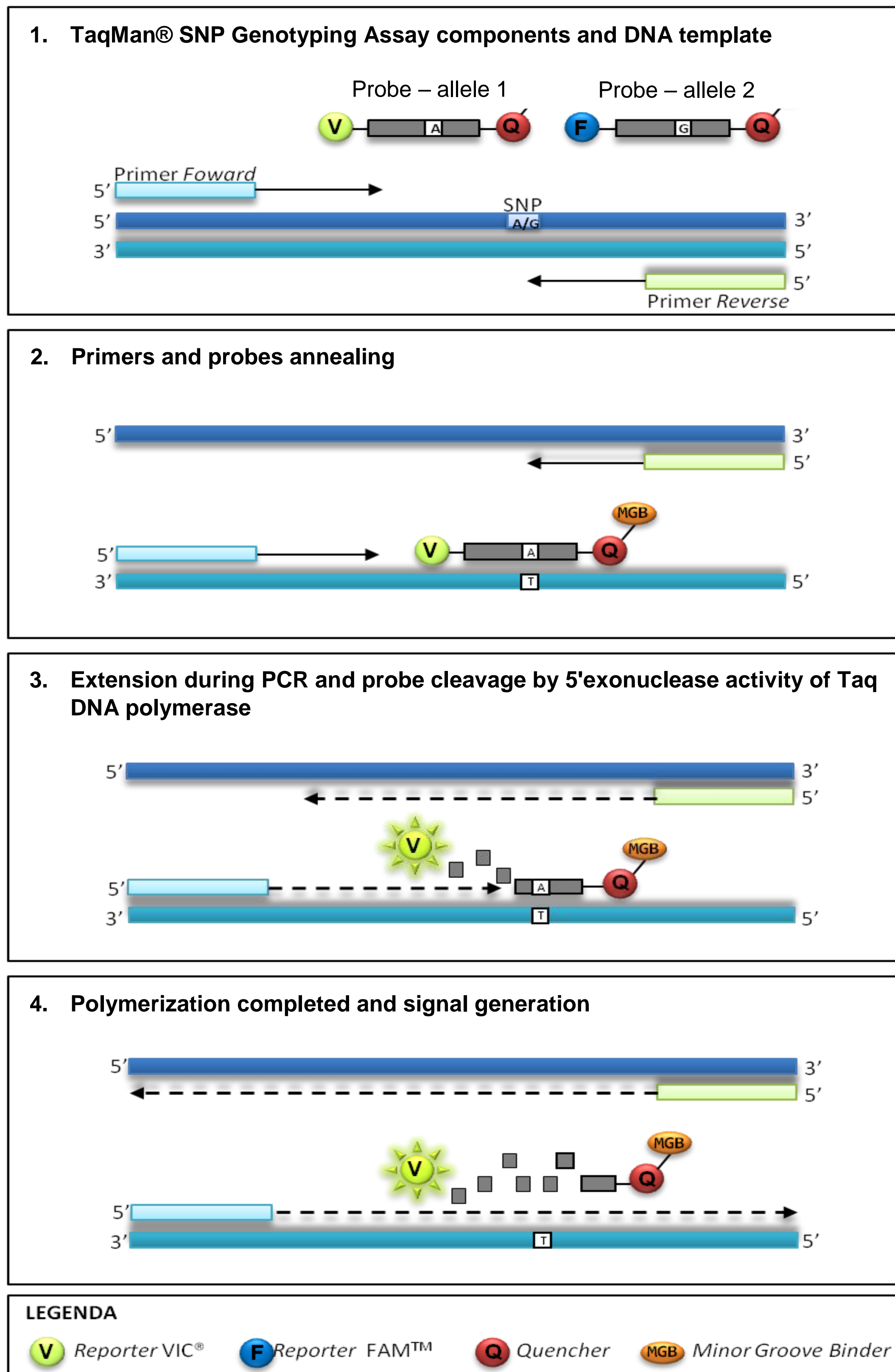

Figure 3. TaqMan® SNP Genotyping Assay

If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5´ nuclease activity of Taq DNA polymerase as this primer

is extended. Upon cleavage of the probe, the dye emits fluorescence as it is no longer quenched. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced. The different alleles were discriminated according to the fluorescence positivity of 6-FAM™ and VIC® probes (Figures 4 and 5). Since there is no PCR product handling, the risk of contamination is substantially reduced.

For *IL4R*, *IL13* and *IL17A* gene polymorphisms, TaqMan® Pre-Designed SNP Genotyping Assays (Table 8) from Applied Biosystem (New Jersey, USA) were used. The samples previously genotyped by sequencing were used for optimization and confirmation of the specificity of the assays.

The PCR reaction was performed in a final volume of 15µl containing 7.5µl of iQTM Supermix (Bio-Rad Laboratories), 0.375µl of TaqMan® SNP Genotyping Assay 40x (Applied Biosystems) and 10-30ng genomic DNA diluted in distilled water for a total of 7.125µl.

For *GSTP1* gene polymorphism, the TaqMan® Drug Metabolism SNP Genotyping Assay (Table 8), also from Applied Biosystems, was used. The PCR reaction was performed in a final volume of 12 µl containing 6 µl of iQTM Supermix, 0.6 µl the TaqMan SNP Genotyping Assay Drug Metabolism 20x (Applied Biosystems) and 10-30ng of genomic DNA diluted in distilled water, totalizing 5.4 µl.

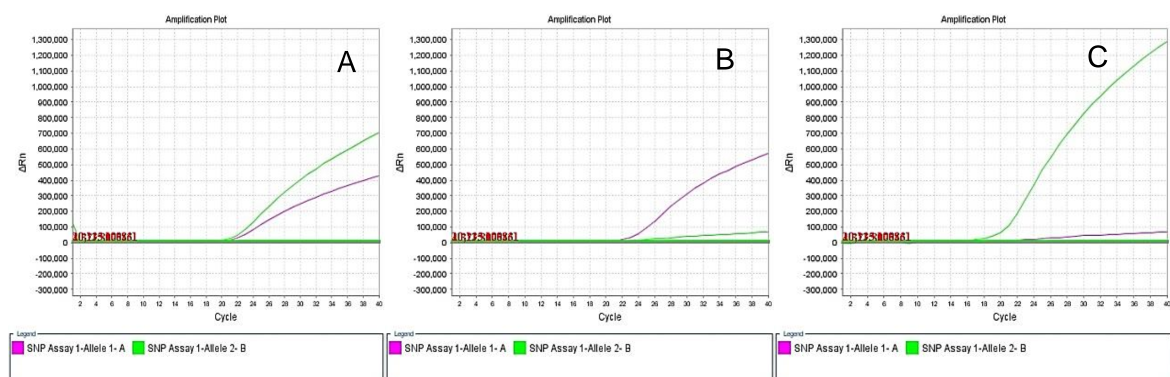

Figure 4. Allelic discrimination using TaqMan® SNP Genotyping Assays

The genotype of each sample is carried out by determining the fluorescence emitted: homozygosity in the presence of only one amplification product corresponding to a single fluorophore VIC (A) or FAM (C); and the presence of two heterozygous amplification products corresponding to the two fluorophores (B).

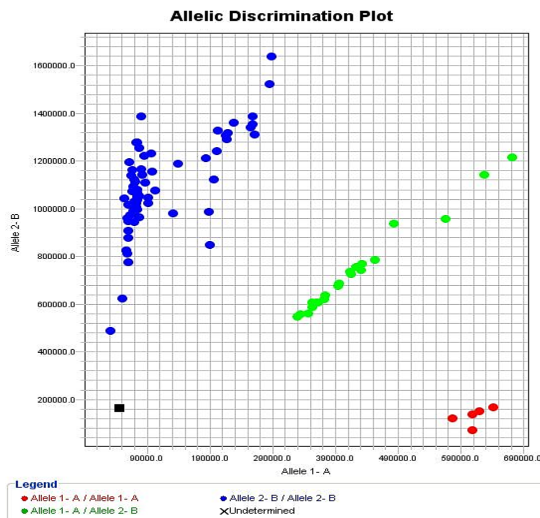

Figure 5. Allelic discrimination plots using TaqMan® SNP Genotyping Assays

The samples in red indicate only the presence of VIC dye fluorescence, corresponding to homozygosity for allele 1; for samples in blue there is only emission of fluorescence of FAM dye, corresponding to homozygosity for allele 2; samples marked in green presented both fluorescence signals, corresponding to heterozygosity.

PCR reaction and the analysis of results were performed in 7500 Fast Real-Time PCR System instrument (Applied Biosystems), using 96-well optical plates. For each reaction plate, four negative controls (No Template Control) and positive controls (previously genotyped samples) were always used. PCR cycling conditions were as follows: initial pre-denaturation 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The amplified products were analysed directly using the allelic discrimination software tool (Figure 4 and 5).

About 10 % of the samples were subsequently randomly selected for genotype confirmation by DNA sequencing.

6. Statistical analysis

Statistical analysis was performed using the IBM® program SPSS® (Statistical Package for Social Sciences) version 20.

The frequency of each variable (age, gender, smoking habits, skin prick test positive) was analysed for controls and patient subgroups. Differences between groups were assessed through a Pearson's test for categorical variables and means were compared by One-Way ANOVA. A significance level set to *p*-value < 0.05 was considered

In what concerns the analysis of genetic polymorphisms, Hardy-Weinberg Equilibrium (HWE) was determined for all populations (controls and patient subgroups).

Polymorphisms' genotypes and alleles frequencies were determined and differences between groups were assessed by a $\chi 2$ test on SPSS (version 20). The test compares the observed distribution of genotypes among cases and controls with the expected distribution. The test has a reasonably high power to detect associations regardless of the underlying risks. The *p*-value of an association test between genotype and phenotype gives information on the statistical evidence of the association [106]. The odds ratio (OR) for each polymorphism (95%CI) were also calculated.

Binary logistic regression models were used to control for potential confounders, and to evaluate the associations between the risk of disease and genetic polymorphisms. The dependent variable (the outcome) was defined as to be positive or negative for the presence of rhinitis and/or asthma while the categorical covariates (predictors) consisted of all possible genotypes of each polymorphism. All ORs with 95% confidence intervals were adjusted for age, sex and smoking habits and $p < 0.05$ was considered statistically significant. When comparing rhinitis and asthma patients, these odds ratios were also adjusted for allergy.

IV. RESULTS

1. Population Characteristic

The populations' sample of this gene candidate study included 424 Portuguese adults, distributed as follows (Figure 6): 83 patients only with rhinitis as respiratory symptoms; 149 patients with asthma (100 had asthma with rhinitis and 49 had asthma without rhinitis); and 192 healthy controls with no prior or currently established diagnosis of rhinitis, asthma or another allergic disease. The association of all patients with asthma, with or without rhinitis in the same group is further explained in section 2. Rhinitis and asthma were defined according to ARIA 2008 [15] and GINA 2008 [102] guidelines. Other immunoinflammatory diseases or cancer were exclusion criteria both for patients and controls selection.

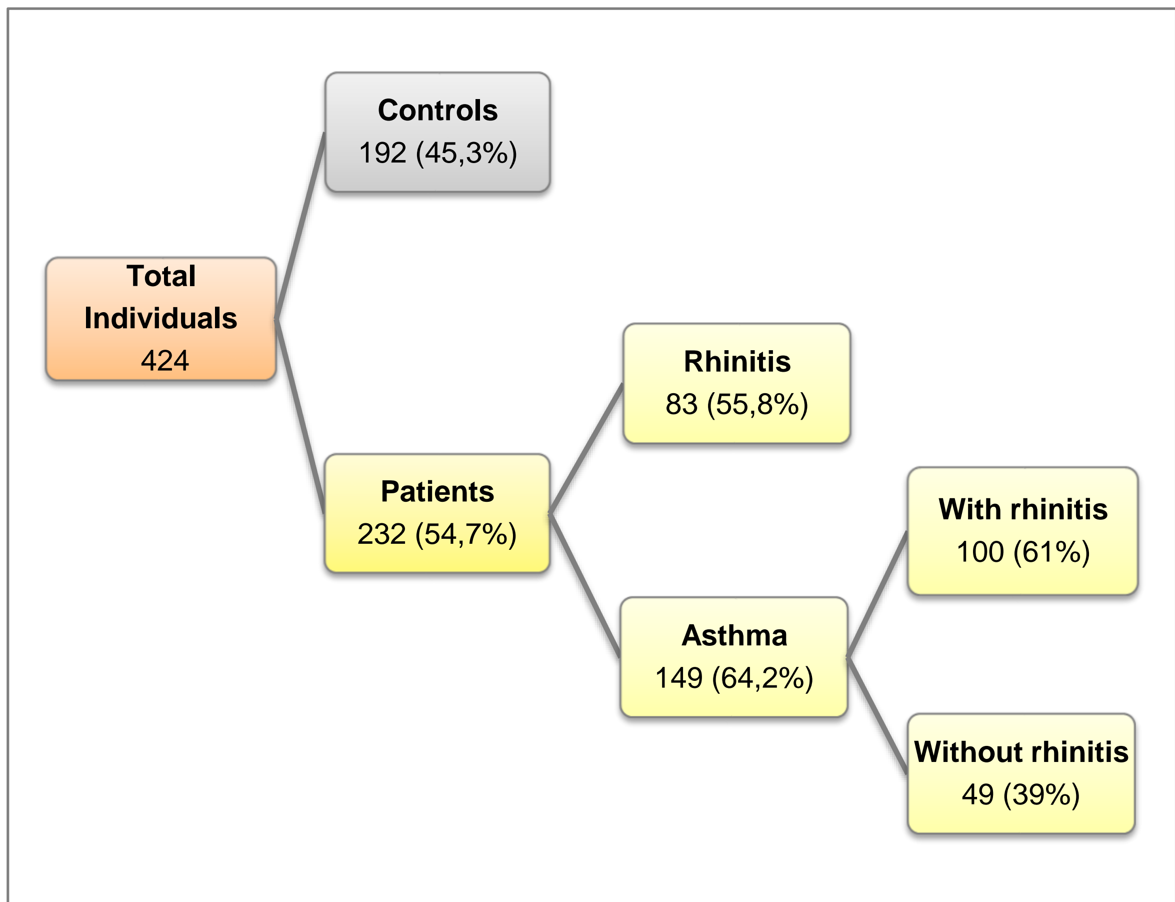

Figure 6. Distribution of phenotypes in the study population

The characteristics of the study participants are presented in Table 3.

Table 10. Characteristics of patients and controls

		Controls	Rhinitis	All Asthma	Asthma with rhinitis	Asthma without rhinitis	p
N		192	83	149	100	49	
Male, (%)		92 (47.9)	30 (36.1)	50 (33.6)	25 (25.0)	25 (51.0)	**<0.001**
Female, (%)		100 (52.1)	53 (63.9)	99 (66.4)	75 (75.0)	24 (49.0)	
Age, mean (SD)		56.06 (19.48)	48.51 (15.17)	39.3 (14.04)	38.7 (13.32)	41.9 (15.24)	**< 0.001**
Smoking habits	**No Smoking (%)**	121 (64.4)	54 (65.9)	104 (71.2)	71 (72.4)	33 (68.8)	0.567
	Smoking (%)	67 (35.6)	28 (34.1)	42 (28.8)	27 (27.6)	15 (31.3)	
Skin test positive (%)		N.A.	37 (46.3)	112 (77.7)	77 (79.4)	35 (74.5)	**< 0.001**

SD – Standard Deviation; N.A. – Non-available data
*All percentages were calculated for the total data available.

1.1. Gender and age

Concerning gender, there were statistically significant differences between control or asthma without rhinitis groups, both with a similar number of men and women, and asthma with rhinitis group, showing a larger number of females (75%) (p<0.001) (Figure 7).

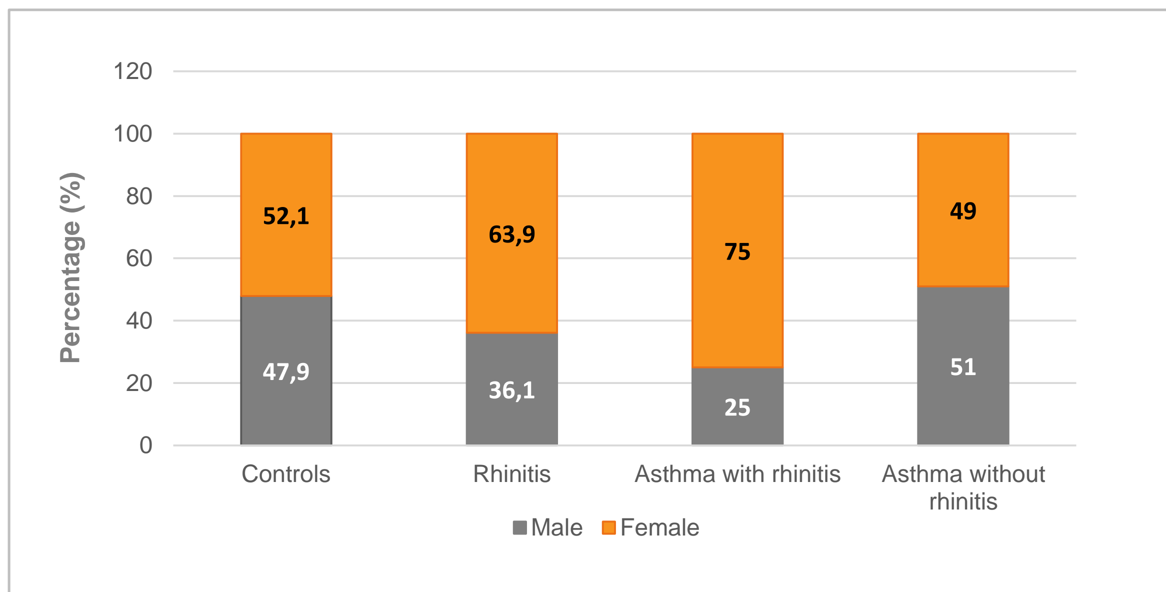

Figure 7. Female and male distribution (%) in patient and control groups

For age, there was a statistically significant difference between the control group and patients ($p < 0.001$), with controls being older [a mean age of 56.06 years with a standard deviation (SD) of 19.48] than patients. Among patients, rhinitis group was the older one, with a mean age of 48.51 years (SD= 15.17) ($p < 0.001$) (Figure 8).

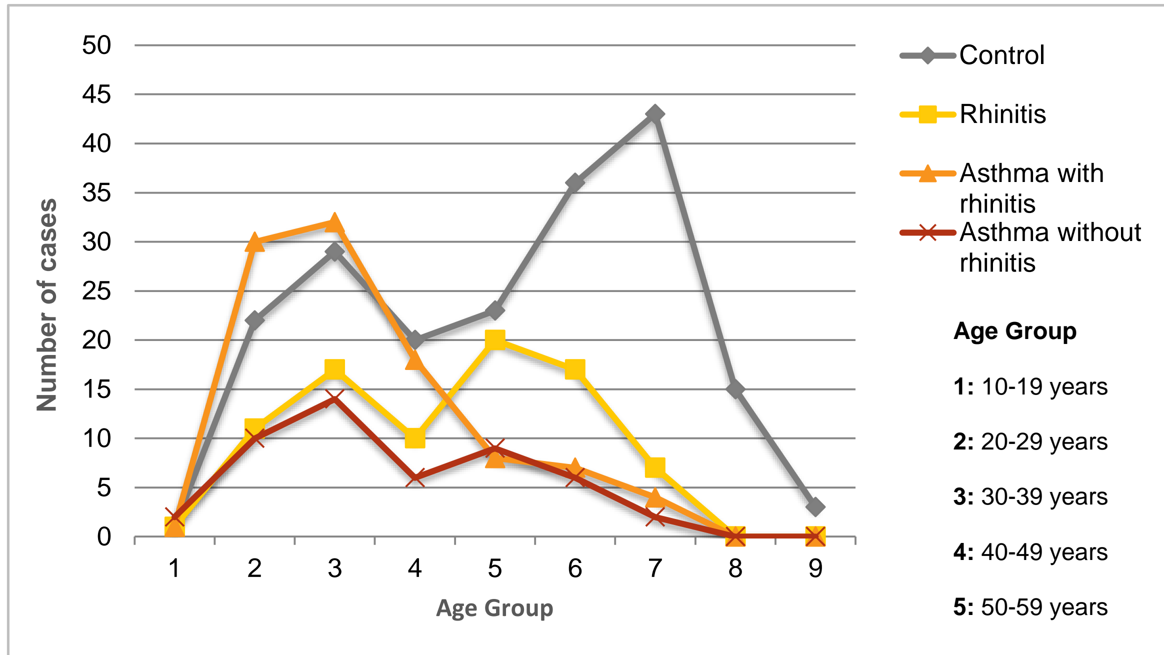

Figure 8. Distribution of age groups in patients and controls

1.2. Smoking habits

No significant differences in the number of smokers and non-smokers were found when comparing the different groups (Table 10). So, smoking habits were not associated with a risk increase for rhinitis or asthma.

1.3. Allergic sensitization

Among patients, 149 had a skin prick test (SPT) positive to at least one of a battery of 18 aeroallergens (Supplemental material 1) and were classified as being skin prick test positive and allergic.

In asthma groups (with or without rhinitis), there was significantly ($p < 0.001$) higher number of patients with positive test results, most of them polysensitized subjects (Figure 9).

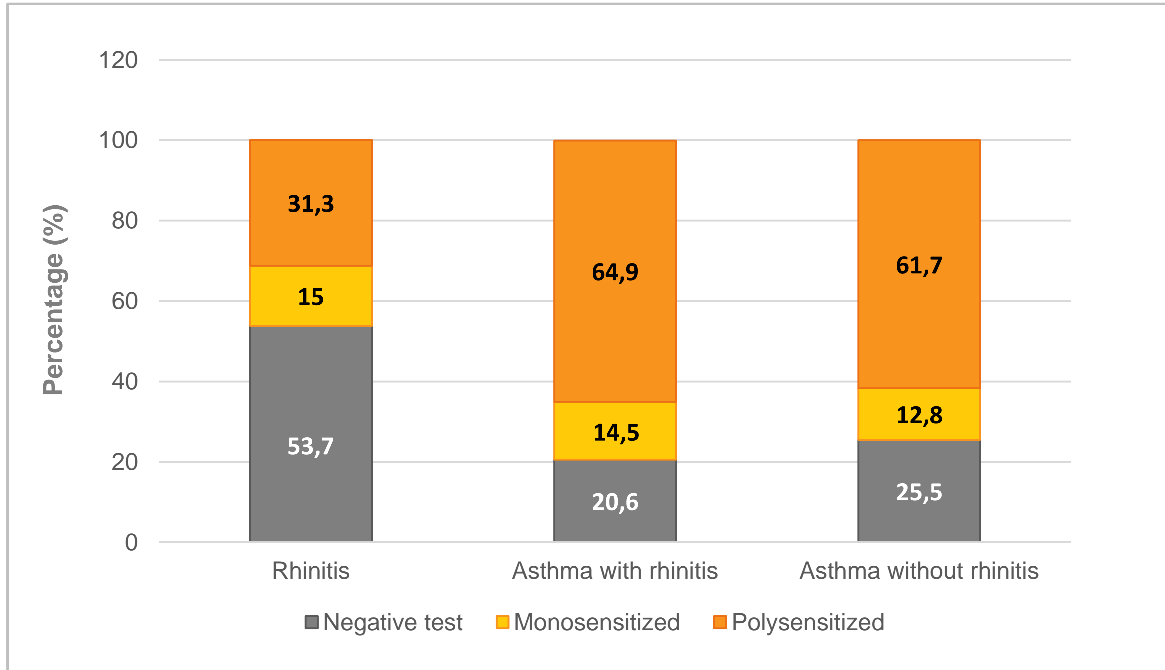

Figure 9. Allergic sensitization in patient groups

2. Allelic and genotypic frequencies

Some results or samples were excluded because of genotyping failure. Therefore, for statistical analysis, results included 423 samples for the *IL4R* rs1805015, 421 samples in the case of *IL13* rs20541, 423 samples for *IL17A* rs2275913 and 424 samples for *GSTP1* rs1695. All percentages represented in tables were calculated for the total number of results for each polymorphism. Associations were considered statistically significant at $p < 0.05$.

All SNPs were in Hardy-Weinberg Equilibrium (HWE) ($p > 0.05$) in control and disease subgroups. Because $p = 0.06$ (Table 12) for *IL4R* SNP, genotyping results were verified by sequencing, as genotyping errors are the most frequent cause of HWE disequilibrium.

When comparing allelic and genotype frequencies in both asthma subgroups (with and without rhinitis), there were no statistically significant differences (Table 11). As for

age and presence of positive skin prick test/allergy, both groups were also similar (p>0.05) (Figure 8 and 9). Based in these, the two subgroups were analysed together as "asthma" patients. So, for further statistical analysis, patients were classified into three groups: control, rhinitis and asthma.

As there were differences in age and gender frequencies between the population groups studied, logistic regression analysis was adjusted for these variables. Although no statistically significant differences were found for smoking habits, as a risk factor, the associations were also adjusted for this.

Table 11. Comparative study of genotypic frequencies between the two subgroups of asthmatic patients, with and without rhinitis

	Asthma with Rhinitis n=100 (%)	**Asthma without rhinitis n=49 (%)**	**p-value**
***IL4R r*s1805015**			
TT	75 (75.0)	40 (81.6)	0.323
CT	21 (21.0)	9 (18.4)	
CC	4 (4.0)	0 (0,0)	
***IL13* rs20541**			
GG	63 (64.3)	29 (59.2)	0.599
AG	31 (31.6)	19 (38.8)	
AA	4 (4.1)	1 (2.0)	
***IL17A* rs2275913**			
GG	46 (46.5)	23 (46.9)	0.432
AG	46 (46.5)	25 (51.0)	
AA	7 (7.1)	1 (2.0)	
***GSTP1* rs1695**			
AA	51 (51.0)	22 (44.9)	0.719
AG	40 (40.0)	21 (42.9)	
GG	9 (9.0)	6 (12.2)	

2.1. Association between genetic polymorphisms and rhinitis

When we comparing rhinitis and control groups (Table 12), there were no significant differences in genotypic frequencies of polymorphisms analysed for *IL4R*, *IL13, IL17A* and *GSTP1* (p> 0.05).

Table 12. Comparative study of genotypic frequencies in control and rhinitis groups

	Controls n (%)	Rhinitis n (%)	p-value	AdjOR* (95% CI)
***IL4R r*s1805015**				
TT	140 (73.3)	65 (78.3)	0.374	NS
CT	43 (22.5)	15 (18.1)	0.447	NS
CC	8 (4.2)	3 (3.6)	0.707	NS
HWE(*p*)	0.06	0.10		
***IL13* rs20541**				
GG	136 (70.8)	60 (73.2)	0.686	NS
AG	52 (27.1)	20 (24.4)	0.694	NS
AA	4 (2.1)	2 (2.4)	0.948	NS
HWE(*p*)	0.71	0.82		
***IL17A* rs2275913**				
GG	94 (49.0)	28 (33.7)	0.068	NS
AG	81 (42.2)	41 (49.4)	0.532	NS
AA	17 (**8.9**)	14 (**16.9**)	**0.072**	NS
HWE(*p*)	0.94	0.88		
***GSTP1* rs1695**				
AA	65 (33.9)	35 (42.2)	0.260	NS
AG	97 (50.5)	40 (48.2)	0.849	NS
GG	30 (15.6)	8 (9.6)	0.501	NS
HWE(*p*)	0.53	0.48		

HWE (*p*) – Hardy-Weinberg Equilibrium *p*-value
*OR Adjusted for age, gender and smoking habit

The *IL17A* rs2275913 AA genotype was twice frequent in rhinitis group (16.9%) than in controls (8.9%), suggesting an increased risk of developing rhinitis (OR – 2.09, 95% CI 0.98 - 4.47), but results did not reach statistical significance (p=0.072).

For alleles' frequencies, assessed by a χ2 test, no significant differences between the groups were also verified (Table 13).

Table 13. Allelic frequencies of the polymorphisms in control and rhinitis groups

Alleles	Controls n (%)	Rhinitis n (%)	p-value (χ2)	OR (95% CI)
IL4R r**s1805015**				
T	323 (84.5)	145 (87.3)	0.535	NS
C	59 (15.5)	21 (12.7)		NS
IL13 **rs20541**				
G	324 (84.3)	140 (85.4)	0.841	NS
A	60 (15.7)	24 (14.6)		NS
IL17A **rs2275913**				
G	269 (70.1)	97 (58.4)	0.075	NS
A	115 (29.9)	69 (41.6)		NS
GSTP1 **rs1695**				
A	227 (59.1)	110 (66.3)	0.303	NS
G	157 (40.9)	56 (33.7)		NS

2.2. Association between genetic polymorphisms and asthma

When comparing asthma and control groups (Table 14), there were no significant differences in the distribution of allelic and genotypic frequencies for *IL4R*, and *IL17A* SNPs (p> 0.05).

Evaluation of the genotype distribution of *GSTP1* rs1695 SNP showed that AA genotype was associated with increased susceptibility to asthma (49.0% is asthma group vs. 33.9% in controls; OR – 1.96, 95% CI 1.18 to 3.25; p=0.010) (Table 14). The A allele was also significantly more frequent in the asthma group (69.5%) than in the control group (59.1%) (p = 0.006; OR – 1.57; 95% IC 1.07 to 1.57) (Table 15). On the other hand, G allele

was more frequent in the control group (40.9% vs.30.5%) (p = 0.006; OR – 1.57; 95% IC 1.07 to 1.57), conferring a lower risk for asthma susceptibility.

For *IL13* rs2054, GG genotype was significantly associated with less susceptibility to asthma (62.6% in asthmatics and 70.8% in controls; OR – 0.55; 95% CI 0.33 - 0.94; p= 0.028) (Table 14).

Table 14. Comparative study of genotypic frequencies of control and asthma groups

	Controls n (%)	**Asthma *n* (%)**	**p-value**	**AdjOR* (95% CI)**
***IL4R r*s1805015**				
TT	140 (73.3)	115 (77.2)	0.939	NS
CT	43 (22.5)	30 (20.1)	0.614	NS
CC	8 (4.2)	4 (2.7)	0.199	NS
HWE (*p*)	0.06	0.25		
***IL13* rs20541**				
GG	136 (**70.8**)	92 (**62.6**)	**0.028**	**0.55 (0.33-0.94)**
AG	52 (27.1)	50 (34.0)	0.908	NS
AA	4 (2.1)	5 (3.4)	0.908	NS
HWE (*p*)	0.71	0.57		
***IL17A* rs2275913**				
GG	94 (49.0)	69 (46.6)	0.552	NS
AG	81 (42.2)	71 (48.0)	0.978	NS
AA	17 (8.9)	8 (5.4)	0.230	NS
HWE (*p*)	0.94	0.06		
***GSTP1* rs1695**				
AA	65 (**33.9**)	73 (**49.0**)	**0.010**	**1.96 (1.18-3.25)**
AG	97 (50.5)	61 (40.9)	0.418	NS
GG	30 (15.6)	15 (10.1)	0.418	NS
HWE (*p*)	0.53	0.67		

HWE (*p*) – Hardy-Weinberg Equilibrium *p*-value
*OR Adjusted for age, gender and smoking habits

Table 15. Allelic frequencies of the polymorphisms in control and asthma groups

	Controls n (%)	Asthma n (%)	p-value ($\chi 2$)	OR (95% CI)
IL4R ***r*****s1805015**				
T	323(84.5)	260 (87.2)	0.535	NS
C	59(15.5)	38 (12.8)		NS
IL13 **rs20541**				
G	234 (79.6)	324 (84.3)	0.353	NS
A	60 (20.4)	60 (15.7)		NS
IL17A **rs2275913**				
G	269 (70.1)	209 (70.6)	1.00	NS
A	115 (29.9)	87 (29.4)		NS
GSTP1 **rs1695**				
A	227 (59.1)	207 (69.5)	**0.006**	**1.57 (1.07-1.57)**
G	157 (40.9)	91 (30.5)		**0.64 (0.46-0.87)**

To analyse whether there was a unique genetic susceptibility profile for allergic asthma (asthma with skin prick positive test), we also compared this subgroup of patients with controls (table 16). For *GSTP1* rs1695 AA genotype, results were similar to those described for the asthma group (OR – 2.18; 95% CI 1.26 to 3.24; p=0.004).

Using a dominant model (GG and AG versus AA), the presence of at least one G allele was associated with a lower risk of developing the disease (OR – 0.496; 95% CI 0.31 to 0.80; p = 0.004). There were no statistically significant differences for other SNPs.

Table 16. Allelic and genotypic frequencies of the *GSTP1* SNP in controls and patients with allergic asthma

	Allergic asthma *n*=117 (%)	Controls *n*=192(%)	p-value	AdjOR* (95% CI)
GSTP1 **rs1695**				
AA	60(**51.3**)	65(**33.9**)	**0.004**	**2.018(1.26-3.24)**
AG	46(39.3)	97(50.5)	0.070	0.648(0.41-1.04)
GG	11(9.4)	30(15.6)	NS	NS
AG + GG	57(48.7)	127 (66.1)	**0.004**	**0.496(0.31-0.80)**

*OR adjusted for age, gender and smoking habits.

2.3. Comparison of allelic and genotypic frequencies between patients

The two groups of patients, one mainly with upper airways disease and the other with a clinically more severe phenotype with lower airways symptoms, were compared in order to evaluate if there were differences in genetic susceptibility profiles (Table 11).

For *IL17A* rs2275913, AA genotype was less frequently associated with asthma than with rhinitis (OR – 0.20, 95% CI 0.07 - 0.56; p= 0.002 (Table 17). *IL13* rs20541, GG genotype was significantly less frequent among asthmatic patients (62.6%) than among rhinitis patients (73.2%) (OR – 0.48, 95% CI 0.25 to 0.93; p=0.031) (Table 17). Analysis of the allelic distribution showed an increased frequency of the *IL17A* rs2275913 G allele in the asthmatic population (70.6% vs 58.4%; OR – 1.71; 95 % CI 1.15 - 2.54).

For *IL4R* and *GSTP1* polymorphisms, no significant differences in the distributions of allelic and genotypic frequencies were highlighted.

Table 17. Comparative study of genotypic frequencies of *IL17A* and *IL13* SNPs between the two patient groups

	Rhinitis ***n*** **(%)**	**Asthma** ***n*** **(%)**	**p-value**	**AdjOR* (95% CI)**
IL4R r**s1805015**				
TT	65 (78.3)	115 (77.2)	0.721	NS
CT	15(18.1)	30 (20.1)	0.599	NS
CC	3 (3.,6)	4 (2.7)	0.720	NS
IL13 **rs20541**				
GG	60 (**73.2**)	92 (**62.6**)	**0.031**	**0.48 (0.25-0.93)**
AG	20 (24.4)	50 (34.0)	0.797	NS
AA	2 (2.4)	5 (3.4)	0.797	NS
IL17A **rs2275913**				
GG	28 (33.7)	69 (46.6)	0.97	NS
AG	41 (49.4)	71 (48.0)	0.97	NS
AA	14 (**16.9**)	8 (**5.4**)	**0.002**	**0.20 (0.07-0.56)**
GSTP1 **rs1695**				
AA	25 (30.1)	49 (32.9)	0.864	NS
AG	48 (57.8)	71 (47.6)	0.143	NS
GG	10(12.1)	29 (19.5)	0.086	NS

* OR adjusted for age, gender, allergy and smoking habits

V. DISCUSSION

In this work we evaluate the role of polymorphisms of four genes, *IL4R*, *IL13*, *IL17A*, and *GSTP1* in the susceptibility to two related phenotypes, asthma and rhinitis.

In agreement with previous studies on population samples having the same mean age [21,22], patients were mostly of the female gender. The prevalence of asthma is known to increase significantly in women after puberty, becoming more common in women by age 20, but the physiologic mechanisms for this gender difference are not well understood [21]. Some studies also report a similar trend in patients with allergic rhinitis [22].

As reported by other authors [107], most of our patients with asthma had allergen sensitization, confirming studies suggesting that allergic sensitization is a major risk factor for asthma [15].

Other clinical risk factors were not evaluated because the complete information was not available. A positive family history is probably the strongest predictor of known risk factors, as for body max index (obesity), exposure to environmental pollutants and allergens or smoking habits, results are more controversial [28,108].

In this study, no significant association was observed between *IL4R* rs1805015 polymorphism with rhinitis and asthma. Although the association of *IL4R* with asthma was described by both gene-candidate and GWA studies [37], a recent meta-analysis did not obtain significant results for this polymorphism [46]. In fact, reports from several studies are contradictory, with results frequently diverging between populations [44-46]. Nevertheless, an important role for IL4 and IL4R in allergic airway disease is recognized [11,74].

For *IL13* rs20541 (p.Gln144Arg; **A**>**G**), our results suggest that homozygosity for G allele, corresponding to arginine (Arg) variant, may decrease susceptibility to asthma. The A allele has been associated with increased *IL13* expression [72] and a lower affinity for the IL13 receptor α2 chain (IL13Rα2), which is a decoy receptor that antagonizes inflammation and tissue remodelling [73]. These observations point out to a lower inflammatory activity of arginine variant (G allele) comparing with glutamine (A allele), supporting the association with a decreased susceptibility to asthma.

IL13 polymorphisms have repeatedly being associated with increased IgE serum levels, allergy and asthma. [44,45,54] The association of rs20541 with allergic rhinitis was

reported in a recent meta-analysis [53,55]. But, on the contrary to other authors [45,55], we could not confirm the same correlation for rhinitis, when comparing to healthy controls. One explanation may be the fact that only 50% of our rhinitis patients were allergic. It also happens that upper airways patency is largely influenced by the vascular tone and tissue edema, whereas, in the lung, airflow is influenced predominantly by smooth muscle function [109]. IL13 was shown to directly interfere with airway smooth muscle cells responsiveness by enhancing agonist-induced contractility and calcium signals, a mechanism that may be mediated by stromal interaction molecule–1 (STIM1), a sarcoplasmic reticulum protein involved in the regulation of intracellular Ca^{2+} concentrations [52].

Comparing both groups of patients, AA genotype of *IL17A* rs2275913 polymorphism was less frequently associated with asthma than with rhinitis, reinforcing the difference between the genetic profiles of these two phenotypes. This SNP is located in 5' regulatory region (c.-197G>A), in close proximity to nuclear factors activated T cell binding motifs (NFAT), and promotes production of high levels of IL-17, which in turn upregulates IL-17-mediated immune responses [81]. Peripheral blood levels determination and gene reporter assay showed that the A variant was associated with increased levels of IL17 expression and displayed a higher affinity for NFAT[82].

For rhinitis, our data are in accordance with the functional relevance of A variant. IL17 is known to be a proinflammatory, leukocyte-derived cytokine that targets epithelial cells [110] and that seems to play a central role in rhinitis [111]. IL17A expression in the nasal mucosa has been associated with disease severity and local eosinophilia [80]. IL17A levels in nasal lavages were also described to be significantly higher in patients with allergic rhinitis than in controls [112]. The same authors found that IL17A enhanced CCL-20 (C-C motif chemokine ligand 20) and IL8 expression in human nasal epithelial cells. The immunologic response is systemic, as serum concentrations of IL17A were shown to be higher in patients with allergic rhinitis compared with healthy controls, and a significant positive relationship between serum IL17A levels and symptom severity was observed [79].

Increased expression of IL17A has been mainly described in severe and non-allergic asthma [77,113]. The majority of patients included in our study had moderate asthma,

which may account for the negative association observed with the higher inflammatory activity A allele. Reinforcing our results, Lei et al.[114] described similar serum levels of IL17 between asthmatic and normal controls. Controversial results have been published, with studies suggesting that different polymorphisms of *IL17A* and *IL17F* may contribute either to susceptibility or to resistance to asthma or allergic rhinitis [115-117].

Comparing patients and controls, homozygosity for *GSTP1* rs1695 A (Ile) allele conferred about two-fold risk for developing asthma and allergic asthma. These results in a sample of Portuguese population are in accordance with previous studies that associated the A variant with a higher risk, [118] or the G variant with a lower risk [91,96] to develop asthma and allergic phenotypes in populations of Asiatic and European ancestry. Yet a meta-analysis [40] only suggested a possible, weak protective effect of the G (Val) allele. As other authors, we found no evidence for a correlation of *GSTP1* SNP with rhinitis [94,95].

GSTs play important roles in airway antioxidant defenses [89] and *GSTP1* contributes to more than 90% of GST-derived enzyme activity in human lung epithelium [88]. The rs1695 missense variant has been associated with differences in GSTP1 substrate affinities [88]. Despite increased level of plasma of GST activity in asthma [99], a recent study [119] showed that asthma patients who have homozygosity for A (Ile) allele are more likely to be affected by air pollutants. These findings substantiate the functional relevance of the SNP [120] and support our results. Yet, we are aware that this genetic effect can be modified by gene-environment interaction [121] and epigenetic modifications that also influence gene expression and ultimately, the level of enzymatic activity [122,123]. Epigenetic regulation is tissue-specific and may account for some of the differences of *GSTP1* role between upper and lower airways.

The difference of results between our data and some previous reports is a common issue in complex disease research. Differences in population genetic backgrounds, in sample size and selection, phenotype classification, in environmental exposure and epigenetic mechanisms, may contribute.

Although promising, our results must be cautiously interpreted since the sample size for patient groups lack robustness, implying that these observations require confirmation in future studies. Larger population sizes and careful phenotyping are major

issues to improve reproducibility. Since association studies compare allele and genotype frequencies, it is important to ensure a common genetic background for cases and controls. In the selection of cases, a strict phenotype definition is important to ensure a homogeneous population. To improve statistical power, the selection of cases can be restricted to include those who are likely to have a higher genetic load, as extreme phenotypes or early onset diseases. [32]

To the natural complexity of the molecular mechanisms involved in biological phenotypes, usually integrating multiple pathways, we have to add environmental and stochastics variables. The third level of complexity is created by the dynamic nature of all these factors, changing over time, and by variables interdependence. This is evidenced by heritability change over time and depending on environment conditions. At the genomic level, the variability of mechanisms regulating gene expression and the high complexity of the organization of genomic information, with gene regulatory sequences locating thousands of bases apart or even in different chromosomes, greatly increase the difficulties to map susceptibility loci and to establish gene-phenotype correlations.

The difficulties found in the study of asthma and related phenotypes are common to other complex diseases. For example, more than 80 loci have been identified for type 2 diabetes, but they only explain less than 20% of disease heritability [124]

In XXI century, research on complex diseases susceptibility loci has not yet translated into clinical useful predicting models. Its major contribution has been to improve our understanding of diseases mechanism and to propose new targets for individualized therapies.

VI. CONCLUSION AND FUTURE PERSPECTIVES

1. For the four SNPs evaluated in our sample, the allelic and genotypic frequencies were similar to those described in other Caucasian populations.

2. For *IL13* SNP rs20541, our results suggest that homozygosity for G allele, corresponding to arginine variant, may decrease susceptibility to asthma.

3. We also describe an association between AA genotype of *IL17A* rs2275913 polymorphism and risk of developing rhinitis, but not asthma.

4. For *IL4R* rs1805015, there were no significant differences in the distribution of allelic and genotypic frequencies between patients and controls nor between the two disease phenotypes, but this result should be confirmed in a larger sample.

5. Comparing patients and controls, *GSTP1* rs1695 AA genotype conferred about the two-fold risk for developing asthma and allergic asthma.

6. Our results support the hypothesis that the genetic susceptibility profiles of asthma and isolated rhinitis are not equivalent.

To confirm our results and better understand these common diseases, following research should include expansion of sample size, better characterization of phenotypes and evaluation of other risk factors, like family history, body mass index and IgE serum levels.

Furthermore, our research should explore the overall importance of gene-environment and gene-gene interactions in the development of phenotypes, disease progression and severity, and response to therapy. For example, gene expression profiles of target human tissues, characterization of disease-associated airways microbiome, and recent approaches such as systems biology, may contribute to the further discovery of risk factors and to understand how they interact.

Additional studies could evaluate the role of the oral microbiome in these diseases, namely the association between poor oral health and asthma and/or rhinitis, especially in patients with periodontitis.

VII. REFERENCES

1. World Health Organization. Chronic respiratory Diseases: Asthma. Retrieved May 2016 from http://www.who.int/respiratory/en/
2. To T, Stanojevic S, Moores G, et al. Global asthma prevalence in adults: findings from the cross-sectional world health survey. *BMC public health* 2012; **12**: 204.
3. Wallace DV, Dykewicz MS, Bernstein DI, et al. The diagnosis and management of rhinitis: an updated practice parameter. *J Allergy Clin Immunol* 2008; **122**(2 Suppl): S1-84.
4. Morais-Almeida M. Epidemiologia da asma. In: Todo-Bom A, ed. Asma. 1st ed. Lisboa: Lidel; 2016: 19-28.
5. Pegas PN, Alves CA, Scotto MG, Evtyugina MG, Pio CA, Freitas MC. [Risk factors and prevalence of asthma and rhinitis among primary school children in Lisbon]. *Rev Port Pneumol* 2011; **17**(3): 109-16.
6. Reddel HK, Bateman ED, Becker A, et al. A summary of the new GINA strategy: a roadmap to asthma control. *Eur Respir J* 2015; **46**(3): 622-39.
7. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention, 2016. Available from: www.ginasthma.com
8. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012; **18**(5): 716-25.
9. Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. *Lancet* 2008; **372**(9643): 1107-19.
10. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008; **8**(3): 183-92.
11. Steinke JW, Borish L. Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. *Respir Res* 2001; **2**(2): 66-70.
12. Orihara K, Dil N, Anaparti V, Moqbel R. What's new in asthma pathophysiology and immunopathology? *Expert Rev Respir Med* 2010; **4**(5): 605-29.
13. Bousquet J, Van Cauwenberge P, Khaltaev N, Aria Workshop G, World Health O. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001; **108**(5 Suppl): S147-334.
14. Boulet LP, Boulay ME. Asthma-related comorbidities. *Expert Rev Respir Med* 2011; **5**(3): 377-93.
15. Bousquet J, Khaltaev N, Cruz AA, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy* 2008; **63 Suppl 86**: 8-160.
16. Sin B, Togias A. Pathophysiology of allergic and nonallergic rhinitis. *Proceedings of the American Thoracic Society* 2011; **8**(1): 106-14.
17. Agache I, Akdis CA. Endotypes of allergic diseases and asthma: An important step in building blocks for the future of precision medicine. *Allergol Int* 2016.
18. Corren J. Asthma phenotypes and endotypes: an evolving paradigm for classification. *Discovery medicine* 2013; **15**(83): 243-9.

19. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention,- Online appendix. 2016. Available from: www.ginasthma.com

20. Sa-Sousa A, Morais-Almeida M, Azevedo LF, et al. Prevalence of asthma in Portugal - The Portuguese National Asthma Survey. *Clinical and translational allergy* 2012; **2**(1): 15.

21. Schatz M, Camargo CA, Jr. The relationship of sex to asthma prevalence, health care utilization, and medications in a large managed care organization. *Ann Allergy Asthma Immunol* 2003; **91**(6): 553-8.

22. Osman M, Hansell AL, Simpson CR, Hollowell J, Helms PJ. Gender-specific presentations for asthma, allergic rhinitis and eczema in primary care. *Primary care respiratory journal : journal of the General Practice Airways Group* 2007; **16**(1): 28-35.

23. Matheson MC, Dharmage SC, Abramson MJ, et al. Early-life risk factors and incidence of rhinitis: results from the European Community Respiratory Health Study--an international population-based cohort study. *J Allergy Clin Immunol* 2011; **128**(4): 816-23 e5.

24. Cazzoletti L, Ferrari M, Olivieri M, et al. The gender, age and risk factor distribution differs in self-reported allergic and non-allergic rhinitis: a cross-sectional population-based study. *Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology* 2015; **11**: 36.

25. Dixon AE, Holguin F, Sood A, et al. An official American Thoracic Society Workshop report: obesity and asthma. *Proceedings of the American Thoracic Society* 2010; **7**(5): 325-35.

26. Lim RH, Kobzik L, Dahl M. Risk for asthma in offspring of asthmatic mothers versus fathers: a meta-analysis. *PLoS One* 2010; **5**(4): e10134.

27. Johansson SG, Bieber T, Dahl R, et al. Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J Allergy Clin Immunol* 2004; **113**(5): 832-6.

28. Sheikh SI, Pitts J, Ryan-Wenger NA, McCoy KS, Hayes D, Jr. Environmental exposures and family history of asthma. *J Asthma* 2016; **53**(5): 465-70.

29. Brozek JL, Bousquet J, Baena-Cagnani CE, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines: 2010 revision. *J Allergy Clin Immunol* 2010; **126**(3): 466-76.

30. Thomsen SF, van der Sluis S, Kyvik KO, Skytthe A, Backer V. Estimates of asthma heritability in a large twin sample. *Clin Exp Allergy* 2010; **40**(7): 1054-61.

31. Willemsen G, van Beijsterveldt TC, van Baal CG, Postma D, Boomsma DI. Heritability of self-reported asthma and allergy: a study in adult Dutch twins, siblings and parents. *Twin research and human genetics : the official journal of the International Society for Twin Studies* 2008; **11**(2): 132-42.

32. Jin L, Zuo XY, Su WY, et al. Pathway-based analysis tools for complex diseases: a review. *Genomics, proteomics & bioinformatics* 2014; **12**(5): 210-20.

33. Hirschhorn JN, Daly MJ. Genome-wide association studies for common diseases and complex traits. *Nature reviews Genetics* 2005; **6**(2): 95-108.

34. Manolio TA, Collins FS, Cox NJ, et al. Finding the missing heritability of complex diseases. *Nature* 2009; **461**(7265): 747-53.
35. Tabor HK, Risch NJ, Myers RM. Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nature reviews Genetics* 2002; **3**(5): 391-7.
36. Vercelli D. Discovering susceptibility genes for asthma and allergy. *Nat Rev Immunol* 2008; **8**(3): 169-82.
37. Moffatt MF, Gut IG, Demenais F, et al. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med* 2010; **363**(13): 1211-21.
38. Tamari M, Tanaka S, Hirota T. Genome-wide association studies of allergic diseases. *Allergol Int* 2013; **62**(1): 21-8.
39. Undarmaa S, Mashimo Y, Hattori S, et al. Replication of genetic association studies in asthma and related phenotypes. *J Hum Genet* 2010; **55**(6): 342-9.
40. Minelli C, Granell R, Newson R, et al. Glutathione-S-transferase genes and asthma phenotypes: a Human Genome Epidemiology (HuGE) systematic review and meta-analysis including unpublished data. *Int J Epidemiol* 2010; **39**(2): 539-62.
41. Bouzigon E, Forabosco P, Koppelman GH, et al. Meta-analysis of 20 genome-wide linkage studies evidenced new regions linked to asthma and atopy. *Eur J Hum Genet* 2010; **18**(6): 700-6.
42. Bonnelykke K, Matheson MC, Pers TH, et al. Meta-analysis of genome-wide association studies identifies ten loci influencing allergic sensitization. *Nature genetics* 2013; **45**(8): 902-6.
43. Ramasamy A, Curjuric I, Coin LJ, et al. A genome-wide meta-analysis of genetic variants associated with allergic rhinitis and grass sensitization and their interaction with birth order. *J Allergy Clin Immunol* 2011; **128**(5): 996-1005.
44. Wang ZD, Lian D, Shen JL, et al. Association between the interleukin-4, interleukin-13 polymorphisms and asthma: a meta-analysis. *Mol Biol Rep* 2013; **40**(2): 1365-76.
45. Bottema RW, Nolte IM, Howard TD, et al. Interleukin 13 and interleukin 4 receptor-alpha polymorphisms in rhinitis and asthma. *Int Arch Allergy Immunol* 2010; **153**(3): 259-67.
46. Zhu N, Gong Y, Chen XD, et al. Association between the polymorphisms of interleukin-4, the interleukin-4 receptor gene and asthma. *Chinese medical journal* 2013; **126**(15): 2943-51.
47. Zhang H, Zhang Q, Wang L, et al. Association of IL4R gene polymorphisms with asthma in Chinese populations. *Human mutation* 2007; **28**(10): 1046.
48. Lee SG, Kim BS, Kim JH, et al. Gene-gene interaction between interleukin-4 and interleukin-4 receptor alpha in Korean children with asthma. *Clin Exp Allergy* 2004; **34**(8): 1202-8.
49. Battle NC, Choudhry S, Tsai HJ, et al. Ethnicity-specific gene-gene interaction between IL-13 and IL-4Ralpha among African Americans with asthma. *Am J Respir Crit Care Med* 2007; **175**(9): 881-7.

50. Wang JY, Liou YH, Wu YJ, Hsiao YH, Wu LS. An association study of 13 SNPs from seven candidate genes with pediatric asthma and a preliminary study for genetic testing by multiple variants in Taiwanese population. *Journal of clinical immunology* 2009; **29**(2): 205-9.
51. Ingram JL, Kraft M. IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. *J Allergy Clin Immunol* 2012; **130**(4): 829-42; quiz 43-4.
52. Jia L, Delmotte P, Aravamudan B, Pabelick CM, Prakash YS, Sieck GC. Effects of the inflammatory cytokines TNF-alpha and IL-13 on stromal interaction molecule-1 aggregation in human airway smooth muscle intracellular Ca(2+) regulation. *Am J Respir Cell Mol Biol* 2013; **49**(4): 601-8.
53. Bunyavanich S, Shargorodsky J, Celedon JC. A meta-analysis of Th2 pathway genetic variants and risk for allergic rhinitis. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology* 2011; **22**(4): 378-87.
54. Nie W, Liu Y, Bian J, Li B, Xiu Q. Effects of polymorphisms -1112C/T and +2044A/G in interleukin-13 gene on asthma risk: a meta-analysis. *PLoS One* 2013; **8**(2): e56065.
55. Ying XJ, Zhao SW, Wang GL, Xie J, Xu HM, Dong P. Association of interleukin-13 SNP rs20541 with allergic rhinitis risk: a meta-analysis. *Gene* 2013; **521**(2): 222-6.
56. Wang M, Xing ZM, Lu C, et al. A common IL-13 Arg130Gln single nucleotide polymorphism among Chinese atopy patients with allergic rhinitis. *Human genetics* 2003; **113**(5): 387-90.
57. Nieters A, Linseisen J, Becker N. Association of polymorphisms in Th1, Th2 cytokine genes with hayfever and atopy in a subsample of EPIC-Heidelberg. *Clin Exp Allergy* 2004; **34**(3): 346-53.
58. Cheng L, Hirota T, Enomoto T, et al. Lack of association between the IL13 variant Arg110Gln and susceptibility to cedar pollinosis in a Japanese population. *Int Arch Allergy Immunol* 2006; **139**(1): 25-30.
59. Kim JJ, Min JY, Lee JH. Polymorphisms in the IL-13 and IL-4 receptor alpha genes and allergic rhinitis. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies* 2007; **264**(4): 395-9.
60. Llanes E, Quiralte J, Lopez E, et al. Analysis of polymorphisms in olive pollen allergy: IL13, IL4RA, IL5 and ADRB2 genes. *Int Arch Allergy Immunol* 2009; **148**(3): 228-38.
61. Black S, Teixeira AS, Loh AX, et al. Contribution of functional variation in the IL13 gene to allergy, hay fever and asthma in the NSHD longitudinal 1946 birth cohort. *Allergy* 2009; **64**(8): 1172-8.
62. Lu MP, Chen RX, Wang ML, et al. Association study on IL4, IL13 and IL4RA polymorphisms in mite-sensitized persistent allergic rhinitis in a Chinese population. *PLoS One* 2011; **6**(11): e27363.
63. Shazia M, Kanza M, Mehwish I, Irum S, Farida A, Asifa A. IL-13 gene polymorphisms and their association with atopic asthma and rhinitis in Pakistani patients. *Iranian journal of allergy, asthma, and immunology* 2013; **12**(4): 391-6.

64. Andiappan AK, Rotzschke O, Wang de Y, Chew FT. Association of interleukin-13 SNP rs20541 (Arg>Gln) to allergic rhinitis in an Asian population of ethnic Chinese in Singapore. *Gene* 2013; **529**(2): 357-8.

65. Howard TD, Whittaker PA, Zaiman AL, et al. Identification and association of polymorphisms in the interleukin-13 gene with asthma and atopy in a Dutch population. *Am J Respir Cell Mol Biol* 2001; **25**(3): 377-84.

66. Donfack J, Schneider DH, Tan Z, et al. Variation in conserved non-coding sequences on chromosome 5q and susceptibility to asthma and atopy. *Respir Res* 2005; **6**: 145.

67. Daley D, Lemire M, Akhabir L, et al. Analyses of associations with asthma in four asthma population samples from Canada and Australia. *Human genetics* 2009; **125**(4): 445-59.

68. Wang XH, Zhao W, Liu SG, Feng XP. [Correlation of IL-4 and IL-13 gene polymorphisms with asthma and total serum IgE levels]. *Zhonghua Jie He He Hu Xi Za Zhi* 2009; **32**(3): 161-4.

69. Dmitrieva-Zdorova EV, Voron'ko OE, Aksenova MG, Bodoev NV. [Association of interleukin-13 gene polymorphisms with atopic bronchial asthma]. *Genetika* 2010; **46**(1): 111-7.

70. Palikhe NS, Kim SH, Cho BY, et al. IL-13 Gene Polymorphisms are Associated With Rhinosinusitis and Eosinophilic Inflammation in Aspirin Intolerant Asthma. *Allergy, asthma & immunology research* 2010; **2**(2): 134-40.

71. Madhankumar AB, Mintz A, Debinski W. Alanine-scanning mutagenesis of alpha-helix D segment of interleukin-13 reveals new functionally important residues of the cytokine. *J Biol Chem* 2002; **277**(45): 43194-205.

72. Arima K, Umeshita-Suyama R, Sakata Y, et al. Upregulation of IL-13 concentration in vivo by the IL13 variant associated with bronchial asthma. *J Allergy Clin Immunol* 2002; **109**(6): 980-7.

73. Lacy ER. Equilibrium and kinetic analysis of human interleukin-13 and IL-13 receptor alpha-2 complex formation. *J Mol Recognit* 2012; **25**(3): 184-91.

74. Huang SK, Xiao HQ, Kleine-Tebbe J, et al. IL-13 expression at the sites of allergen challenge in patients with asthma. *J Immunol* 1995; **155**(5): 2688-94.

75. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity* 2004; **21**(4): 467-76.

76. Maalmi H, Beraies A, Charad R, Ammar J, Hamzaoui K, Hamzaoui A. IL-17A and IL-17F genes variants and susceptibility to childhood asthma in Tunisia. *J Asthma* 2014; **51**(4): 348-54.

77. Agache I, Ciobanu C, Agache C, Anghel M. Increased serum IL-17 is an independent risk factor for severe asthma. *Respir Med* 2010; **104**(8): 1131-7.

78. Newcomb DC, Peebles RS, Jr. Th17-mediated inflammation in asthma. *Curr Opin Immunol* 2013; **25**(6): 755-60.

79. Ciprandi G, De Amici M, Murdaca G, et al. Serum interleukin-17 levels are related to clinical severity in allergic rhinitis. *Allergy* 2009; **64**(9): 1375-8.

80. Makihara S, Okano M, Fujiwara T, et al. Local expression of interleukin-17a is correlated with nasal eosinophilia and clinical severity in allergic rhinitis. *Allergy Rhinol (Providence)* 2014; **5**(1): 22-7.
81. Liu XK, Lin X, Gaffen SL. Crucial role for nuclear factor of activated T cells in T cell receptor-mediated regulation of human interleukin-17. *J Biol Chem* 2004; **279**(50): 52762-71.
82. Espinoza JL, Takami A, Nakata K, et al. A genetic variant in the IL-17 promoter is functionally associated with acute graft-versus-host disease after unrelated bone marrow transplantation. *PLoS One* 2011; **6**(10): e26229.
83. Zhu M, Wang T, Chen R, Wang C, Liu S, Ji Y. Association between interleukin-17a gene polymorphisms and asthma risk: a meta-analysis. *Asian Pac J Allergy Immunol* 2016.
84. Wang JY, Shyur SD, Wang WH, et al. The polymorphisms of interleukin 17A (IL17A) gene and its association with pediatric asthma in Taiwanese population. *Allergy* 2009; **64**(7): 1056-60.
85. Chen J, Deng Y, Zhao J, et al. The polymorphism of IL-17 G-152A was associated with childhood asthma and bacterial colonization of the hypopharynx in bronchiolitis. *Journal of clinical immunology* 2010; **30**(4): 539-45.
86. Schieck M, Michel S, Suttner K, et al. Genetic variation in TH17 pathway genes, childhood asthma, and total serum IgE levels. *J Allergy Clin Immunol* 2014; **133**(3): 888-91.
87. Du J, Han JC, Zhang YJ, et al. Single-Nucleotide Polymorphisms of IL-17 Gene Are Associated with Asthma Susceptibility in an Asian Population. *Medical science monitor : international medical journal of experimental and clinical research* 2016; **22**: 780-7.
88. Watson MA, Stewart RK, Smith GB, Massey TE, Bell DA. Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. *Carcinogenesis* 1998; **19**(2): 275-80.
89. Sahiner UM, Birben E, Erzurum S, Sackesen C, Kalayci O. Oxidative stress in asthma. *World Allergy Organ J* 2011; **4**(10): 151-8.
90. Piacentini S, Polimanti R, Simonelli I, et al. Glutathione S-transferase polymorphisms, asthma susceptibility and confounding variables: a meta-analysis. *Mol Biol Rep* 2013; **40**(4): 3299-313.
91. Aynacioglu AS, Nacak M, Filiz A, Ekinci E, Roots I. Protective role of glutathione S-transferase P1 (GSTP1) Val105Val genotype in patients with bronchial asthma. *Br J Clin Pharmacol* 2004; **57**(2): 213-7.
92. Hanene C, Jihene L, Jamel A, Kamel H, Agnes H. Association of GST genes polymorphisms with asthma in Tunisian children. *Mediators Inflamm* 2007; **2007**: 19564.
93. Tamer L, Calikoglu M, Ates NA, et al. Glutathione-S-transferase gene polymorphisms (GSTT1, GSTM1, GSTP1) as increased risk factors for asthma. *Respirology* 2004; **9**(4): 493-8.
94. Iorio A, Polimanti R, Piacentini S, Liumbruno GM, Manfellotto D, Fuciarelli M. Deletion polymorphism of GSTT1 gene as protective marker for allergic rhinitis. *Clin Respir J* 2014.

95. Fuertes E, Brauer M, MacIntyre E, et al. Childhood allergic rhinitis, traffic-related air pollution, and variability in the GSTP1, TNF, TLR2, and TLR4 genes: results from the TAG Study. *J Allergy Clin Immunol* 2013; **132**(2): 342-52 e2.
96. Fryer AA, Bianco A, Hepple M, Jones PW, Strange RC, Spiteri MA. Polymorphism at the glutathione S-transferase GSTP1 locus. A new marker for bronchial hyperresponsiveness and asthma. *Am J Respir Crit Care Med* 2000; **161**(5): 1437-42.
97. Oh JM, Kim SH, Suh CH, et al. Lack of association of glutathione S-transferase P1 Ile105Val polymorphism with aspirin-intolerant asthma. *The Korean journal of internal medicine* 2005; **20**(3): 232-6.
98. Plutecka H, Sanak M, S. D, Mastalerz L, Szczeklik A. Reactive oxygen species metabolism and allergy. *Allergy Clin Immunol Int* 2006; **18**: 158-64.
99. Mak JC, Ho SP, Leung HC, et al. Relationship between glutathione S-transferase gene polymorphisms and enzyme activity in Hong Kong Chinese asthmatics. *Clin Exp Allergy* 2007; **37**(8): 1150-7.
100. Castro-Giner F, Kunzli N, Jacquemin B, et al. Traffic-related air pollution, oxidative stress genes, and asthma (ECHRS). *Environmental health perspectives* 2009; **117**(12): 1919-24.
101. Piacentini S, Polimanti R, Moscatelli B, Re MA, Manfellotto D, Fuciarelli M. Lack of association between GSTM1, GSTP1, and GSTT1 gene polymorphisms and asthma in adult patients from Rome, central Italy. *J Investig Allergol Clin Immunol* 2012; **22**(4): 252-6.
102. Bateman ED, Hurd SS, Barnes PJ, et al. Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J* 2008; **31**(1): 143-78.
103. Fokkens W, Lund V, Mullol J, European Position Paper on R, Nasal Polyps g. European position paper on rhinosinusitis and nasal polyps 2007. *Rhinology Supplement* 2007; (20): 1-136.
104. Heinzerling LM, Burbach GJ, Edenharter G, et al. GA(2)LEN skin test study I: GA(2)LEN harmonization of skin prick testing: novel sensitization patterns for inhalant allergens in Europe. *Allergy* 2009; **64**(10): 1498-506.
105. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**(3): 1215.
106. Lewis CM, Knight J. Introduction to genetic association studies. *Cold Spring Harbor protocols* 2012; **2012**(3): 297-306.
107. Craig TJ. Aeroallergen sensitization in asthma: prevalence and correlation with severity. *Allergy Asthma Proc* 2010; **31**(2): 96-102.
108. Burke W, Fesinmeyer M, Reed K, Hampson L, Carlsten C. Family history as a predictor of asthma risk. *American journal of preventive medicine* 2003; **24**(2): 160-9.
109. Rimmer J, Ruhno JW. 6: Rhinitis and asthma: united airway disease. *Med J Aust* 2006; **185**(10): 565-71.
110. Eyerich S, Eyerich K, Cavani A, Schmidt-Weber C. IL-17 and IL-22: siblings, not twins. *Trends Immunol* 2010; **31**(9): 354-61.

111. Liu Y, Zeng M, Liu Z. Th17 response and its regulation in inflammatory upper airway diseases. *Clin Exp Allergy* 2015; **45**(3): 602-12.
112. Xu G, Zhang L, Wang DY, et al. Opposing roles of IL-17A and IL-25 in the regulation of TSLP production in human nasal epithelial cells. *Allergy* 2010; **65**(5): 581-9.
113. Wang YH, Wills-Karp M. The potential role of interleukin-17 in severe asthma. *Curr Allergy Asthma Rep* 2011; **11**(5): 388-94.
114. Lei Z, Liu G, Huang Q, et al. SCF and IL-31 rather than IL-17 and BAFF are potential indicators in patients with allergic asthma. *Allergy* 2008; **63**(3): 327-32.
115. Bazzi MD, Sultan MA, Al Tassan N, et al. Interleukin 17A and F and asthma in Saudi Arabia: gene polymorphisms and protein levels. *J Investig Allergol Clin Immunol* 2011; **21**(7): 551-5.
116. Wang M, Zhang Y, Han D, Zhang L. Association between polymorphisms in cytokine genes IL-17A and IL-17F and development of allergic rhinitis and comorbid asthma in Chinese subjects. *Hum Immunol* 2012; **73**(6): 647-53.
117. Jin Y, Deng Z, Cao C, Li L. IL-17 polymorphisms and asthma risk: a meta-analysis of 11 single nucleotide polymorphisms. *J Asthma* 2015; **52**(10): 981-8.
118. Imboden M, Rochat T, Brutsche M, et al. Glutathione S-transferase genotype increases risk of progression from bronchial hyperresponsiveness to asthma in adults. *Thorax* 2008; **63**(4): 322-8.
119. Sada M WH, Inui T, Nakamoto K, Honda K, Nakamura M, Horie M, Mikami Y, Noguchi S, Takizawa H. Influence Of Air Pollutants And Glutathione S-Transferase P1 Gene Polymorphism In Patients With Bronchial Asthma. *Am J Respir Crit Care Med* 2015; **191**: A1070.
120. Hayes JD, Strange RC. Glutathione S-transferase polymorphisms and their biological consequences. *Pharmacology* 2000; **61**(3): 154-66.
121. Hollman AL, Tchounwou PB, Huang HC. The Association between Gene-Environment Interactions and Diseases Involving the Human GST Superfamily with SNP Variants. *International journal of environmental research and public health* 2016; **13**(4).
122. Miller RL, Ho SM. Environmental epigenetics and asthma: current concepts and call for studies. *Am J Respir Crit Care Med* 2008; **177**(6): 567-73.
123. Child F, Lenney W, Clayton S, et al. The association of maternal but not paternal genetic variation in GSTP1 with asthma phenotypes in children. *Respir Med* 2003; **97**(12): 1247-56.
124. Prasad RB, Groop L. Genetics of type 2 diabetes-pitfalls and possibilities. *Genes* 2015; **6**(1): 87-123.

VIII. SUPPLEMENTAL MATERIAL

Supplemental Material 1

Table 18. Diagnostic criteria for asthma in adults, adolescents, and children 6–11 years (GINA 2016)[7]

DIAGNOSTIC FEATURE	CRITERIA FOR MAKING THE DIAGNOSIS OF ASTHMA
1. History of variable respiratory symptoms	
Wheeze, shortness of breath, chest tightness and cough Descriptors may vary between cultures and by age, e.g. children may be described as having heavy breathing	• Generally more than one type of respiratory symptom (in adults, isolated cough is seldom due to asthma) • Symptoms occur variably over time and vary in intensity • Symptoms are often worse at night or on waking • Symptoms are often triggered by exercise, laughter, allergens, cold air • Symptoms often appear or worsen with viral infections
2. Confirmed variable expiratory airflow limitation	
Documented excessive variability in lung function* (one or more of the tests below) **AND** documented airflow limitation*	The greater the variations, or the more occasions excess variation is seen, the more confident the diagnosis At least once during diagnostic process when FEV1 is low, confirm that FEV1/FVC is reduced (normally >0.75–0.80 in adults, >0.90 in children)
Positive bronchodilator (BD) reversibility test* (more likely to be positive if BD medication is withheld before test: SABA ≥4 hours, LABA ≥15 hours)	*Adults*: increase in FEV1 of >12% and >200 mL from baseline, 10–15 minutes after 200–400 mcg albuterol or equivalent (greater confidence if increase is >15% and >400 mL). *Children*: increase in FEV1 of >12% predicted
Excessive variability in twice-daily PEF over 2 weeks*	*Adults:* average daily diurnal PEF variability >10%** *Children*: average daily diurnal PEF variability >13%**
Significant increase in lung function after 4 weeks of anti-inflammatory treatment	*Adults:* increase in FEV1 by >12% and >200 mL (or PEF by >20%) from baseline after 4 weeks of treatment, outside respiratory infections
Positive exercise challenge test*	*Adults*: fall in FEV1 of >10% and >200 mL from baseline *Children*: fall in FEV1 of >12% predicted, or PEF>15%
Positive bronchial challenge test (usually only performed in adults)	Fall in FEV1 from baseline of ≥20% with standard doses of methacholine or histamine, or ≥15% with standardized hyperventilation, hypertonic saline or mannitol challenge
Excessive variation in lung function between visits* (less reliable)	*Adults*: variation in FEV1 of >12% and >200 mL between visits, outside of respiratory infections *Children*: variation in FEV1 of >12% in FEV1 or >15% in PEF between visits (may include respiratory infections)

BD: bronchodilator (short-acting SABA or rapid-acting LABA); FEV1: forced expiratory volume in 1 second; LABA: long-acting beta2-agonist; PEF: peak expiratory flow (highest of three readings); SABA: short-acting beta2-agonist.

*These tests can be repeated during symptoms or in the early morning. **Daily diurnal PEF variability is calculated from twice daily PEF as ([day's highest minus day's lowest] / mean of day's highest and lowest), and averaged over one week.

Supplemental Material 2

Table 19. Standard prick test panel for aeroallergens [104].

Allergen/control	
Histamindihydrochloride 0,1 % (positive control)	
NaCl 0.9% (negative control)	
Hazel	*Corylus avellana*
Alder	*Alnus incana*
Birch	*Betula alba*
Plane	*Platanus vulgaris*
Cypress	*Cupressus sempervirens*
Grass mix	smooth meadow grass/*Poa pratensis*, cock's foot grass/*Dactilis glomerata*, perennial rye grass/*Lolium perenne*, timothy grass/*Phleum pratense*, meadow fescue/*Festuca pratensis*, meadow oat grass/*Helictotrichon pretense*
Olive	*Olea europaea*
Mugwort	*Artemisia vulgaris*
Ragweed	*Ambrosia artemisiifolia*
Alternaria	*Alternaria alternata* (*tenuis*)
Cladosporium	*Cladosporium herbarum*
Aspergillus	*Aspergillus fumigatus*
Parietaria	*Parietaria*
Cat	
Dog	
Dermatophagoides pteronyssinus	
Dermatophagoides farinae	
Blatella	*Blatella germanica*