Asthma Polymorphic *Loci* Genotyping in Madeira Population

Identification of potential genetic markers of disease susceptibility, severity and clinical relevance

DOCTORAL THESIS

Anabela Gonçalves Berenguer
DOCTORATE IN BIOLOGICAL SCIENCES

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SUPERVISOR
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“There is no subject so old that something new cannot be said about it.”

Fyodor Dostoevsky

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Vassili "there is always some madness in love. But there is also always some reason in madness". [1]

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1Nietzche FW (2008). Assim falou Zaratustra (de Campos M, Trans.). Publicações Europa-América, Sintra. (Original work published 1883–1885).

Abbreviations

ADAM33	Disintegrin and metalloprotease domain 33
ADRB2	Beta2 adrenergic receptor
AHR	Airway hyperresponsiveness
BHR	Bronchial hyperresponsiveness
BMI	Body mass index
CAMP	Childhood Asthma Management Program
Can f 1	*Canis familiaris* allergen 1
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
DCs	Dendritic cells
Df	*Dermatophagoides farinae*
DNA	Deoxyribonucleic acid
Dpt	*Dermatophagoides pteronyssinus*
Der p 1	*Dermatophagoides pteronyssinus* group 1 allergen
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FEV_1	Forced expiratory volume in the first second
FVC	Forced vital capacity
GINA	The Global INitiative for Asthma
GSDML	Gasdermin-like
GWA	Genome-wide association
HDM	House dust mites
HWE	Hardy-Weinberg Equilibrium
IgE	Immunoglobulin E
Il4Rα	Interleukin 4 receptor, subunit alpha
IL4	Interleukin 4
IL4-RP2	IL4 Repetitive Polymorphism 2
IL5	Interleukin 5
IL13	Interleukin 13
ISAAC	International Study of Asthma and Allergies in Childhood
JNK	c-Jun-N-terminal kinase
KIDMED	Mediterranean Diet Quality Index for children and adolescents
LD	Linkage disequilibrium
LGH	Human Genetics Laboratory
mtDNA	Mitochondrial DNA
NaCl	Sodium chloride
EPR-3	Expert Panel Report 3
NCBI	National Center for Biotechnology Information
NF-kB	Nuclear factor kB
NHLBI	National Heart, Lung and Blood Institute
$N0_2$	Nitrogen dioxide
OR	Odds ratio
ORMDL3	Orosomucoid like 3
PAC	Portuguese Study of Allergic Diseases in Childhood
PCR	Polymerase chain reaction
PEF	Peak expiratory flow
rs	Reference SNP ID number
s.d.	Standard deviation
SDS	Sodium dodecyl sulfate
SNPs	Single nucleotide polymorphisms
STAT6	Signal transducer and activator of transcription 6

TDT	Transmission disequilibrium test
Th1	T helper 1 type cell
Th2	T helper 2 type cells
UPR	Unfolded-protein response
VNTR	Variable Number of Tandem Repeats
WHO	World Health Organization

Abstract

Asthma is a complex disease, influenced by both environmental and genetic factors. In this study, the analysis of multiple environmental factors assessed by questionnaire and the genotyping of SNPs *IL13-c.144 G/A, IL4-590 C/T, IL4-RP2 253183, ADRB2-c.16 A/G, ADAM33-V4 C/G, ADAM33-S1 c.710 G/A, GSDML-236 C/T* and *STAT6-21 C/T* were performed in a sample of Madeiran asthmatic patients and their families, and their association to asthma susceptibility and severity was assessed. Family, environmental, social and individual factors such as the presence of rhinitis in one of the parents, the habitation conditions, the family smoking habits, individual food habits and allergen sensitivity, were found to account for asthma severity. *IL4-590*T* and *IL4-RP2*183* alleles as well as the combined genotypes *IL4-590*CT/IL4-590*TT* and *IL4-RP2*253183/IL4-RP2*253183* were associated to both asthma susceptibility and severity. *GSDML-236*TT* was found associated only to asthma severity. Allele *ADAM33-V4*C* was significantly over-transmitted to asthmatic offspring being linked with the disease by TDT. These findings suggest that in addition to environmental influences, *IL4-590 C/T, IL4-RP2 253183, ADAM33-V4 C/G* and *GSDML-236 C/T* SNPs may constitute important genetic factors contributing to asthma susceptibility and/or severity in Madeira population.

Keywords: Asthma, Madeira, SNPs, Environment, Susceptibility, Severity

Resumo

A asma é uma doença complexa, cuja génese depende de factores ambientais e genéticos. No presente trabalho, a análise de múltiplos factores ambientais obtidos através de questionário e a análise genética dos SNPs *IL13-c.144 G/A, IL4-590 C/T, IL4-RP2 253183, ADRB2-c.16 A/G, ADAM33-V4 C/G, ADAM33-S1 c.710 G/A, GSDML-236 C/T* e *STAT6-21 C/T* foi efectuada numa amostra de asmáticos da Madeira e respectivas famílias tendo-se determinado a sua associação à susceptibilidade e severidade da asma. Factores de ordem familiar, ambiental, social e individual, tais como a presença de rinite num dos pais, as condições de habitação, hábitos tabágicos da família, hábitos alimentares e sensibilidade a alergénios foram associados ao grau de severidade da doença. Os alelos *IL4-590*T* e *IL4-RP2*183* assim como a combinação de genótipos *IL4-590*CT/IL4-590*TT* e *IL4-RP2*253183/IL4-RP2*253183* foram associados à susceptibilidade e severidade da asma. O genótipo *GSDML-236*TT* foi associado apenas ao grau de severidade da asma. O alelo *ADAM33-V4*C* foi significativamente transmitido numa maior frequência à descendência asmática, verificando-se a sua associação à doença através de TDT. Adicionalmente aos factores ambientais, os SNPs *IL4-590 C/T, IL4-RP2 253183, ADAM33-V4 C/G* e *GSDML-236 C/T* poderão constituir importantes factores genéticos, contribuindo para a susceptibilidade e/ou severidade da asma na população da Madeira.

Palavras-chave: Asma, Madeira, SNPs, Ambiente, Susceptibilidade, Severidade

List of publications

1. Berenguer AG, Câmara RA, Brehm AD, Oliveira S, Fernandes AT (2012). Distribution of polymorphisms IL4-590C/T and IL4-RP2 in the human populations of Madeira, Azores, Portugal, Cape Verde and Guinea-Bissau. Int J Mol Epidemiol Genet. 3(2): 179-83.
2. Berenguer AG, Rosa A, Brehm A. Asthma-snapshot or motion picture? Manuscript submitted for publication.
3. Berenguer AG, Câmara RA, Rosa A, Brehm A: Association of gene polymorphisms *IL13-c.144, IL4-590, IL4-RP2, ADRB2-c.16, ADAM33-V4, ADAM33-S1 c.710, GSDML-236* and *STAT6-21* with asthma in Madeira Population. Manuscript submitted for publication.

1. Introduction

In the year 750 B.C., Homer narrates in the Iliad, a warrior who died of *asthma and perspiration* in the end of a battle, this being the first known written record of the word (Marketos & Ballas 1982). The term asthma comes, in fact, originally from the Greek meaning short of breath or panting (Marketos & Ballas 1982; Holgate 2010).

Asthma is a common respiratory disorder characterized by frequent episodes of coughing, wheezing and shortness of breath (Van Eerdewegh *et al*. 2002; Holgate 2011). Its key features include airway hyperresponsiveness, excessive airway mucus production, airway inflammation and elevated serum immunoglobulin E (IgE) levels (Wills-Karp & Ewart 2004). Asthma results from the complex interaction of multiple genetic and environmental factors (Cookson 2002; Su *et al.* 2012) and affects more than 300 million people worldwide (Himes *et al*. 2009). It represents the most common chronic disease among children and despite its relatively low fatality rate compared to other chronic diseases, 255,000 people died of asthma in 2005, according to the World Health Organization- WHO (Makino & Sagara 2010).

Asthma has increased in prevalence over the past 30 years in all Westernized societies, as a possible consequence of the decline of childhood infections (Cookson & Moffatt 2000). The inverse relation between the incidence of infectious diseases and the prevalence of allergic diseases and asthma constitutes the Hygiene Hypothesis (Martinez 2001; Okada *et al.* 2010; Brooks *et al.* 2013) proposed to explain the increasing prevalence of allergic diseases in Western societies in the last decades (Vercelli 2006).

According to GINA (the Global INitiative for Asthma) the clinical asthma prevalence in the world, defined as 50% of the prevalence of "current wheezing" in 13 to 14-year-old children over a 12-month period, is highly variable, ranging from 18.4 % in Scotland to 4.8% in Portugal and 0.7% in Macau (Masoli *et al*. 2004). More recently, the World Health Survey designed by the WHO found a percentage of 7.83 for clinical asthma and 8.72 for wheezing symptoms in Portugal (To *et al.* 2012) (Figures 1 and 2).

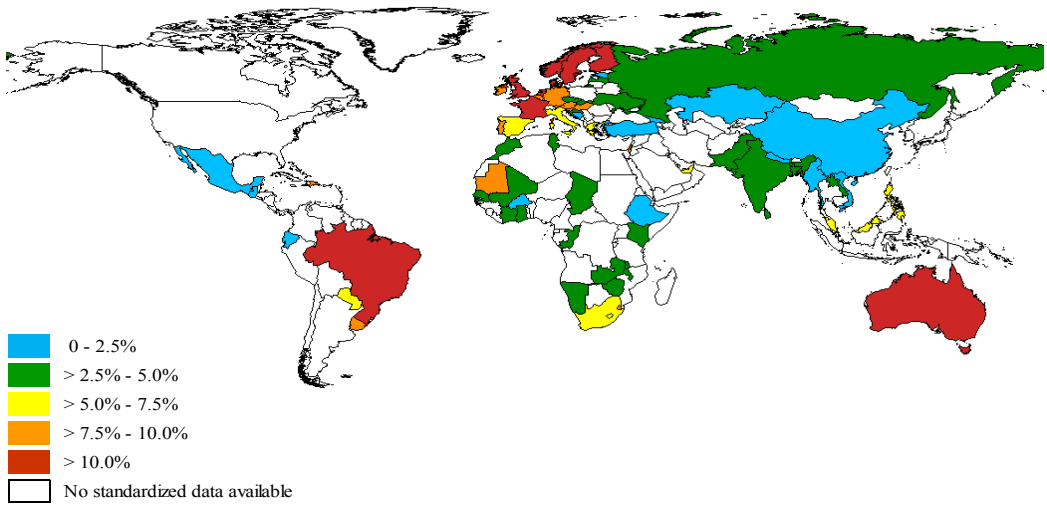


Figure 1. World map of clinical asthma prevalence (To *et al*. 2012).

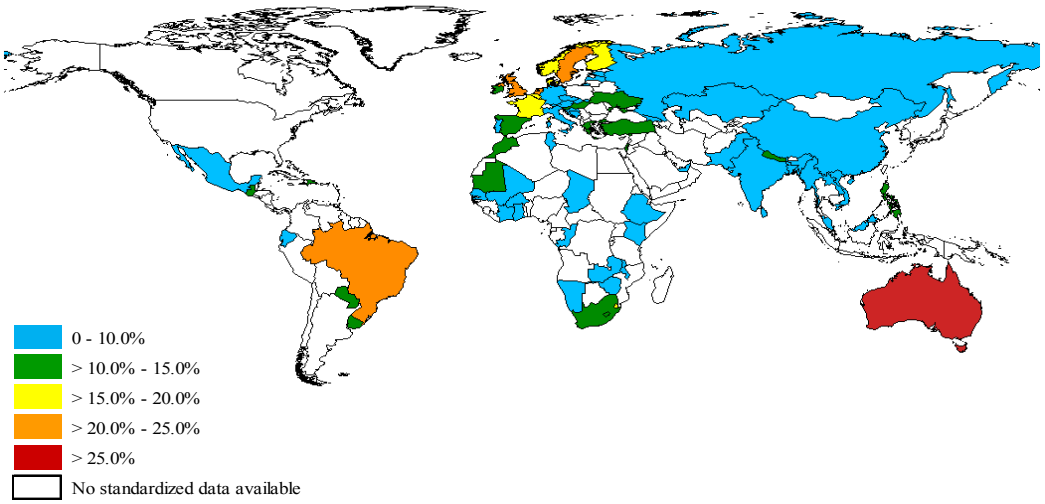


Figure 2. World map of wheezing prevalence (To *et al.* 2012).

Earlier, in 1998, the ISAAC (International Study of Asthma and Allergies in Childhood) compared asthma prevalence symptoms in two age groups (13–14 and 6–7 years) in 56 and 38 countries respectively and also found large differences between populations. The findings suggest a role for the environmental component to account for the discrepancies (Asher 1998), which represents an opportunity for prevention (Patel *et al.* 2008). Nevertheless, such dissimilarities may also be explained by geographic variation and methodological heterogeneity in defining asthma symptoms (Patel *et al.* 2008). Wheezing is considered the most important factor for identifying asthma in epidemiological studies (Lee *et al.* 1983; Masoli *et al.* 2004; Hansen *et al.* 2012). However, despite the use of standardized written questionnaires, some languages do not have an equivalent of "wheezing" as understood by English speakers (Asher 1998). Both the definition and identification of asthma by questionnaire still remains a controversial issue (Patel *et al*. 2008). However, asthma is mostly defined based on the clinician diagnosis. Under these circumstances, the clinician must determine the presence of partially reversible symptoms of airway flow obstruction and airway hyperresponsiveness, through a detailed medical history and a physical exam to the upper respiratory tract, chest, and skin (EPR-3 2007). The evaluation of airway dysfunction can either be assessed by a significant change in forced expiratory volume in the first second (FEV_1) after bronchodilator administration or with airways hyperresponsiveness (Gjevre *et al*. 2006). Airway hyperresponsiveness is measured through inhalation challenges using constrictor agonists, like histamine and methacholine (Cockcroft 2010). Also, a significant change in forced vital capacity (FVC) postbronchodilator may be used (Gjevre *et al*. 2006). According to Ukena *et al.* (2008), the demonstration of obstruction ($FEV_1/VC < 70\%$) and FEV_1 increase by >15% (at least 200 mL) with respect to the initial value is one of the criteria for asthma diagnosis.

Asthma is also a serious public health problem with economic implications. Recurrent asthma symptoms frequently cause sleeplessness, daytime fatigue, reduced activity levels and school and work absenteeism (WHO 2011). According to the NHLBI (National Heart, Lung and Blood Institute), the estimated cost for asthma has raised from 6.2 billion dollars in 1990 to 11.3 billion dollars in 1998 (Leigh *et al*. 2002). The projected

economic cost of asthma for 2010 only in the USA was 20.7 billion dollars, 15.6 of which represent direct costs, 3.1 due to morbidity and 2.0 due to mortality (NHLBI 2009). In Europe, the expected total cost of asthma for people aged 15-64 years, was estimated in 19.3 billion euros (25.7 billion dollars) (Accordini *et al.* 2013).

1.1. Asthma definition and physiopathology

The current definition of asthma proposed by GINA in 2012 describes the condition as an airways chronic inflammation in which inflammatory cells such as mast cells, eosinophils T cells, dendritic cells, macrophages and neutrophils play an active role (GINA 2012). In susceptible individuals, this inflammation is responsible for generalized yet variable airway obstruction, frequently reversible both spontaneously or after treatment, associated to bronchial hyperresponsiveness, leading to wheezing, breathlessness and coughing (GINA 2012).

During an asthma attack, the lining of the bronchial tubes swells, causing airways to narrow and reducing the airflow into and out of the lungs (WHO 2011). The contraction of the bronchial smooth muscle, know as bronchoconstriction (EPR-3 2007) constitutes the main physiological event leading to clinical symptoms (EPR-3 2007) caused by the release of inflammatory mediators such as histamine and leukotrienes from mast cells (Busse & Lemanske 2001) and occurring as a result of an increased sensitivity of the airways to a variety of stimuli, known as bronchial hyperresponsiveness (Sterk & Bel 1989; Scichilone *et al.* 2006; Kang *et al.* 2012a). As a result of permanent chronic inflammation, airway remodeling takes place (Bergeron *et al.* 2007), though it has been recognised that it may happen earlier in asthma, in some cases, before clinical symptoms (Murphy & O'Byrne 2010). It consist of a number of structural changes occurring in the asthmatics airways, including thickening of the sub-basement membrane, subepithelial fibrosis, airway smooth muscle hypertrophy and hyperplasia, increased airway vascularity and dilation and mucous gland enlargement and hypersecretion (Bergeron *et al.* 2007; EPR-3 2007; Murphy & O'Byrne 2010).

1.1.1. Classification of asthma

Asthma can be classified as extrinsic or intrinsic. Extrinsic (atopic) asthma is characterized by infiltration of the bronchial mucosa with eosinophils and T helper 2 type cells (Th2), circulating specific IgE antibodies and positive skin tests to common aeroallergens whereas intrinsic (non-atopic) asthma shows negative skin tests, no clinical or family history of allergy and furthermore, serum total IgE concentrations within the normal range and no evidence of specific IgE antibodies response against common allergens (Humbert *et al*. 1999). On the other hand, atopy can be defined as the personal or familial predisposition to overproduce IgEs as a response to allergen exposure (Barata 2007). Allergens are antigens causing allergies. They consist of low-molecular weight proteins, though some glycoprotein and even some carbohydrates might also act as allergens (Barata 2007). At the molecular level, allergens activate Th2 lymphocytes in genetically predisposed individuals, leading to the production of IgEs antibodies (via interleukin 4- IL4 and interleukin 13- IL13) and eosinophilia (via interleukin 5- IL5), leading to the allergic inflammation (Ngoc *et al*. 2005).

Asthma can be further divided into four categories, according to its severity: intermittent, mild persistent, moderate persistent and severe persistent. This clustering is determined by the frequency of asthma symptoms, the therapeutic approach and the lung function, this last shown by both the FEV_1 and the peak expiratory flow (PEF) (Nunes *et al*. 2003). Therefore, intermittent asthma is characterized by intermittent symptoms (less than once a week), nocturnal symptoms less than twice a month, normal PEF between crisis, PEF or FEV_1 over 80% from the normal predicted values and a PEF variation inferior to 20%. Mild persistent asthma includes asthma symptoms happening more than once a week, but less than once a day and nocturnal symptoms occurring more than twice a month; PEF presents changes between exacerbations and PEF or FEV_1 values depart equally over 80% from the normal predicted values while PEF varies between 20 and 30%; additionally asthma exacerbations may affect sleep and daily activity. Moderate persistent asthma comprises persistent symptoms, nocturnal symptoms more than once a week, PEF or FEV_1 between 60% and 80% higher than normal, PEF variations higher than 30%, leading to interference in sleep and daily activity. Besides, it includes the daily use of short-term inhaled beta-agonists. Finally, persistent severe asthma implies persistent symptoms every day of the week and daily or frequent nocturnal symptoms, PEF or FEV_1 60% less than normal, and PEF variations superior to 30%. Additionally it is characterized by frequent attacks and/or exacerbations, limited physical activity and daily use of beta-agonists (Nunes *et al*. 2003).

Though it has been shown that PEF is more commonly used by general practitioners to assess asthma severity, the American National Asthma Education and Prevention Program Expert Panel Report 3 (NAEPP EPR-3) from 2007 does no longer recommended PEF in the assessment of asthma severity due to poor correlation with asthma frequency symptoms, suggesting the use of FEV_1/FVC and the percentage of predicted FEV_1 (EPR-3 2007). According to these authors, the normal values for FEV_1/FVC in 8 to 10 year-old is 85%, more 5% than in the age group between 20 and 39 years old (80%), 75% for individuals aged between 40-59 years old and finally 70% for 60 to 80 year-old (EPR-3 2007). For the age group over 12 years-old, the predicted FEV_1 for both intermittent and mild asthma is higher than 80%, with normal FEV_1/FVC ratio, while for moderate and severe asthma FEV_1 varies between 60-80% and less than 60%, respectively, and FEV_1/FVC are reduced in 5%, in both cases (EPR-3 2007). However, a study in a group of German children found FEV_1 and FVC reference values to be 101±14.9% and 95.4±13.6%, respectively (Kalhoff *et al*. 2009). Lung function in children recruited from the NHLBI Severe Asthma Research Program (SARP) revealed FEV_1 and FVC percentages of 102 ± 15% and 105 ± 18%, respectively, for mild to moderate asthma and significantly lower values for severe asthma (FEV_1 and FVC of 78 ± 20% and 96 ± 19%, p-values 0.001 and 0.168, correspondingly); for FEV_1/FVC a value of 97 ± 9% *versus* 82 ± 13 $p < 0.001$ was found between mild to moderate and severe asthma (Fitzpatrick *et al*. 2011). However, severity, the intrinsic intensity of the disease process, must have into account the frequency and intensity of symptoms and the likelihood of either asthma exacerbations, or risk of adverse effects from medication (EPR-3 2007). Recently, a new approach to the definition of asthma severity, based on the level of current clinical control and risks has been proposed; however given the heterogeneity of factors influencing the disease, global comparisons are still limited (Bush & Zar 2011).

1.2. Risk factors

Many cross-sectional studies have confirmed increases in the incidence and prevalence of asthma over the past 2 to 3 decades, but much remains unknown as to the fundamental immunologic, genetic and environmental mechanisms underlying the development of this condition and its increased expression, especially in the developed world (Subbarao *et al.* 2009). However, some risk factors, such as sex, ethnicity, allergen exposure and genetics, have been consistently identified as contributors to the disease etiology (Subbarao *et al.* 2009).

1.2.1. Gender

The incidence of asthma has a strong sex bias (Melgert and Postma 2009). There are evidences pointing to a higher prevalence of asthma among boys in childhood and a higher occurrence of new cases among girls around and following puberty (Ober *et al*. 2008). This appears to be related to a more reduced caliber of the male children's airways and lung volume that progressively recedes towards adolescence (Gaspar & Almeida 2003). As for girls and adult women, increased levels of sex hormones by puberty are thought to account for a higher prevalence of the disease (Melgert & Postma 2009). Even at the intrauterine environment, the sex of the fetus may affect the course of asthma during pregnancy. Pregnant asthmatic women with girls are more likely to have increased symptoms of asthma during pregnancy than those pregnant with boys (Beecroft *et al*. 1998).

Sex differences in asthma may also result from differences in use of healthcare as both men and women respond differently to their disease (Sundberg *et al*. 2009). However, because some polymorphisms are particularly related to asthma in females, genetic studies should be stratified by sex (Postma 2007).

1.2.2. Ethnicity

Significant differences regarding asthma incidence and prevalence amongst different ethnic groups have been reported in the literature. Asthma morbidity and mortality is excessively high and continues to increase among African Americans and low-income-housing residents (Barnes 2006). However, in the Bronx, New York, Hispanic children (mostly Puerto Rican ancestry) showed a prevalence of 10% of asthma over a 12-month period, compared to 6.9% among African-American and 7.6% among Caucasians (Lara *et al*. 1999).

A review on ethnic variation in asthma frequency compared three ethnic groups living in UK, namely South Asian, African and Caucasian children, and revealed that both wheeze frequency and clinician-diagnosed asthma were less common in South Asian children compared to the other groups; however, African children showed significantly greater rate of clinician-diagnosed asthma in previous 12 months than Caucasian children (Netuveli *et al*. 2005).

More recently, a study in a multicultural birth cohort in Netherlands, by Gabriele *et al*. (2012) found Antilleans were more likely to have doctor-diagnosed asthma at 12 months and wheezing at 24 months compared to Dutch; also, Turkish infants had an

increased risk of lower respiratory symptoms at 12 and 24 months compared to their Dutch peers (OR 1.14, 95 % CI 1.02–1.27 and 1.21, 95 % CI 1.07–1.38, respectively). However, no differences were found for Cape Verdean, Moroccan or Surinamese, when compared to Dutch infants (Gabriele *et al*. 2012).

Although ethnic differences in disease incidence and prevalence have often been dismissed as a mix of environmental, social, cultural, or economic factors in cause, genetic factors cannot be ignored (Barnes 2006; Forno & Celedón 2009).

1.2.3. Allergen exposure

Amongst the environmental variables associated to the increased prevalence of asthma, the allergenic sensitization is pointed as the most frequent risk factor (Gaspar & Almeida 2003; Baxi & Phipatanakul 2010). Exposure to indoor allergens, such as house dust mites (HDM), pets and cockroaches are among the most common factors of sensitization and respiratory allergy everywhere in the world (Mascia *et al*. 2002; Perfetti *et al*. 2004; Gent *et al.* 2012). HDM Dpt species is responsible for the Der p 1 (*Dermatophagoides pteronyssinus* group 1 allergen) protein synthesis, which is, from an epidemiological perspective, one of the top candidates for an allergy vaccine (Wolfowicz *et al*. 2003; Chen *et al.* 2012). This is particularly pertinent in Westernized countries where people spend most of their time indoors (Perfetti *et al*. 2004), exposed to increasing allergen levels due to sedentary habits in warmer houses, with a wide-range of furniture and deficient ventilation (Platts-Mills *et al*. 1997; Hägerhed-Engman *et al.* 2009). Once sensitized, the repeated allergen exposure will lead to disease persistence (Holgate *et al*. 2010).

On the other hand, over the past decade, there have been many studies demonstrating a lower prevalence of asthma and allergies in children brought up in a farming environment (Alfvén *et al*. 2006; Wong & Chow 2008; von Mutius & Vercelli 2010). Studies in an experimental model of ovalbumin-sensitized mice revealed that inhalation of stable dust extract was also found to suppress the development of airway hyperresponsiveness and airway eosinophilia (Wong & Chow 2008). The understanding of how genes may interact with specific environmental factors and the identification of the genetic determinants is important for the future development of primary prevention in asthma (Wong & Chow 2008).

1.2.4. Genetics

Twin studies support a strong genetic component for asthma (especially childhood asthma), with heritability -the proportion of phenotypic variation in a population that is attributable to genetic variation- estimates suggesting that 48–79% of the disease risk is attributable to genetic susceptibility (Pinto *et al*. 2008).

On the other hand, monozygotic twins studies showed relatively modest concordance rates (the probability that one sibling is affected, given that the other sibling is affected (Visscher 2002) between 14.7–19%, suggesting that environmental factors play an important role in phenotypic expression of the disease (Mrazek *et al*. 1999). More than one hundred susceptibility genes have been tested for association to asthma (Pinto *et al*. 2008; Zhang *et al*. 2008). Different approaches have been used to outstand these genes:

positional cloning and candidate gene and, most recently, genome-wide association (GWA) studies (Koppelman *et al*. 2008). Seven asthma genes have been discovered through positional cloning, the first of which *ADAM33*- a disintegrin and metalloprotease domain 33 (Koppelman *et al*. 2008); *IL4*, *IL13* and *ADRB2*- beta-2-adrenergic receptor- are amongst those found through candidate gene studies (Hoffjan & Ober 2002). However, replication studies have been inconsistent across different populations (DeMeo *et al*. 2002; Sakagami *et al*. 2007; Rad *et al*. 2010; Isaza *et al*. 2012). Variants regulating orosomucoid like 3 (*ORMDL3)*- member of a family of genes responsible for encoding transmembrane proteins of the endoplasmic reticulum (ER)-expression were first found to be determinants of susceptibility to childhood asthma, through a GWA study (Moffatt *et al.* 2007). So far, association of polymorphisms within this gene or neighboring genes regulating its expression with asthma, have been successfully replicated in a number of studies on different populations (Tavendale *et al*. 2008; Bisgaard *et al*. 2009; Yu *et al*. 2011; Kang *et al*. 2012b; Wan *et al*. 2012).

Most of the genetic variability in the human genome comprises changes in DNA (deoxyribonucleic acid) single nucleotide bases, with approximately 10 to 15 million of these variants being found in more than 1% of the studied populations. These are therefore referred to as single nucleotide polymorphisms – SNPs (Steinke *et al*. 2008). Their very low mutation rate together with their compatibility with high-throughput analysis has made them the genetic markers of choice for the study of many Mendelian disorders and complex traits (Hsu *et al*. 2001). Most known SNPs are assigned with a RefSNP rs- accession ID number, for standardized nomenclature, and defined by their genomic position and genotype (Vercelli 2008).

Positional cloning, biologically plausible candidate gene approaches and GWA studies have been performed over the past 20 years to search for the genetic background of asthma and have led to the discovery of several asthma genes and related phenotypes (Lee *et al*. 2011).

Positional cloning and candidate gene association studies have been two of the approaches for the identification of genetic SNPs for complex diseases. Positional cloning begins with linkage association studies, identifying chromosomal regions that are transmitted within families along with the disease phenotype of interest, and further narrows down the candidate region, until the gene and mutations are found. On the other hand, association studies analyse candidate genes, known to be enrolled in the disease mechanism, by testing the association of particular SNP alleles to one or more phenotypic traits (Palmer & Cookson 2001). GWA studies, in which a dense set of SNPs across the genome is genotyped to survey the most common genetic variation for association with a disease in hundreds or thousands of persons (Wang *et al.* 2005; Manolio 2010) have been responsible for identifying a large number of robust associations between specific chromosomal *loci* and complex human disease (Hardy & Singleton 2009; Cookson & Moffatt 2011). The Exome Sequencing is a recent method that is being widely adopted and consists of reducing a genomic DNA sample to the protein-coding regions of the genome (exons), followed by very high-throughput sequencing of the exon-enriched sample (Singleton 2011). In complex diseases, exome sequencing is used to identify common exonic risk alleles and also rare risk alleles and is

quicker and cheaper than traditional sequencing approaches (Singleton 2011), justifying its application to complex disorders such as asthma (Cookson & Moffatt 2011).

The identification of new asthma susceptibility genes suggests that many genes with small effects rather than just a minority with strong effects contribute to the disease susceptibility (von Mutius 2009). Many of these genes are illustrated in Figure 3 and cluster into four main functional categories: i) innate immunity and immunoregulation, including genes encoding pattern recognition receptors such as the cluster of differentiation 14 (*CD14*), toll-like receptor 2, 4, 6 and 10 (*TLR2*, *TLR4*, *TLR6* and *TLR10*), intracellular receptors such as nucleotide-binding oligomerization domain containing 1 and 2 (*NOD1*, and *NOD2*), immunoregulatory cytokines *IL10* (interleukin 10) and transforming growth factor-β1 (*TGFB1*), the transcription factor signal transducer and activator of transcription 3 (*STAT3*), *HLA* (Human Leucocyte Antigen) class II, specifically, *HLA-DR*, *-DQ* and *-DP* alleles and variants of the prostaglandin E_2 receptor (PTGER2); ii) Th2-cell differentiation and effector functions, like, for example, *GATA*-binding protein 3 (*GATA3*), *IL4*, interleukin 4 receptor, alpha (*Il4Rα*), signal transducer and activator of transcription 6 (*STAT6*), interleukin 12B (*IL12B*) and *IL13*; iii) epithelial biology and mucosal immunity, such as chemokine C-C motif ligand 5, 11, 24 and 26, (*CCL5*, *CCL11*, *CCL24, CCL26* –the last three known as eotaxins 1–3-, secreted by the epithelium) and factors involved in maintaining the integrity of the epithelial-cell barrier- serine protease inhibitor, Kazal-type, 5 (*SPINK5*) and filaggrin (*FLG*); and iv) lung function, airway remodeling and disease severity, including *ADRB2*, tumour necrosis factor (*TNF*) and *ADAM33* (Vercelli 2008). A wide number of genetic variants in these locations are believed to contribute to the disease pathophysiology and furthermore, to synergize with others to amplify the impact on disease risk (Figure 3) (Vercelli 2008).

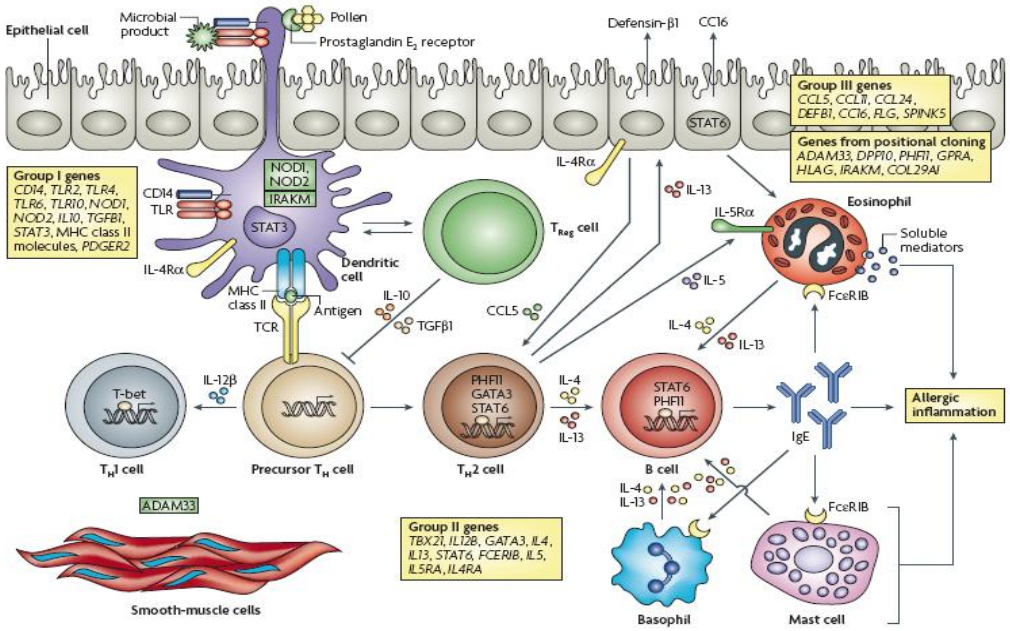


Figure 3. Susceptibility genes for asthma and asthma-related traits (Vercelli 2008).

T-cell activation is initiated by the engagement of the T-cell receptor (TCR) on the surface of T-cell precursors with a complex on the dendritic cell (DC) surface, which is composed of allergenic peptides bound to the major histocompatibility complex II (MHCII) molecule (Wills-Karp & Ewart 2004). The Th2-cell pathway is strongly linked to asthma (Le Souëf *et al*. 2006), playing an important role in promoting the bronchial inflammation observed in the disease (Moller *et al*. 2007). Their CD4+ (cluster of differentiation 4) Th2-cells subtype preferentially produce cytokines such as IL4, IL5, interleukin 9 (IL9- not represented in Figure 3) and IL13, crucial for the promotion of an IgE-based response (Levine & Wenzel 2010). This mechanism is opposite to that of T helper 1 type cells (Th1), preferentially promoters of cellular immune responses, with the activation of cytolytic T cells and the killing of intracellular pathogens by macrophages (Renauld 2001). The joint action of both IL4 and IL13 pleiotropic interleukins leads to B-cell proliferation and their differentiation towards IgE production (Kruse *et al*. 2002), with the subsequent activation of eosinophils, basophils and mast cells and the release of soluble mediators that trigger the allergic inflammation process (Figure 3).

Cytokines are humoral immunomodulatory signalling proteins or glycoproteins, which control or modulate the activities of target cells, generally those within the haematopoietic system (Bidwell *et al*. 1999). Interleukins are a subgroup of cytokines produced by pleiotropic leukocytes and which may exhibit redundant characteristics since different cytokines can have the same function (Cardoso 2007). In response to allergens, T-cells produce a restricted array of cytokines. In particular, the pro-inflammatory cytokines are synthesized by the Th2- cells (Malerba & Pignatti 2005).

Despite the heterogeneity of asthma, clinical and genetic studies imply that both IL4 and IL13 interleukins are central to the pathogenesis of atopic asthma (Tomkinson *et al*. 2010). Clinical studies with the soluble recombinant human IL4 receptor (rhuIL4R) proved to be promising, safe and effective in the treatment of patients with asthma (Steinke & Borish 2001). Pitrakinra, a recombinant protein derived from human IL4 that binds to IL4Rα and acts as a competitive antagonist of IL4 and IL13 has also provided evidences as a potential therapeutic agent for asthma (Tomkinson *et al*. 2010).

Therefore, polymorphisms in cytokine genes might determine the efficacy of medications and the progress of the disease and degree of asthma control. Increasing knowledge on cytokine genetics might help managing asthma (Daneshmandi *et al*. 2012).

The IL13 is typically produced by Th2 cells and acts as a central mediator in allergic inflammation (Vercelli 2008). Together with IL4 it is responsible for inducing IgEs synthesis. *IL13* gene maps at 5q31, and is regarded as a strong candidate for asthma and allergy since IL13 cytokine and its receptors are highly expressed in the respiratory tract of asthma patients (Vercelli 2008). Furthermore, this cytokine is sufficient to induce the main features of Th2 responses in animal models of experimental asthma in such as airway hyperresponsiveness and mucus hypersecretion (Vladich *et al*. 2005; Brightling *et al*. 2010). For the *IL13-c.144G/A* (Gln/Arg, rs20541) variant it is accepted that *IL13-c.144*A* allele is associated with increased levels of IgEs (p-value=2 x 10^{-6}, Graves *et al*. 2000; Arima *et al*. 2002; He *et al*. 2003; Heinzmann *et al*. 2003). In addition,

functional studies indicate that the same allele is significantly more biologically active than the wildtype *IL13-c.144*G*, therefore enhancing the allergic mechanisms in asthma (Chen *et al*. 2004; Vladich *et al.* 2005; Chu *et al.* 2012). *IL13-c.144*A* allele has been associated with asthma in both British and Japanese populations (p-value=0.003, odds ratio (OR) 2.14, 95% confidence interval (CI) 1.28-3.60 and p-value=0.013, OR 1.81, 95%CI 1.11-2.93, respectively (Heinzmann *et al*. 2000).

The IL4 cytokine is involved in several interactions of the immune system, namely it induces naive T cells (Th0) to assume the Th2 cell phenotype, represses Th1 cells inducing signals and stimulates lymphocyte B to secrete IgEs (Rockman *et al*. 2003). The *IL4* gene also mapped on chromosome 5q31 has been consistently linked to asthma and related phenotypes in linkage association and candidate gene association studies (Rosenwasser *et al*. 1995; Noguchi *et al*. 1997; Noguchi *et al*. 2001; Kabesch *et al.* 2003; Donfack *et al*. 2005). Its promoter region harbours the SNP *IL4-590 C/T* (rs 2243250), whose T allele was found associated to asthma risk in Chinese (Wang *et al*. 2004), Russian (Gervaziev *et al*. 2006), a German group of children (Kabesch *et al*. 2006), Taiwanese (Chiang *et al*. 2007) and more recently in Filipinos (de Guia & Ramos 2010). A meta-analysis by Li *et al.* (2008), determined an overall OR of 0.86, 95% CI 0.78-0.94, p-value=0.002), therefore suggesting a strong evidence of association of increased asthma risk and T allele, relatively to C allele. Furthermore, *in vitro* studies show that the *IL4-590*T* allele allows an extra binding site to the nuclear factor for activated T cells (NFAT), which favours the *IL4* transcription and expression by 3 fold (Rockman *et al*. 2003). The Variable Number of Tandem Repeats (VNTR) *IL4-RP2* (*IL4* Repetitive Polymorphism 2) consists of tandem repeats of a 70 bp motif located in the second intron of *IL4* gene. Two different alleles have been described for *IL4-RP2*, namely the ancestral 253bp allele (three repeats of the 70pb motif) and the derived 183bp allele (two repeats of the 70bp motive, by deletion, RP2del; Mout *et al*. 1991). Interestingly, *IL4-590*T* and *IL4-RP2*183* haplotypes were found to be significantly more frequently transmitted to asthmatic Japanese children (χ^2 p-value=0.002, Noguchi *et al*. 2001).

The *ADRB2* is an intronless gene also located at chromosome 5q31-32 (Drysdale *et al*. 2000), as mentioned, a region consistently linked to asthma and related phenotypes (Ortega 2007). The protein belongs to the G protein-coupled receptor (GPCR) family and is highly expressed on the bronchial smooth muscle (Taylor 2007). Hence the administration of beta-2 agonist medication for asthma results in bronchial relaxation and dilation (Liggett 1997; Lucas *et al*. 2004; Taylor 2007). Thirteen SNPs have been identified within *ADRB2* gene, three of which alter protein function at nucleotide protein (np) 46, 79 and 491 corresponding to residues 16, 27 and 164 of the protein (Drysdale *et al*. 2000; Hawkins *et al*. 2006). The extracellular NH_2 terminal domain of *ADRB2* contains the non-synonymous polymorphism *ADRB2-c.16A/G* (Arg/Gly, rs 1042713) (Turki *et al*. 1995; Lucas 2004). The *ADRB2-c.16*G* allele has been associated to nocturnal asthma (p-value=0.007, OR 3.8) (Turki *et al*. 1995; Fukui *et al*. 2006), asthma in a Chinese population (p-value<0.05, OR 2.918, 95% CI 1.256-6.781) (Gao *et al*. 2002) and asthma severity (Turner *et al.* 2004). A meta-analysis by Contopoulos-Ioannidis *et al*. (2005) demonstrated an overall increased risk for nocturnal asthma (p-value<0.001, OR 2.20), asthma susceptibility (p-value=0.025, OR 1.19) and severe forms of asthma (p-value=0.028, OR 1.42), for *ADRB2-c.16*G* allele. On the other hand, the study of Palmer *et*

al. (2006) on a Scottish population suggested that *ADRB2-c.16*A* allele predisposes to asthma exacerbation in children and young adults regularly treated with salmeterol (p-value=0.022, OR 3.40, 95% CI 1.19 - 9.40).

The *ADAM33* gene, located at chromosome 20p13, was the first asthma susceptibility gene to be identified by positional cloning (Van Eerdewegh *et al*. 2002). ADAM33 is expressed in airway smooth muscle cells and lung fibroblasts (Umland *et al*. 2003; Schedel *et al*. 2006; Holloway *et al.* 2010), which plays an important role in airway remodeling and bronchial hyperresponsiveness (BHR) (Lee *et al*. 2004; Yang *et al.* 2012). Considering that other ADAM proteins such as ADAM 10 and 17 appear to interact with inflammatory cytokines, it is possible that ADAM33 may also stimulate cytokine network (Raby *et al*. 2004). About 55 SNPs have been identified within *ADAM33* (Van Eerdewegh *et al*. 2002) though only a few were significantly linked to asthma and/or associated phenotypes: in a UK population, six SNPs of *ADAM33* including the exonic *S1* and *S2* were significant at p-value<0.03, the intronic ST+4 at p-value=0.02 and haplotypes composed by *S2* and *ST+4* greatly increased the level of significance (p-value=$3x10^{-6}$–$5x10^{-4}$) (Holgate *et al*. 2003). Results are however rather diverse, possibly due to population's heterogeneity and/or differences in asthma definition in the studied populations, such as proposed by Schedel *et al*. (2006). The C allele of the polymorphism *ADAM33-V4 C/G* (3'UTR, rs2787094) has been initially associated to asthma in a UK and US/UK dataset (p-value=0.03) (Van Eerdewegh *et al*. 2002) and later in a Dutch Caucasian population (p-value=0.0009) (Howard *et al*. 2003). These same authors found an association with atopy in African Americans (p-value=0.017). According to Schedel *et al*. (2006), the carriers of the 3'UTR *ADAM33-V4*G* allele were found to be more prone to the risk of developing non-atopic asthma, in a German population set (p-value=0.031, OR 1.44, 95%CI 1.03–2.01). Similarly, allele *ADAM33-V4*G* was found to have a significantly higher frequency among a group of asthmatic Chinese children, compared to the control group (p-value<0.05, OR 2.187, 95%CI 1.768-2.705) (Qu *et al.* 2011). The nonsynonymous polymorphism *ADAM33-S1 c.710 G/A* (Val/Ile, rs3918396; Howard *et al*. 2003) is located at the 19th exon. The *ADAM33-S1c.710*G* allele has been significantly associated to asthma in UK and US/UK combined population sets (p-value<0.03) (Van Eerdewegh *et al*. 2002), as well as to atopy in a Dutch Caucasian population (p-value=0.037) (Howard *et al*. 2003). However, the risk of developing non-atopic asthma was increased in carriers of the *ADAM33-S1 c.710*A* allele in a German population (p-value=0.042, OR 1.53, 95%CI 1.01–2.31) (Schedel *et al*. 2006).

GSDML (gasdermin-like) is located at chromosome 17q21 and encodes the protein gasdermin B, from gasdermins family, implicated in epithelial barrier function and skin differentiation (Tavendale *et al*. 2008). The *GSDML-236 C/T* (rs7216389) SNP is located within the first intron of GSDML and *GSDML*T* is considered as a risk allele given it is significantly enriched in asthma patients (p-value<0.05, OR 1.45, 95%CI 1.17-1.81), according to Moffatt *et al*. (2007), p-value<0.05, OR 1.50, 95% CI 1.24–1.81, according to Tavendale *et al*. (2008) and p-value=0.01, OR 1.88, 95%CI 1.15–3.07, as reported by Bisgaard *et al*. (2009). Interestingly, this derived allele is also strongly associated with the expression of the neighbouring gene *ORMDL3*. By its turn, ORMDL3 is abundantly expressed in the lymphocytes, which may reflect that these genes, individually or

epistatically related, play a role in the immunologic determinants of asthma (Moffatt *et al*. 2007; Tavendale *et al*. 2008).

STAT6 gene is located at 12q13–24 and is a critical transcription factor involved in the Th2 response mediated by IL4 and IL13 cytokines (Kaplan *et al*. 1999; Gao *et al*. 2000a; Xia *et al*. 2003; Nofziger *et al.* 2011). Therefore, an interaction between polymorphisms in this metabolic pathway is essential for IgE production and asthma development (Kabesch *et al*. 2006). Several studies restricted to this chromosomal region have provided evidence for linkage to asthma-related phenotypes (Weidinger *et al*. 2004). Furthermore, mice lacking *STAT6* gene were found to be protected against allergic pulmonary manifestations (Gao *et al*. 2004). *STAT6-21 C/T* (rs324011) (Pykäläinen *et al*. 2005) is located in the second intron of *STAT6*, within a nuclear factor *k*B (NF-*k*B) transcription factor binding site, and therefore a functional role of this polymorphism is anticipated (Schedel *et al*. 2004). In fact, *STAT6-21*T* allele has been found to significantly increase *STAT6* promoter activity in vitro (p-value<$1x10^{-5}$) and gene expression of STAT6 splice variants *ex vivo* (p-value< 0.01), compared to wild type C allele (Schedel *et al*. 2009). Furthermore, the presence of *STAT6-21*T* allele has been associated to elevated levels of IgE (p-value=0.015) (Weidinger *et al*. 2004; Schedel *et al*. 2009).

1.3. Asthma in Madeira Archipelago

According to the Portuguese Study of Allergic Diseases in Childhood (PAC study), Madeira shows the highest active asthma prevalence in Portugal (14.6%), with atopy levels rising up to 54% (Pinto & Almeida 2005).

The Madeira Archipelago, located in the Northeast Atlantic (32ºN, 17ºW), was discovered and settled by the Portuguese in the first half of the 15th century, playing an important role in the complex Atlantic trade network (Gonçalves *et al*. 2005). Despite of the great Caucasian influence, both mitochondrial DNA (mtDNA) and Y-chromosome studies show an important sub-saharan and northern African influence in the population's genetic background as sub-Saharan mtDNA haplogroups L1-L3 were found to constitute about 13% of the Madeiran lineages, while Y-chromosome haplogroup E3b2, comprising about 75% of the Y-lineages in North Africa, is present in 5-6% in Madeira population and Y-haplogroup E3b1-M78 shows an increased frequency of 4.6% (Brehm *et al*. 2003; Gonçalves *et al*. 2005). In spite of the initial ethnic diversity, as in any other Island, inbreeding levels are high (Câmara & Marques 2003). In fact, inbreeding might be a positive predictor for complex diseases such as asthma (Rudan *et al*. 2003).

Air pollution has long been considered as a risk factor for the expression of allergic diseases (Almeida *et al*. 2002). In 2002, Madeira (Funchal) was found to have the highest outdoor concentration of nitrogen dioxide ($N0_2$) -22.5 $\mu g/m^3$- compared to the mean rate level found in the three studied Portuguese centers, namely Lisbon, Portimão and Funchal (17.5 $\mu g/m^3$; Almeida *et al*. 2002). The PAC study also found high levels of cigarette smoke exposure in 6-10 year-old children's homes and house dust mites sensitization as the most common amongst Madeiran asthmatic children (80%), closely

followed by cockroaches (*Periplaneta americana*) in 41% of the cases (Câmara & Marques 2003).

In Madeira the death rate from asthma, per 100,000 inhabitants, registered in 2003 was 1.23 but has varied from 4.80 in 1994 to 9.36 in 1996, in average being globally higher than the national rate (Gaspar *et al.* 2006). Furthermore, a higher degree of asthma severity has been associated with increased mortality risk (Omachi *et al.* 2008). Despite the fact that most asthma lies between mild and moderate, severe asthmatics are an important subgroup, since they suffer a stronger impact on their quality of life, and are responsible for more than 50% of the total disease-related costs (Gaspar *et al.* 2006). The clinical status and the severity level of the disease are fundamental features important to asthma control being the main factors influencing diagnosis and treatment (Ferrante & La Grutta 2012).

2. Aims

Globally, the present work aims to explore both the environmental and genetic factors leading to the risk of asthma in Madeira population, contributing to a better understanding of its pathogenesis and development and, hopefully, allowing, in the future, an early intervention in terms of primary prevention for at risk children.

Specifically, one of the purposes of the study is to identify possible environmental factors that may contribute, isolated or synergistically to the disease severity as well as to analyse the role of the family in the prediction of the disease and disease related phenotypes.

A second objective is to establish, for the first time, for Madeira population, a quality DNA biobank that can be a reference to further methodical studies on asthma genetics, with the purpose of allowing a continuous understanding of the genetic bases of asthma.

Finally, and most importantly, this work aims to identify the contribution of each studied polymorphism in asthma susceptibility and severity in Madeira population as well as to identify a predictor genetic profile for asthma patients. The determination of a risk profile could be used, in the future, as an important auxiliary in medical diagnosis, allowing a personalized intervention and consequently a targeted therapy with expected improved results.

3. Material and Methods

3.1. Selection and sampling of asthma patients and families

One hundred and one (n=101) children adolescents and adults, aged between 6 and 25 years old (mean age 13.5 ± 4.3; sex ratio M/F=58.8/41.2%) and followed in the Immunoallergology consultation at Dr. Nélio Mendonça Hospital for well-established asthma (referred to as Patients), were recruited together with one of their siblings (n=86, referred to as siblings) and parents (fathers n=88, mothers n=95, referred to as Mother and Father, respectively). The study and procedures were approved by Hospital's Ethics Committee. All participants also gave their written informed consent.

A comprehensive questionnaire adapted from ISAAC was administered by a nurse or doctor to all patients to assess their asthma symptoms (Questionnaire B, see Appendix). In addition, the asthmatics underwent skin prick testing for a number of common aeroallergens. A positive skin reaction was defined as a wheal size ≥ 3 mm after subtraction of the negative control, as defined by the standardized ISAAC Phase II protocol (Weiland *et al*. 1999). Asthma patients were divided into different groups according to the disease severity: intermittent (n=23), mild persistent (n=43), moderate persistent (n=26) and severe persistent (n=4) asthma, according to the clinician diagnosis. No data regarding asthma severity was available for 5 patients and, as a consequence, these samples were not used in the analysis by severity categories.

Approximately 7 ml of blood were collected from each asthma patient by venipuncture in 8ml ethylenediaminetetraacetic acid (EDTA) blood collection tubes by qualified professionals while saliva samples from siblings and both parents (father and mother) were collected with the Oragene Kit ™/Saliva, according to the manufacturer's instructions (DNA Genotek Inc.).

3.2. Selection and sampling of a Madeira population reference sample set

Blood samples from the Blood Sample Bank of the Human Genetics Laboratory (LGH) were used as a reference population dataset. These samples were collected for the purpose of population genetics and have therefore been explored in multiple studies (Fernandes *et al.* 2002, Brehm *et al*. 2003, Gonçalves *et al*. 2005, Spínola *et al*. 2009, Berenguer *et al*. 2012). They constitute a random representative subset of the healthy male population of the Archipelago, whose ancestors for the last three generations were reported to be locals (to exclude individuals with recent history of immigration).

3.3. DNA extraction by salting-out method

The salting-out method consists of a rapid, safe and inexpensive method for DNA extraction, based on dehydration and precipitation of cellular proteins with a saturated sodium chloride (NaCl) solution (Miller *et al*. 1988).

After separating the plasma components, 500 μl of collected anticoagulated whole blood from asthmatics, were ressuspended in 1 ml of a Red Cell Lysis Buffer (0.5M Tris-HCl, 25mM $MgCl_2$, 1M Sacharose and Triton X100, pH 7.5), in a 1.5 ml eppendorf tube. The mix was vortexed and centrifuged at 13,000 rpm for 2 minutes. The supernatant was discharged and centrifugation was repeated until a clear supernatant was obtained. After eliminating the final supernatant, the sample was washed with 1 ml of distilled water. Both cell and nuclear membranes were digested by incubation at 55º C for 10 minutes in 244 μl of distilled water, 80 μl of proteinase buffer, 40 μl of sodium dodecyl sulfate (SDS) 10% and 8 μl of proteinase K (50 mg/ml), with occasional vortexing. About 120 μl of NaCl 6M saturated solution were added and the sample was then vigorously shaken and placed put on ice for 5 minutes. This last step aims to dehydrated and precipitated cellular proteins. Following centrifugation at 13,000 rpm for other 5 minutes, the DNA-containing supernatant was transferred into a new 1.5 ml tube. An equal volume of pure ethanol at -20ºC was added and gently mixed by inversion until DNA precipitation. After discarding the ethanol, DNA was ressuspended in 200 μl of distilled water and placed at -20ºC for long-term storage (adapted from Miller *et al*. 1988).

3.4. DNA extraction by Oragene Kit™/Saliva

The protocol for DNA extraction from saliva samples followed the recommendation from the Oragene Kit™/Saliva manufacturer (DNA Genotek Inc.). In brief, after gentle mixing by inversion, a volume of 500 μl of Oragene/Saliva sample was taken from the OGR-250 vial to a 1.5 ml eppendorf tube. Samples were then incubated at 50ºC for one hour, prior to the addition of 20 μl of Oragene Purifier (OG-L2P). The mixes were vortexed, incubated on ice for 10 minutes and later centrifuged at room temperature for 5 minutes at 13,000 rpm. The supernatant was then transferred into a new 1.5ml tube and the pellet was discarded. An equal volume of room temperature 100% ethanol was added to the volume of supernatant and gently mixed by inversion, about 10 times. The samples were then left standing at room temperature for 10 minutes to allow full DNA precipitation. The tube was later placed in the microcentrifuge in a known orientation and centrifuged at room temperature for 2 minutes at 13 000 rpm. The supernatant was carefully removed with a pipet and discarded, to avoid disturbing the DNA pellet. A volume of 100 μl of TE buffer (10mM Tris-HCL and 1 mM EDTA, pH 8.0) was added to dissolve the DNA pellet and the sample was vortexed for a few seconds. Samples were stored at -20ºC for posterior use.

3.5. DNA extraction by Phenol-Chloroform method

A volume of 250 μl of Hillis solution (NaCl 0.1M; Tris-HCL 0.05M, pH 7.5; EDTA 0.001M), 30 μl of SDS 10% and 15 μl of proteinase K (20 mg/ml) were placed in a 1.5 ml tube, to which 100 μl of the stored blood sample from Madeira population reference set were added. An extra volume of 250 μl of Hillis solution was added and the tubes were incubated at 65º C for three hours. Posteriorly, in the fume hood, tubes were placed on ice and 500 μl of phenol was added, followed by a quick vortexing. Next, the samples were centrifuged at 7,000 rpm for 5 minutes and the supernatant was transferred to a

new 1.5ml eppendorf tube. Volume of 250 μl of phenol and 250 μl of chloroform were added and again the mixes underwent a 5 minutes centrifugation at 7,000 rpm. DNA-enriched supernatant was withdrawn to new 1.5ml eppendorf tubes and 500 μl of chloroform were added, followed by centrifugation and transfer into a new tube. Subsequently, 50 μl of NaAc 3M and 1 ml of absolute ethanol (at -20ºC) were added to each tube and the samples were gently mixed to allow DNA precipitation. Tubes were placed on ice for 20 minutes, followed by centrifugation at 7,000 rpm for 5 minutes. Finally, the ethanol was discarded and 150 μl of distilled water was added. The samples were then stored at -20ºC for posterior use (adapted from Sambrook *et al*. 1989).

The DNA integrity was assessed by a standard 1% agarose gel electrophoresis.

3.6. Genotyping of selected polymorphisms

A panel of eight polymorphisms in candidate genes in asthma susceptibility genes were genotyped (Table 1) namely: *IL13-c.144G/A* (Gln/Arg, rs20541), *IL4-590 C/T* (promoter, rs2243250), *IL4-RP2 253183* (intron), *ADRB2-c.16 A/G* (Arg/Gly, rs1042713), *ADAM33-V4 C/G* (3'UTR, rs2787094), *ADAM33-S1 c.710 G/A* (Val/Ile, rs3918396), *GSDML-236 C/T* (intron, rs7216389) and *STAT6-21 C/T* (intron, rs324011).

Except for *IL4-RP2* VNTR analysis, all genotyping was performed by using TaqMan® SNP Genotyping Assays (Applied Biosystems), in a Real-Time Polymerase Chain Reaction (PCR) 7300 thermocycler (Applied Biosystems), with the 7300 System SDS Software v1.4. For each reaction 2.5 μl of TaqMan Universal PCR Master Mix (2x), 0.125 μl of TaqMan SNP Genotyping Assay (20x) and 1.375 μl of water were used. PCR cycling conditions were as follows: 50°C for 2 min; 95°C for 10 min, followed by 40 cycles of 95°C for 15 s; and 60°C for 1 min. The different alleles were discriminated according to the fluorescence positivity of FAM and VIC probes, respectively indicating the presence of mutant and wildtype alleles.

For the genotyping of *IL4-RP2* VNTR polymorphism, the genomic region of interest was amplified by conventional PCR with the primers described by Mout *et al.* (1991): *IL4-RP2* $P_{Forward}$ 5' TAG GCT GAA AGG GGG AAA GC 3' and *IL4-RP2* $P_{Reverse}$ 5' CTG TTC ACC TCA ACT GCT CC 3' (*MWG Biotech)*. For a total PCR volume of 15 μl, 9.3 μl of distilled water (*Versol*), 1.5 μl of PCR buffer 25mM, 1.5 μl of $MgCl_2$ 25 mM, 1.5 μl dNTPs 2.0 mM (*Promega*), 0.5 μl of each primer 5 ρmol/μl (*MWG Biotech*) and 0.2 μl of *Taq* – polimerase *FirePol* 5 U/μl (*Solis Biodyne*) were added to individual PCR microtubes. About 3 μl of DNA (from salting-out and oragene kit extraction methods) and 4 μl of DNA (obtained from phenol-chloroform) was added to the mixture and the samples were amplified in the TRIO-Thermoblock thermocycler (*Biometra*). PCR cycling conditions were as follows: initial denaturation at 94ºC for 3 minutes, followed by 38 cycles of 94ºC for 30 seconds, annealing at 55ºC for 45 seconds and extension at 72ºC for 1 minute, and a final extension at 72ºC for 3 minutes. For the analysis of this VNTR, PCR products were separated by electrophoresis in a T9C5 polyacrylamide gel for 30 minutes at 200 V, and visualized by silver staining. Both described alleles were observed; in more detail the 253bp three repeat allele and the 183bp two repeat allele (*IL4-RP2del*).

Table 1. Details of the polymorphisms selected for analysis.

Gene	Polymorphism	dbSNP rs ref (NCBI)	Chromosome location	Alleles	Assay ID
IL13	*c.144*Gln/Arg	rs20541	5q31 exon 4	G>A	C__2259921_20
IL4	*590*	rs2243250	5q31 promoter	C>T	C__16176216_10
IL4	*RP2*	-	5q31 intron 2	253>183(del)	-
ADRB2	*c.16*Arg/Gly	rs1042713	5q31 exon	A> G	C__2084764_20
ADAM33	*V4*	rs2787094	20p13 3'UTR	C> G	C__11201381_1_
ADAM33	*S1 c.710*Val/Ile	rs3918396	20p13 exon 19	G> A	C__1276547_20
GSDML	*236*	rs7216389	17q21 intron 1	C>T	C__29062108_10
STAT6	*21*	rs324011	12q13-24 intron 2	C>T	C___620399_10

3.7. Statistical analysis

In the epidemiological evaluation of asthma by questionnaire (Questionnaire B, see Appendix), patients were divided by gender and age groups defined as children (6-10), pre-adolescents (11-13), adolescents (14-17) and adults (18-25) years old. Their family members were also inquired and the same parameters were assessed. Frequencies of each parameter regarding the demographics on the clinical profile of asthma patients, the environmental factors including the inhabitancy conditions, the dietary habits and lifestyle comprising the body mass index (BMI) and the allergy profile were calculated with SPSS (IBM SPSS Statistics, version 19.0.0). The frequency of each factor was analysed for overall asthma and according to asthma severity for the subgroups mild persistent (referred to as mild), moderate persistent (referred to as moderate), severe persistent (referred to as severe), persistent and intermittent asthma and differences between groups were assessed through a Pearson's χ^2 test for categorical variables, while means were compared through One-Way ANOVA, at a significance level set for p-value<0.05. OR indexes were also determined with 95% CI. Results were also illustrated as charts built on Excel (Microsoft® Excel® for Mac 2011, version 14.1.4).

The BMI (kg/m^2) was adjusted for age and sex and the percentiles were determined according to the Portuguese Health Ministry normative No: 05/DSMIA of 21/02/06 concerning the update on the growth charts for boys and girls aged between 2 and 20 year old ("Curvas de Crescimento" 2006). The percentile intervals were defined as normal weight (<85th percentile), overweight (85th–95th percentile) and obesity (>95th percentile). For ages over 20, the BMI was compared to the reference values defined by the Portuguese Program Against Obesity under the normative No: 03/DGCG from 17/03/05 ("Combate à Obesidade" 2005) and posteriorly included in the respective equivalent percentile.

In what concerns the analysis of genetic polymorphisms, Hardy-Weinberg Equilibrium (HWE) was determined for both Madeira population reference sample set and asthma patients (overall asthma and by asthma severity categories). Linkage disequilibrium

(LD) for polymorphisms in genomic regions 5q31 and 20p13 was also assessed by using ARLEQUIN version 3.11 (Excoffier *et al.* 2005).

Genotype and allele frequencies were also determined and differences between groups (Madeira population reference sample set *versus* overall asthma and each asthma severty category, as well as, within overall asthma, comparisons between degree of asthma severity) were tested by a χ^2 test on SPSS. The allelic OR for each polymorphism (95%CI) was determined. Differences between genotypic and allelic frequencies in mild, moderate and severe, as well as between persistent and intermittent asthma were assessed for each polymorphism by a χ^2 test.

The genotype frequencies across overall asthma and the Madeira population reference sample set were compared by a Binary Logistic Regression (SPSS). The dependent variable (the outcome) was defined as to be positive or negative for the presence of asthma while the categorical covariates (predictors) consisted of all possible genotypes of each polymorphism. The Exp (B) and 95% CI were determined.

In order to determine possible linkage between disease and markers, the transmission disequilibrium test (TDT) was performed by using data from heterozygous parents and by comparing the alleles transmission frequency amongst the asthmatic children, through a McNemar test (Lowry 2013). Both the p-value and the OR 95% CI were determined, considered at a 0.05 significance level.

The genetic profile for the eight genotyped polymorphisms, consisting of all possible combinations of pairs of *loci*, was established for asthmatics and Madeira population reference group. The frequencies were plotted in a bar chart and comparisons between groups were determined by Fisher's exact test (ARLEQUIN version 3.11). The 5q31 region was analysed separately (in combinations of 2 and 4 *loci*) and the haplotype frequencies were determined for each group. Differences between groups with the p-value threshold set to 0.05 were assessed by PHASE 2.1. (Stephens *et al.* 2001; Stephens & Scheet 2005).

The combined contribution of environmental and genetic factors in the prediction of asthma severity was assessed by exploring logistic regression models (SPSS) whose covariates were found to be significantly associated to asthma severity, when analysed separately. The Exp (B) and 95% CI were determined.

4. Results

4.1. Demographics

The results of the demographic elements of asthma patients, namely their clinical profile by severity of asthma, forced expiratory volume (FEV_1) and mean forced vital capacity (FVC) are summarized in Table 2. It should be noted, however, that given the very low number of samples assessed as severe asthma (n=4), the analysis regarding this category should be considered with caution. Overall, asthma patient are aged 13.5 +/- 4.3 years old, with nearly 60% of those being males. Among the surveyed groups, skin prick test positivity was found to be higher in intermittent asthma (90.9%) and lower in severe asthma (75%). In what concerns FEV_1 and FVC indexes, both were higher in mild asthma and lower in severe asthma. Differences between categories of asthma severity were assessed for each phenotype by One-Way ANOVA. Significant differences were found for mean FEV_1 - F (3, 39)=3.776, p-value= 0.018) and FVC – F (3, 39)=2.994, p-value=0.042. A LSD (Least Square Difference) post-hoc test revealed that both FEV_1 and FVC means were significantly different between mild and moderate asthma (p-value=0.013 and p-value=0.031, respectively) and between mild and severe asthma (p-value=0.016 and p-value=0.025, correspondingly), opposite to moderate and severe (p-value=0.238 and p-value=0.245). Similarly, no statistically significant difference was found between persistent and intermittent asthma for either FEV_1 or FVC – F (1, 41)=0.263, p-value=0.611 and F (1, 41)=0.209, p-value=0.650, respectively.

Table 2. Characterization of the clinical phenotype of asthmatics (overall asthma and subdivided by asthma severity). Relative frequencies are shown in percentage (%), with correspondent standard deviation (s.d.).

Phenotype and severity categories	n	Male /Female[b] (%)	Positive skin test (%)	Mean age (years+-s.d.)	Mean FEV_1 (%)[c]	Mean FVC (%)[c]
Overall asthma	101[a]	58.8/41.2	85.4	13.5+-4.3	95.0+-12.6	93.1+-10.4
Mild asthma	43	53.5/46.5	88.4	14.2+-4.6	99.7+-11.8*	96.4+-10.3*
Moderate asthma	26	57.7/42.3	76.9	12. 0+-4.2	88.6+-11.1*	88.3+-10.2*
Severe asthma	4	100/0	75	12.3+-3.8	78.0 +-5.7*	79.5+-2.1*
Persistent asthma	73	57.5/42.5	83.6	13.3 +- 4.5	94.3+-12.9	92.5+-11.0
Intermittent asthma	23	60.9/39.1	90.9	14.0+-3.6	96.7+-12.3	94.2+-8.8

[a] No data available for 5 patients regarding asthma severity; [b] No data available for 4 patients regarding gender; [c] Data available for 44 patients; *FEV_1 and FVC Mild *versus* Moderate (p-value=0.013 and 0.031); Mild *versus* Severe (p-value=0.016 and 0.025).

4.1.1. Epidemiological evaluation of asthma by questionnaire

The epidemiological evaluation of asthma and frequency of asthma symptoms assessed by Questionnaire B (see Apendix), are shown in Figure 4 for both asthmatics (divided by age group and gender) and family members. Further details are shown in Supplementary Table 1, 1.1 and 1.2.

Overall, sneeze attacks, runny nose or nasal congestion apart from having a cold or flu is quite frequent amongst asthmatics 97.9% and a high frequency of these symptoms has been registered in the last 12 months (94.8%). Amongst the age groups, the symptoms are less frequent within the 11 to 13 year-old (91.7 and 87.5 %, respectively), but no significant differences were found between subgroups (χ^2 p-value >0.05).

When asked about whether the previous symptoms were accompanied by itchy and watery eyes in the last year, a percentage of 76.3% of overall asthmatics answered positively. For this criterion, both 11 to 13 and 14 to 17 year-old differ from 18 to 25 year-old, as a significant lower number of adolescents within the first two groups presents the symptoms (66.7% and 61.5%), compared to the second (100%) (χ^2 p-values =0.006 and 0.003, OR 2.125, 95%CI 1.488-3.035, correspondingly-Table 3).

When asked about how much their daily activities were affected by sneeze attacks, runny nose or nasal congestion, the majority of overall asthmatics reported to be moderately affected (51.7%-Figure 4). As for the age groups, children between 6 and 10 years-old and adults aged 18 to 25 appear to be very much affected by this symptomatic triad in their daily activities (11.5% *versus* 11.1%, correspondingly), comparably to the pre-adolescent (0%) and adolescent groups (4.2%). In fact, significant differences were found between 14 to 17 and 18 to 25 year-old as a lower number of non-affected was found amongst adults, compared to adolescents (χ^2 p-value= 0.030, OR 0.175, 95%CI 0.033-0.938-Table 3). Also noteworthy is the absence of non-affected 6 to 10 year-old children, contrasting with the percentage of reported cases of non-affected amongst adolescents (41.7%-Figure 4).

As for rhinitis, 94.8% of overall asthmatics replied to have experienced the condition. A similar higher percentage of cases was detected amongst all age groups and no significant differences were found (χ^2 p-value >0.05).

The percentage of wheezing or whistling in the chest ever was 97.9% for overall asthmatics, but showed a decrease over the last 12 months (74.2%). Amongst age groups the same pattern occurs, with children aged 6 to 10 exhibiting the highest number of cases (82.8%) and adolescents the lowest (69.2%), over the last year. The number of wheezing attacks more commonly experienced over the previous 12 months was between 1 to 3 for overall asthma (57.9%), similarly to all age groups, except for 6-10 year-old in which the most common number of wheezing attacks was 4-12 (53.8%)-Figure 4. In fact, significant differences were found between 6 to 10 and pre-adolescents, adolescents and adults, as a significant lower number of wheezing attacks between 1 to 3 was found amongst children, when compared to the remaining age groups (χ^2 p-value= 0.021, OR 0.221, 95%CI 0.059-0.825; χ^2 p-value =0.025, OR 0.244, 95%CI 0.069-0.862 and χ^2 p-value =0.026, OR 0.12, 95%CI 0.052-0.870, respectively). Additionally, 6

to 10 year-old differ from 14 to 17 year-old for the subgroup 4-12 wheezing attacks, as the first presents a higher frequency of occurrences compared to the second (χ^2 p-value = 0.027, OR 4.375 (1.139-16.804)-Table 3.

Sleep disturbance over wheezing in the last 12 months was more frequently reported as less than one night per week (57.9%), for overall asthma as well as for each age group. However, children aged 6 to 10 were the most frequently disturbed in their sleep, for one or more nights a week (30.8%), when compared to the remaining age groups. No significant differences were however found between age clusters (Figure 4).

Wheezing was only severe enough to limit speech to just one or two words at a time between breathes, over the last year in 30.9%, of overall asthmatics. Once again, children aged 6 to 10 were the most affect age group (44.8%), compared to 14 to 17 and 18 to 25 year-old (19.2 and 16.7%, respectively-Figure 4).

Wheezing in the chest during or after exercise in the last 12 months was registered in almost 60% of the overall patients. Age group 6-10 was the most affected (72.4%), while adolescents presented the lowest percentage of occurrences (46.2%-Figure 4). As for dry cough at night apart from a cough associated with a cold or respiratory infection, over the previous 12 months, it was observed in nearly half (48.5%) of asthmatics, and more frequently reported in 18 to 25 year-old, opposite to 6 to 10 year-old (37.9%-Figure 4).

The frequencies of each criterion are evenly distributed by gender (χ^2 p-value>0.05) and follow broadly the tendency verified for overall asthmatics. Despite that, when asked about how much their daily activities were affected by sneeze attacks, runny nose or nasal congestion, 11.1% of asthmatic females reported to be very much affected, against only 3.8 % of male (Figure 4). However, no significant differences were found between groups (χ^2 p-value =0.175, OR 0.314, 95%CI 0.054-1.813). Inversely, 26.4% of male patients were little affected by these problems, whereas only 13.9% of asthmatic females reported little or no affection. Nevertheless, these observations did not prove to be sustained by statistical significance (χ^2 p-value =0.157, OR 2.226, 95%CI 0.723-6.854 and (χ^2 p-value=0.491, OR 1.463, 95%CI 0.493-4.341, correspondingly).

Table 3. Significant diferences observed between age groups for asthma symptoms. The p-value and OR (95%CI) are shown.

Age group	Itchy and watery eyes last 12 months	Daily activities very much affected	Wheezing or whistling last 12 months (1-3)	Wheezing or whistling ever (4-12)
11-13 *vs.* 18-25	0.006 2.125 (1.488-3.035)	-	-	-
14-17 *vs.* 18-25	0.003 2.125 (1.488-3.035)	0.030 0.175 (0.033-0.938)	.	.
6-10 *vs.* 11-13	-	-	0.021 0.221 (0.059-0.825)	.
6-10 *vs.* 14-17	-	-	0.025 0.244 (0.069-0.862)	0.027 4.375 (1.139-16.804)
6-10 *vs.* 18-25	-	-	0.026 0.120 (0.052-0.870)	-

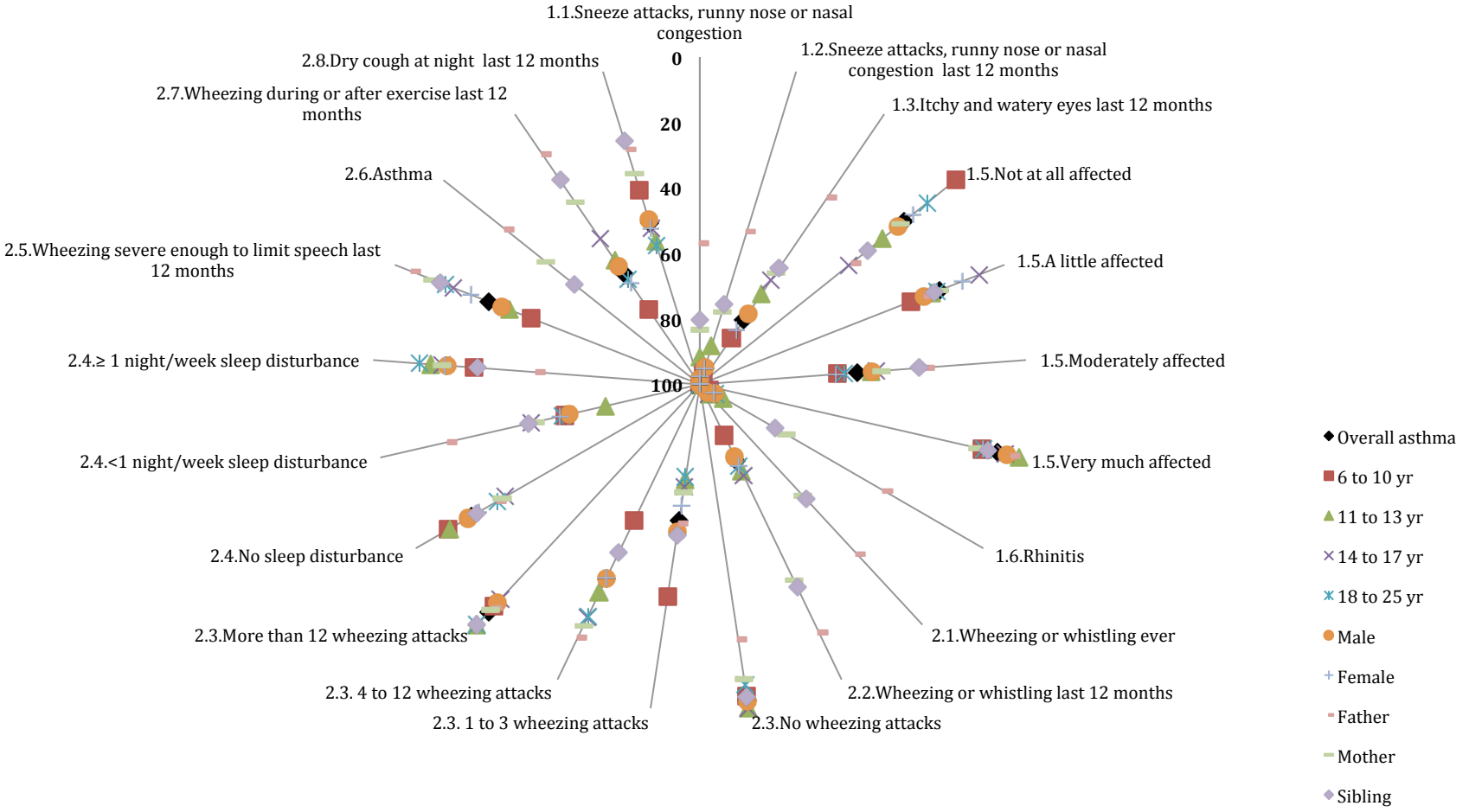


Figure 4. Frequency of asthma symptoms (in percentage, %), for overall asthma, by age groups, gender and family members. Differences between gender, overall asthmatics and family members are further described in Supplementary Tables 1.1. and 1.2.

When analysing the occurrence of the nasal problem in asthmatics by month, there's an overall tendency of occurrences starting in January and gradually rising towards March and April, where the peak occurs (71.1 and 75.3%, respectively-Figure 5). Afterwards, there is a progressive decay in the percentage of cases, reaching to a minimum around July (34.0%) and August (30.9%). There is a slow increase in the percentage of cases towards the end of year (Figure 5).

Within the age groups, a similar trend occurs, as the peak takes place in March and April for each cluster except for 6 to 10 year old, where 82.8 and 86.2% of cases happen in April and May, respectively. The lowest number of cases also occurs in July and August for every group, except for 18 to 25 year-old, where the lowest percentage of occurrences (50%) takes place in August and September, *ex-aequo* (Figure 5).

Significant differences, regarding the occurrence of the nasal symptoms, were found in the distribution of frequencies between age groups, by month. Therefore, both in May and June, a significant difference was found between age group 6 to 10 and both 11 to 13 and 14 to 17, as a significantly higher percentage of cases occurs in the first group compared to the latter two (χ^2 p-value =0.010, OR 5.288, 95%CI 1.404-19.920 and χ^2 p-value=0.018, OR 4.583, 95%CI 1.235-17.008, for May; χ^2 p-value=0.037, OR 3.273, 95%CI 1.054-10.158 and χ^2 p-value=0.004, OR 5.455, 95%CI 1.674-17.770, for June). For the latter month and July, 14 to 17 year-old present the lowest frequency of nasal problems (23.1 and 11.5%, respectively), exhibiting significant differences from the 18 to 25 year-old (χ^2 p-value =0.011, OR 5.238, 95%CI 1.406-19.519 (June) and χ^2 p-value =0.001, OR 12.048, 95%CI 2.605-55.722 (July). For July, significant differences were also established between 14 to 17 and 6 to 10 year-old (χ^2 p-value=0.013, OR 5.412, 95%CI 1.319-22.210-Table 4).

In August, differences were again found between children and 14 to 17 year-old adolescents, suggesting a significantly higher number of occurrences in the first group (χ^2 p-value =0.025, OR 4.685, 95%CI 1.135-19.340). Adolescents present again the lowest frequency (11.5%), differing significantly from the 18 to 25 year-old group, with 50% of cases (χ^2 p-value =0.005, OR 7.667, 95%CI 1.682-34.947). Finally in November, the age group 6-10 exhibits, once more, a significantly higher frequency of nasal problems compared to pre-adolescents (χ^2 p-value = 0.042, OR 3.167, 95%CI 1.026-9.770-Table 4).

No significant differences were found between male and female patients regarding the frequency and/or time of occurrence of the nose problem (χ^2 p-value >0.05) and, similarly to the overall asthma, the peak occurs in March/April (70.2 *versus* 72.5% and 71.9 *versus* 80%, for male and female, respectively) while the lowest percentages are registered in July/August (35.1 *versus* 32.5% and 31.6 *versus* 30%, for male and female, correspondingly-Figure 5 and Supplementary Table 1.1).

Table 4. Statistically significant differences observed between age groups regarding the frequency of occurrence of nose congestion over the previous 12 months. The p-value and OR (95%CI) are shown.

Age group	May	June	July	August	November
6-10 *vs.* 11-13	0.010 5.288 (1.404-19.920)	0.037 3.273 (1.054-10.158)			0.042 3.167 (1.026-9.770)
6-10 *vs.* 14-17	0.018 4.583 (1.235-17.008)	0.004 5.455 (1.674-17.770)	0.013 5.412 (1.319-22.210)	0.025 4.685 (1.135-19.340)	
14-17 *vs.* 18-25		0.011 5.238 (1.406-19.519)	0.001 12.048 (2.605-55.722)	0.005 7.667 (1.682-34.947)	

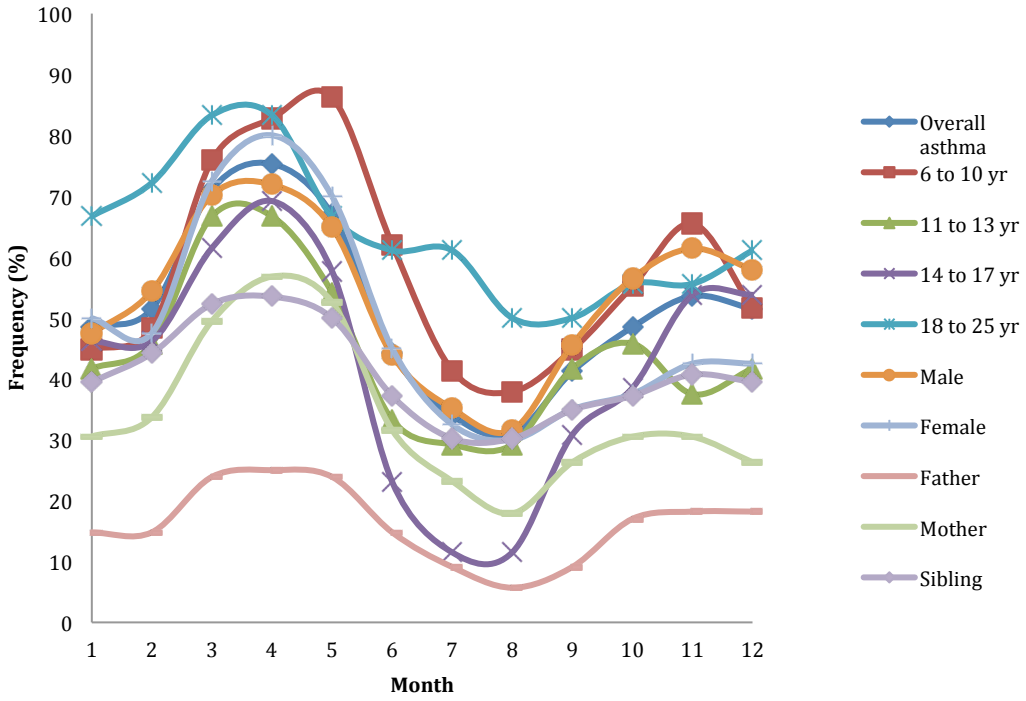


Figure 5. Percentage of occurrence of nose congestion over the previous 12 months, for overall asthma, age groups, gender and family members.

Comparisons of the asthma symptom's frequencies between family members and overall asthmatics, reveal that both fathers, mothers and siblings present significant differences for all criteria (χ^2 p-value <0.05), except for the frequency in which daily activities are affected, the frequency of wheezing attacks and sleep disturbance in the last 12 months (Supplementary Table 1.2). In addition, mothers also do not differ in the frequency in which the nose symptoms occur, but only for June and July (χ^2 p-value=0.069 and χ^2 p-value=0.096, respectively). In addition siblings, do not differ from overall asthmatics in the months in which the nose problem arises: there seems to be a concordance in the frequency of occurrences in January and February and from June to December (χ^2 p-value >0.05. Further details are explored in Supplementary Table 1.2.

4.1.2. Family asthma symptoms in asthma severity

Frequencies of patients by categories of asthma severity, wheezing ever and rhinitis ever, were determined considering each parent and sibling status regarding asthma as follow: i) only mother with asthma; ii) only father with asthma; iii) both parents with asthma; iv) parents without asthma; v) sibling with asthma; vi) sibling without asthma (Figure 6). Similar premises were analysed for rhinitis and wheezing parameters.

A general analysis reveals that, from mild asthma to rhinitis ever, nearly half of the patients present, at least, one asthmatic parent, while the other half presents both parents without the disease, except for severe asthma, where 75% of patients have either their mother or father presenting the condition (Figure 6 and Supplementary Table 2). As for siblings, the frequencies of both positive and negative history of asthma are equally distributed for each category of asthma severity, wheezing and rhinitis. Intermittent asthmatics have, however a higher percentage of siblings with asthma (70%), than persistent asthmatics (46.7%) though no significant association was found between the family member's presence of asthma and the offspring's asthma severity (χ^2 p-value>0.05).

When considering the presence of rhinitis among family member's, a dissimilar pattern occurs. Both mild and persistent asthmatics as well as wheezers and positive asthmatics for rhinitis, have preponderantly “only mothers with rhinitis” (61.1, 50, 43.6 and 45.3% respectively)-Figure 6. In fact, when considering "only mother with rhinitis" *versus* all other groups clustered together, a significant association was found as patients belonging to the first group are three times more likely to experience mild asthma than patients in the other groups (χ^2 p-value =0.030, OR 3.143, 95% CI 1.066-9.267), and four times more prone to present persistent asthma (χ^2 p-value =0.002, OR 4.000, 95%CI 1.196-13.372, Table 5).

In severe asthma, half of the patients present “only mother with rhinitis” while the other half both parents with the disorder. In intermittent asthma, "only mother with rhinitis" and “both parents with rhinitis”, comprise together the majority of cases (50%), though no further significant associations were found between rhinitis in the family and the patient's asthma severity (χ^2 p-value >0.05).

Both wheezers and positive asthmatics for rhinitis also show a significantly higher number of mothers with rhinitis compared to the other groups (χ^2 p-value=0.047, OR 3.125, 95%CI 2.086-4.681 and χ^2 p-value=0.014, OR 3.267, 95%CI 2.141-4.979, correspondingly-Table 5).

Siblings with rhinitis represent a frequency equal or superior to 70% across all groups, from mild (77.8%) to moderate (70%) and severe asthma (100%), persistent (76.7%), intermittent (70%), wheezers (75.6%) and positive for rhinitis (77.3%)-Figure 6. No significant association was found between the sibling's rhinitis and any category of asthma severity (χ^2 p-value >0.05).

As for family members reporting a positive history of wheezing, the number of cases across categories of asthma severity shows that mild asthmatics are equally likely to have both mother only or none of the parents with the symptoms (36.1%); moderate

asthmatics have a higher percentage of "both parents without wheezing" (40%), but no significant association was found between the two variables (χ^2 p-value>0.05). Severe asthmatics present a null percentage of "both parents without wheezing", contrasting with the other groups.

Table 5. Significant p-values and OR (95% CI) for associations between family status regarding asthma symptoms and asthma severity, wheezing and rhinitis in patients.

	Mild asthma	Persistent asthma	Wheezing (+)	Rhinitis (+)
Only mother	0.030	0.002	0.047	0.014
with rhinitis	3.143	4.000	3.125	3.267
vs. all	(1.066-9.267)	(1.196-13.372)	(2.086-4.681)	(2.141-4.979)

Both persistent and intermittent asthmatics show the highest frequencies for "wheezing ever mother only" (33.3 and 35%) but also for "both parents without wheezing" (35%). Similarly, wheezers and positive asthmatics for rhinitis also present 32.1 and 33.3% of "wheezing ever mother only" while 35.9 and 34.7% have "both parents without wheezing" (Figure 6).

As for wheezing history in siblings, the frequency distribution is relatively homogeneous across the distinct subgroups of asthma severity, with nearly half of siblings having reported a positive history of symptoms. In intermittent asthma, however, the percentage of positive history of wheezing in siblings as opposed to negative cases, is relatively higher (70% *versus* 30%)-Figure 6; nevertheless, no further significant association was found between the family's history of wheezing and asthma severity in patients (χ^2 p-value >0.05).

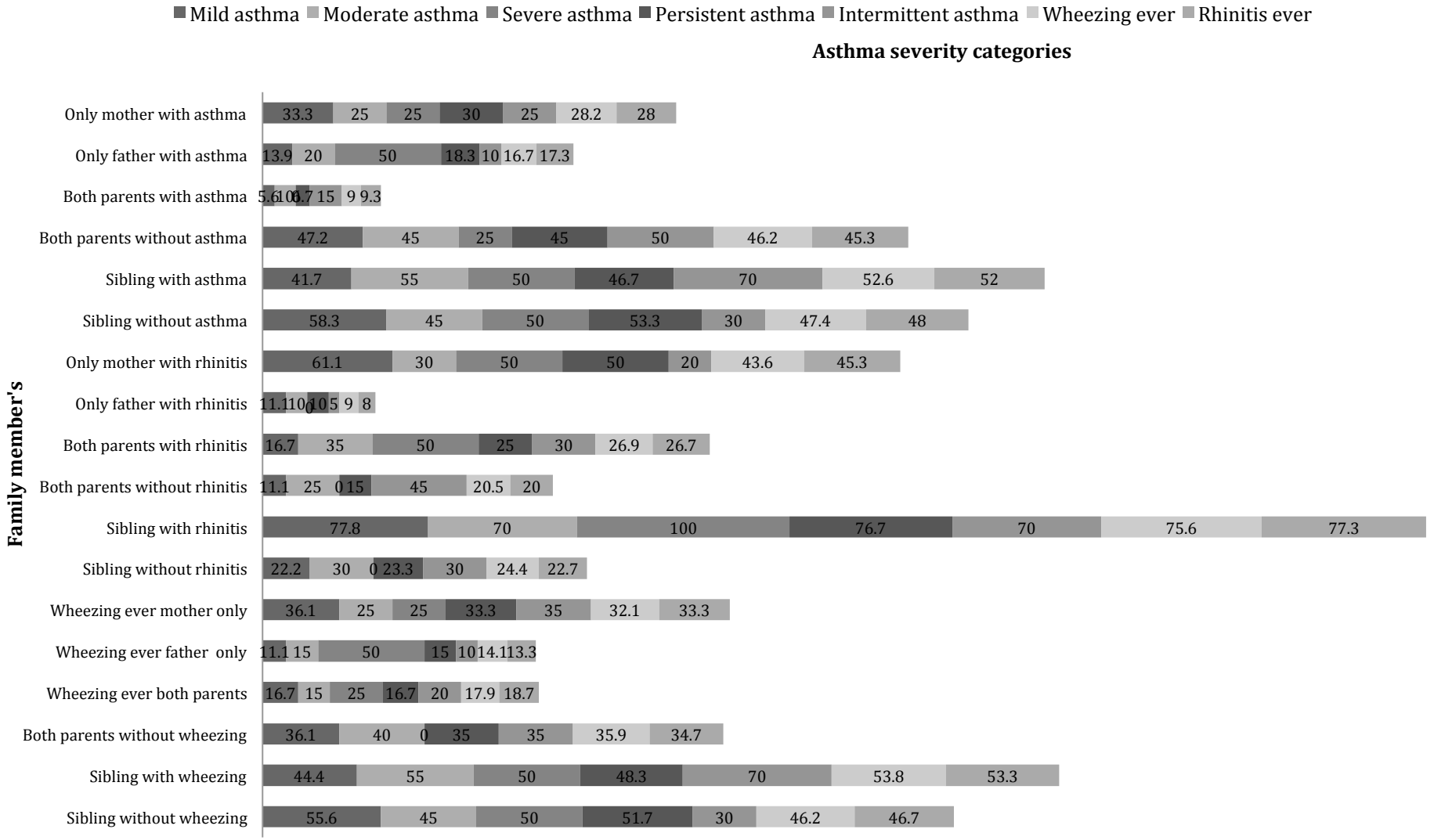


Figure 6. Frequency of asthma severity (mild, moderate, severe, persistent and intermittent), wheezing and rhinitis (%) according to the family member's status for asthma, rhinitis and wheezing.

4.1.3. Environmental factors

The analysis of the inhabitancy conditions of asthmatic patients reveals information regarding the type of environment in which asthmatic live, from their location (urban, suburban, rural), humidity conditions (outdoor and indoors), socioeconomic factors (such as basic sanitation, potable water, electricity, number of years of the house and number of years living in the house, number of rooms and residents, room and living room area, type of construction material used for the house, room floor and walls, type and age of mattress and pillow, type of blankets, number of people sleeping in the room and in bed as well as bedroom contiguity), to the presence of pets and pests ever and in the last 12 months, fuel used to heat the room and kitchen and also smoking habits in the family (Figures 7, 8, 9, 10 and 11 and Supplementary Table 3).

Among overall asthmatics, there is a homogeneous distribution concerning their location, as 30.9% live in an urban environment, 35.1% in a suburban environment and 34.0% in a rural environment. Mild and moderate asthmatics follow a similar distribution pattern, while severe asthmatics live either in urban (75%) ou suburban (25.0%) environments, albeit no significant differences were found between groups (χ^2 p-value >0.05). However, a statistically significant difference was found in the distribution of persistent asthma according to the location. In more detail, there is a significantly higher frequency of asthmatics with persistent asthma living in an urban environment when compared to the frequency of intermittent asthma patients in the same location (χ^2 p-value=0.031, OR 3.913, 95%CI 1.063-14.404)-Table 6.

More than 60% of asthmatics across all groups, reported to live in a humid area, while only half of the severe asthmatics answered the same. However, when questioned about house humidity, the percentages dropped by half across all groups, except for intermittent asthmatics, as still 60.9% reported to live in a humid house, opposite to persistent asthmatics (30.1%, Figure 7). In fact, a significant association was found between intermittent asthma and house humidity, as a significantly higher number of intermittent asthmatics reside in a humid house (χ^2 p-value =0.008, OR 3.606, 95%CI 1.360-9.563)-Table 6.

The majority off overall asthmatics has basic sanitation (94.8%), whereas only 75% of severe asthmatics reported the same. As for potable water and electricity, the reported percentages are equal, with nearly 70% of overall asthmatics having answered positively, while only 50% of severe asthmatics gave a positive response. The totality of the group comprising intermittent asthma presents both features, contrasting with only 58.9% registered for persistent asthma (Figure 7). A significant association was found between both potable water and electricity and intermittent asthma, as a significantly higher number of intermittent asthmatics appear associated to the presence of both settings (χ^2 p-value < 0.05, OR 1.698, 95%CI 1.402-2.056)-Table 6.

Most families live in a house with more than 10 years (64.6 for overall asthmatics, 79.1,46.2, 64.4 and 63.3% for mild, moderate, persistent and intermittent asthma, respectively), except for severe asthmatics whose majority (75%) lives in a house between 5 to 10 years-old. Similarly, most patients have been living in the house for

more than 10 years (overall-40.7%, mild-47.6% and persistent asthma-38.8%) or between 5 to 10 (moderate-30.4%, severe-100% and intermittent-52.2%)-Figure 7.

Both the house and room floor are mainly made of wood (nearly 70% across all groups) and tiles varying from 25% in severe asthma, 34.6% in moderate, and 41.1% in persistent, 46.5 and 48.5% in mild and overall asthma and 69.6% in intermittent asthma, for the house floor and around 20% for the room floor in each category of asthma severity (Figure 7). A significant association was found between house floor of tiles and intermittent asthma as a significantly higher number of asthmatics belonging to the group is associated with this particular feature (χ^2 p-value 0.017, OR 3.276, 95%CI 1.202-8.934)-Table 6.

Only a very small percentage of patients reported to have a carpet (or rug) in the room (4.1% in overall asthmatics, 4.7 in mild, 2.7 for persistent and 8.7 for intermittent asthma). Both moderate and severe asthmatics did not report the presence of rugs (Figure 7). Nearly 97% of asthmatics in all groups reported the presence of a window in the room, except for severe asthmatics where the percentage dropped to 75%, whereas the presence of air conditioning was not reported by any of the groups (Figure 7).

As for the walls, the most common paint is water-based, for nearly 70% of overall, mild and persistent asthmatics, 61.5% of moderate asthmatics, 100% in severe asthma and 91.3% in intermittent asthma (Figure 7). No significant differences were detected between groups.

The majority of patients reports the use of light curtains (nearly or more than 80% across all groups), while only 12.4% of overall asthmatics use heavy curtains, similarly to mild asthmatics (11.6%), opposite to moderate asthma (23.1%) or severe asthma (0%)-Figure 7. Persistent asthmatics use heavy curtains in 15.1% of the cases, while in intermittent asthma the percentage drops to 4.3%, though these numbers do not reflect a statistically significant difference.

The presence of teddy bears varies from 32% in overall asthma, 25.6% in mild, 30.8 in moderate and 25% in severe asthma. Compared to intermittent asthma (43.5%), only 27.4% of persistent asthmatics have teddy bears, albeit this does not reflect a statistically significant difference. Most of these stuffed toy bears are, in number, less than 5 (67.7, 72.7, 62.5, 65 and 80%, for overall, mild, moderate, persistent and intermittent asthma, respectively). Only for severe asthma the number is higher than 10 (100%)-Figure 7.

As for the bed clothing, the most popular is the duvet, as over 95% of patients across all groups report to use it, except for severe asthma (75%).

Over 40% of all bedrooms are contiguous with the W.C., across groups, except for severe asthma (25%), while around 10% are contiguous with the kitchen, with exception for the severe asthmatics group (25%).

The heat source for both the room and kitchen was either electricity (2.1 and 2%, respectively) or gas (9.3 and 69.1%, in this order), for overall asthma. All groups follow the same tendency and no significant differences were detected between clusters.

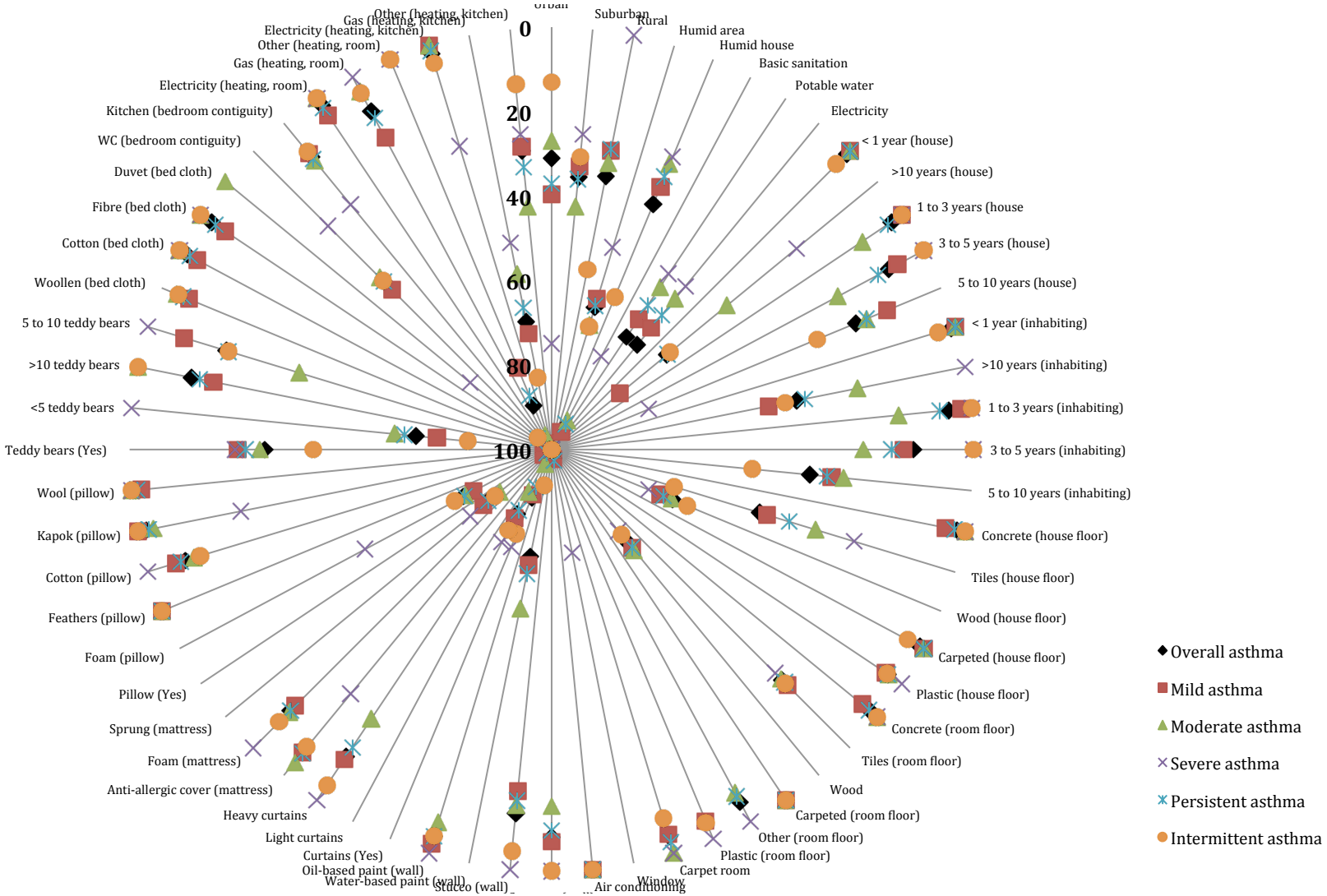


Figure 7. Environmental factors (%) including inhabitancy conditions, namely location, basic sanitation, number of years of the house and inhabiting, house and bed materials and type of heating for overall asthma and by subgroup of asthma severity.

The mean number of rooms is about 3 across all groups and the number of residents about 4. The average of people sleeping in the room is about 1.6 for overall and moderate asthma, 1.5 for mild, severe and persistent asthma and 1.7 for intermittent asthma, while the number of people sleeping in bed is about one, in average across all groups (Figure 8).

The room area is approximately 13 m^2 for overall, moderate and persistent asthma, about 14 for mild asthma and 15 for intermittent asthma. The living room area is around 25 m^2 for overall and persistent asthma, 23 for mild asthma, 26 for moderate, 30 for severe, and 28m^2 for intermittent (Figure 8). No statistically significant differences were found between groups.

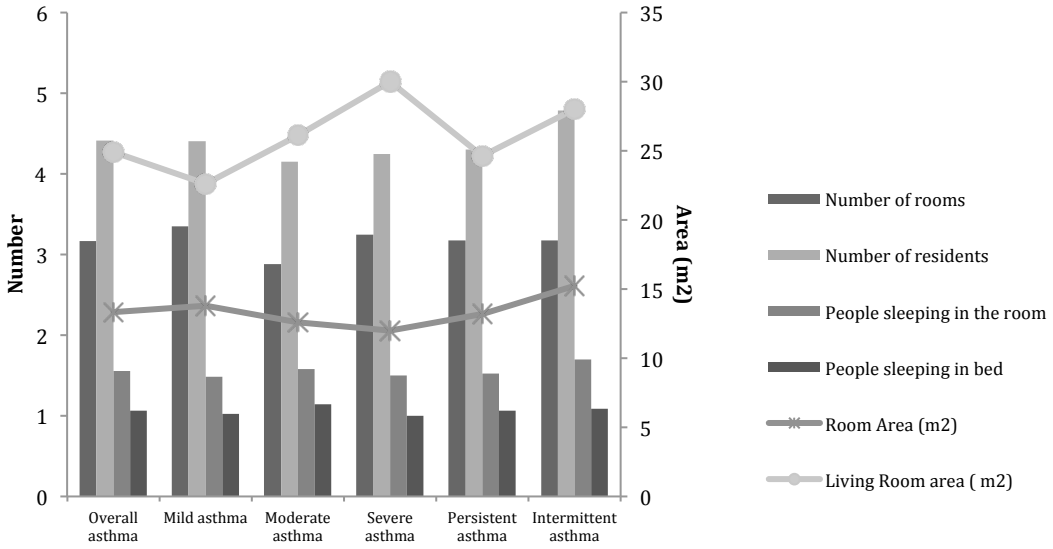


Figure 8. Mean number of rooms, residents, people sleeping in the room, and in bed and both the room and the living room area (m^2) are show for overall asthma and for each asthma severity category.

As for the mattress, most asthmatics reported to use a sprung mattress (nearly 80% across all groups) while only 7.3% of overall asthmatics reported the use of a mattress with anti-allergic cover. However, amongst severe asthma this percentage rised to 25% (Figure 7). In average, the mattress was about 4.3 years old in overall asthma, almost 5 years in mild, 4 in moderate and 2.75 years in severe asthma. Within persistent asthma the mean was almost 4.5 years, whereas for intermittent asthma the mattress was, in average, 4 years (Figure 9). No statistical significant difference was registered between groups.

The most used type of pillow was foam, in all studied groups, in a percentage close to 80%, except for severe asthma (50%) -Figure 7. In average, the pillow was about 2 years old in overall and mild asthma, over 2.6 years in moderate and 2.75 years in severe asthma. Within persistent asthma the mean was nearly 2.5 years, whereas for

intermittent asthma the pillow was, in average, 1.6 years (Figure 9). No statistical significant difference was registered between groups.

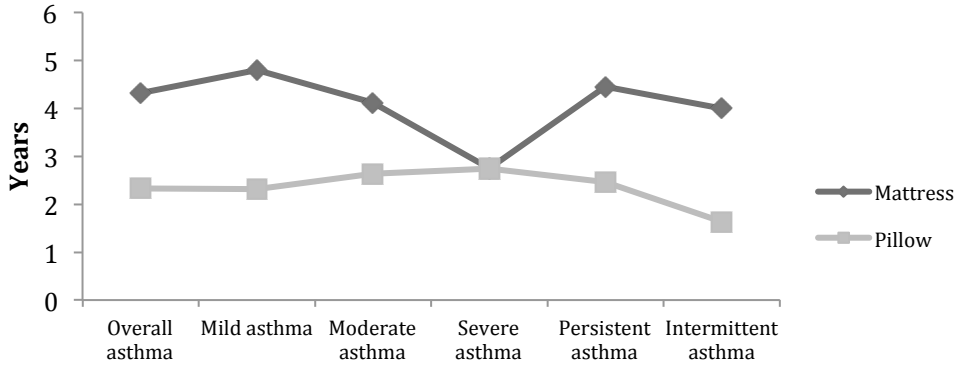


Figure 9. Mean age of the matress and pillow (years) for overall asthma and by asthma severity.

As for pets, over 60% of overall, mild, moderate, severe and persistent asthma groups and 52.2% of intermittent asthmatics reported to have at least one pet. Dogs are the most common pets - 45.4% for overall asthma, 48.8, 46.2 and 50% for mild, moderate and severe asthma, correspondingly and 47.9 and 34.8% for persistent and intermittent asthmatics, respectively. Cat is the second most common pet, in overall, mild, moderate and persistent asthma (18.6, 23.3, 19.2 and 20.5%, respectively), while birds are the second most common amongst severe and intermittent asthmatics (25 and 17.4%). Cockroaches are present in about 20% of the overall asthmatics house, 23.3% in mild asthmatics, 7.7% in moderate asthmatics but there are no registers of their presence amongst severe asthmatics. Amongst the cluster comprising persistent asthma, 17.8% reported the existence of cockroaches in their homes, but the highest percentage was registered for intermittent asthmatics (30.4%). In all groups, the presence of big cockroaches was notoriously higher than that of small cockroaches (Figure 10).

When analizing the presence of pets in the last 12 months, the scenario is not different from the one reported above, with dog and cat being the first and second most common pets amidst all groups, except in severe asthma where there are no registers of cat as a pet and in intermittent asthma, where the second most common pet are birds (17.4%). Cockroaches are also present in 15.5% of overall asthmatics, 14% of mild asthmatics and 11.5% of moderate asthmatics houses. No cockroaches were reported in the last 12 months for severe asthmatics. Persistent asthmatics reported 12.3% of coackroches in the previous 12 months, less than half the registered in intermittent asthma (26.1%, Figure 10). However, no statistically significant associations were found.

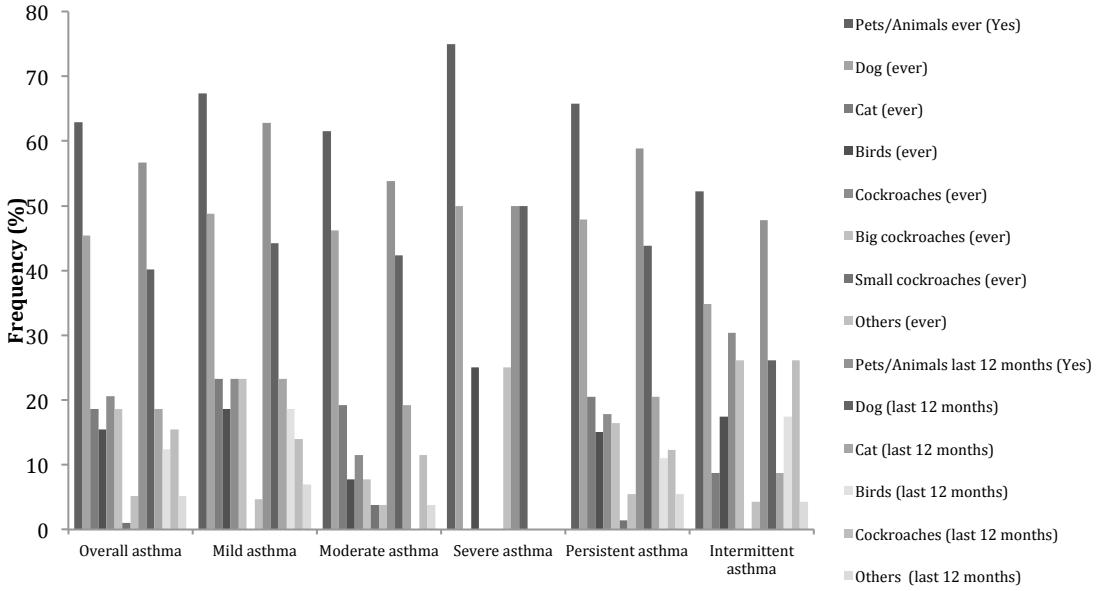


Figure 10. Presence of pets, animals or pests ever and in the last 12 months shown for overall asthma and by category of asthma severity.

Finally, the frequency analysis of smoking habits in the family revealed that the father is the main contributor for passive smoking, as 69.4% of fathers smoke amongst overall asthmatics. Amongst mild asthmatics, the percentage rises to 88.9%, dropping for moderate (57.1%) and severe asthma, where there is no register of cases (Figure 11). In fact, an association was found for passive parental smoking and asthma severity. There is a significant relationship between smoking fathers and light asthma as a significantly higher number of smoking fathers were found amongst mild asthmatics (χ^2 p-value =0.036, OR 3.269, 95%CI 1.047-10.205- Table 6). The persistent asthma cluster shows 76.9% of smoking fathers while intermittent asthma only 50%, but no further significant differences were detected.

Only 2.8% of mothers are smokers amongst overall asthmatics, and the highest registered percentage is found amongst moderate asthma (14.3%). Both mild, severe and intermittent asthma groups have no register of smoking mothers, while persistent asthmatics show a percentage of 3.8. As for both mother and father as jointly contributors for passive smoke, the percentages vary from 5.6 in overall and mild asthma to 14.3 in moderate asthma, 0% for severe asthma, 7.7% in persistent and no cases for intermittent asthma (Figure 11). Siblings also contribute for passive smoking amongst overall asthmatics in 8.3%, 5.6% amongst mild asthma, 3.8 in persistent and with the highest frequency amongst intermittent (20%), but not in moderate or severe asthma. Other family members such as grandfather comprises 2.8% of overall asthma passive smoking and 10% amongst intermittent asthma, while uncle represents 5.6% of passive smoking amongst overall asthma, 14.3% amongst moderate, 3.8% amidst persistent and 10% in intermittent asthma, with no contributions amongst the remaining groups. Other non-specified individuals represent 5.6 of passive smoke amidst overall asthma, 100% amidst severe asthma, 3.8 amongst persistent and 10% in intermittent asthma (Figure 11).

The number of cigarettes per day is, in average 17.655 for overall asthma, 17.692 for mild, 27.167 for moderate, 5 for severe, 19.900 for persistent and 12.667 for intermittent asthma.

As for active smoking, it is factual for 1% of overall asthmatics, due to 25% amongst severe asthmatics, representing 1.4% amongst persistent asthma. The number of cigarretes per day is 8 (Figure 11).

Significant associations between asthma severity and the depicted environmental factors are show in Table 6.

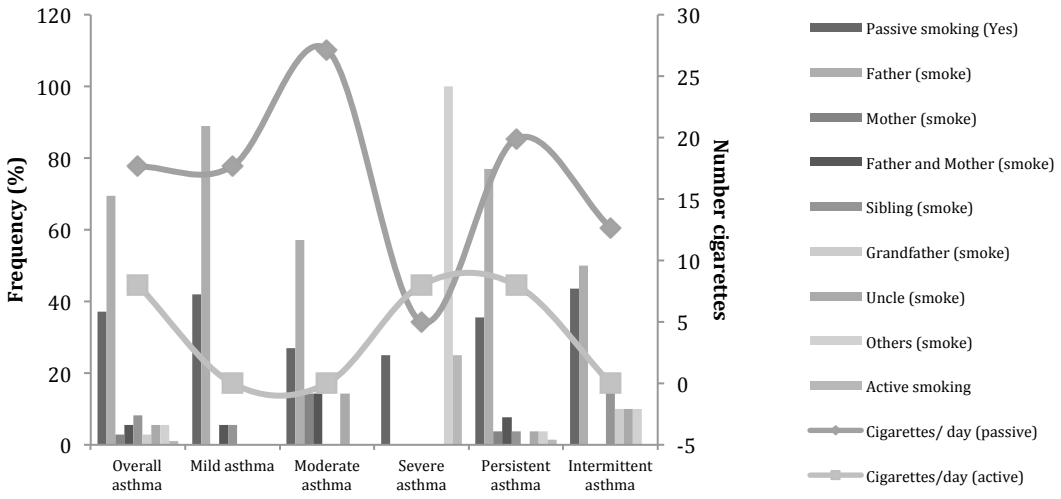


Figure 11. Passive and active smoking. The presence of passive smoking, subdivided by father, mother, sibling, and other family members is shown (%) for overall asthma and by asthma severity. The percentage of active smoking amongst asthmatics (%) and the number of cigarretes from both passive and active smoking is shown.

Table 6. Summary of the significant interactions observed between asthma severity and environmental elements. χ^2 p-values and OR (95%CI) are reported.

	Rural/Suburban environment	Humid house	Potable water and electricity	House floor tiles	Passive smoking
Light *vs.* Moderate and Severe asthma	-	-	-	-	0.036 3.269 (1.047-10.205)
Intermitent *vs.* Persistent	0.031 3.913 (1.063-14.404)	0.008 3.606 (1.360-9.563)	< 0.05 1.698 (1.402-2.056)	0.017 3.276 (1.202-8.934)	-

4.1.4. Dietary habits and lifestyle

The dietary habits and lifestyle in the previous 12 months, depicted by asthma severity is described in Figure 12 and Supplementary Table 4.

Most males amongst overall asthma present a normal BMI, as 80% are under the 85th percentile. Amongst mild, moderate and severe asthma, the percentages are similar (73.3, 75.0 and 78.0%) and no differences were found between groups. Similarly, 84.6% of females also present a BMI under the 85th percentile, analogous to the one found amongst mild (89.5%) and moderate asthma (90.9%)-Figure 12. There are no registers for severe asthma, as all severe asthmatics were male.

A close analysis of the results reveals the existence of a lower percentage of male patients with persistent asthma below the BMI 85th percentile (78%) compared to the proportion of male intermittent asthma in the same percentile category (92.3%).

The inverse situation seems to occur in female patients where a higher percentage of persistent asthma patients are below the BMI 85th percentile (90%), compared to intermittent asthma (66.7%). Nevertheless, and despite these differences, no statistical significance was found (χ^2 p-value>0.05).

About 16 % of overall male asthmatics are overweighted, as they fall into the 85th -95th BMI percentile category. There is a modest increase in the frequency of overweighted from mild (18.2%), to moderate (20%) and severe asthma (25%), amongst males, thought this does not reflect a statistically significant difference. As for female, the overall percentage of overweighted is 12.8% and similarly, a subtle increase from mild to moderate (5.3 to 9.1%) can be observed (Figure 12).

Still in the BMI 85th-95th percentile category, comparisons between persistent and intermittent asthma, reveal a higher number of overweighted in the first category compared to the second for male patients (19.5 *versus* 7.7%). In asthmatic females this percentage appears inverted (6.7 *versus* 33.3%, respectively). As for the above comparisons, these numbers do not reflect any statistically significant differences (χ^2 p-value >0.05).

Obesity was observed only for 3.6 of overall male asthmatics, only in moderate asthma (6.7%), reflecting a percentage of 2.4% amongst the persistent asthma cluster. As for females, the percentage falling over the 95th percentile is 2.6, for overall asthma coming from mild asthma (5.3%) and representing 3.3 % in persistent asthma.

When considering the food habits, more than 70% of overall asthmatic patients consume fruit, vegetables, cereals, pasta, rice, potatoes and milk three or more times a week (Table 5). This tendency is also broadly verified across asthma severity groups, except for vegetables in intermittent asthma (60.9%), pasta in severe (50%) and intermittent asthma (56.5%), rice in intermittent asthma (65.2%) and potatoes in severe (50%) and intermittent asthma (60.9%)-Figure 12.

Legumes are consumed at a lower weekly frequency (53.2% three or more times a week and 29.8% once or twice a week), a similar scenario for that of fish (49.5% once or twice a week, 47.4% three or more times a week), butter (27.4 % affirms not to consume it at

all or only sporadically while 46.3% use it three or more times a week) and egg consumption (63.2% once or twice a week-Figure 12). Amongst the asthma severity categories, a similar scenario can be observed.

Asthma patient's consumption of fast food is relatively low as only 15.1% consumes it once or twice a week and only 4.3 % at least 3 times a week. The remaining 80.6% are non-consumers. The percentage of non-consumers rises substantially for margarine (73.7%), and dried fruits (72.6%) and these numbers do not significantly differ between the asthma severity category groups.

However, and despite the similarities reported above between asthma severity clusters, when analysing the weekly food consumption by asthma severity, through χ^2 tests and OR indexes, some significant association were highlighted:

i) Weekly intake of fish and persistent asthma (when compared to intermittent asthma), as in the first group a significantly lower consumption of fish (one or two times a week) is reported, (χ^2 p-value =0.015, OR 0.244, 95%CI 0.086-0.692);
ii) Legumes intake and asthma severity, the first being more often consumed (once or twice a week) in moderate and severe asthma, compared to mild (χ^2 p-value=0.008, OR 5.204, 95%CI 1.746-15.508);
iii) Weekly intake of pasta is significantly associated to moderate (when compared to severe asthma) and persistent asthma (when compared to intermittent) as within these categories, a higher consumption of pasta (three times a week or more) can be observed (χ^2 p-value=0.024, OR 11.500 95%CI 1.007-131.287 and χ^2 p-value =0.035, OR 2.872, 95%CI 1.054-7.823, correspondingly);

As for intense physical activity most of the overall asthma patients practice it one or two times a week (53.7%). Mild asthmatics also practice intense physical activity more often once or twice a week (57.1%), similarly to the persistent and intermittent cluster (52.1 and 56.5%, respectively). The highest percentage of moderate asthmatics practices it three or more times a week (48%), while 44% do it only once or twice a week; severe asthmatics are as likely to do it in either temporal frequencies (50/50%).

Overall asthmatics watch TV more frequently between one and three hours per day in a normal week (55.9%). An identical scenario can be spotted across the different categories of asthma severity, except for severe asthma in which most patients only watch TV less than one hour per day (50%, Figure 12). No further statistical significant differences were, however, found between groups (χ^2 p-value >0.05).

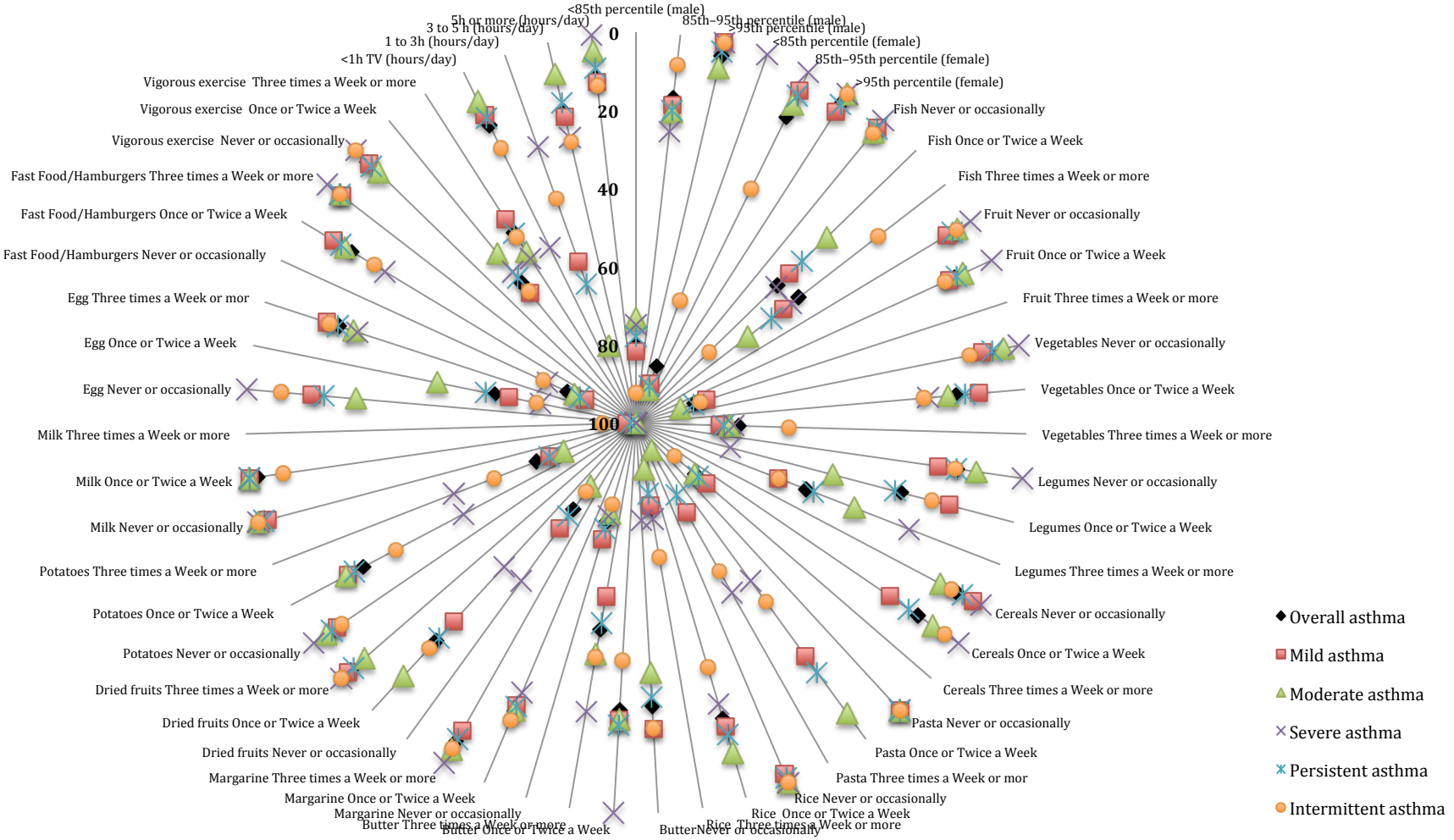


Figure 12. Food habits and lifestyle depicted for overall asthma and for each subgroup of asthma severity (%).

4.1.5. Allergy profile

The allergy profile for asthma patients, according to asthma severity, is described in Figures 13 and 14 and Supplementary Table 5. Patients were tested for:

i) house dust mite extracts, namely *Dermatophagoides pteronyssinus* (Dpt), *Dermatophagoides farinae* (Df), *Blomia sp.*, *Lepidoglyphus sp.*, *Glycifagus sp.*, *Tyrophagus sp.* and *Euroglyphus sp.*;
ii) pollens from Gramineae (grass), *Parietaria sp*. and *Urtica sp*. (Gramineae, (Urticaceae family); *Artemisia sp*., *Aster sp*. and *Taraxacum sp*. (Gramineae, Asteraceae family), *Chenopodium sp*., *Pinus sp*., *Cupressus sp*., *Juglans sp*., *Quercus sp*., *Castanea s*p., *Salix sp*., *Mimosa sp*., *Platanus sp*. and *Tilia sp*.);
iii) moulds like *Penicillium sp*., *Aspergillus sp*., *Alternaria sp*., *Cladosporium sp*., *Mucor sp*., *Candida sp*.);
iv) cockroach's extracts such as *Periplaneta americana*, *Blattella germanica* and *Blatta orientalis*;
v) domestic animals like dog and cat extracts.

Histamine was used as a positive control for the skin prick testing.

Globally, the majority of overall asthma patients were sensitive to Dpt (89.0%), closely followed by Df (81.7%) and *Blomia sp.* (65.9%). *Lepidoglyphus sp.*, *Glycifagus sp*, *Tyrophagus sp.* and *Euroglyphus sp.* extracts produced a lower number of positive tests (42.7, 31.7, 9.8 and 22.0%, correspondingly-Figure 13). Amongst asthma severity categories, a similar pattern was observed, except in severe asthma where only half of the patients were responsive to Dpt, Df, *Blomia sp*., *Lepidoglyphus sp.* and *Euroglyphus sp.* and no positive reaction was detected for *Tyrophagus sp.* No significant differences were found between groups (χ^2 p-value>0.05).

The mean wheal size reported for Dpt was 9.007+-4.306 mm, the highest mean value registered amongst all tested allergens, amongst overall asthma, not differing significantly from mild (9.457 mm), moderate (9.150 mm), severe (9.500 mm), persistent (9.351 mm) or intermittent asthma (7.900 mm) (One-Way ANOVA p-value>0.05). Df presented the second highest wheal diameter amongst house dust mite extracts (7.940 mm), followed by *Euroglyphus sp.* (7.333 mmm), *Blomia sp*. (7.204 mm), *Lepidoglyphus sp.* (6.514 mm), *Glycifagus sp* (6.440 mm) and *Tyrophagus sp.* (5.125 mm). Noteworthy is the mean wheal size registered for *Blomia sp*. in severe asthma (11.500 mm), the highest value observed amongst asthma severity categories (Figure 14).

When analysing the mean wheal size between groups a significant difference was found between mild (5.675 mm) and severe asthma (9.500 mm) for *Lepidoglyphus sp.* (One-Way ANOVA F (1,20)= 6.598, p-value=0.018).

As for pollens, Gramineae allergens (grass) were responsible for the highest percentage of sensitization (23.2%), followed by *Parietaria sp.* (13.4%), *Urtica sp.* (6.1%), *Artemisia sp.* (2.4%), *Aster sp.* and *Taraxacum sp.* (1.2%) and *Chenopodium sp.* (2.4%). Mild asthmatics follow this distribution broadly, however for moderate asthma no positive

skin prick tests were observed for *Artemisia sp*., *Aster sp*., *Taraxacum sp.* and *Chenopodium sp*. In severe asthma, no positive reaction was detected for any of the aforementioned pollen extracts. Persistent asthmatics follow the tendency of overall asthmatics, while intermittent asthma shows the lowest number of positive cases for Gramineae (8.7%) and no positive reaction for *Urtica sp*., *Artemisia sp*., *Aster sp*., *Taraxacum sp*. and *Chenopodium sp*. (Figure 13).

Amongst the above mentioned pollen extracts, *Aster sp.* registered the highest mean wheal size in overall asthmatics (7.000 mm), followed by *Chenopodium sp.* (6.500 mm), Gramineae (grass, 5.632 mm), *Artemisia sp.* and *Taraxacum sp.* (5.000 mmm), *Parietaria sp.* (4.180 mm) and *Urtica sp.* (3.800 mm)-Figure 14. No significant differences were found for mean wheal size between groups (One-Way ANOVA p-value>0.05).

Positive skin prick tests to *Pinus sp.* and *Cupressus sp.* tree pollen were only observed in 3.7% of overall asthmatics. Positive reactions to *Juglans sp.* and *Quercus sp.* extracts were also registered in a small percentage (2.4 and 1.2%, respectively). Aditionally, 4.9% reacted to *Castanea sp*., 1.2% to *Salix sp*., 2.4% to *Mimosa sp*., 7.3% to *Platanus sp.* and 1.2% to *Tilia sp*. Amongst mild asthma there is a similar frequency distribution pattern, but in moderate asthma the only positive reactions occurred in the presence of *Pinus sp*., *Castanea sp*. and *Mimosa sp*. (3.8%), while for severe asthma 25% of the patients were sensitized only by *Platanus sp*. (Figure 13).

The persistent asthma cluster shows the same allergy profile as overall asthmatics. However, the intermittent asthma group show no reaction to *Juglans sp*., *Quercus sp*., *Salix sp*., *Mimosa sp*. and *Tilia sp.* (Figure 13). Nevertheless, intermittent asthmatics show a significantly higher frequency of sensitization to *Platanus sp*., when compared to persistent asthma (χ^2 p-value=0.011, OR 7.474, 95%CI 1.271-43.932).

As for the mean wheal size, the highest value registered amidst tree pollens was for *Salix sp.* and *Tilia sp*. (6.000 mm), followed by *Platanus sp.* (5.500 mm), *Cupressus sp.* (5.333 mm), *Castaneae sp.* (4.750 mm), *Pinus sp.* (4.667 mm), *Juglans sp.* (3.500 mm) and finally, *Quercus sp.* and *Mimosa sp*. (3.000 mm, Figure 14). No differences were found between groups (One-Way ANOVA p-value>0.05).

The three most common positive skin prick tests amongst moulds were registered for *Alternaria sp.* (20.7%), *Aspergillus sp.* (13.4%) and *Cladosporium sp.* (8.5%). Sensitization to *Mucor sp*., *Candida sp*. and *Penicillium sp*. were the least frequent (6.1, 2.4 and 1.2%, respectively). For mild asthma, *Mucor sp*. was the third most frequent extract to cause a positive reaction (7%), while for moderate, both *Aspergillus sp*. and *Cladosporium sp.* were the second most frequent allergens to cause sensitization, *ex-aequo* (11.5%) and no reaction was detected for *Penicillium sp*. For severe asthma, only *Aspergillus sp.* and *Alternaria sp*. caused a positive response (25%). Once again, persistent asthma follows the tendency verified for overall asthma. In intermittent asthma, *Penicillium sp.* caused no positive reaction and *Aspergillus sp.* and *Cladosporium sp.* were the second most frequent allergens to cause sensitization (8.7%)- Figure 13.

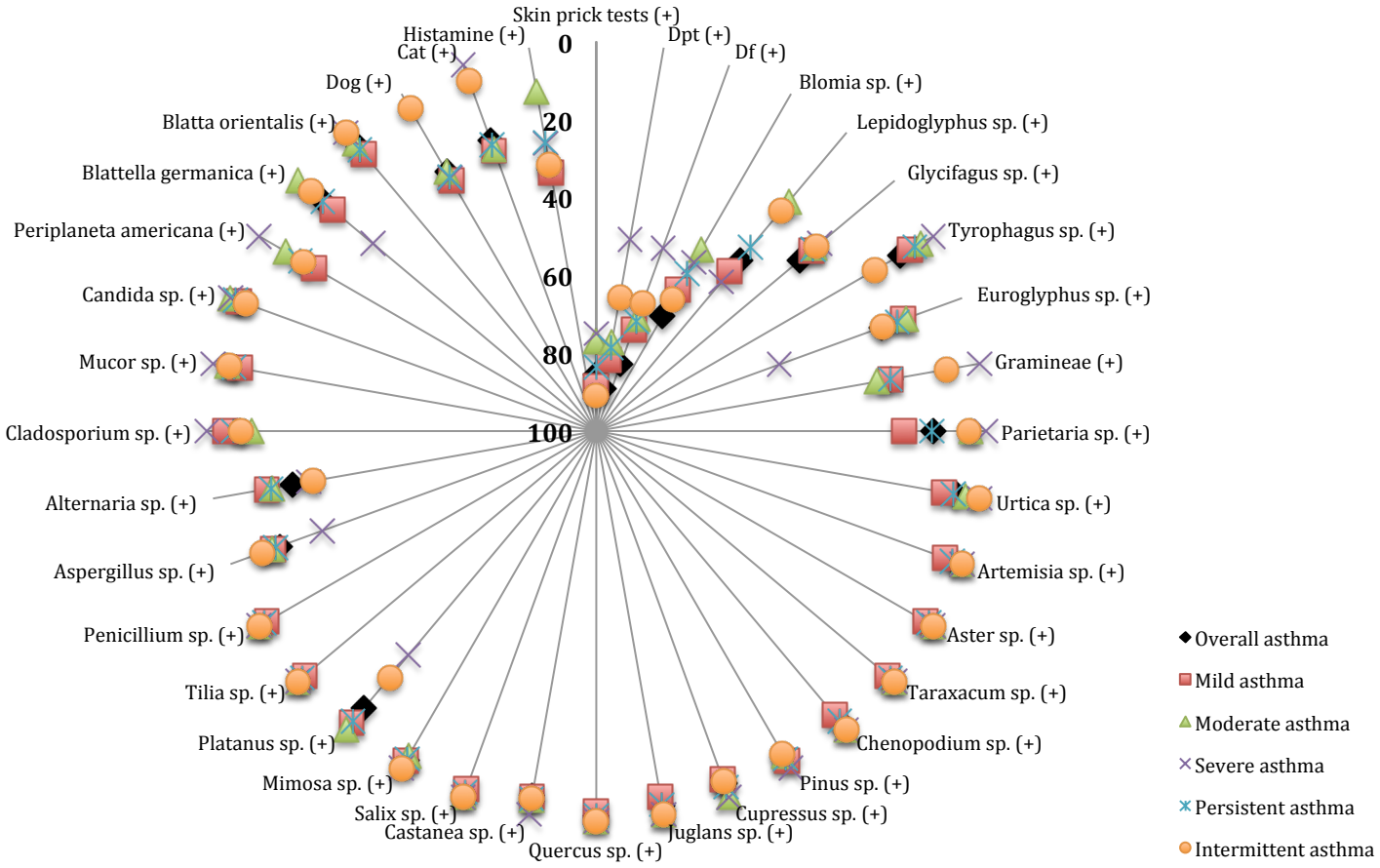


Figure 13. Allergic profile for overall asthma and by subgroup of asthma severity. Frequencies are given in percentage (%) of positive skin prick test.

Amidst mould extracts, *Mucor sp.* registered the highest mean wheal size (5.800 mm), followed by *Penicillium sp.* and *Candida sp.* (5.000 mm), *Alternaria sp. (*4.853 mm), *Aspergillus sp.* (4.045 mm) and *Cladosporium sp. (*3.929 mm)-Figure 14. Amongst asthma severity groups, the mean wheal diameter is roughly identical, and no statistically significant differences were found between clusters.

As for cockroaches, *Periplaneta americana* was accountable for 14.6% of positive skin prick tests, followed by *Blattella germanica* (8.5%) and *Blatta orientalis* (4.9%), for overall asthma, and a similar pattern was registered in mild asthma. Moderate asthma group was not reactive to *Blattella germanica*, contrary to severe asthma, only responsive to this last cockroache allergen (25%). In the intermittent asthma cluster, there was no reaction to *Blatta orientalis.* No significant differences were found between groups (χ^2 p-value>0.05).

Blattella germanica, was responsible for a larger mean wheal size (5.140 mm), compared with both other cockroaches species (*Blatta orientalis* 4.250 mm and *Periplaneta americana* 3.625 mm)-Figure 14. Asthma severity categories present similar mean values and therefore no significant differences in wheal size were found between groups (One-Way ANOVA p-value>0.05).

Finally the percentage of overall asthmatics sensitized for dog and cat allergens were 23.2 and 20.7%, correspondingly (Figure 13). Similar numbers were registered for mild (25.6 *versus* 23.3%) and moderate asthma (23.1% for each). Severe asthmatics did not react to cat allergen, only to dog extract (25%). Among persistent asthma, 24.7% experienced a positive reaction to the allergen, while in intermittent asthma, only 4.3% responded positively. In fact, a significant association was found between dog allergen and intermittent asthma as this group is significantly less likely to experience a positive reaction compared to persistent asthma (χ^2 p-value=0.033, OR 0.754, 95%CI 0.632-0.900). The number of positive skin prick tests in persistent asthma, compared to intermittent asthma for cat extract is also higher however, no significant differences were found between both categories for this allergen extract (χ^2 p-value>0.05).

The mean wheal diameter for dog's allergen was 4.895 mm for overall asthmatics (Figure 14). When comparing the mean values amongst categories of asthma severity, a significant difference was found as the registered mean wheal size in severe asthma is significantly higher than the one in mild asthma (8.000 mm *versus* 4.364 mm), One-Way ANOVA F (1,10)=11.494, p-value=0.007. Cat allergen extract produced a mean wheal size of 5.735 mm, in overall asthmatics. Despite the findings of a higher wheal diameter amongst intermittent asthmatics (8.500 mm), when compared to persistent asthma (5.563 mm), no significant differences were found (One-Way ANOVA p-value>0.05).

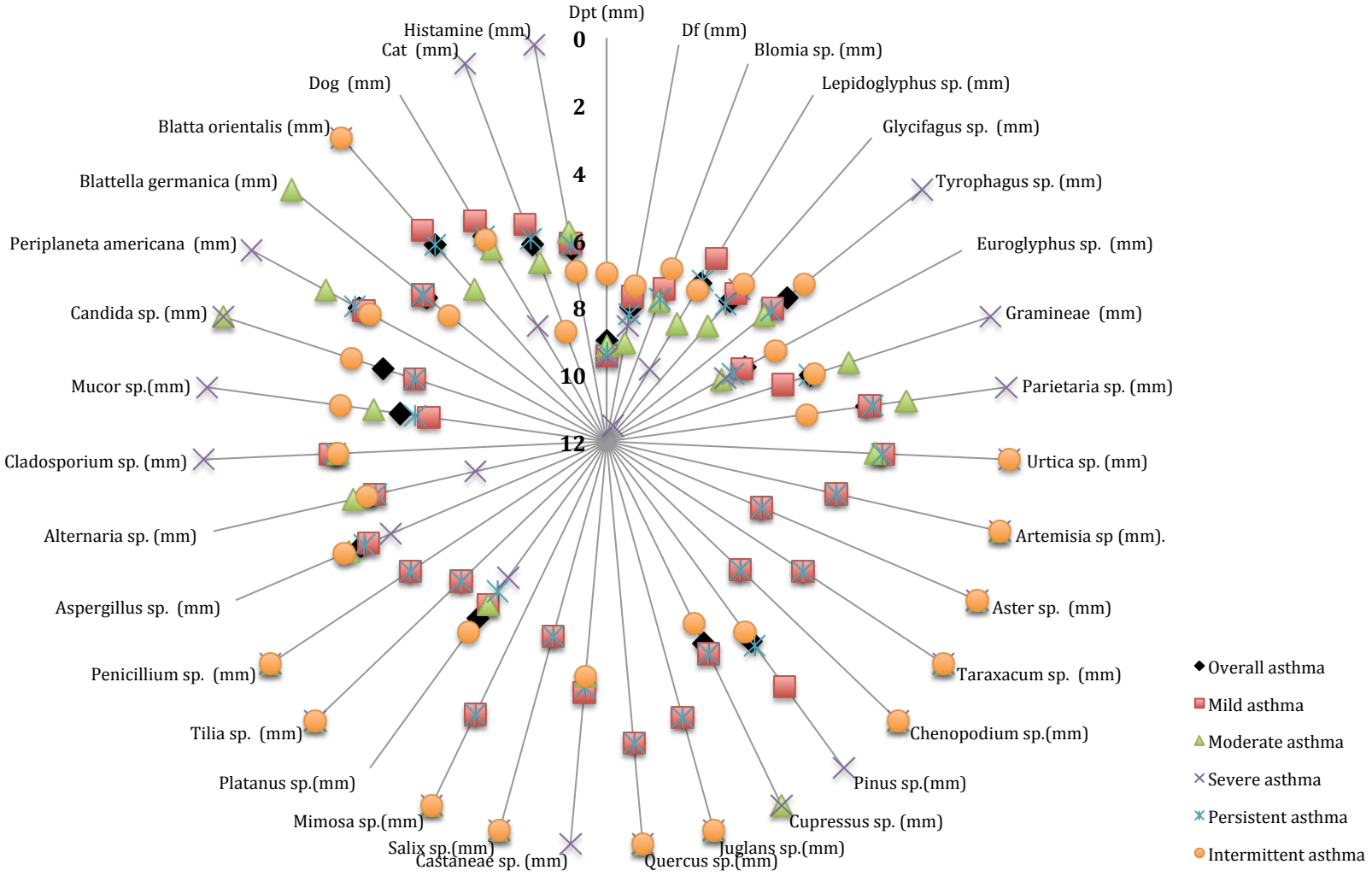


Figure 14. Mean wheal size for positive skin prick tests (mm) for overall asthma and by asthma severity.

4.2. Analysis of genetic polymorphisms

4.2.1. Case-Madeira reference set study

Regarding the analysis of the genetic polymorphisms, Table 7 shows the analysis of HWE for each polymorphism on both asthmatics and Madeira reference sample set. Both groups are in HWE for all polymorphisms (p-value>0.05). However, when analysing the patient's genetic profile by asthma severity, significant deviation from HWE were found for moderate asthma at *ADRB2-c.16 locus* (p-value=0.042) and for mild asthma at *STAT6-21 locus* (p-value=0.033).

Table 7. HWE analysis p-values for genetic polymorphism in asthma patients (overall and by asthma severity categories). Statistically significant p-values are highlighted in bold.

Asthma severity	n	*IL13-c.144*	*IL4-590*	*IL4-RP2*	*ADRB2-c.16*	*ADAM33-V4*	*ADAM33-S1 c.710*	*GSDML-236*	*STAT6-21*
Madeira reference	105*	0.733	1.000	0.094	0.552	0.383	1.000	0.838	0.385
Overall asthma	101	1.000	1.000	0.735	1.000	1.000	1.000	1.000	0.662
Mild asthma	43	0.519	0.567	1.000	0.065	0.372	1.000	0.312	**0.033**
Moderate asthma	26	1.000	1.000	1.000	**0.042**	1.000	1.000	0.695	0.195
Severe asthma	4	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Persistent asthma	73	1.000	0.719	1.000	1.000	1.000	1.000	0.810	0.430
Intermittent asthma	23	1.000	0.518	0.311	0.676	1.000	1.000	1.000	1.000

* Except for *IL4-590* and *IL4-RP2* (n=110)

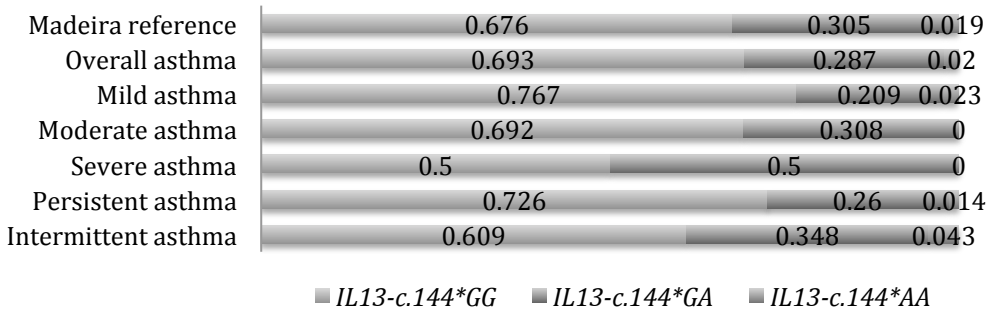
Furthermore, the *IL4-590*/*IL4-RP2*, *IL4-RP2*/*IL13-c.144*, *IL4-590*/*IL13-c.144* and *ADAM33-V4*/*ADAM33-S1 c.710* pairs of polymorphism were found to be in LD in both Madeira reference sample set and overall asthma (p-value<0.05). Both the pairs *IL4-590 / ADRB2-c.16* and *ADRB2-c.16 / IL13-c.144* were found to be in LD only for the Madeira reference set (p-value=0.010, for each). The combination of polymorphisms *IL4-RP2*/*ADRB2-c.16* was not found in LD for neither Madeira reference group nor overall asthma (p-values=0.070 and 0.560, respectively). Further details are shown in Supplemmentary Table 6.

The genotypic and allelic frequencies obtained for asthmatic patients (overall and according to asthma severity) and for the Madeira reference sample set are summarized in Figures 15 and 16 and in Supplementary Tables 7 and 8, respectively. Both tables depict χ^2 statistics conducted to assess significant differences on genotypic or allelic frequencies between groups. Both *ADRB2-c.16* and *STAT6-21* genetic markers were not considered for the analysis of moderate and mild asthma, respectively, since their genotypic proportions violate the HWE. When considering the overall asthma group, significant differences were found for *IL4-590* and *IL4-RP2* genotype counts, with *IL4-590*CT*/*IL4-590*TT* and *IL4-RP2*253183*/*IL4-RP2*183183* genotypes being the most

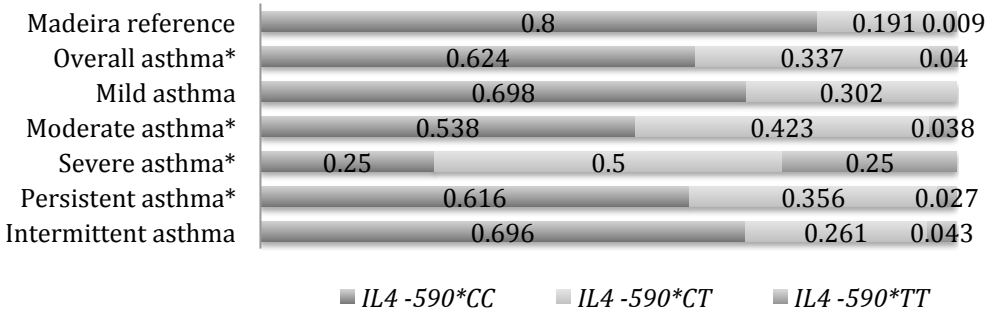
frequent amongst asthmatics, compared to the Madeira reference sample set (p-value=0.010, OR 2.413, 95% CI 1.302-4.470 and p-value=0.048, OR 2.184, 95% CI 1.153-4.135, respectively-Supplementary Table 7.1).

Also when comparing each severity subgroup of asthmatics with the Madeira reference sample set, significant differences were found for moderate, severe and persistent asthma concerning *IL4-590* and *IL4-RP2* genotype counts. Therefore, once again**,** *IL4-590*CT* / *IL4-590*TT* and *IL4-RP2*253183*/*IL4-RP2*183183* genotype counts were found in a significantly higher frequency amongst each one of the above mentioned clusters, when compared to the Madeira reference group (χ^2 p-value=0.015, OR 3.429, 95% CI 1.392-8.446; χ^2 p-value=0.009, OR 12, 95%CI 1.190-121.006; χ^2 p-value=0.016, OR 2.489, 95%CI 1.281-4.835, for *IL4-590*CT*/ *IL4-590*TT* and χ^2 p-value=0.024, OR 3.300, 95%CI 1.320-8.252; χ^2 p-value=0.015, OR 13.500, 95%CI 1.334-136.618 and χ^2 p-value=0.033, OR 2.344, 95%CI 1.182-4.647, for *IL4-RP2*253183* and *IL4-RP2*183183* genotype counts (Figures 15b and 15c and Supplementary Table 7.1).

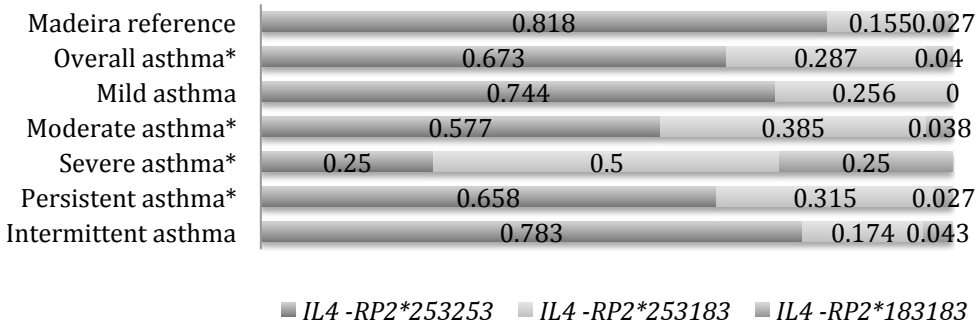
a *IL13-c.144*



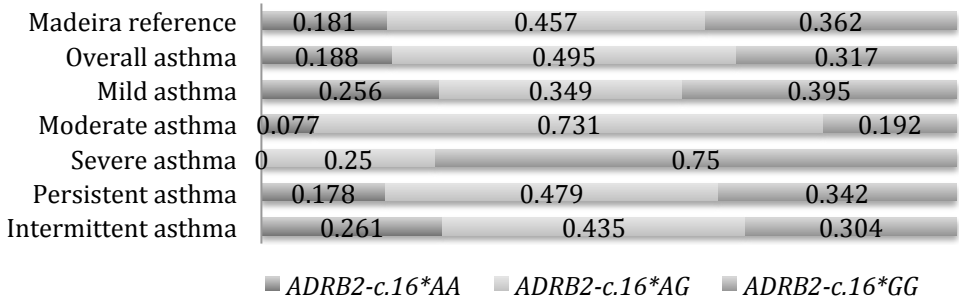
b *IL4 -590*



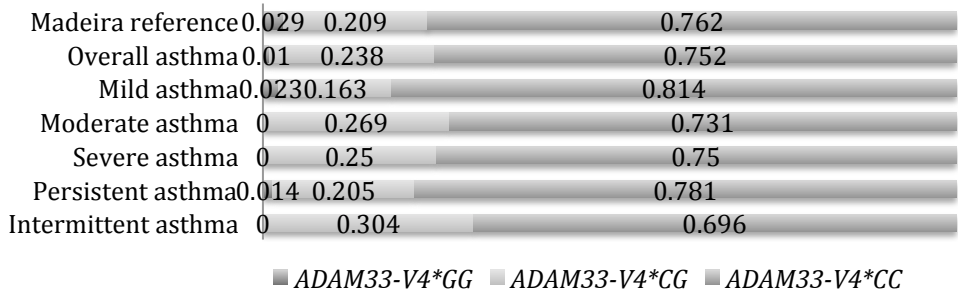
c ***IL4-RP2***



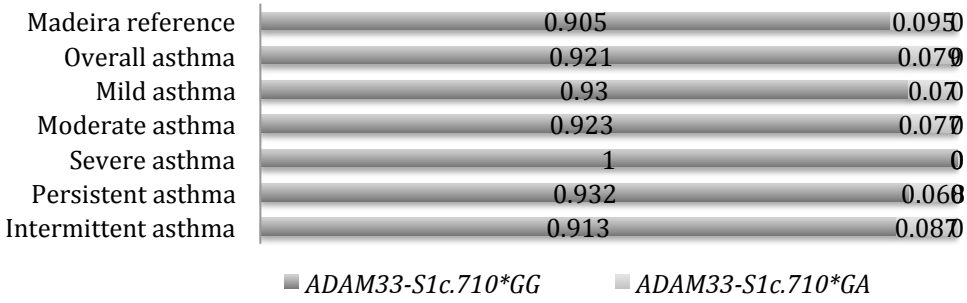
d ***ADRB2-c.16***



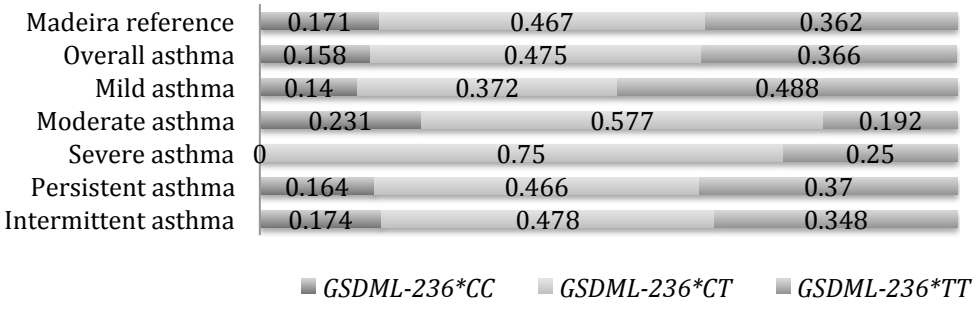
e ***ADAM33-V4***



f ***ADAM33-S1c.710***



g ***GSDML-236***



h ***STAT6-21***

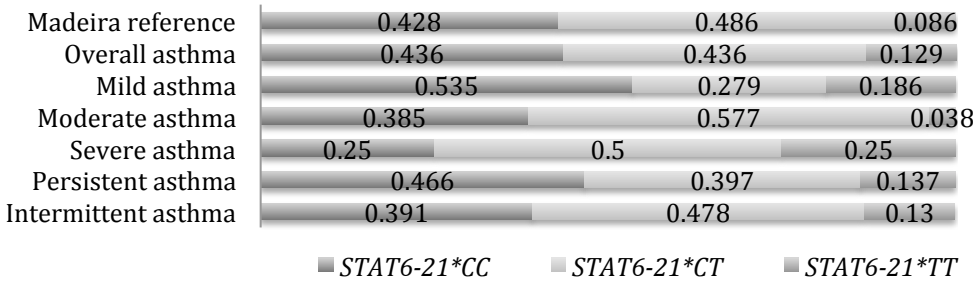
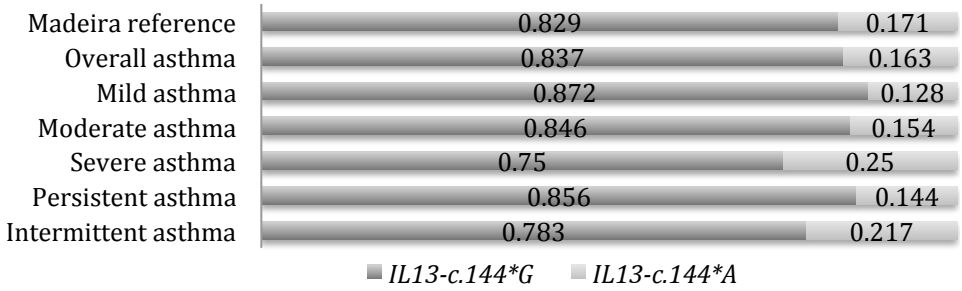


Figure 15. Genotype frequencies for the Madeira reference sample set, overall asthma and subgroup of asthma severity: a) *IL13-c.144* ; b) *IL4-590*; c) *IL4-RP2*; d) *ADRB2-c.16*; e) *ADAM33-V4*; f) *ADAM33-S1 c.710*; g) *GSDML-236*; h) *STAT6-21*. Statistically significant differences in relation to the Madeira reference set are signaled (*).

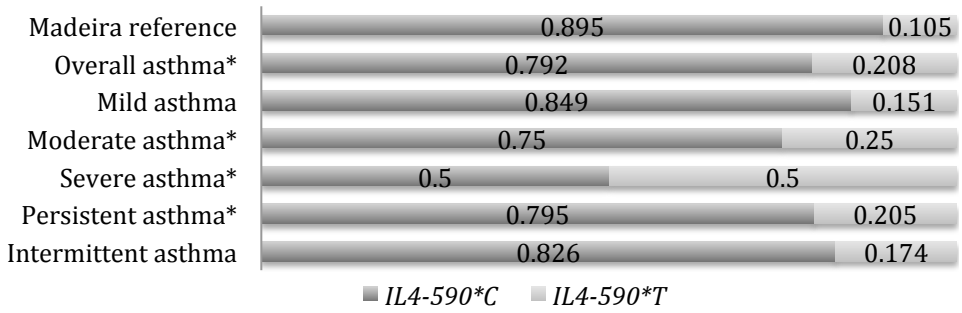
Similarly, *IL4-590*T* and *IL4-RP2*183* alleles were found to be significantly more common in overall asthma, when compared to the Madeira reference sample set (χ^2 p-value= 0.005, OR 2.207, 95%CI 1.301-2.743 and χ^2 p-value=0.025, OR 1.902, 95%CI 1.102-3.285, respectively). Furthermore, an increasing gradient in the frequency of both *IL4-590*T* and *IL4-RP2*183* alleles from mild to severe asthma was observed (Figures 16b and 16c and Supplementary Table 8).

χ^2 p-values<0.05 and OR for allelic frequencies of each polymorphism shown in Supplementary Table 8.1, further suggest significant associations of *IL4-590*T* and *IL4-RP2*183* alleles to moderate asthma (χ^2 p-value=0.009, OR 3.532, 95%CI 1.450-8.604 and χ^2 p-value= 0.024, OR 3.070, 95%CI 1.247-7.558), severe asthma (χ^2 p-values=0.009 and 0.008, OR 43.439, 95% CI 4.918-383.717 for both alleles) and persistent asthma (χ^2 p-value=0.009, OR 2.254, 95%CI 1.245-4.082 and χ^2 p-value=0.031, OR 1.973, 95% CI 1.074-3.626-Supplementary Table 8.1).

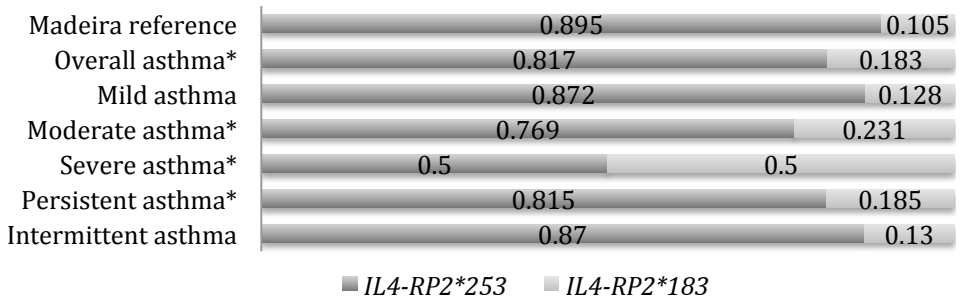
a ***IL13-c.144***



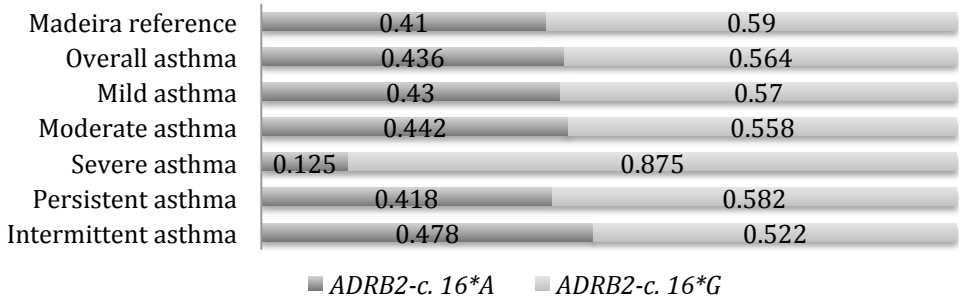
b ***IL4-590***



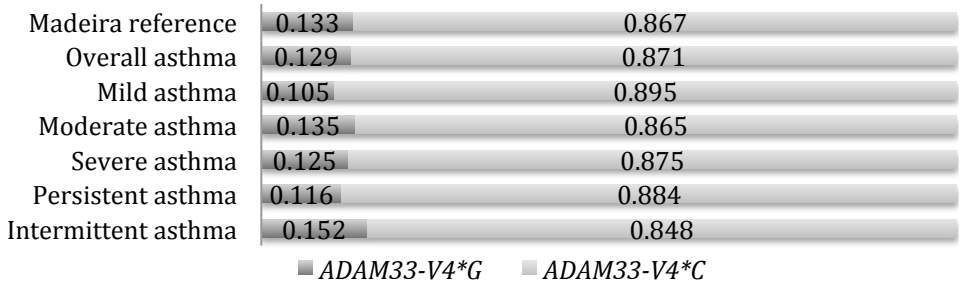
c ***IL4-RP2***



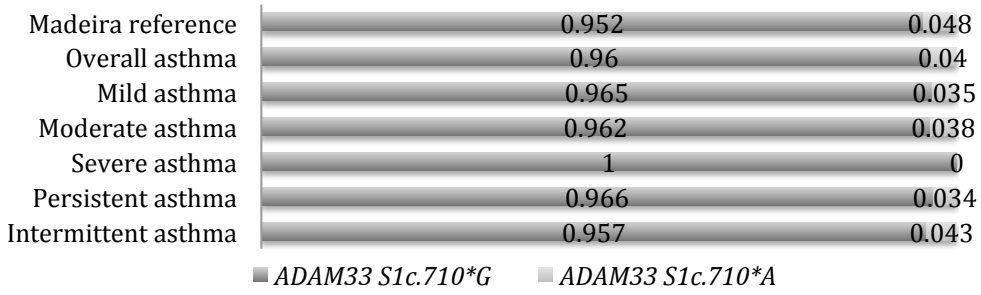
d ***ADRB2-c.16***



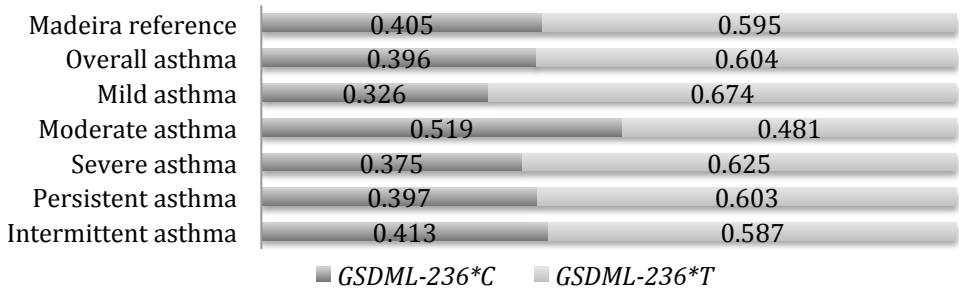
e ***ADAM33-V4***



f ***ADAM33 S1c.710***



g ***GSDML-236***



h ***STAT6-21***

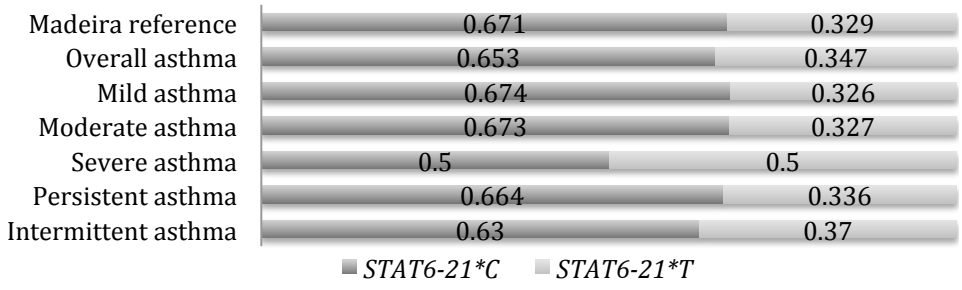


Figure 16. Allele frequencies for the Madeira reference sample set, overall asthma and subgroups of asthma severity: a) *IL13-c.144*; b) *IL4 -590*; c) *IL4-RP2*; d) *ADRB2-c.16*; e) *ADAM33-V4*; f) *ADAM33-S1 c.710*; g) *GSDML-236*; h) *STAT6-21*.

Statistically significant differences in relation to the Madeira reference set are signaled (*).

When excluding from the analysis the Madeira reference sample set further significant differences were found for both genotype and allele frequencies within the asthma severity categories.

Both *IL4-590* and *IL4-RP2* polymorphisms appear to contribute to severe asthma, when compared to mild and moderate forms clustered together (χ^2 p-values=0.012 and 0.010, OR 22.667, 95%CI 1.125-456.809, for genotype count and for allele count, χ^2 p-value=0.034, OR 4.308, 95%CI 1.010-18.367 and χ^2 p-value=0.018, OR 5.000, 95%CI 1.165-21.543, respectively for each polymorphism. Genotypes *IL4-590*TT* and *IL4-RP2*183183* and allele *IL4-590*T* and *IL4-RP2*183* are more frequent amongst the severe group, compared with mild to moderate forms of asthma. However, these polymorphisms are no longer associated to moderate or persistent asthma (p>0.05).

When comparing mild with moderate asthma, significant differences were found for *GSDML-236*, as a significantly lower count of *GSDML-236*TT* genotype and *GSDML-236*T* allele were found amongst the second group, compared to the first (χ^2 p-value=0.048, OR 0.249, 95%CI 0.079-0.783 and χ^2 p-value=0.024, OR 0.447, 95%CI 0.221-0.906) for genotype and allele count, respectively). Similarly, the comparison between mild and the combined subgroups comprising moderate and severe asthma revelead that for *GSDML-236* there is a significantly lower number of both *GSDML-236*TT* genotype and *GSDML-236*T* allele amongst the group including more acute forms of the disease (χ^2 p-value=0.042, OR 0.254, 95%CI 0.082-0.786 and χ^2 p-value=0.034, OR 0.483, 95%CI 0.245-0.951, respectively), when compared to mild forms.

A summary of the significant associations found amongst asthma severity groups is reported in Table 8.

Table 8. χ^2 p-value and OR (95%CI) to test for genotypic and allelic differences within the group of asthmatics for each polymorphism. Statistically significant relationships are shown.

	IL4-590	***IL4-RP2***	***GSDML-236***
Severe *vs.* Mild - Moderate (genotype)	0.012 **OR** 22.667 (1.125-456.809)	0.010 **OR** 22.667 (1.125-456.809)	
Severe *vs.* Mild - Moderate (allele)	0.034 **OR** 4.308 (1.010-18.367)	0.018 **OR** 5.000 (1.165-21.543)	
Mild *vs.* Moderate-Severe (genotype)			0.042 **OR** 0.254 (0.082-0.786)
Mild *vs.* Moderate (genotype)			0.048 **OR** 0.249 (0.079-0.783)
Mild *vs.* Moderate (allele)			0.024 **OR** 0.477 (0.221-0.906)
Mild *vs.* Moderate-Severe (allele)			0.034 **OR** 0.483 (0.245-0.951)

The genotype frequencies across overall asthma and the Madeira reference sample set were also analysed by binary logistic regression (Table 9). The dependent variable (the

outcome) was defined as to be or not asthmatic while the categorical covariates (predictors) consisted of all possible genotypes of each polymorphism. Only the variable *IL4-590*CC* was found to significantly contribute to the prediction of the outcome, as these homozygotes are more likely to belong to the group of non-asthmatics. Combined haplotypes of *IL4-590*CC* and *IL4-RP2*253253* are also significantly more frequent in the Madeira reference sample set (Table 9).

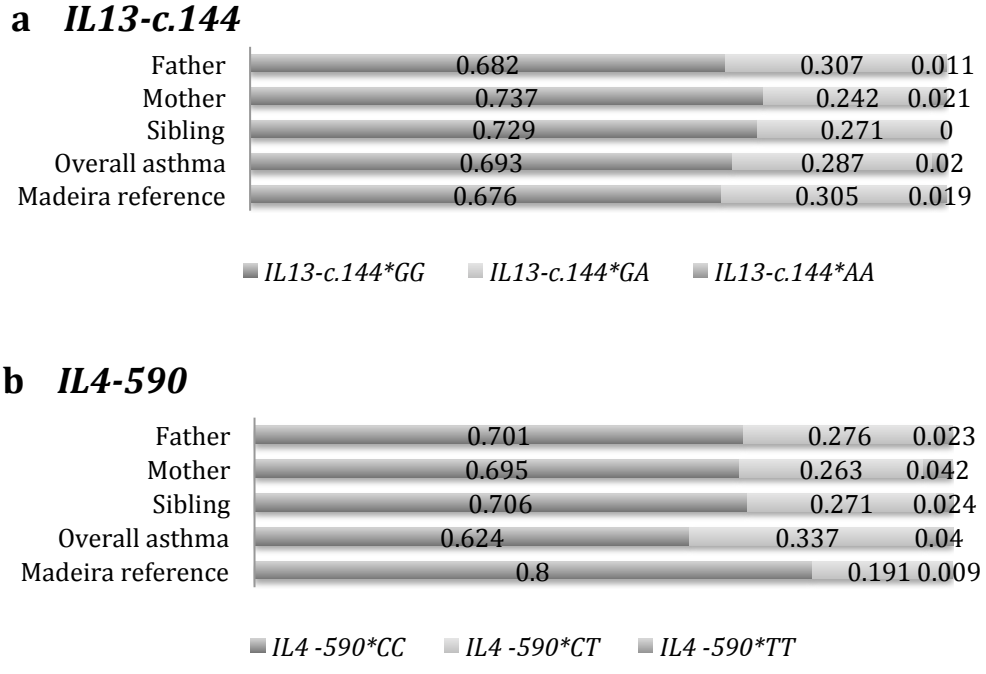
Table 9. Binary logistic regression for genotypic frequencies in asthma patients and Madeira reference sample. Odds ratio (Exp B) at 95% CI is shown.

		p-value	95% CI for Exp (B)		
	B(SE)		Lower	Exp (B)	Upper
Constant	-0.226 (0.169)				
*IL4-590*CC*	0.645 (0.308)[a]	0.035	1.042	1.906	3.486
Constant	-0.199 (0.164)				
*IL4-590*CC /IL4-RP2*253253*	0.651 (0.324)[b]	0.042	1.017	1.918	3.616

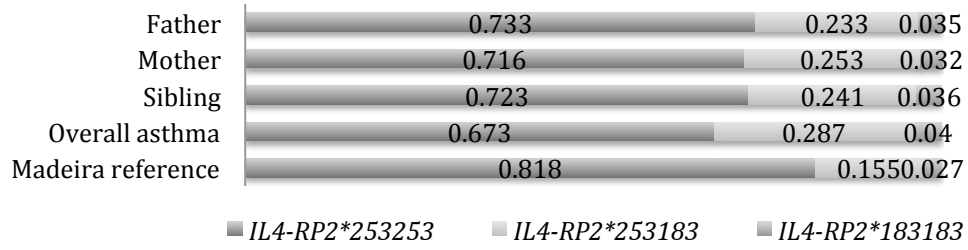
[a]Note: R^2= 0.022 (Cox &Snell); 0.029 (Nagelkerke). Model $\chi 2$ (1)= 4.465; [b]R^2= 0.020 (Cox &Snell); 0.027 (Nagelkerke). Model $\chi 2$ (1)= 4.465.

4.2.2. Family-based study

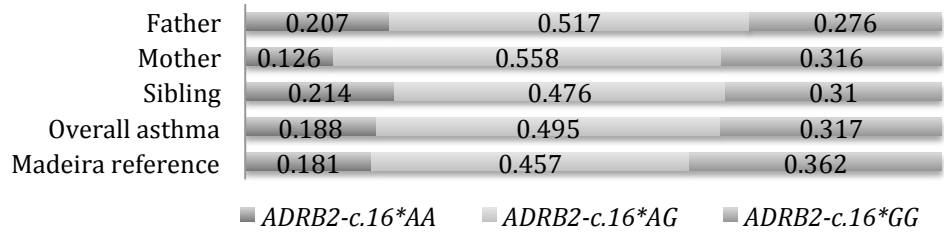
Both the genotype and allele frequencies for each family member were found to be similar to those reported for overall asthmatics (χ^2 p-value >0.05, Figure 17 and Supplementary Tables 9, 9.1,10 and 10.1).



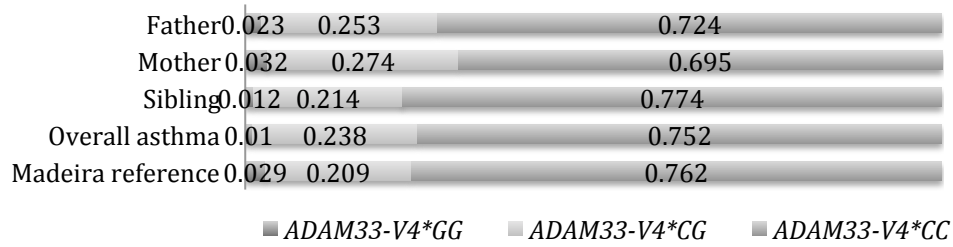
c ***IL4-RP2***



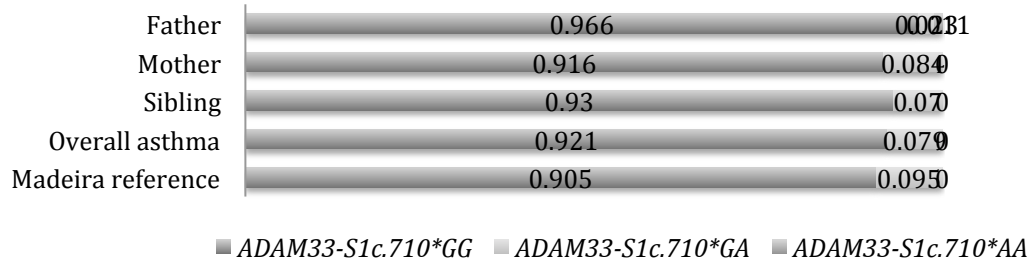
d ***ADRB2-c.16***



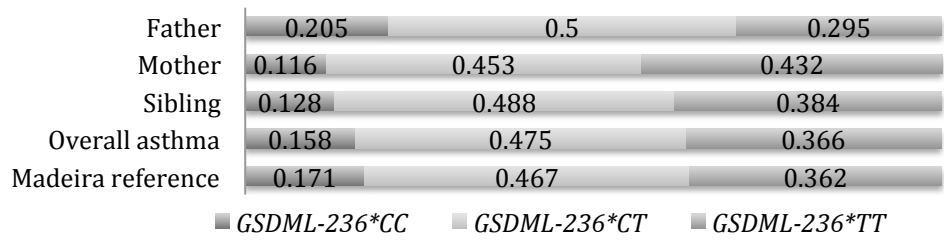
e ***ADAM33-V4***



f ***ADAM33-S1c.710***



g ***GSDML-236***



h *STAT6-21*

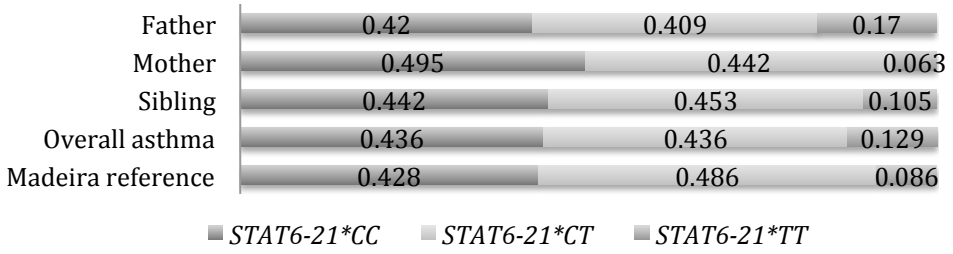
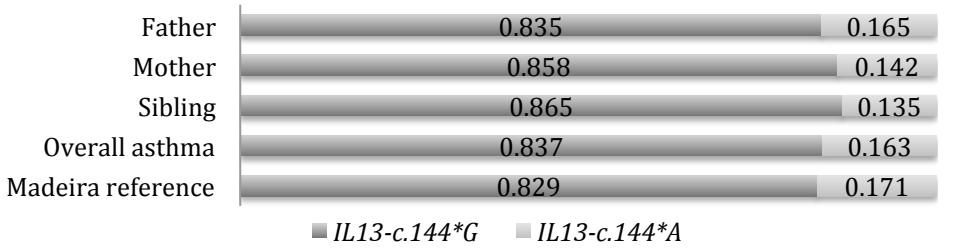


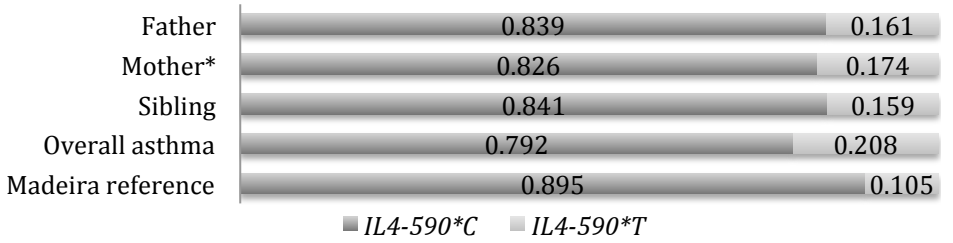
Figure 17. Genotype frequencies for the family members- father, mother and siblings. Overall asthma and Madeira reference genotypes are also shown for comparisons: a) *IL13-c.144*; b) *IL4 -590*; c) *IL4-RP2*; d) *ADRB2-c.16*; e) *ADAM33-V4*; f) *ADAM33-S1 c.710*; g) *GSDML-236*; h) *STAT6-21*. Statistically significant differences are signaled with (*), with reference to the Madeira reference set.

No statistically significant differences were found between overall asthma and each family member regarding the studied polymorphisms. However, when comparing the family members with the Madeira reference set, a significant difference was found for the allelic frequency of *IL4-590*, regarding the Mother group (χ^2 p-value=0.042, OR 1.800, 95%CI 1.06-3.190), where a significantly higher than expected number of *IL4-590*T* alleles was found (Figure 18).

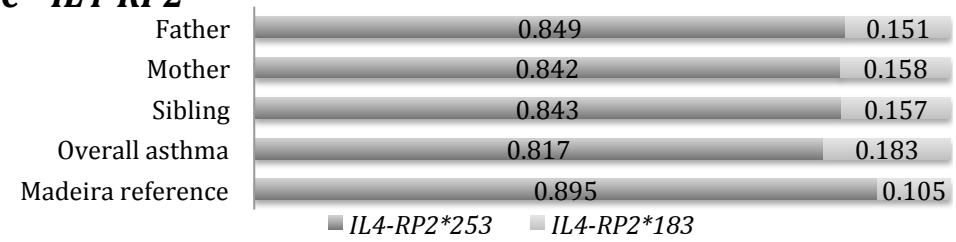
a *IL13-c.144*



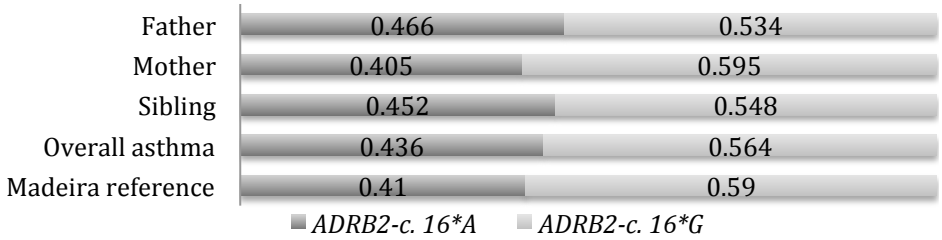
b *IL4-590*



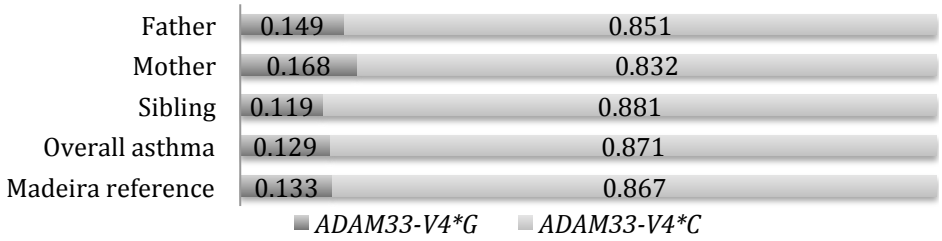
c *IL4-RP2*



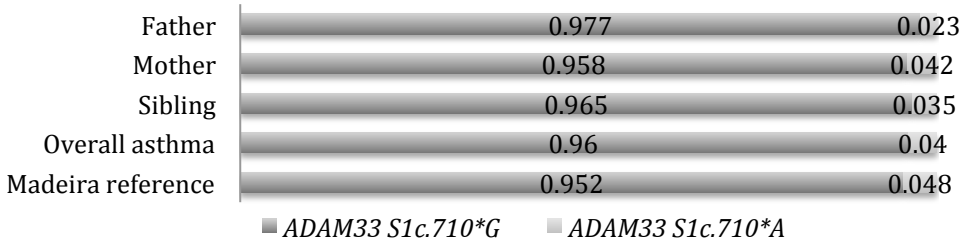
d *ADRB2-c.16*



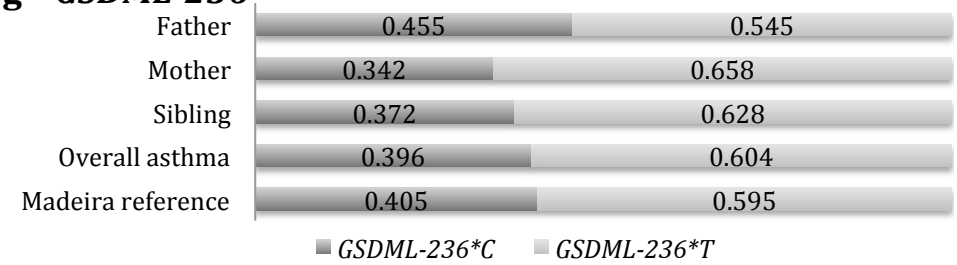
e *ADAM33-V4*



f *ADAM33-S1c.710*



g *GSDML-236*



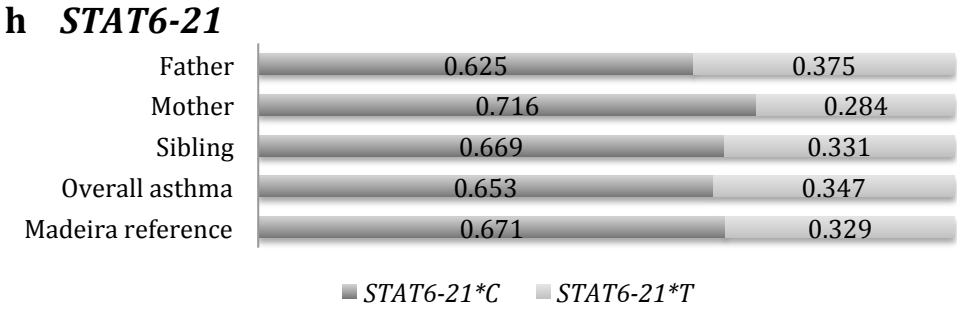


Figure 18. Allele frequencies for the family members- father, mother and siblings. Overall asthma and the Madeira reference set are also shown for comparisons: a) *IL13-c.144*; b) *IL4-590*; c) *IL4-RP2*; d) *ADRB2-c.16*; e) *ADAM33-V4*; f) *ADAM33-S1 c.710*; g) *GSDML-236*; h) *STAT6-21*. Statistically significant differences are signaled with (*), with reference to the Madeira sample set.

The TDT was performed by using data from heterozygous parents for each marker and by comparing the allele transmission frequency among asthmatics (Table 10). The TDT intends to test for linkage between disease and each genetic marker, by over-transmission of an allele from heterozygous parents to the affected offspring.

Only one significant TDT p-value was found for the genetic marker *ADAM33-V4* (p-value=0.033), suggesting linkage to asthma, despite the previous association tests resulted non-significant (Figures 15e, 16e and Supplementary Tables 7.1 and 8.1). Contrariwise both *IL4-590* and *IL4-RP2*, systematically reported as associated to the disease (Figures 15b, 15c and 16b, 16c, correspondingly) were not significant for the TDT (p-value= 0.085 and 0.092, respectively-Table 10).

Table 10. TDT from heterozygous parents to asthmatic offspring for each marker. The p-value and the OR (95%CI) are shown.

Marker	n (Families)	TDT p-value	OR (95%CI)
IL13-c.144	39	0.437	1.105 (0.594-2.056)
IL4-590	48	0.085	1.524 (0.879-2.642)
IL4-RP2	42	0.092	1.556 (0.861-2.812)
ADRB2-c.16	58	0.500	1.029 (0.646-1.638)
ADAM33-V4	42	0.033	1.867 (0.997-3.495)
ADAM33-S1 c.710	11	0.274	1.750 (0.512-5.978)
GSDML-236	57	0.358	1.125 (0.699-1.811)
STAT6-21	61	0.171	1.290 (0.807-2.062)

4.3. Genetic profiles and haplotypes

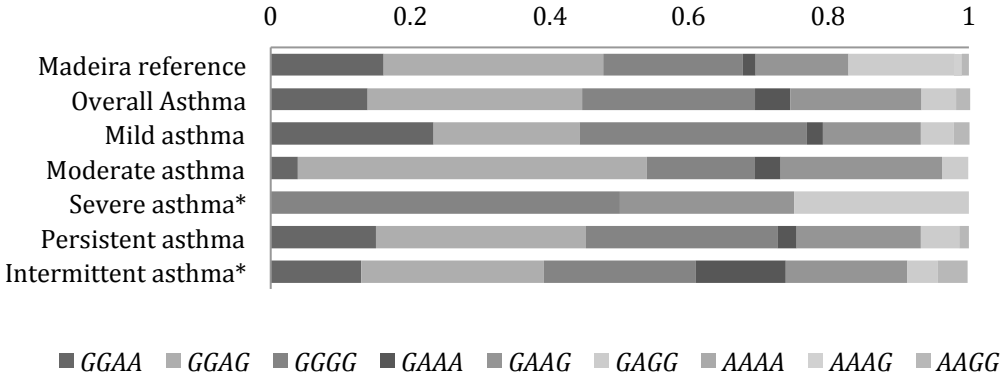
The genetic profiles for all possible pairs of polymorphisms were determined and the profiles obtained are described for overall asthmatics and by category of asthma severity, compared with the Madeira reference set. Significant differences were found in the distribution of frequencies for *IL13-c.144* and *ADRB2-c.16* pair of *loci* for severe and intermittent asthma (Fisher p-values <0.05, Figure 19a), when compared against the Madeira reference sample set. In severe asthma the profile *GGGG* is the most common (0.500), against a frequency of 0.200 in the Madeira reference sample set. As for intermittent asthma, the most frequent profile is *GGAG* (0.261), the same as in the Madeira reference set (0.314). However, the profile *GAAA* is approximately seven times higher in frequency amongst intermittent asthma when compared to the Madeira reference sample set (Figure 19a and Supplementary Table 11). No other significant differences were detected for the remaining *loci* paired with *IL13-c.144*. As for *IL4-590*, significant differences were also observed when combined with *IL4-RP2* (for overall asthma, moderate and severe asthma, Figure 19b) and *STAT6-21* (for severe asthma, Figure 19c). Regarding the first pair of *loci*, the profile *CT183253* is more frequent amongst overall asthma (0.287) when compared to the Madeira sample set (0.145). Similarly this profile is also higher amongst moderate (0.385) and severe asthma (0.500), in comparison to the reference set. As for the second pair of *loci*, the most common profile amongst severe asthma is *CTCT* (0.500), in contrast with the frequency of 0.124 in the Madeira reference set and where the most commom profile is *CCCT* (0.352).

The combined inheritance of *IL4-RP2* with each *ADRB2-c.16, ADAM33-S1 c.710, GSDML-236* and *STAT6-21*, seems to suggest an association to severe asthma each (Fisher p-values <0.05) (Figures 19d, 19e, 19f and 19g, respectively). For the first combination of polymorphisms, the most common profile represented in the Madeira reference set is *253253AG* (0.356), with no expression in the severe asthma group. In fact, amongst severe asthma, the frequencies are equally distributed by four profiles (*253253GG*, *183253AG*, *183253GG* and *183183GG*). However, this last profile is in proportion twenty five times more common amongst severe asthma (0.250), when compared to the frequency registered for the Madeira reference set (0.010). As for the combination of *IL4-RP2* and *ADAM33-S1 c.710*, the profile *183253GG* acounts for 0.500 among severe asthma, while it represents only 0.173 in the Madeira reference set, where the most commom profile is *253253GG* (0.712). The pair *IL4-RP2* and *GSDML-236* is mainly represented by the profile 253253CT amongst the Madeira reference set (0.375), with severe asthma registering a frequency of 0.250. The profile 183253TT, however, is almost three times more frequent amongst severe asthma when compared to the reference sample. The interaction between *IL4-RP2* and *STAT6-21* has the profile *253253CT* as the most commom occurring in the Madeira reference sample (0.375), but with null expression in the severe asthma subgroup. However, amongst this last subgroup the profile *183253CT* is the most frequent (0.500), opposing to the frequency of 0.096 amidst the Madeira reference group.

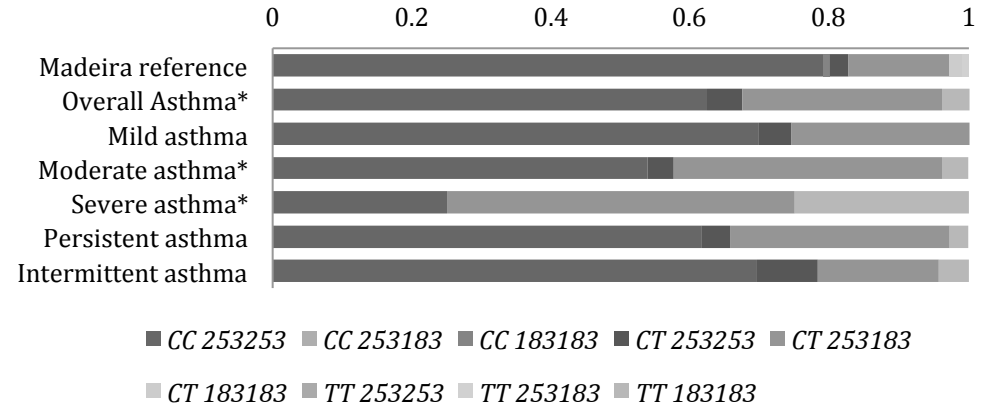
ADRB2-c.16 and *GSDML-236* pair of *loci* also seem to account for the risk of mild asthma (Fisher p-value<0.05, Figure 19h). Amongst the mild asthma subgroup the profile *AGTT* is the most frequent (0.233), against 0.162 in the reference set group. For this last group,

the most commom profile is *AGCT* (0.238) contrasting with a frequency of 0.023 amongst mild asthma. Frequencies are further described in Supplementary Table 11.

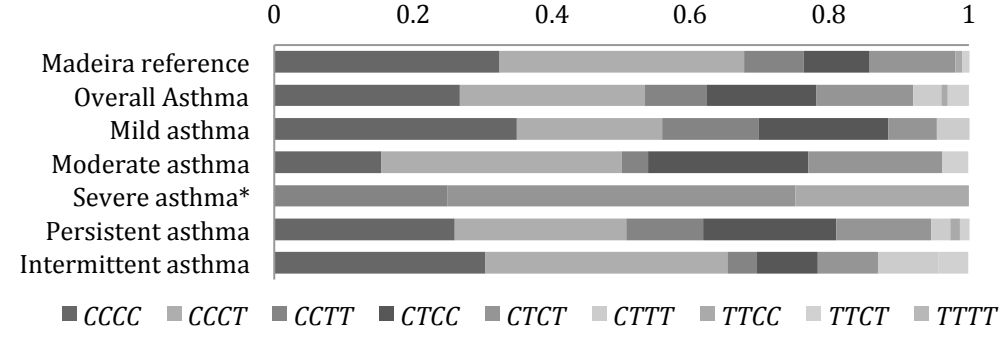
a ***IL13-c.144/ADRB2-c.16***



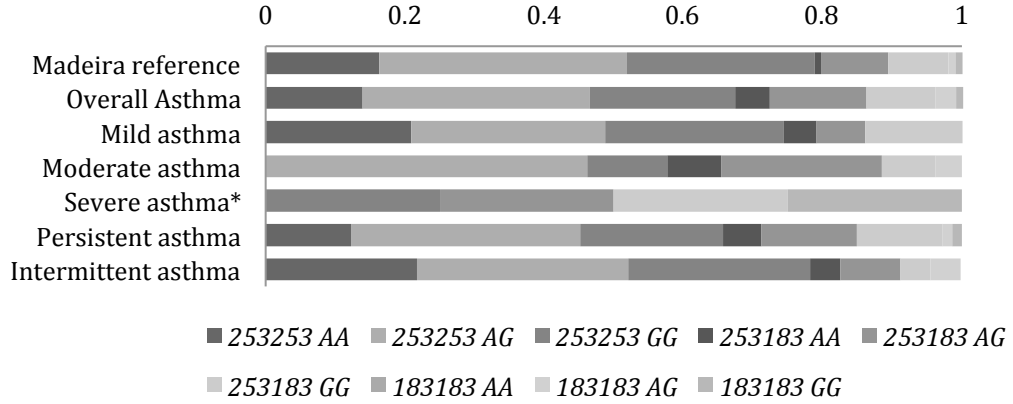
b ***IL4-590/IL4-RP2***



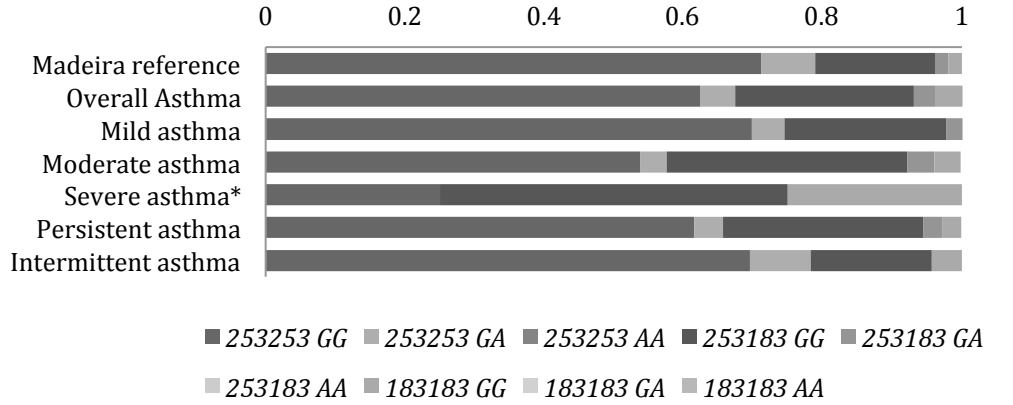
c ***IL4-590/STAT6-21***



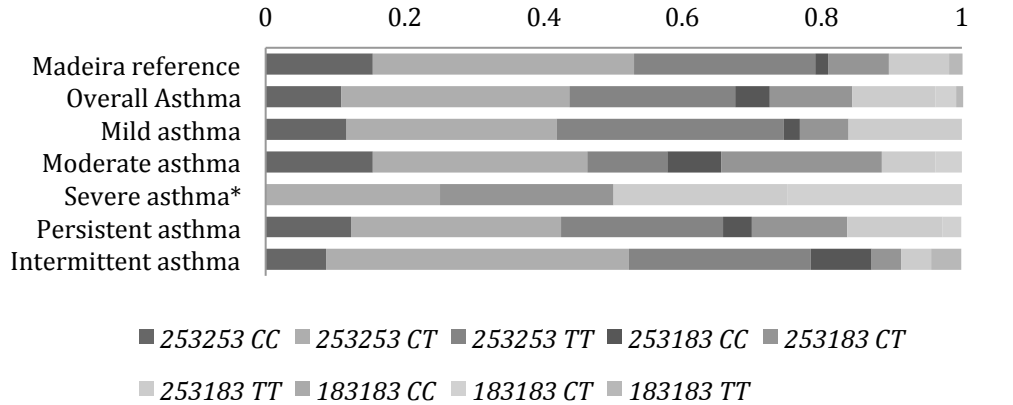
d ***IL4-RP2/ADRB2-c.16***



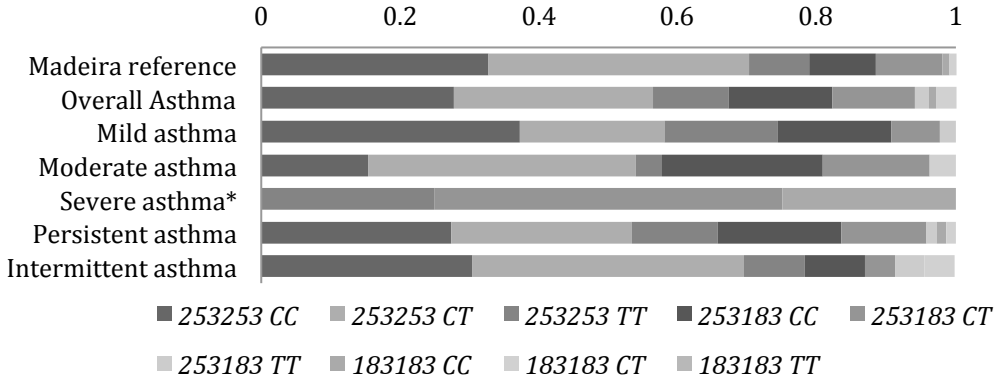
e ***IL4-RP2/ADAM33-S1c.710***



f ***IL4-RP2/GSDML-236***



g ***IL4-RP2/STAT6-21***



h ***ADRB2-c.16/GSDML-236***

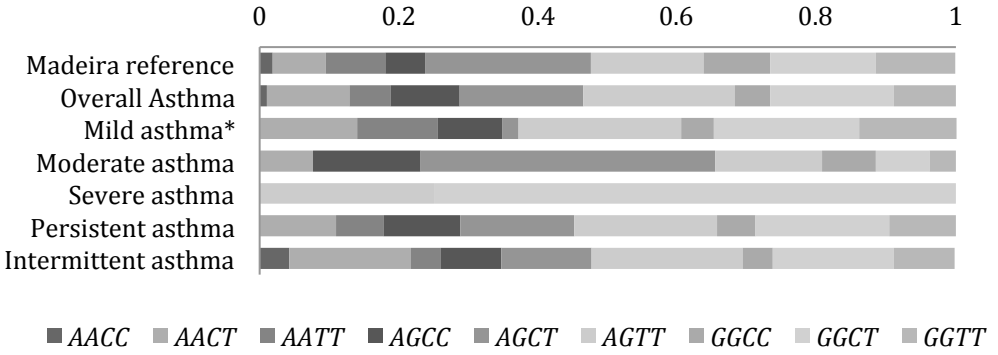


Figure 19. Genetic profile frequencies for the pairs of loci a) *IL13-c.144 /ADRB2-c.16*; b) *IL4-590/IL4-RP2*; c) *IL4-590/STAT6-21*; d) *IL4-RP2/ADRB2-c.16*; e) *IL4-RP2*/ADAM33-S1 c.710; f) *IL4-RP2/GSDML-236*; g) *IL4-RP2/STAT6-21*; h) *ADRB2-c.16/GSDML-236*, for the Madeira reference set, overall asthma and categories of asthma severity. Statistically significant differences, having as reference the Madeira sample set, are signaled by (*).

The polymorphisms at 5q31 were analysed as a block haplotype and its association with asthma was determined through PHASE 2.1. The frequency distribution of the genetic profile at 5q31 in asthma patients (overall asthma and categories according to the severity) is shown in Figure 20 and Supplementary Table 12. No significant differences were found between groups when compared to the Madeira reference set (p-value>0.05).

The most frequent profile amongst the Madeira reference sample set, resulting from the combination of the four polymorphisms at 5q31 is *GGCC253253AG* (0.260), a tendency also registered amidst moderate asthma (0.346). The overall asthma group, the mild asthma subgroup and the persistent asthma cluster share the profile *GGCC253253GG* as occurring in a higher frequency in each (0.168, 0.209 and 0.575, respectively), differing only at the *ADRB2-c.16 locus*. Amongst severe asthma the frequency is equally distributed by four distinct profiles, the first being the above mentioned for overall and mild asthma together with *GGCT183253GG*, *GACT183253AG* and *GATT183183GG*.

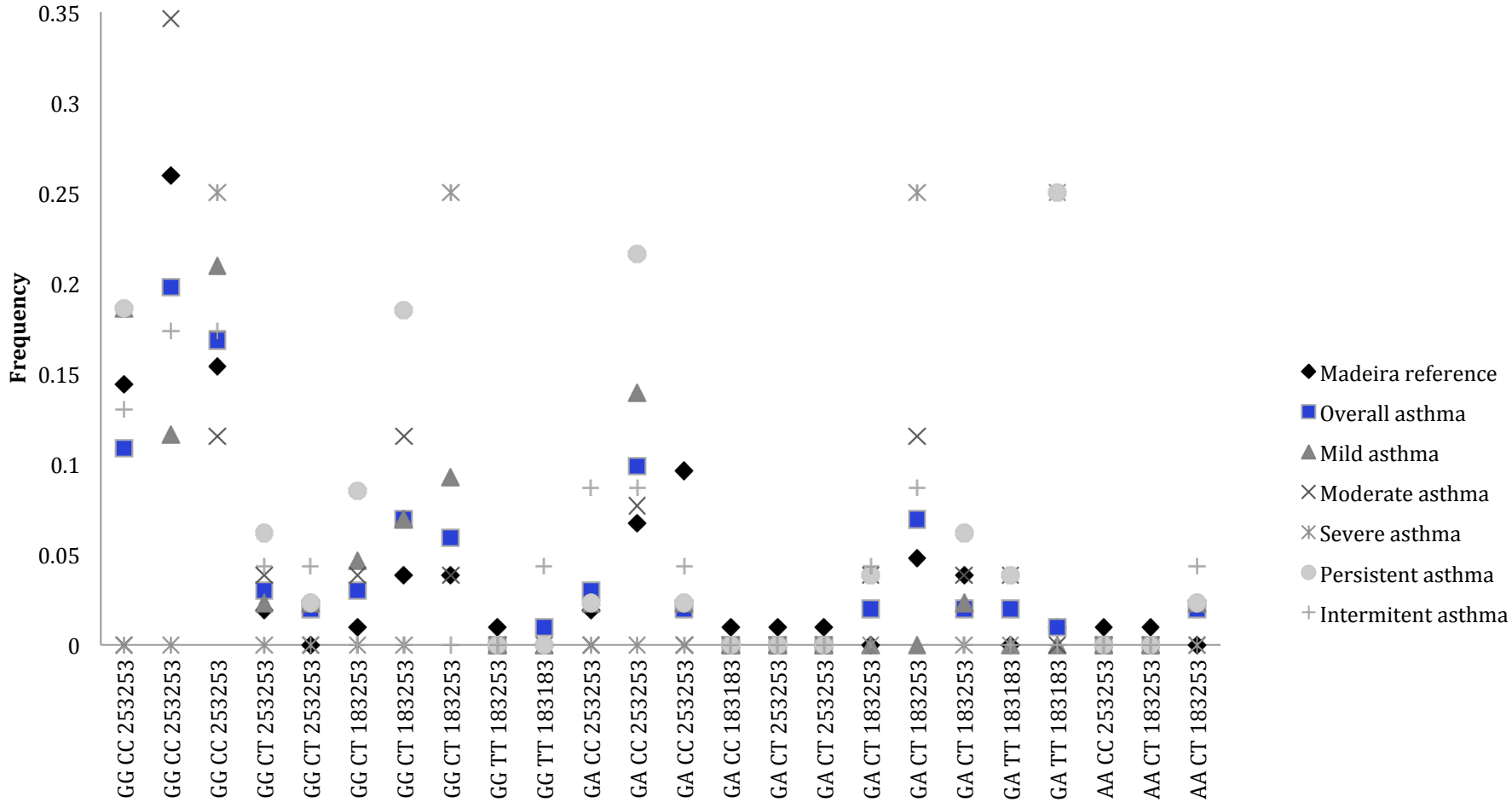


Figure 20. Genetic profile frequencies for genetic markers *IL13-c.144*, *IL4-590*, *IL4-RP2* and *ADRB2-c.16* at 5q31, for the Madeira reference set, overall asthma and each asthma severity category.

The intermittent asthma subgroup presents two genetic profiles in the same frequency, which had already been described as the most frequent amongst the remaining groups (*GGCC253253AG* and *GGCC253253GG*- 0.174, *ex-aequo*) (Figure 20).

In addition, the genetic profiles including all eight polymorphism were determined and their frequency is shown in Figure 21 for the Madeira reference set and overall asthma.

The top section of the Figure illustrates the genetic profiles found to be common to Madeira reference group and asthma patients, despite different frequencies are found. Profile *GGCC253253AACCGGTTCC* is the most frequent amongst the Madeira reference set, (0.048) compared to *GGCC253253GGCCGGCCCT* (0.040) in overall asthma. These last profiles differ for *ADRB2-c.16*, *GSDML-236* and *STAT6-21 loci* (Figure 21).

The lower sections of the figure illustrate genetic profiles exclusively found in the Madeira reference set and overall asthma patients, respectively. The most common profile occurring only in Madeira reference sample set is *GGCC253253AGCCGGCTCT* (0.058), while in asthma patients the exclusive profile *GGCC253253AACCGGCTCC* appears with the highest frequency (0.040). These profiles differ on the *ADRB2-c.16* and *STAT6-21* SNPs, as in the Madeira population reference set both polymorphisms appear in the heterozygous form, compared to the homozygous form in overall asthma (Figure 21).

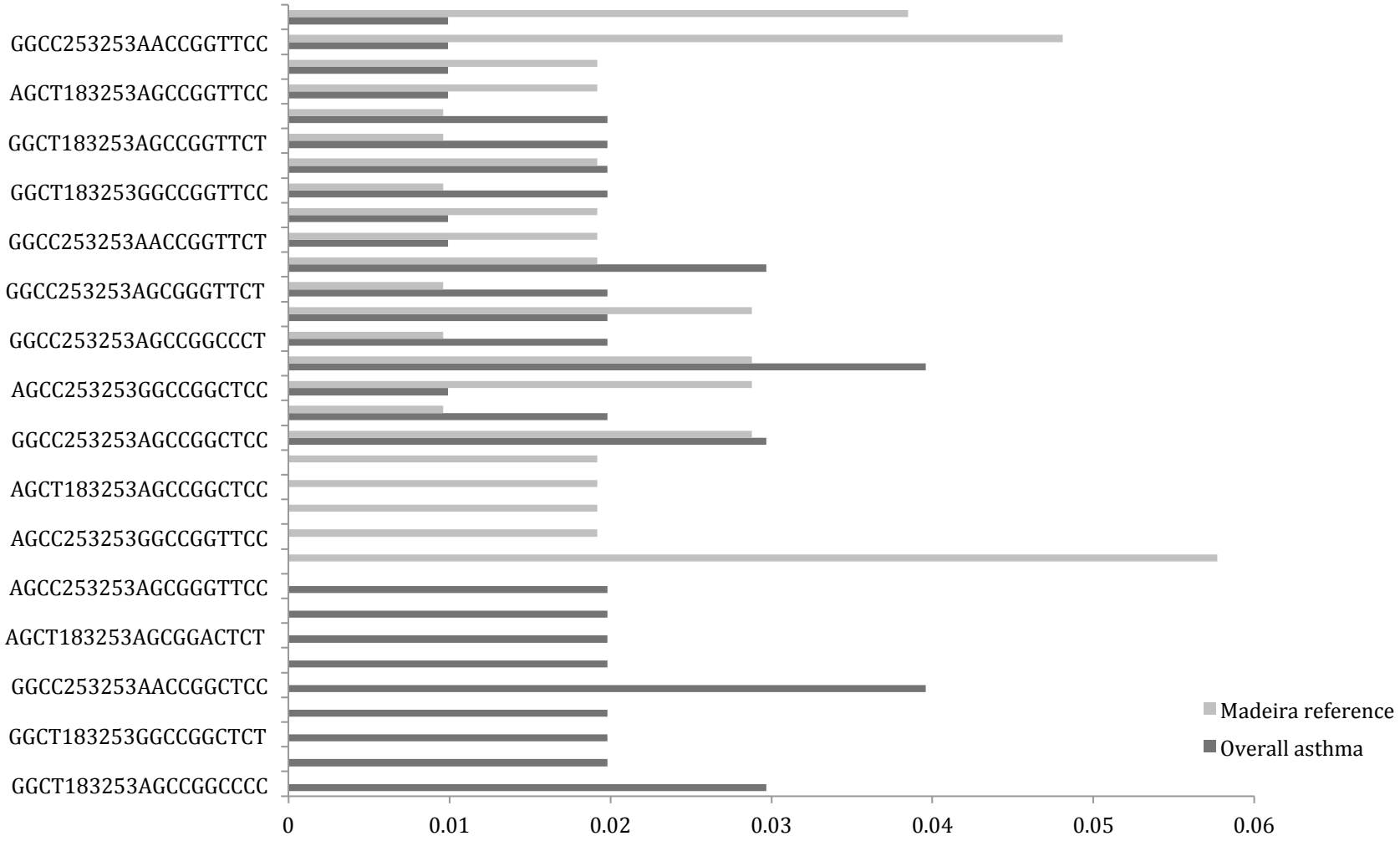


Figure 21. Genetic profiles (Madeira reference set *versus* overall asthma) based on the eight studied polymorphisms (*IL13-c.144*, *IL4-590*, *IL4-RP2*, *ADRB2-c.16*, *ADAM33-V4*, *ADAM33-S1 c.710*, *GSDML-236*, *STAT6-21*). Exclusive profiles and commom profiles presenting each a frequency <0.0099 are not represented.

4.4. Environmental and genetic factors

Taking into account the significant associations detected between asthma severity and both environmental and genetics factors, several models of logistic regression were explored in order to predict asthma severity based precisely on combinations of environmental and genetic variables whose association to asthma severity were previously found to be significant.

Therefore, the factors and covariates considered for the categories of mild, moderate and severe asthma were the following: "only mother with rhinitis", "father passive smoking", *GSDML-236*; "pasta intake", "legume intake", *IL4-590*, *IL4-RP2*, *GSDML-236*; "parents with wheezing", "*Lepidoglyphus sp.* wheal size", "dog allergen wheal size", *IL4-590* and *IL4-RP2*", respectively. For the categories of persistent and intermittent asthma, the factors taken into account were: "only mother with rhinitis", "urban environment", "weekly fish intake", "weekly pasta intake", *IL4-590*, *IL4-RP2*; "house humidity", "potable water", "electricity", "house floor of tiles", and "positive skin prick tests for *Platanus sp.*".

A binary logistic regression model for the dependent variables "Mild *versus* Moderate asthma", Mild *versus* Severe asthma", "Moderate *versus* Severe asthma", Mild *versus* Moderate/Severe asthma", and "Persistent *versus* Intermitent asthma" was explored. The combined effect of the environmental and genetic factors in analysis was also subject of examination.

For the dependent variable defined as to have either persistent or intermittent asthma, three variables were validated as potencial predictors. First, living in a urban environment was found to significantly contribute to the presence of persistent asthma (p-value= 0.030, Exp (B)=5.435 (1.173-25.173), while the presence of "house floor of tiles" setting is less likely to occur amongst persistent asthma (p-value=0.013, Exp (B)= 0.244, 95%CI (0.080-0.746), similarly to "positive skin prick tests for *Platanus sp.*", whose presence is significantly less common amongst the above mentioned group (p-value=0.020, Exp (B)= 0.104, 95% CI (0.016-0.700). These outcomes support the results previously obtained through the χ^2 test (see section 5.1.3. and 5.1.5.) However, no interactions between the environmental and genetic factors taken in consideration for this analisys were found to be predictors of asthma severity through the explored model of binary logistic regression. Significant associations are summarized in Table 11.

Table 11. Binary logistic regression including environmental variables involved in the prediction of asthma severity. The B coefficient and the respective standard error (SE), significant p-values and the odds ratio (Exp B) at 95% CI are shown.

			95% CI for Exp (B)		
	B(SE)	**p-value**	**Lower**	**Exp (B)**	**Upper**
Persistent *vs.* Intermittent					
Constant	0.833 (0.268)				
[a)] Urban environment	1.693 (0.782)	0.030	1.173	5.435	25.173
Constant	1.483 (0.979)				
[b)] House floor tiles	-1.410 (0.570)	0.013	0.080	0.244	0.746
[c)] Positive skin prick tests *Platanus sp.*	-2.259 (0.970)	0.020	0.016	0.104	0.700

a) $\mathbf{R^2}$= 0.067 (Cox &Snell); 0.101 (Nagelkerke). Model χ^2 (2)= 6.667; **b)** R^2= 0.156 (Cox &Snell); 0.234 (Nagelkerke). Model χ^2 (4)=16.285; **c)** $\mathbf{R^2}$= 0.156 (Cox &Snell); 0.234 (Nagelkerke). Model χ^2 (4)=16.285.

Following, an ordinal logistic regression model, comprising the mild, moderate and severe asthma subgroups as the categories of the dependent variable was performed against each studied environmental variable, filtered by polymorphism (Table 12).

First, the presence of birds as pets, ever, is inversely related to the presence of more severe forms of asthma, for individuals with *ADAM33-V4*CC* genotype (p-value**=** 0.008, Exp (B)= -6.511, 95%CI (-11.331 to -1.690) and genotype *ADAM33-S1c.710*GG* (p-value= 0.009, Exp (B)=-5.328, 95%CI (-9.324 to -1.332). The presence of cockroaches, however, increases the odds of the outcome, within the subgroups *ADAM33-V4*CC* (p-value<0.05, Exp (B)= 13.863, 95%CI (6.080-21.646), *ADAM33-S1c.710*GG* (p-value<0.05, Exp (B)=13.400, 95%CI (6.501-20.314) and *IL13-c.144*GG* (p-value=0.006, Exp (B)= 12.201, 95%CI 3.434-20.968)-Table 12.

Individuals within the 85th-95th percentile, with the *ADAM33-S1c.710*GG* genotype, also seem to significantly contribute to the prediction of asthma severity (p-value=0.006, Exp (B)= 7.789, 95%CI (2.270-13.307). Similarly, the odds of the outcome occurring are increased by legumes intake only once or twice a week, either for subgroup *ADAM33-S1c.710*GG* (p-value=0.010, Exp (B)= 5.372, 95%CI (1.394-10.071) and *IL13-c.144*GG* (p-value=0.020, Exp (B)= 19.460, 95%CI (3.128-35.792); pasta intake (either once or twice a week or three or more times per week, p-value= 0.015, Exp (B)= 8.964 (1.744-16.184) and fast-food once or twice a week (p-value=0.028 Exp (B)= 9.730, 95%CI (1.062-18.397) are also positively related to an increase in asthma severity, given the presence of genotype *ADAM33-S1c.710*GG*. However, both butter comsumption once or twice a week and margarine intake three or more times per week, for patients exhibiting the aforementioned genotype, are inversely correlated with the display of severe forms of asthma (p-value=0.006, Exp (B)= -8.041, 95%CI (-13.721 to -2.360) and p-value=0.022, Exp (B)= -15.336, 95%CI (-28.431 to -2.241), respectively (Table 12).
Positive skin prick tests for *Blomia sp.* house dust mite extract, are positively correlated with more severe forms of asthma (p-value=0.030, Exp (B)= 13.229, 95%CI (1.266-25.192), as well as fruit comsumption only one or twice a week (p-value=0.024; Exp (B)= 22.181, 95%CI (2.935 - 41.427), for subgroup *IL13-c.144*GG* (Table 12).

No further significant interactions between predictors were found accounting for asthma severity (p-value>0.05).

Table 12. Ordinal logistic regression in the prediction of asthma severity. The outcome (mild, moderate, severe) was analysed in relation to the environmental variables, by subgroup of polymorphism. Significant p-values, Exp (B) and 95% CI for Exp (B) are shown.

		95% CI for Exp (B)		
ADAM33-V4	**p-value**	**Lower**	**Exp (B)**	**Upper**
[a)] CC*Birds	0.008	-11.331	-6.511	-1.690
[a)] CC*Cockroaches	<0.05	6.080	13.863	21.646
ADAM33-S1 c.710				
[b)] GG*Birds	0.009	-9.324	-5.328	-1.332
[b)] GG*Cockroaches	<0.05	6.501	13.400	20.314
[c)] GG*85th-95th	0.006	2.270	7.789	13.307
[c)]GG*Legumes 1-2x/week	0.010	1.394	5.372	10.071
[c)]GG*Pasta (1-2 and 3x-more/week)	0.015	1.744	8.964	16.184
[c)]GG*Butter 1-2x/week	0.006	-13.721	-8.041	-2.360
[c)]GG*Margarine 3x-more/week	0.022	-28.431	-15.336	-2.241
[c)]GG*Fast Food 1-2x/week	0.028	1.062	9.730	18.397
IL13-c.144				
[d)]GG*Cockroaches	0.006	3.434	12.201	20.968
[e)]GG**Blomia sp.*	0.030	1.266	13.229	25.192
[f)]GG*Fruit 1-2x/week	0.024	2.935	22.181	41.427
[f)]GG*Legumes 1-2x/week	0.020	3.128	19.460	35.792

[a)] $\mathbf{R^2}$=0.550 (Cox &Snell);0.682 (Nagelkerke).Model χ^2(23)= 45.494; [b)] $\mathbf{R^2}$= 0.482 (Cox &Snell); 0.591 (Nagelkerke). Model χ^2 (25)= 44.794; [c)] $\mathbf{R^2}$= 0.819 (Cox &Snell); 1.000 (Nagelkerke). Model χ^2 (38)=112.941; [d)] $\mathbf{R^2}$= 0.772 (Cox &Snell); 0.974 (Nagelkerke). Model χ^2 (25)=78.286; [e)] $\mathbf{R^2}$= 0.750 (Cox &Snell); 0.946 (Nagelkerke). Model χ^2 (33)=73.372; [f)] $\mathbf{R^2}$= 0.795 (Cox &Snell); 1.000 (Nagelkerke). Model χ^2 (36)=82.296.

5. Discussion

5.1. Epidemiological evaluation of asthma by questionnaire

5.1.1. Demographics

The evaluation of both the FEV_1 and FVC indexes has shown significant differences between mild with each moderate and severe asthma, but not between moderate and the severe form of the disease. Though a number of studies suggest the FEV_1 and FVC indexes as good indicators of the disease severity (Ramsey *et al.* 2005, Kim *et al.* 2009, Forno & Celedón 2012), other studies have reported the opposite, as abnormal values of both indicators have been found amongst children with severe asthma (Ratageri *et al.* 2001) and interestingly, when classifying asthma severity based on the more severe frequency of symptoms, both the predicted percentage of FEV_1 and FVC did not show differences between the most severe forms of asthma (Bacharier *et al.* 2004). A possible explanation for these observations lies in the complexity of the asthma severity classification, since it is influenced by a number of factors varying over time and possibly confounded by the present asthma treatment (Bacharier *et al.* 2004). Therefore and given the intricacy underlying the definition of asthma severity allied to the importance of the severe asthma subgroup in what it concerns the disease burden (Gaspar *et al.* 2006) the subgroups were analysed as according to the doctor's diagnosis, as the reliance in a sole parameter was not considered sufficient to encompasse the many factors probably outlining the severity of the disease.

5.1.2. Asthma symptoms

Although the identification of asthma by questionnaire remains contentious, asthma surveys conducted in the community have relied greatly on responses to standardized questionnaires (Smeeton *et al.* 2006). The epidemiological evaluation of asthma, assessed by questionnaire adapted from ISAAC was applied to both patients and their family members.

Some interesting aspects have been remarked. The symptom regarding the presence of itchy and watery eyes over the previous 12 months has found to be significantly lower both in 11-13 and 14-17 year-old, when compared to the adult subgroup. Similarly, the 14-17 age group has reported a significantly higher number of non-affected individuals in their daily activities by the symptomatic triad sneeze attacks, runny nose or nasal congestion, compared to the 18-25 age subgroup. In addition, 6 to 10 year-old are the most affected regarding the number of wheezing attacks in the last 12 months, when compared to the remaining age groups.

First, it should be mentioned that the understanding of ''wheezing'' in the community, regardless of cultural background, is essential in the assessment of asthma prevalence and its risk factors (Smeeton *et al.* 2006). A study of parents who had asthmatic children showed wide variation in the conceptualization of the word ''wheeze''(Smeeton *et al.* 2006). Second, the interpretation of the symptoms might vary if reported by adults, adolescents or mothers reporting on behalf of their children (Smeeton *et al.* 2006).

Given the age range of the Madeiran patients, it is possible that these three situations might have occurred, conferring potential biased results.

In addition, adolescents seem to be the age group where the above mentioned asthma symptoms are reported in a lower frequency, compared to the remaining groups. In fact, despite adolescents have a better understanding of the disease than children, they tend to underestimate the severity of their symptoms as they prefer not to admit it causes interferences with their daily life, as it might affect their relationship with their pairs (Câmara & Marques 2003).

The PAC study conducted in Madeira, in 1995, agreed with the ISAAC evaluation of wheezing in the last 12 months of 14.7% amongst 6-10 year-old and only 10.6% within 13-14 year-old adolescents (Câmara & Marques 2003). In Madeira patients, the percentages are evidently much higher, but a similar tendency in the difference registered between 6 to 10 year-old (82.2%) and adolescents (69.2%) may further support the previous explantion for the observed differences between subgroups.

Despite not reflecting a statistically significant interaction, female patients reported to be very much affected by sneeze attacks, runny nose or nasal congestion (apart from having a cold or flu ever and in the last 12 months) almost 3 times more than male asthmatics, whereas the frequency of female patients reporting to be very little affected in their daily activities by these symptoms was in proportion only half of that reported by male asthmatics. In fact, perception of asthma symptoms is subjective and varies widely (Cydulka *et al.* 2001). According to these authors, women were more likely than men to report "severe" complaints in terms of symptom frequency, intensity, and resulting activity limitations (Cydulka *et al.* 2001). Women with moderate exacerbations were especially likely to describe their exacerbation as causing "severe" activity limitations (Cydulka *et al.* 2001). These distinctions between genders may be the result of cultural patterns in which men could tend to underestimate their illness (Nowobilski *et al.* 2011). In fact, social issues might explain these differences. It has been postulated that men may be more reluctant to seek health care or to acknowledge symptoms because they believe that they are too busy working, the symptoms may not seem severe enough to them, or they ignore symptoms or illnesses until the health problem is so acute that they are unable to ignore it any longer, while it has been suggested that women have greater attentiveness to bodily changes (Cydulka *et al.* 2001). Other studies suggest that women may experience increased symptoms and distress in response to a given level of reduced pulmonary function compared to men (Schatz *et al.* 2006).

When asked about in which of the previous 12 months the asthma patient's nose problem had occurred, a pattern emerged: a peak around March and April, a gradual decay until August and a new gradual increase on the percentage of cases towards the end of the year was transversal to all analysed subgroups. This is not surprising, considering Madeira's large pollinic season (Câmara & Marques 2003). In fact, the highest pollen concentration occurs on springtime and autumn, with *Urticaceae* and *Pteridophyta* being the most prevalent pollen families with a mean grain pollen/m^3/year of 1246 (Oliveira *et al.* 2004). Additionally, the highest incidence of mould spores, being the most prevalent *Cladosporium* occurs on springtime, contributing to the allergic

disease prevalence in Madeira population (asthma 14% and rhinitis 20% - ISAAC – 1st phase) (Oliveira *et al.* 2004). There is a consensus about the strong association between the increase in pollens and the increase in allergic diseases in relation to the sensitizing, being rhinitis and asthma amongst those manifestations (Chappard *et al.* 2004).

The analysis of the nose problem by age group revelead that children aged 6 to 10 register systematically a higher number of cases in May, June, and November, compared to pre-adolescents and to adolescents in May, June, July and August. Once again from June to July, adolescents report a significantly lower frequency for the nasal symptoms when compared to adults. Once again these differences between children and both pre-adolescents and adolescents, may be a reflection of the way each age group perceives and deals with the disease. Alternatively, a decline in the asthma symptoms, in the group of adolescents may have occurred as the prevalence of respiratory symptoms has been observed to be significantly lower during the summer (Koster *et al.* 2011). However this does not explain the higher number of cases amongst 6-10 and 18-25 year-old. Again, factors associated to the perception of the social burden of the disease by adolescents may take them to undervalue their symptoms in order not to affect their social life, as previously proposed. Besides, as also explained before the different age groups also assess their symptoms diversely, leading to possible discrepancies.

Looking at the families' demographics regarding the epidemiological evaluation of asthma, the frequencies for most parameters and for each family member are significantly lower than the ones registered in overall asthma, with exception for the frequency in which daily activities are affected, the frequency of wheezing attacks, the frequency of sleep disturbance in the previous year and the frequency in which the nose symptoms ocurr, thought only in specific months and only for mother and sibling. These specific symptoms may effectively ocurr with a similar frequency in every family member and in the asthmatic reflecting a concordance for these indicators in the analysed asthmatic families. However, this agreement, may be related to specific family habits affecting, in a colective way, the expression of asthma symptoms or alternatively, with the family's perception, as a whole, of their symptoms. In fact, the parenting attitudes and the mother's stress related conditions, has been shown to affect their children's asthma (Nagano *et al.* 2010). Alternatively, when mothers report asthma symptoms on behalf of their children, their perception on their children's symptoms, is always subjected to a certain degree of subjectivity (Smeeton *et al.* 2006, Ringlever *et al.* 2012).

5.1.3. Family's contribution to asthma severity, rhinitis and wheezing.

The frequency of asthma severity, rhinitis, and wheezing according to the family member's asthma status, presence of rhinitis and wheezing was subsequently assessed.

A significant association between mild asthma, persistent asthma, the presence of wheezing and the presence of rhinitis amongst asthmatics and "only mother with rhinitis" was found. However, parental atopy (defined as mother or father reporting ever having had rhinitis and/or eczema) has been found to be a poor predictor for asthma in children from Northern Sweden (Bjerg *et al.* 2007).

On the other hand, despite not addressing to severity, Litonjua *et al.* (1998) refers to a study in the United Arab Emirates (UAE) reporting findings of both maternal allergic rhinitis and eczema significantly increasing the risk for childhood asthma. Dold *et al.* (1992) described, in a German population, that the risk of the child developing asthma was increased by asthma in a parent, but not by parental allergic rhinitis. Nonetheless, Madeiran patients with severe asthma have mostly "only father with asthma" and "only father with wheezing " which could corroborate the observation that for asthma in the child only paternal asthma has a strong influence (Dold *et al.* 1992). Curiously, in our sample all severe asthmatics were male. Fergusson *et al.* (1983) also described that asthma in parents was associated with asthma in boys in a New Zealand birth cohort. However, such observation cannot be established in the present case.

More recently it has been established that the severity of maternal allergic rhinitis is associated with an increased risk of the offspring developing allergic conditions (Wang *et al.* 2012), however, this study did not assess the severity of the allergic manifestations in the offspring.

As for siblings, no significant association was found. This is in agreement with Bjerg *et al.* (2007) whose findings point to a non significant association between sibling asthma and asthma in the child. In addition, when parental history of asthma was positive, there was no additional effect of having a sibling with asthma, concluding that sibling asthma is only a marker of parental disease (Bjerg *et al.* 2007).

5.2. Environmental factors

5.2.1. Inhabitancy conditions

A significantly higher frequency of persistent asthmatics rather than intermittent asthmatics was found living in a non-rural (urban or suburban) environment. In recent decades, a global increase in the prevalence of asthma and other allergic diseases, has been observed specially among children living in the urban areas of economically developed countries (Renzetti *et al.* 2009). Additionally, numerous studies have emphasized the relation between outdoor air pollution and asthma (Wong & Lai 2004; McConnel *et al.* 2010; Patel *et al.* 2011; Tzivian *et al.* 2011). With rapid urbanization in many communities, traffic exhausts have become the major source of pollution, which has been associated with increased asthma morbidity (Wong & Lai 2004).

Madeira Island has experienced in the 20th century a rapid development of its road network, leading to an increasing number of motorized vehicles and a fuel consumption that has tripled in 20 years and higher levels of air pollution in the urban areas (Câmara & Marques 2003). In 2002, the city of Funchal, Madeira Island's capital, was found to have the highest outdoor concentration of $N0_2$ (22. 5 μg/m^3) compared to the mean rate national level (17. 5 μg/m^3) found amongst three studied Portuguese centres (Almeida *et al*. 2002).

In fact, various studies have demonstrated that inhalation of air pollutants such as ozone (O_3), $N0_2$ and sulphur dioxide (SO_2), either individually or in combination, can enhance the airway response inducing asthma exacerbations (D'Amato *et al.* 2002; Peden *et al.*

2002; Zervas *et al.* 2012). Therefore, it could be argued that the higher frequency of Madeiran persistent asthmatics associated to an urban or suburban environment could be interpreted as a potential consequence of the negative effect of air poluttants present in those environments, contributing to asthma exacerbations.

On the other hand, it has been demonstrated in a number of epidemiological studies that children who grow up in rural environments amidst traditional farms are protected from asthma, hay fever and allergic sensitization (Alfvén *et al*. 2006; Wong & Chow 2008; von Mutius & Vercelli 2010). However, the timing of exposure is crucial, with the strongest effects observed for exposures occurring *in utero* and during the first years of life (von Mutius & Vercelli, 2010). An intense microbial exposure and possibly xenogeneic signals delivered before or soon after birth seem to point to the activation and modulation of innate and adaptive immune responses, which probably in turn favours non-Th2-type immune responses (von Mutius & Vercelli 2010), contributing to the reduction in risk for developing asthma. In which concerns the Madeiran asthmatics we do not have, however, specific information regarding the early exposure of patients to a rural environment.

With respect to the house humidity, a significantly higher number of intermittent asthmatics compared to persistent asthmatics were found living in a humid house. Humidity of the inspired air has been variously studied in relation to asthma symptoms, control of the disease and airway response to exercise (Singh *et al.* 2002). There is evidence, on one side, which demonstrate the attenuation of broncho-provocative response to exercise when administered along with humidity increase in the inspired air, whereas other evidences show that an increase in humidity of the ambient air in the natural habitat of asthmatic individuals increases asthma symptoms due to an increase in the mould and house dust mite concentration in the environment (Singh *et al.* 2002). In Madeira, the mean relative humidity is high (65-80%)- Câmara & Marques 2003. It should perhaps be expected that persistent asthmatics, presenting a higher frequency in symptoms, would be found associated to a humid house environment rather than intermittent asthmatics, with less frequent disease manifestations. A possible explanation could be that relative humidity may not directly contribute to asthma exacerbations as it might be merely the figurehead of another associated variable, this last justifying the observed difference between both groups. Another possible justification is that given the higher frequency in the severity of the symptoms, persistent asthmatics and their families are more aware of the need to avoid environmental factors that might aggravate their condition, therefore avoiding living in a humid house. This hypothesis gains some weight, by looking at the frequency of teddy bears in persistent and intermittent asthma (27.4 *versus* 43.5%), despite the absence of significant differences between groups for this variable. It has been shown that the presence of a stuffed animal, like a teddy bear in the bed, increases bed endotoxin, an amphiphilic outer-cell-wall component of gram-negative bacteria that is a potent inflammatory agent and asthma trigger (Thorne *et al.* 2009).

Plus, soft toys are a major source of HDM allergens, and at a very early age, sleeping with soft toys is strongly associated with HDM sensitization, which in turn, is associated with the development of asthma in children (Chang *et al.* 2011).

Another noteworthy association was verified, again, between persistent asthma and both potable water and electricity, as a lower frequency of persistent asthmatics (compared to intermittent) is associated to the presence of these two variables. The presence of both potable water and electricity are associated to better living conditions. But while all intermittent asthmatics have potable water and electricity, only 58.9% of persistent asthmatics live in identical conditions. It has been suggested that early exposure to mainly innocuous mycobacteria in soil and water with low or no pathogenicity may protect against later atopic disease, but this exposure has been greatly reduced by water treatment and sanitation in Western urban environments (Bloomfield *et al.* 2006). Despite the fact that both persistent and intermittent groups present asthma, this condition varies in frequency of symptoms, for which distinct environmental exposure, namely the soil mycobacteria, given the living conditions, might have been a possible contributor.

Therefore, supposing that children who live in unsanitary environments tend to have a lower incidence of asthma and other allergic illnesses due to childhood infections that are rare in developed countries (Brugge *et al.* 2011), it should be perhaps expected that persistent asthmatics were to be found associated within a more sanitary environment. However, as the two variables, water and electricity, are somehow indicators of socio-economic factors, it could be hypothesized, as a possible explanation, that persistent asthmatic might have less economic possibilities to acquire medication, for example, necessary to control their asthma symptoms, exhibiting, therefore, more frequent symptoms of the disease.

The frequency of tiles on the house floor was found to be lower in persistent asthma, compared to intermittent. In persistent asthma there is a higher percentage of wooden floor, followed by plastic, in a lower percentage and concrete.

The materials used in interior decoration, such as phthalates, other chemicals, and organic and inorganic particles are potential sources of indoor air pollutants and exposure may induce harmful immunologic responses in the airways and increase the risk of bronchial hyperresponsiveness and asthma (Jaakkola *et al.* 2006). The phthalate BBzP used in the vinyl tile flooring and phthalates in general are associated with allergic symptoms in children, within the concentration usually found indoors (Bornehag *et al.* 2004). Another study indicates that exposure to plastic flooring may increase the risk of respiratory conditions in children (Jaakkola *et al.* 2004). However, for Madeira patients, this percentage is very low and similar to the one registered in intermittent asthma.

It is important to refer that patients used no carpet in their room-floor. This is particularly important because it has been suggested that levels of endotoxin found within the living room carpets of children with asthma may in part be responsible for an increased risk of asthma development (Tavernier *et al.* 2005).

Passive parental smoking was also found to be related to asthma severity, as a higher percentage of smoking fathers was present in mild asthma, compared to moderate and severe.

According to the PAC study, the levels of domestic smoking in Madeira, reached almost 50% of the homes of children aged between 6 to 10 (Câmara & Marques 2003). The levels of passive smoke were even higher, reaching 55% and the incidence of passive smoking in young asthmatics at the immunoalergology consultation was 46% (Câmara & Marques 2003). According to our findings, the current percentage of passive smoking amongst overall asthmatics is 37.1%, with the percentage decaying from mild (41.9%) to moderate (26.9%) and severe asthma (25%).

Exposure to environmental tobacco smoke is associated with increased wheezing and increased symptoms in asthmatics and has negatively affecting the health of children from conception through adolescence having detrimental effects in respiratory health throughout life (Landau *et al.* 2001; Burke *et al.* 2012; Reinius *et al.* 2013). However, in the present study, the patient's sample presents a higher frequency of smoking fathers in mild asthma lowering in moderate asthma and non-existent in severe asthma. Given these facts, it could be proposed as a potential explanation that perhaps fathers are more conscious when acknowledge the presence of more severe asthma symptoms in their children, reducing the smoking as a measure of prevention. However, the exposure of children with asthma to environmental tobacco smoke has a highly negative effect on the severity of their asthma (Vergara *et al.* 2013).

5.2.2. Food habits and lifestyle

5.2.2.1. BMI

In the Madeira patients, no significant correlation was found between BMI and asthma severity, similarly to the study of Pelegrino *et al.* (2007) who also failed to find a significant correlation between obesity and asthma severity for either gender, in a Brazilan group of patients. However, and despite the absence of a significant relationship between asthma severity and the BMI, still, a lower frequency of male persistent asthmatics was observed below the 85th percentile, when compared to intermittent asthma, opposite to frequencies within the 85th -95th percentile, an observation that requires some consideration.

Literature suggests that obesity is a pro-inflammatory state (von Mutius *et al.* 2001; Ghanim *et al.* 2004; Kapiotis *et al.* 2006; Sheu *et al.* 2008; Emanuela *et al.* 2012). The inflammatory condition unique to obese individuals, including an increase in tumor necrosis factor alpha and other pro-inflammatory cytokines, such as IL4, IL5 and interleukin 6 (IL6), determines the superimposition of these inflammatory mechanisms on those involved in asthma, increasing the influence on airway muscle contractility (Pelegrino *et al.* 2007).

According to a study performed in different Hispanic populations in the USA when national origin and other factors were taken into account, children who were overweight or at risk for overweight were about 60% more likely to have asthma than other children (Jacobson *et al.* 2008). Another study in an Iranian population took asthma severity into account and showed that amongst asthmatic patients (>10 years) with increasing asthma severity, the BMI also increased (Behmanesh *et al.* 2010). Among Brazilian adolescents the increase in body mass index was associated with the

increase in the prevalence of wheezing, but not with the increase in the prevalence and severity of asthma (Cassol *et al.* 2005). In a Canadian group of patients, higher BMI scores had higher asthma control and lower asthma quality of life scores, assessed by questionnaire, independent of age, sex and asthma severity (Lavoie *et al.* 2006). It is therefore plausible that for Madeira male patients pre-obesity might be associated to persistent asthma, contributing to an aggravation of the symptom's frequency, when compared to intermittent asthma. However, this association might also be explained by the fact that persistent asthmatics might be less physically active due to their condition and tend, therefore to gain more weight. This hypothesis could be supported by the fact that amongst persistent asthmatics, 5.6% don't practice intense physical activity or do it only occasionally, unlike intermittent asthmatics. However, this small percentage is unlikely to reflect a real effect.

In studies stratified according to sex, associations of BMI with respiratory symptoms and lung function have been more pronounced in women and girls than in men and boys (von Mutius *et al.* 2001). For Madeira female patients, however, and contrary to male, a higher frequency of persistent asthmatics present normal weight (i.e. are under the 85th percentile), compared to intermittent asthmatics, while the opposite happens amongst overweight (i.e. belonging to the 85th to 95th percentile category).

This result might seem inconsistence given the evidences pointed so far, prompting higher BMI scores associated to more pronounced forms of asthma. However, approximately 10% of females with persistent asthma under the 85th percentile are in fact below the 5th percentile, and are therefore, underweight (data not shown). According to Lusky *et al.* (1996) asthma is amongst the functional disorders associated to underweight. Nonetheless, the percentage of males below the 5th percentile is approximately the same as in females, so this might not be an adequate justification. However, when analyzing by age group, the 11-13 year-old pre-adolescent group, shows a much higher frequency of underweight girls (28.6%) than boys (10%) and no cases were detected for intermittent asthma, which, again, might help explain the differences between genders.

Only persistent asthmatics fall into the 95th percentile category. In relation to males, it accounts for 6.7% of moderate asthmatics, while for females it represents 5.3 of mild asthmatics. Despite the absence of a significant association and taking no other variable into account, one could hypothesize that in Madeira patients, pre-obesity and obesity seems to be orientated to more severe forms of asthma in male, but not in female. Although, in most studies, association of asthma incidence with body mass index (BMI) and weight gain has been reported in women but not in men (Beckett *et al.* 2001; Chen *et al.* 2002; Guerra *et al.* 2002; Varraso *et al.* 2005). A higher sample size could be necessary to confirm these observations in future studies.

5.2.2.2. Food habits

It has been suggested that the rise in asthma prevalence may partly reflect changes in the population susceptibility resulting from alteration in diet, especially a fall in antioxidant intake (Arvaniti *et al.* 2010). In Madeira asthmatics a significant higher

consumption of fish one or two times a week was observed amongst intermittent asthma, compared to persistent.

The increase in the consumption of polyunsaturated fatty acids omega-6 found in vegetable oils and the lower consumption of omega-3, found in fish, have been associated to the rise in prevalence of asthma and atopy in developed countries (Sampaio & Romeira 2003). There seems to be a competitive interplay between pro-inflammatory omega-6 fatty acids and the less pro-inflammatory omega-3, suggesting the latter could contribute to prevent or treat asthma (Reisman *et al.* 2006).

For Madeira patients it seems that the higher weekly intake of fish is associated to intermittent asthma. It could be argued, therefore, that the more frequent inclusion of fish amongst the intermittent asthma group, compared to the persistent cluster, may have beneficial effects over the frequency by which the asthma symptoms ocurr. However, there are large inconsistencies regarding the benefic role of omega-3 in asthma, either in children or adults and more evidence is necessary (Broadfield *et al.* 2004; Reisman *et al.* 2006; Almqvist *et al.* 2007; Simopoulos 2008).

Also, a significant association was found between weekly consumption of legumes and asthma severity, as there is a higher consumption of legumes only once or twice a week in moderate and severe asthma, compared to mild. According to a study in a Spanish pediatric population, lentils and chickpeas were the legumes that caused most allergic reactions assessed by skin tests (San Ireneo *et al.* 2008). However, the Mediterranean diet including fish, fruits, vegetables, legumes, nuts and cereals, is known to have a protective effect over asthma-like symptoms and atopy in early life (Chatzi & Kogevinas 2009). According to Pandey & Rizvi (2009), besides fruits, vegetables and cereals, dry legumes also contribute to the polyphenolic intake. Epidemiological and functional studies have revealed that polyphenols provide a significant protection against development of several chronic diseases among which is asthma (Knekt *et al.* 2002; Jung *et al.* 2009; Pandey & Rizvi 2009). Polyphenols might protect against obstructive lung disease and increased consumption of the soy isoflavone, has been associated with better lung function in asthmatic patients (Pandey & Rizvi 2009). Further evidence is therefore necessary to support the observations regarding the relationship between asthma severity and legume intake amongst the Madeiran patients.

Finally, another association was found between the weekly consumption of pasta and both moderate (compared to severe) and persistent asthma (compared to intermittent) as a more frequently consumption ocurrs amongst both groups. In a study in North India, children from Lucknow urban area were found to have increased risk of asthma or wheeze with the consumption of pasta, noodles fast-food or meat once or more a week (Awasthi *et al.* 2004). However, in Madeira asthmatics, the consumption of fast food was relatively low, as 80.6% answered not to consume it at all.

According to the KIDMED index (Mediterranean Diet Quality Index for children and adolescents), pasta or rice should be consumed at least five times per week and one-unit increase in the KIDMED score was associated with 14% lower likelihood of having asthma symptoms (Arvaniti *et al.* 2011). Therefore, the association of weekly pasta intake with both moderate and persistent asthma might simply reflect the patient's

and/or the families' knowledge about healthy food habits that could potentially contribute to improve their asthma symptoms.

It is interesting to note that the consumption of dried fruits is quite low amongst patients. Nuts, for instance, are also part of the KIDMED index and its weekly intake should be of at least 2–3 servings (Arvaniti *et al.* 2011). Another product of lower consumption is margarine. It has been proposed that the formation of arachidonic acid–derived eicosanoids from omega-6 fatty acids leads to enhanced production of IgE, thus promoting allergic sensitization (Bolte *et al.* 2001). Besides, a sex-specific association of margarine consumption with allergic sensitization assessed by specific IgE serum levels and with symptoms of allergic rhinitis, has been found, but not for asthma (Bolte *et al.* 2001).

5.2.3. Allergy profile

5.2.3.1. House dust mites

In the Madeiran sample of patients, skin prick tests were performed and, globally, the highest percentage of sensitization was found for Dpt, followed by Df and *Blomia sp*. However, no significant association was found between any of these house dust mites and the asthma severity status. Nevertheless, throughout most of the world, the most common inhalant allergens associated with asthma are those from the house dust mite (Mueller *et al.* 1998). It is believed that the major allergen of HDM, (Der p 1), may influence allergic responses by suppressing the secretion of interferon-gamma (IFN-γ) thus promoting a Th2 response (Maneechotesuwan *et al.* 2009).

According to the PAC study, the most frequent sensitization for the Madeira population was also for house dust mites, in about 30% and in about 80% of asthmatic children analysed under the study's scope (Câmara & Marques 2003), close to the value registered for this sample of patients (89.0%). Indoor conditions consisting of a high relative humidity and mean HDM concentrations of 19.76 μg/g of dust, for Der p 1 in mattresses and 2.11 μg/g of dust for Der p 1 on the floor, contribute to atopy and allergic disease prevalence in Madeira population (Oliveira *et al.* 2004). It has been reported that children growing up in houses with greater than 2 μg of Group I mite allergen per gram of dust are at risk for developing positive skin test responses and serum IgE antibodies (Platts-Mills *et al.* 1995). Levels above 2 μg/g for Der p 1, have, in fact, been associated with more severe asthma (Gent *et al.* 2009). However, no data regarding these parameters were available for our Madeiran patient's sample.

Lepidoglyphus sp. wheal size, however, was found to be significantly larger in severe asthma, when compared to mild. The *Lepidoglyphus destructor* is one of the most abundant nonpyroglyphid storage mite species (Olsson & van Hage-Hamsten 2000) and a major source of allergy in Europe, especially in rural populations, but also in urban areas (Saarne *et al.* 2003). A number of allergens has been isolated from this HDM namely Lep d 2, Lep d 5, Lep d 7 and Lep d 13 and its recombinant tropomyosin allergen rLep has revelead an IgE binding frequency of 13% (Saarne *et al.* 2003). Despite the fact that literature regarding the prediction of asthma severity based on the skin prick test wheal size of *Lepidoglyphus destructor* was not found, to our knowledge, it could be

argued that, in fact, for Madeira patients this mite may be a possible indicator of asthma severity, similarly to the relationship between cockroach wheal size found to be the single strongest predictor of the severity of asthma in a population from Baltimore, USA (Platts-Mills *et al.* 1997).

5.2.3.2. Pollens

Amongst pollen allergens, Gramineae (grass) was found to be responsible for the highest percentage of sensitization, followed by *Parietaria sp*. Past studies have revealed similar results, as grass pollen was found to be the most prevalent allergen amongst a group of allergic rhinitis patients from Madeira who also had the highest *Parietaria sp.* sensitization prevalence in Portugal (Pereira 2004). *Parietaria* is a genus of dicotyledonous weeds belonging to the Urticaceae family, which is composed of several allergenic species, namely *Parietaria judaica* and *Parietaria officinalis* the most important immunological species (Cortes *et al.* 2006). The major allergens of both species are small glycoproteins with molecular weights ranging between 10 and 14 kDa, with high cross-reactivity (Cortes *et al.* 2006). It has been proposed that Pj-peptidase, released from *P. judaica* pollen, may be involved in the initial steps of sensitization by disruption of epithelium barrier, which may enhance the delivery of allergenic protein to dendritic cells, playing therefore a significant importance in asthma among other respiratory complications (Cortes *et al.* 2006). However, amongst the Madeiran patients, no significant relationship was observed between asthma severity and the frequency of sensitization of neither Gramineae nor *Parietaria sp.* similarly to the reported by Zureik *et al.* (2002), where no association between asthma severity and sensitization to pollens was found.

Nevertheless, a significant higher sensitization to *Platanus sp*. amongst intermittent asthma, compared to persistent asthma was found. The capability of *Platanus sp.* pollen grains to trigger instant hypersensitivity reactions has been long recognized (González et al. 2010). In the *Platanus acerifolia*, three allergens have been identified, one of which (Pla a 2) accounting for 52% of the total IgE binding capacity of the species (Fernández-González *et al*. 2010). In Cova da Beira, Portugal, *Platanus acerifolia* was one of the most representative aeroallergen sensitization (11.4%) (Loureiro *et al.* 2005). Given these facts, a possible relationship verified amongst the Madeira asthma patients regarding the relationship between asthma severity and the frequency of *Platanus sp.* sensitization could be considered.

Findings of a clear relationship between increased risk of childhood asthma and levels of ambient grass pollen below 20 grains/m^3 have been reported in Australia, with important implications for patient care, such as asthma management programs (Erbas *et al.* 2012). In Portugal, the Polleniferous Bulletin, promoted by The Portuguese Society of Allergology and Clinical Immunology (SPAIC), provides information about the type of pollen by region and period of time, offering useful advice to avoid pollen exposure.

5.2.3.3. Moulds

Athought no significant relationship has been found between asthma severity and the frequency of sensitization determined by skin prick tests for any of the studied mould extracts, some important aspects have been observed.

The highest frequency of sensitization amongst moulds belongs to *Alternaria sp.*, followed by *Aspergillus sp.* and *Cladosporium sp*. For the first two, the frequency of positive skin prick tests was higher in severe asthma than in mild or moderate, while for the latter, a higher frequency of positive skin prick tests was detected in moderate asthma, compared to mild, despite absence of statistical significance. A study by Lyons *et al.* (2011) despite having failed to find an association between *Alternaria* skin test reactivity and increasing asthma severity, has reported an association of the mould with severe persistent asthma.

In Madeira, a high prevalence of sensitization to mould has been described (Câmara & Marques 2003). According to Oliveira *et al.* (2004), the airborne level of mould spores varies between 6584 to 11925 spores/m^3/year with *Cladosporium sp.* being the most prevalent genus. Sensitization to *Alternaria sp.* and *Cladosporium sp.* has been reported to be 3% to 30% in European countries (Bavbek *et al.* 2006).

A study on the Island of Wight registered 6% of positive reactions to *Alternaria alternata* and *Cladosporium herbarum* and at the age of 4, *Alternaria sp.* and *Cladosporium sp.* were the third most common causes of sensitization, after house dust mite and grass pollen (Tariq *et al.* 1996). In addition, exposure to *Alternaria alternata* was associated with active asthma symptoms in US homes (Salo *et al.* 2006) and it was found to be the major allergen associated with the development of asthma in children in Arizona (Halonen 1997).

5.2.3.4. Cockroaches

The highest sensitization for Madeira patients with respect to cockroaches was registered for *Periplaneta americana*. In Madeira, it has been previously described under the ambit of PAC study that the high populational density in the urban and suburban areas, and the inadequate and unorganized lodging provided the conditions for the appearance of cockroaches (*Periplaneta americana*) responsible for a high number of sensitization cases in the studied group of asthmatic children (41%) (Câmara & Marques 2003). However, the percentage of positive skin prick tests cases detected for this specie in the present group of asthmatics is only 14.6%. An improvement in the housing conditions and /or a better pest control might have conditioned the patients exposure to *Periplaneta americana* and, therefore, their sensitization to the allergen. However, there might be other factors responsible for these differences. Some concerns have been addressed about the skin prick test solutions, as they may vary by manufacturer and may be non-specific (Woodcock 2007).

According to Asturias *et al.* (1999), inhalation of allergens produced by *Periplaneta americana* induces IgE production and the development of asthma in genetically predisposed individuals.

It has been proposed that cockroach-derived protease can disturb airway epithelial integrity and lead to an increased penetration of cockroach allergen, resulting in activation of innate immune cells (like dendritic cells -DCs) via binding to either *TLRs* or C-type lectin receptors (*CLRs*). The activated DCs can direct cells of the adaptive immune

system to facilitate promotion of Th2 cell response and subsequently increase risk of sensitization (Gao 2012).

Periplaneta americana was found to account for 34.0% of positive skin prick tests in a population of asthmatic adolescents from Northeast of Brazil (Sarinho *et al.* 2009) while in a group of children from Turkey, aged 2-16 years-old, where 77.7% were atopic, *Periplaneta americana* registered only 7.4% of positive reactions (Yilmaz *et al.* 2004). The variation on frequency across populations probably reflects both the incidence of cockroaches as well as the individual predisposition to develop sensitization. In addition, given the ocurrence in Madeira Island of the endemic cockroache species *Rhyparobia maderae* (Robinson 2005), future studies should perhaps test for the degree of sensitization to the specific allergens present in the species for the Madeira population.

5.2.3.5. Dog and Cat

The dog allergen frequency of sensitization was significantly positively associated to persistent asthma and it was estimated in 23.2%. Interestingly, among persistent asthma, the percentage of families who owned a dog was 47.9%. *Canis familiaris* allergen 1 (Can f 1) is a protein with molecular weight ranging from 21 to 25 kD, binding IgE from the majority of dog-allergic subjects. Immunoaffinity-purified Can f 1 was found to elicit a high frequency of positive skin prick test reactions (Konieczny *et al.* 1997).

The percentage of sensitization for cat allergen amongst persistent asthma patients was also found to be higher compared to intermittent asthma, but no significant relationship was found. However, the dander of the domestic cat (*Felis domesticus*) is the main source of cat allergens, and five allergenic proteins have been identified so far, namely Fel d 1–Fel d 5, with Fel d 1 being the major immunodominant protein accounting for 60–90% of the total allergenicity of cat dander (Emara *et al.* 2011).

Nevertheless, a number of studies has reported conflicting results on both cat and dog allergens (Perzanowski *et al.* 2002; Wu & Takaro 2007; Carlsen *et al.* 2012). A systematic review concluded that early childhood exposure to cat or dog does not have an impact on the development of asthma and wheezing symptoms up to school age. On the contrary, dog exposure protects children from developing sensitization against aeroallergens with indication that it was associated with wheezing symptom later in life (Chen *et al.* 2010).

Again, the individual susceptibility determined by the genetic background, and varying across populations, might determine sensitization to allergens.

Next, the genetic polymorphisms and their relation with both asthma susceptibility and severity are addressed.

5.3. Genetic polymorphisms: case-Madeira reference set

5.3.1. *IL13*

In this study, eight polymorphisms described in the literature as have being associated to asthma were analysed for the first time in the Madeira population.

The analysis of *IL13-c.144* did not reveal any significant differences between the Madeira population reference set and asthmatics, despite the findings of an increasing gradient in allele's *IL13-c.144*A*, from mild, to moderate and severe asthma, though no significant association to asthma severity was found. Still, this fact may support earlier observations suggesting that this particular allelic variant is the more biologically active in enhancing the allergic mechanisms of asthma (Vladich *et al.* 2005; Chen *et al.* 2004).

Despite these findings there is a large number of studies proposing the *IL13-c.144*A* variant as an important polymorphism associated to asthma and/or atopic asthma and related phenotypes (Heinzmann *et al.* 2000; Vladich *et al.* 2005; Vercelli 2008; Bottema *et al.* 2010; Brightling *et al.* 2010; Cui *et al.* 2012). Hence, Graves *et al.* (2000) reported in 3 different populations (from Munich, Leipzig and Arizona) a strong association of allele *IL13-c.144*A* with increased levels of IgEs, while Liu *et al.* (2000) also reported in a German group of children the association of the same allele with higher levels of IgEs and atopic dermatitis. The *IL13-c.144*A* variant was also found to be associated to asthma in a British population and to atopic and non-atopic asthma in a Japanese population (Heinzmann *et al.* 2000). Another study conducted in a British cohort by Maier *et al.* (2006) reported an association of the same variant with total IgEs. More recently, Bottema *et al.* (2010) remarked that one or two copies of the *IL13-c.144*A* allele were significantly more prevalent in asthmatics than in controls and were also associated to elevated levels of IgEs. A meta-analysis in the Chinese population revealed an association between the genotypes *IL13-c.144*AA* and *IL13-c.144*AG* clustered together against genotype *IL13-c.144*GG* and the risk of asthma in Chinese adults (Li *et al.* 2010). Thus, according to Yang *et al.* (2011) *IL13-c.144*A* allele carriers had a 40% increased risk of asthma compared to homozygotes *IL13-c.144*GG*. The same authors found in a meta-analysis that *IL13-c.144*A* allele was associated with an increased risk of asthma among Asians but not among Caucasians.

Nevertheless, a study conducted in a Dutch Caucasian population failed to report a significant relationship between the SNP *IL13-c.144* and asthma, BHR or skin tests (Howard *et al.* 2001). Leung *et al.* (2001) reported a positive association of *IL13-c.144*AA* to total IgEs, specific IgEs to Der p 1, mixed cockroaches and dog, but not for diagnosed asthma or asthma severity, in a chinese population. Similarly, DeMeo *et al.* (2002) studied a group of asthmatic children from the Childhood Asthma Management Program (CAMP) and despite having established a positive relationship between the polymorphism *IL13-c.144* and eosinophils, IgE and positive skin tests, found no evidence of association to asthma diagnosis or asthma severity.

Despite the discrepancies of the results across populations, as reviewed by Hunninghake *et al.* (2007) it appears that *IL13-c.144* SNP association to total IgEs and eosinophil count are the most consistent in effect and direction in the literature, so far, suggesting that

the polymorphism might have a predominant genetic effect in allergy and not asthma *per se*. However, it has been previously demonstrated that there is strong evidence that IL13 is crucial for induction of an asthma-like phenotype in animal models (Heinzmann *et al.* 2000). Then, the lack of reproducibility across studies might be related to limited sample size, absence of data regarding certain phenotypic aspects of asthma, mismatch in terms of age and/ or gender between case and control subjects such as proposed by Hunninghake *et al.* (2007). Other reasons pointed out as a possible explanation for these diverse results is that each study is based on population samples that are ascertained differently; moreover, studies are performed in different population groups and, consequently, LD, accounting for allergy phenotype, with other markers in 5q31 region, where *IL13* is located, might differ in each population (Howard *et al.* 2001).

Regarding the allele *IL13-c.144*A* frequency across populations and comparing it to the one registered for Madeira population reference set (0.171), frequencies vary between 0.180 and 0.267 amongst Caucasians (Graves *et al.* 2000; Heinzmann *et al.* 2000; Liu *et al.* 2000; Howard *et al.* 2001; Maier *et al.* 2006; Bottema *et al.* 2010) while a marginally higher frequency of 0.290 was found in a Chinese population (Lu *et al.* 2011). However, the highest frequency of allele *IL13-c.144*A* (0.430) was registered in a Japanese control group (Heinzmann *et al.* 2000). Among an African population of Mali, the observed frequency of *IL13-c.144*A* was 0.246 (Kouriba *et al.* 2005). These variations in allele frequency between populations might be explained by processes of natural selection, varying LD patterns at causal *loci* and/or distinct pathways contributing to asthma expression phenotypes (Baye *et al.* 2011).

The Th2 pathway is strongly linked to asthma and the same genes that are candidates for host protection from helminthic disease are also candidate genes for asthma (Le Souëf *et al.* 2006). IL13 is an important Th2 cytokine and as an example the *–1111T* allele from polymorphism *C-1111T* has been associated with allergy to inhaled antigens and atopic dermatitis. The frequency of this allele was much lower in Caucasians (0.120) compared to Africans (0.480) (Le Souëf *et al.* 2006).

5.3.2. *IL4*

Significant differences were found between the Madeira reference sample set and asthmatics for both *IL4-590* and *IL4-RP2* polymorphisms, suggesting a possible relationship between the polymorphisms and the disease, which is in agreement with a number of studies previously published in diverse populations (Rosenwasser *et al.* 1995; Noguchi *et al.* 1998; Burchard *et al.* 1999; Noguchi *et al.* 2001; Kabesch *et al.* 2003; Wang *et al.* 2004; Hosseini-Farahabadi *et al.* 2007) but differs from several others (Cui *et al.* 2003; Attab *et al.* 2008; Rad *et al.* 2010). It is known by reporter gene transfer experiments in Jurkart cells and EMSA experiments, that *IL4-590*T* allele presents a threefold higher promoter activity contrarily to wild type *IL4-590**C allele (Wierenga & Messer 2000). In fact, the allele *IL4-590*T* allows for an extra binding site for the NFAT at the promoter of the human *IL4* gene leading to a synergistic effect on the transcription rate by more than threefold (Rockman *et al.* 2003).

Despite being present in an intronic region, the association of *IL4-RP2* polymorphism to asthma could be explained by two different mechanisms. First, it is possible that this

SNP could be directly involved in the metabolic pathway of asthma and/or associated phenotypes, since, it has been reported that the mouse *IL4* intron 2 contains a specific enhancer for mast cells, acting as a regulator of the *IL4* expression (Chouchane *et al.* 1999). The activation of mast cells is important in initiating the acute bronchoconstrictor responses to allergen, playing a key role in asthma (Kumari & Rana 2012). The second possibility is that polymorphism within this intron do not directly contribute to asthma but are in LD with a proximate *locus* or *loci*, accounting for asthma phenotypes (Chouchane *et al.* 1999). Thus, it has been reported that, the haplotype between allele *IL4-RP2*183* and other SNPs within the *IL4* gene promoter region was found to allow for high IL4 production (Nakashima *et al.* 2002).

IL4 was also found to be increased in the serum and bronchoalveolar lavage of allergic individuals; plus, nebulized administration of IL4 to patients with mild asthma resulted in a significant increase in airway hyperresponsiveness (Steinke & Borish 2001). Altogether, this might explain both allele's *IL4-590*T* and *IL4-RP2*183* and genotypes *IL4-590*CT/IL4-590*TT* and *IL4-RP2*253183/IL4-RP2*183183* significantly higher frequency in the group of asthmatics when compared to the Madeira reference set, accounting for a higher disease risk.

It is pertinent to note that the alleles *IL4-590*T* and *IL4-RP2*183* are in LD for our samples and therefore it is possible that a synergic effect may occur, contributing to the pathophysiology of asthma (Berenguer *et al.* 2012) or, alternatively, that allele *IL4-RP2*183* is no more than the marker for *IL4-590*T*, as proposed by Nakashima *et al.* (2002), due to it's intronic location.

Both alleles also show an increasing frequency cline from the Madeira population reference set to mild, moderate and severe asthma groups, but significant differences were only found between the Madeira reference set and each moderate, severe and persistent asthma cluster, where again the frequency of alleles *IL4-590*T* and *IL4-RP2*183* as well as genotypes *IL4-590*CT/IL4-590*TT* and *IL4-RP2*253183/IL4-RP2*183183* was found to be significantly higher amongst each asthma severity subgroup, constituting potential genetic risk factors to the disease severity. Previous studies have reported an association of *IL4-590*T* allele to mild asthma (Hosseini-Farahabadi *et al.* 2007) and the same allele was observed more frequently in mild to moderate compared to severe disease, modulating asthma's severity (Kamali-Sarvestani *et al.* 2007). In the present study a significant association was found not for mild but for moderate and severe asthma. Differences in the definition of severity between studies may explain the dissimilar outcomes. While Hosseini-Farahabadi *et al.* (2007) categorized mild, moderate and severe asthma according to FEV_1 as >80%, 60-80% and <60%, respectively, Kamali-Sarvestani *et al.* (2007) divided patients in two groups as mild to moderate (FEV_1> 50%) and severe (FEV_1 <50%), while in the present study, mild, moderate and severe asthmatics present mean FEV_1 values of 99.7%, 88.6% and 78.0%. These dissimilar categorizations do not allow a clear comparison, providing conflicting results.

The TDT results regarding *IL4* polymorphisms indicate no linkage between disease and markers an unpredicted fact considering the initial associations to disease susceptibility

and severity. It is know that, for example, systematic differences in the ancestry of cases and controls are one source of false positive associations (Freedman *et al.* 2004) but the TDT, using within family comparisons, it is not affected by aspects of population structure that can lead to association in the absence of linkage (Spielman & Ewens 1998). Therefore, it could be argued that asthmatics might present partially or to a greater extent, a different genetic background from the Madeira population reference set. For this last group, all recruited individual were from local provenance for at least three generations. However, for patients, this parameter might have been disregarded and so, a possible recent phenomenon of migration from their ancestors, for instance, might have introduced confounding factors into the patient's genetic background. According to Freedman *et al.* (2004), stratification is probably most problematic in populations whose ancestors recently mixed due to intercontinental migrations and for diseases that have different prevalence rates across these ancestral populations. It is also important to mention that a disease that is most prevalent in one subpopulation will be associated with any alleles that are in high frequency in that subpopulation (Pritchard & Rosenberg 1999). According to Pinto & Almeida (2005), Madeira presented the highest prevalence of active asthma in the country (14.6%). On the other hand, mtDNA and Y-chromosome studies show an important sub-saharan and northern African influence in the population's genetic background (Brehm *et al*. 2003; Gonçalves *et al*. 2005) and despite the fact that genotype *IL4-RP2*183183 IL4-590*TT* prevails in African populations while *IL4-RP2*253253 IL4-590*CC* appears in a higher frequency among typically Caucasian populations (Berenguer *et al.* 2012), perhaps it could be hypothesized that the frequency of alleles *IL4-590*T* and *IL4-RP2*183* within Madeira population, as a result of the African contribution, (in addition to the circumstance of being a relatively small island where founder effect cannot be excluded) is higher than expected for this specific population. Consequently, given the coincident high prevalence of asthma and the potential higher frequency of both *IL4-590*T* and *IL4-RP2*183* alleles, the association of the respective polymorphisms towards the disease might be an artefact rather than a true association.

However, the frequency of allele *IL4-590*T* across other Caucasian populations seems to be relatively approximate to the one detected in the Madeira population reference set (0.105) as frequencies vary from 0.113 to 0.152 amongst the Caucasian populations of South of England, Czech Republic, Spain and North West of England (Howell 2004; Pravica *et al.* 2004; Slavcev & Striz 2004; Leon *et al.* 2006). However, the frequency of allele *IL4-590*T* was relatively higher, reaching 0.270 in a population from west Australia (Walley & Cookson 1996). When considering other populations such as African Americans, the allele frequency increases strikingly to 0.544 (Burchard *et al.* 1999) and to 0.592 in Cabo-Verde and 0.765 in Guinea-Bissau (Berenguer *et al.* 2012), with the highest frequencies registered in a Japanese and Chinese population (0.700 and 0.770, respectively) (Noguchi *et al.* 1998; Cui *et al.* 2003).

Such a variation in frequency across populations might be explained by the fact that polymorphisms with effects on the immune system are thought to be under selection in many organisms including primates (Casto & Feldman 2011). This is particularly relevant if one considers that *IL4* genes have been associated with differential susceptibility to specific infections and with an increased likelihood to develop

autoimmune or allergic/atopic diseases (Fumagalli *et al.* 2009). In fact, risk alleles for autoimmune diseases are sometimes selected to improve resistance to virus and malignancy, despite the risk of autoimmunity (Rajagopalan & Long 2005), which constitutes the hygiene hypothesis. A study conducted in the Fulani ethnic group from West Africa showed that *IL4-590*T* allele, significantly more common among the Fulani group, was associated with elevated anti-malarial IgG levels making them more immunologically responsive to the disease (Luoni *et al.* 2001). Therefore, the higher frequency of *IL4-590*T* allele in other African populations where malaria or other viral infections are still common pathologies (Murray *et al.* 2012), might result from a selective pressure, by playing a protective role against the disease progression, while in westernized societies, where exposure to pathogens is reduced, such percentage could tend to be lower. This hypothesis is supported by Le Souëf *et al.* (2006) that point out examples of a higher frequency of alleles that either directly or indirectly promote Th2 activities in populations whose most recent long-term ancestry was in the tropics, such as the *IL4-590*T*, which had an allele frequency of 0.544 in African-Americans (whose origins lie mostly in tropical West Africa) compared with only 0.183 in European-Americans (Le Souëf *et al.* 2006).

Regarding the eventual clinical relevance of *IL4*, the *IL4-590*T* allele was found to be associated with increased *IL4* gene transcription and glucocorticoid (GC)-resistant asthma (Szalai 2008). Given the significant higher frequency of *IL4-590*T* allele amongst Madeiran patients, perhaps *IL4-590* could in the future be a useful auxiliary in medical diagnosis and intervention in the Madeira population.

5.3.3. *ADRB2*

The *ADRB2-c.16* polymorphism was found in Hardy-Weinberg proportions for both the Madeira reference sample set and the patients group, as a whole. However, a departure from HW proportions was found for *ADRB2* in the group comprising the moderate asthmatics. The Hardy-Weinberg principle states that, in the absence of natural selection, mutation, migration, non-random mating, random genetic drift, gene flow and meiotic drive, the observed genotype frequencies should agree to the expected frequencies (Wang & Shete 2012). However, a significantly higher observed heterozygosity (0.731, data not shown) than expected (0.503) justifies the deviation from Hardy Weinberg's proportions (p-value=0.042). Ye *et al.* (2009) also reported in a sample of South Korean patients an absence of Hardy-Weinberg proportions for the same SNP, having excluded it from the study.

However, despite the deviation from HW proportions for moderate asthma, when grouping moderate and severe asthma, the HWE was not disturbed (p-value=0.0607-data not shown). Therefore, when analyzing the genotype frequencies between asthma severity subgroups (i.e. in mild *versus* moderate and severe), an unexpected high frequency of *ADRB2-c.16*AG* genotype was found amongst moderate and severe asthmatics, when compared to mild.

According to Sato (2000) only the homozygotic form *ADRB2-c.16*GG* was found to be higher in moderate to severe asthma when compared to the mild form of the pathology. Turner *et al.* (2004) reported that a significantly higher percentage of homozygotic

individuals *ADRB2-c.16*GG* were admitted in the hospital with asthma, compared to the percentage of individual with the remaining genotypes. A meta-analysis by Contopoulos-Ioannidis *et al.* (2005) concludes that *ADRB2-c.16*G* allele doubles the risk for nocturnal asthma and modestly increases asthma severity, also most noticeable for *ADRB2-c.16*GG* homozygotes. A possible explanation lies in the fact that enhanced receptor downregulation caused by the *ADRB2-c.16*G* allele may lead to unfavorable asthma phenotypes (Contopoulos-Ioannidis *et al.* 2005). However, a different meta-analysis performed by Thakkinstian *et al.* (2005) suggests that children with the *ADRB2-c.16*GG* genotype had about 29% lower risk of having asthma than did children with the *ADRB2-c.16*AA* and *ADRB2-c.16*AG* genotypes. Again, these conflicting results might arise from different approaches in the study design and /or to gene–gene interaction, gene–environment interaction and inter-ethnic variability as proposed by Hizawa (2009).

ADRB2-c.16 was not found to account for asthma susceptibility in the Madeira population. The lack of association between *ADRB2-c.16* and asthma susceptibility has also been reported by other scholars. Turner *et al.* (2004) found no association between the polymorphism and atopy or diagnosed asthma in an Australian population; Isaza *et al.* (2012) also failed to found any differences between asthmatics and non-asthmatics or a relationship to asthma severity in Colombian children. Our study was also unable to find an obvious association between disease and *ADRB2-c.16* alleles by TDT, similarly to the findings of Migita *et al.* (2004).

When comparing the frequency of *ADRB2-c.16*G* registered in the Madeira population reference set (0.590) to the one found in other populations, there are less striking differences, contrarily to the ones registered for both *IL13* and especially for *IL4* polymorphisms discussed before. Therefore, frequencies fluctuate from 0.430 in a sample of Mexican mestizos (Santillan *et al.* 2003) to 0.490 both in a healthy Japanese population (Sato 2000) and in a group of Han ethnicity from Northern China (Gao *et al.* 2000b) to 0.500 both in a group of non-asthmatic African-Americans and Colombian children (Weir *et al.* 1998; Isaza *et al.* 2012). Slightly higher frequencies were registered for Israeli Jews and amongst the total Israeli population, 0.510 and 0.540, respectively (Shachor *et al.* 2003), while the highest values were found in a non-asthmatic control group from New Zealand (0.600) (Holloway *et al.* 2000) and among a group from Tucson Children's Respiratory Study, Arizona (0.620) (Martinez *et al.* 1997) as well as amongst Arab groups (Shachor *et al.* 2003).

Although there is some variation in allele frequency across populations, it is interesting to note that for *ADRB2-c.16*, this variation may be considered mild. In all the studies mentioned, the frequency for *ADRB2-c.16*G* varies between 0.620 (Martinez *et al.* 1997) and 0.430 (Shachor *et al.* 2003), which represents, in average, a frequency of 0.500. This value also implies a somewhat higher frequency of heterozygotes across populations. In Madeira the observed heterozygosity was 0.457. Martinez *et al.* (1997) reported 0.470; in Binaei *et al.* (2003) it was 0.432 and in Santillan *et al.* (2003) 0.530. For Shachor *et al.* (2003) non-asthmatic controls showed 0.460 of *ADRB2-c.16*AG* allele. This might suggest a potential heterozygote advantage or balancing selection. Thakkinstian *et al.* (2005), points out three motives as a possible explanation: advantages in having variation in a multimeric protein, an allele with a selective advantage that is detrimental when homozygous (e.g. sickle cell and falciparum malaria) and a greater range of

expression of gene products and plasticity with heterozygotes than homozygotes. In fact, according to Castellano *et al.* (2003) functional relevance and a high degree of heterozygosity, are the bases for a possible pathophysiological role in the modulation of complex traits. However, given the similarity between of the heterozygotes frequencies among both the Madeira reference set and overall asthma, perhaps the possible heterozygote advantage may be related to a different metabolic pathway, other than asthma.

Variation in allele frequency across populations might also be a consequence of prolonged exposure to helminthic infection. Although not regarded as involved in IgE production, the *ADRB2-c.16*A* allele has been associated with increased levels of ascaris-specific IgE in a tropical Caribbean population, being more frequent in African-North Americans than European-North Americans (Le Souëf *et al.* 2006).

Beta-adrenergic receptors, such as ADRB2, are important targets of pharmacological therapy for asthma. Inhaled beta-receptor agonists (e.g. albuterol) and antagonists (e.g. beta-blockers) remain among the mostly commonly prescribed medications for asthma treatment in adults (Taylor 2007). *ADRB2-c.16*G* has been linked to differential response to beta-agonist therapy (albuterol) in asthmatics (Taylor 2007). The bronchodilating response to inhaled short-acting β-agonist was found to be significantly higher in subjects with the homozygous *ADRB2-c.16*AA* than in those with the homozygous *ADRB2-c.16*GG* configuration (Cho *et al.* 2005). In a different study, *ADRB2-c.16*AA* homozygotes and *ADRB2-c.16*AG* heterozygotes were 5.3 times and 2.3 times more likely than *ADRB2-c.16*GG* homozygotes to exhibit a positive response to albuterol (Martinez *et al.* 1997). This last study suggests that because heterozygotes show intermediate levels of response to beta-2-adrenergic agonists, it is possible that receptors with different downregulation properties may be expressed in the cell surface of these subjects (Martinez *et al.* 1997). Again, this might be related to a presumed balanced selection, favoring the heterozygous genotype. Comings & MacMurray (2000) illustrate by giving an example of the firing of the norepinephrine neurons and the symptoms of the Attention Deficit Disorder (ADHD) where heterozygotes would show optimal performance with an intermediate level of norepinephrine output, compared to both homozygotic forms. Given the fact that there is no data available for asthma patients from Madeira regarding the use of bronchodilators and/or other types of medication, it is not possible to infer about the role of *ADRB2-c.16* genotypes in the clinical outcome. However, given the *ADRB2-c.16*AG* genotype's higher frequency amongst more severe forms of asthma, and assuming a positive response of heterozygotes to albuterol and/or other short-acting beta-agonists (SABAs), as suggested by literature (Martinez *et al.* 1997), this could provide useful insight for future approaches on the clinical level, for this specific population.

5.3.4. *ADAM33*

No significant associations with asthma susceptibility or severity were found for *ADAM33-V4* or *ADAM33-S1 c.710* polymorphisms in the Madeira population sample. However, Qu *et al.* (2011), reported a strong association of *ADAM33-V4*G* allele to childhood asthma in a Han Chinese population in northern China, while Su *et al.* (2008),

described a significant association between allergic asthma and *ADAM33-V4*G* allele, but not for *ADAM33-S1 c.710*, also in a Chinese population. Regarding asthma severity and *ADAM33-V4*, our results are in accordance to Sakagami *et al.* (2007) who also failed to report it in a sample of Japanese asthmatics.

In the Madeira asthma group the allele *ADAM33-V4*C* was significantly, yet marginally, over-transmitted to the asthmatic offspring when performing the TDT (transmitted C/ non-transmitted-28/15), an observation supported by Van Eerdewegh *et al.* (2002), who also found a significant higher transmission of C allele (43) compared to the non-transmitted (28) in a group of Caucasian children with asthma and BHR. As for *ADAM33-S1 c.710*, the TDT was non-significant for the Madeiran patients, an observation also reported by Van Eerdewegh *et al.* (2002), although only for the group of asthma and BHR; in the group comprising only asthmatics, *ADAM33-S1c.710*G* allele was significantly over-transmitted and also a significant value for the case-control study was found, contrary to our observations.

Again, the non-significant associations in the case-Madeira population reference set study and the significant association found by TDT for *ADAM33-V4* to asthma in the Madeira asthma sample might result from population admixture, resulting in spurious associations as proposed by Lander & Schork (1994), and as extensively discussed earlier for *IL4* gene.

However, other studies have failed to report a significant TDT p-value for *ADAM33-V4* and /or *ADAM33-S1 c.710*. Thus, a study conducted in Puerto Rican and Mexican populations, concluded that both *ADAM33-V4* and *ADAM33-S1 c.710* were not important factor for asthma or associated phenotypes, as a significant over-transmission of the risk alleles was not observed for either polymorphism in neither group (Lind *et al.* 2003). Another study performed in White, African-Americans and Hispanic trios, recruited from the CAMP study also reported a non-significant TDT association in all groups, regarding *ADAM33-V4* (Raby *et al.* 2004). Noguchi *et al.* (2006) also described a non-significant TDT in a Japanese population, for both *ADAM33-V4* and *ADAM33-S1 c.710*, but revealed a significant over-transmission of other minor alleles at *S+1*, *ST+4* and *T2* SNPs in *ADAM33* to asthma-affected offspring.

Once again, the inconsistencies about the role of *ADAM33* polymorphisms in asthma may be due to racial and ethnic differences in gene and environmental factors (Burchard *et al.* 2003).

Frequencies for *ADAM33-V4*G* allele vary between 0.146, among Chinese (Su *et al.* 2008) to 0.172 in Mexican (Lind *et al.* 2003) and to 0.184 in Caucasians from CAMP study (Raby *et al.* 2004). Among Germans, Hispanics and a combined UK /US Caucasian population frequencies range from 0.220 to 0.233 (Van Eerdewegh *et al.* 2002; Raby *et al.* 2004; Schedel *et al.* 2006). Puerto Ricans present a slightly higher allele frequency (0.242- Lind *et al.* 2003) together with African Americans (0.311) (Raby *et al.* 2004) and a Japanese population from Niigata (0.316) (Sakagami *et al.* 2007). However, in a different Japanese population from Tsukuba the reported frequency for *ADAM33-V4*G* was null (Noguchi *et al.* 2006).

As for the polymorphism *ADAM33-S1 c.710*, the frequency of allele *ADAM33-S1c.710*G* detected in the Madeira population reference set is quite high (0.952), which is in within the allele frequency range detected in other populations, namely in a combined US/UK Caucasian population (0.895- Van Eerdewegh *et al.* 2002), a German population (0.910- Schedel *et al.* 2006), amongst Puerto Ricans (0.944) and Mexicans (0.972)- Lind *et al.* 2003- and also Japanese (0.997-Noguchi *et al.* 2006.). Chinese, however, have a considerably lower frequency for this allele (0.136- Su *et al.* 2008).

All members of the ADAM family are transmembrane proteins with a unique domain structure composed of a signal sequence and pro, metalloprotease, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic domains (Yoshinaka *et al.* 2002). These domains suggest roles in adhesive interactions, cell fusion, proteolysis and/or intracellular signaling and implicate this family of proteins in numerous biological processes including fertilization, neurogenesis, myoblast fusion and protein-ectodomain shedding of cytokines and other cell surface proteins (Gunn 2002).

The structure of the protease/cleavage domain of ADAM33 is dominated by amino acids with uncharged side chains, such as valine/serine or leucine/alanine and is unfavourable to amino acid substitutions, exhibiting decreased catalytic activity. Thus, all SNPs potentially modulating the catalytic domain of ADAM33 can contribute to asthma pathogenesis (Sampsonas *et al.* 2007). The *ADAM33-S1 c.710* polymorphism is located at exon 19, a transmembrane domain, (Sampsonas *et al.* 2007; Howard *et al.* 2003), resulting from a substitution of a Val to Ile, by a change from G to A nucleotide. Given that allele G is the ancestral (Choudhry *et al.* 2006), it is possible to presume that the relatively high frequency of G allele across most populations is preserved due to the need of maintaining ADAM33 catalytic activity necessary for all processes previously described. This, of course, may contradict the observation of an over-transmission of allele *ADAM33-S1c.710*G* to affected offspring, as reported by Van Eerdewegh *et al.* (2002). However, it is difficult to speculate whether mutations located in the transmembrane region cause changes that affect the proteolytic domain (Orth 2004). Additionally, both Val and Ile are nonpolar, highly hydrophobic amino acids (NCBI amino acid explorer 2013) and, therefore, it is not clear whether a change of aminoacid would have a substantial impact in the protein function. It is therefore possible that the over-transmitted allele at *ADAM33-S1 c.710* might be in linkage with another proximal SNP affecting the protein function and therefore implicated in asthma phenotypes. A similar explanation might be applied to *ADAM33-V4.*

5.3.5. *GSDML*

GSDML-236 was not found to be associated to asthma susceptibility in our Madeira population. Though, when analyzing asthma severity, a significant lower frequency of homozygous *GSDML-236*TT* in moderate and severe asthma was found when compared to mild asthma. These results contradict previous findings putting allele *GSDML-236*T* as associated to asthma susceptibility, severity and related phenotypes.

The first study to ascertain *GSDML-236*T* allele as strongly associated to asthma in a UK and German Caucasian population was Moffatt *et al.* (2007), through a GWA analysis. A

replication study, by Madore *et al.* (2008) obtained similar results in a French Canadian population. Tavendale *et al.* (2008), in a group of Scottish patients recruited from the BREATH study found allele *GSDML-236*T*, in one or two copies, significantly associated to the risk of childhood asthma and asthma exacerbations. Galanter *et al.* (2008) analyzed a group of Puerto Ricans and Mexicans from the Genetics of Asthma in Latino Americans (GALA) study and using a family based approach found an association between allele *GSDML-236*T* and asthma, asthma severity and levels of IgEs, for the first group. In a case-control study, the same authors were however unable to demonstrate a relationship between the risk allele and the previous parameters in a group of African-Americans. Wu *et al.* (2009), contrarily to Galanter *et al.* (2008) showed that there is an increased risk of asthma for *GSDML-236*TT* homozygous or *GSDML-236*CT* heterozygous individuals in a population from Mexico. Bisgaard *et al.* (2009) linked the genotype *GSDML-236*TT* with increased risk of asthma, acute severe exacerbations, wheezing and increased BHR. The role of *GSDML-236*T* allele in asthma was also assessed by Halapi *et al.* (2010) in diverse populations. The authors discovered a significant association between allele *GSDML-236*T* and all asthma in Iceland, The Netherlands and Germany study groups. *GSDML-236*T* was also found associated with childhood asthma in Germany and early onset of adult asthma (at or younger than 18) in Iceland, Australia, The Netherlands and Korea. In the combination of early-onset cases (for Iceland, Australia and UK), *GSDML-236*T* correlated positively with asthma severity. In the same study, *GSDML-236*T* allele was significantly associated to *ORMDL3* and *GSDML* expression. Binia *et al.* (2011) reported a significant association between the *GSDML-236*T* allele and adult asthma severity, as well as with susceptibility to childhood asthma-onset in a UK population. Yu *et al.* (2011) described *GSDML-236*TT* genotype, as associated to asthma, atopic asthma, increased levels of IgEs and severity of BHR in a sample of Korean children.

All studies mentioned present *GSDML-236*T* allele as the risk allele for asthma. In fact, it is known that ORMDL3 expressed by a neighbouring gene, modifies the endoplasmic reticulum (ER) mediated Ca^{2+} homeostasis and enables the unfolded-protein response (UPR) (Cantero-Recasens et al. 2010).The UPR consists of the set of responses by the endoplasmic reticulum to the burden of unfolded proteins in its lumen, also know as the endoplasmic reticulum stress, since most membrane proteins must first enter the ER, where the folding and assemblance process takes place (Walter & Ron 2011).

Increased ORMDL3 expression promotes stronger activation of the UPR transducing molecules and target genes, triggering the activation of NF-kB and c-Jun-N-terminal kinase (JNK), key molecules in the onset of inflammation, which may be particularly relevant in chronic inflammatory diseases such as asthma (Cantero-Recasens *et al.* 2010). Two independent studies confirmed the association of *GSDML-236*T* with expression levels of ORMDL3 (Moffatt *et al.* 2007; Hapali *et al.* 2010). Given these facts, the results observed for Madeira asthmatics, in which, as initially reported, a lower percentage of *GSDML-236*TT* genotype was observed in moderate and severe asthma, compared to mild are atypical. If one excludes the possibility of genotyping error, it could be proposed that, in our Madeira sample this polymorphism might be in LD with another, affecting it's expression. SNP rs7212938 is a non-synonymous polymorphism affecting the function of another gasdermin protein (GSDMA), belonging to GSDM family

and also located at 17q21.2. As proposed by Yu *et al.* (2011), although *GSDML-236* in GSDMB is located in an intron, rs7212938 might affect asthma susceptibility and intermediate phenotypes of asthma by acting in concert.

Aditionally, the absence of information in our sample regarding the asthma-age of onset, a relevant factor according to Bouzigon *et al.* (2008) and Halapi *et al.* (2010) and also levels of IgEs as well as BHR and distinct definition regarding asthma severity might have contributed to dissimilar results, compared to the ones obtained for other populations.

It is important to note, however, that the frequency of the *GSDML-236*T* allele is slightly augmented in the patients (0.604), compared to the Madeira population reference set (0.595), though it is not sufficient to reach the critical value of significance; plus the frequency of *GSDML-236*CC* genotype in severe madeiran asthma patient is null and severe asthmatics show the highest frequency of *GSDML-236*CT* genotype compared to moderate and mild asthma. Also, Tavendale *et al.* (2008) found allele *GSDML-236*T* in either one or two copies as significantly associated to asthma exacerbations. Hence, another possible explanation for the incongruous results might come from the lack of statistical power, conditioned by an insufficient sample size and producing spurious results.

One of the challenges to understand disease risk is still the lack of knowledge regarding the frequency of the polymorphisms in the populations (Cross et al. 2010). In fact, factors that could affect the association of the allele with disease, either positively or negatively, may not be possible to determine without population based allele frequencies (Cross *et al.* 2010). Therefore, the reported frequency for *GSDML-236*T* in the Madeira population reference set was 0.595, which is nearly within the range of other frequencies reported amongst Caucasian populations (0.460 to 0.578) (Moffatt *et al.* 2007; Madore *et al.* 2008; Tavendale *et al.* 2008; Halapi *et al.* 2010; Binia *et al.* 2011) but diverging from Puerto Ricans and Mexicans (0.668 and 0.732) and also African Americans (0.784) (Galanter *et al.* 2008), as well as Koreans, who present the highest frequency for *GSDML-236*T* allele (0.750 and 0.730 (Halapi *et al.* 2010; Yu *et al.* 2011). Again, the differences for the risk allele frequency across populations suggest that this SNP is not likely to act as a single contributor associated to asthma related phenotypes.

5.3.6. *STAT6*

It has been shown that STAT6 is essential for the induction of allergic asthma, playing a pivotal role for the development of pulmonary eosinophilia, airway hyper-responsiveness (AHR) and mucus hypersecretion in a Th2 cell-transfer model of allergic asthma in mice (Pernis & Rothman 2002; Hoshino *et al.* 2004).

However, *STAT6-21* revealed no association to asthma susceptibility in our sample from Madeira. Kavalar *et al.* (2012) reported similar results in a population from Slovenia, despite having found a significant association with recurrent wheezing as this group presented a lower frequency of allele *STAT6-21*C* compared to patients without recurrent wheezing in early childhood. Duetsch *et al.* (2002) also failed to report a significant association to asthma among a Caucasian sample from Germany and Sweden,

but found a positive effect on total IgEs levels by allele *STAT6-21*C*. Both Schedel *et al.* (2004) and Weidinger *et al.* (2004) also reported elevated serum IgEs levels, in German populations, but associated to *STAT6-21*T* allele. The Haplotype TCA, resulting from a combination of *STAT6-21*T* with two other SNPs in the same gene (rs3024974 and rs4559), was also found to have a major influence on total IgE level, according to Godava *et al.* (2012) in a population of Czech Republic. Kabesch *et al.* (2006), found haplotypes composed by *STAT6-21*T* and *IL13-1112*T* to be overrepresented in the combinations conferring the greatest risk for elevated IgE and asthma in a German population. Daley *et al.* (2009) reported an association between *STAT6-21*T* allele and both AHR and atopic asthma in a Caucasian population.

As described above, literature suggests in a number of studies, a relationship between variant *STAT6-21*T* and asthma or asthma related traits, opposite to our findings in the Madeira population. It is worthy to note that in asthmatics, the frequency of *STAT6-21*TT* genotype is 1.5 times higher than in the Madeira reference set, thought no significant difference was found. Furthermore, it has been demonstrated that the levels of *STAT6* isoforms are increased in individuals carrying the polymorphic *STAT6-21*T* allele (Schedel *et al.* 2009) and increased phosphor STAT6 (and also phosphor STAT1) expression have been found in asthmatic patients, compared with that seen in healthy control subjects (Gernez *et al.* 2007). In fact, allele *STAT6-21*T* disrupts a presumed nuclear factor kB site within a cis-regulatory region, potentially affecting the regulation of *STAT6* gene (Kabesch *et al.* 2006).

A deviation from Hardy-Weinberg proportions was observed for mild asthma, as a significant higher frequency of heterozygotes was expected (0.444), compared to the observed (0.279). For this reason the mild asthma group has not been further considered in the remaining analysis. Yet, a comparison between moderate and severe asthma revealed no differences between groups resulting in a non-contribution of SNP *STAT6-21* for asthma severity in the Madeira population. However, allele's *STAT6-21*T* frequency among severe asthmatics was also 1.5 times higher compared to moderate asthma. Findings of increased levels of epithelial STAT6 expression, in subjects with severe asthma in comparison with subjects with mild asthma and normal controls have been previously reported by Mullings *et al.* (2001).

Again, the lack of reproducibility in Madeira population might arise from a number of factors. First, given the number of studies emphasizing the relationship between this polymorphism and levels of IgEs, the absence of data regarding this parameter, for the study population, might have concealed a possible association. Additionally to the previously exposed reasons, such as diverse studies design, different definitions of asthma in different populations, exposure to distinct environmental factors, the sample size might have lacked sufficient statistical power to detect eventual associations, such as discussed by Cardon & Bell (2001).

Of all studied polymorphisms, *STAT6-21* shows the highest value of observed heterozygosity, and a frequency of 0.329 for the *STAT6-21*T* allele, within the Madeiran population. In fact, in German populations the *STAT6-21*T* allele frequency varies from 0.370 (Schedel *et al.* 2004; Kabesch *et al.* 2006), to 0.380 (Weidinger *et al.* 2004) to 0.410 in a Czech population (Godava *et al.* 2012) and 0.420 in a Slovenian control

population, (Kavalar *et al.* 2012). Amongst Caucasian sib-pair families from Germany and Sweden, the registered frequency was 0.439 (Duetsch *et al.* 2002). Chinese were found to present a lower allele frequency (0.273-Wu *et al.* 2010).

If for a particular allele, a difference in allele frequencies is observed between two different populations, there is no easy way to determine whether this difference arose from a founder effect, genetic drift or natural selection; however, patterns of allele frequencies in genes related to respiratory status may provide new insight into the genetic factors underlying respiratory disease susceptibility (Le Souëf *et al.* 2006). Particularly, alleles in genes related to the Th2 lymphocyte responses could become more frequent in tropical climates, owing to the need for defence against helminthic infection, and less frequent in cool or dry climates, owing to a modest long- term increase in mortality from allergic disease (Le Souëf *et al.* 2006). With respect to STAT6 the same variant of the gene associated with a reduced burden of ascaris infection has been associated with asthma (Le Souëf *et al.* 2006). Therefore, frequencies might vary in accordance.

Despite disagreeing results verified in the Madeira population, it appears that *STAT6-21*, influences *STAT6* regulation and may help explain the underlying biological mechanisms of the observed associations with increased total serum IgE levels and the risk for asthma at the population level; however its impact on the transcriptional activity of *STAT6* is subtle, an expected fact from a regulatory SNP involved in a complex pathway of such a heterogeneous disease (Schedel *et al.* 2009).

5.4. Gene-Gene interaction

5.4.1. The 5q31.1 region

Chromosome 5q31 has been studied by many groups (Holloway *et al.* 2001;Palmer *et al.* 2001; Parate *et al.* 2010) following an original observation of genetic linkage to total serum IgE concentrations in extended Amish pedigrees and the confirmation of linkage to the same region (Cookson 2002).

Regarding our Madeira population, *IL13-c.144* and *ADRB2-c.16* pair of polymorphisms was associated to asthma severity. A study conducted in a Chinese population, found that the relative risk of developing childhood asthma in carriers of both *IL13-c.144*GG* and *ADRB2-c.16*AA* was significantly higher than that in the carriers of either, suggesting an enhancing effect between both (Hua *et al.* 2008). Leung et al. (2007) reported a significant 3-*loci* interaction between *IL13-c.144*, *ADRB2-c.16* and *STAT6 C1570T* for determining change in FVC in a group of Chinese asthmatic children supporting epistasis among the genes.

Also, a significant combined effect was found for *IL4-590* and *IL4-RP2* polymorphism affecting asthma susceptibility and severity. These polymorphisms are in LD for Madeira population, as previously discussed (see section 6.3.2) and both the *IL4-590*TT, IL4-RP2*183183* and *IL4-590*CT, IL4-RP2*183253* are significantly higher amongst patients (and increasingly higher in moderate and severe asthma) than in the Madeira population reference set, opposite to *IL4-590*CC, IL4-RP2*253253*, whose role as a

predictive factor of belonging to the Madeira population reference set group was confirmed by binary logistic regression. A significant haplotype transmission composed of *IL4-RP2*183*), *IL4-590*T* and *IL4-33*T* alleles was found amongst asthma-affected children from Japan (Noguchi *et al.* 2001). Haplotypes from *IL4-590* and *IL4-RP2* were also found to be significantly different between chronic obstructive pulmonary disease (COPD) and a control group in a Japanese population (Hegab *et al.* 2004). Despite the fact that asthma and COPD show substantial differences, they might also share similarities (Postma *et al.* 2011). Hence, according to the Dutch hypothesis, various forms of airway obstruction are different expressions of a single disease entity, suggesting that genetic factors, endogenous factors and exogenous factors all play a role in the pathogenesis of chronic nonspecific lung disease (Postma & Boezen 2004). Moreover, according to Postma *et al.* (2011) there are examples from the candidate-gene approach that may illustrate a genetic overlap in asthma and COPD, including the genes *IL4* and *ADRB2*.

A significant interaction between *IL4-RP2* and *ADRB2-c.16* was observed when comparing the Madeira population reference set to severe asthma. In an Egyptian population, *ADRB2-c.16* and the proximate polymorphisms *ADRB2-c.27* in combination with *IL4-RP2* showed more significant differences than the analysis of the *ADRB2-c.27C/G,* for COPD (Hegab *et al.* 2004). Lonjou *et al.* (2000) analyzed the cytokine region on chromosome 5q31–33 and confirmed that the estimated effects at *IL4* and *ADRB2* correspond to substantial risk for asthma attributable to the 5q cytokine region.

5.4.2. Other gene-gene interactions

Besides the 5q31 region, other gene-gene interactions related to asthma were identified in Madeira population. Therefore, *IL4-590* or *IL4-RP2* combined with *STAT6-21* contribute to differences in severe asthma when compared to the Madeira population reference set. Both *IL4* and *STAT6* genes, together with *IL13* form the *IL4/IL13/STAT6* pathway playing a key role in asthma pathogenesis (Oh *et al.* 2010). According to Vercelli (2008) the risk of developing asthma is, in fact, synergistically increased by combinations of SNPs in individual genes of the Th2-cell-associated signalling pathway. In a group of German children, each of the individual polymorphisms in *IL4-590*, *IL13* (rs1800925) and *STAT6-21* had a modest effect in isolation on asthma susceptibility, but the risk was synergistically enhanced by carriers with combinations of two or three SNPs within the Th2-cell associated pathway (Vercelli 2008). Polymorphism within these genes cause aberrant binding and signaling activities that result in over-activation of the IL4/IL13 pathway (Oh *et al.* 2010). *IL4* and *IL13*, which share the IL4Rα subunit in their cognate receptors, activate *STAT6*, required for IL4 production. Moreover, activation of *STAT6* is critical for the differentiation of naive T-cells into Th2 effector cells and regulates IL4 and IL13-induced production of Th2 chemokines, from airway epithelial cells, fibroblasts and smooth muscle cells (Oh *et al.* 2010).

IL4-RP2 and *ADAM33-S1 c.710* grouping also showed significant differences between Madeira group and severe asthma. Although literature regarding the association of these two genes is scarce, Cui *et al.* (2003) genotyped *IL4-590* and *IL4R∝Q576R* and concluded that the last confered genetic susceptibility to allergic asthma in Chinese, but added that other genes, namely *ADAM33* could be possible contributors to the genetic

interaction accounting for an additional risk of asthma in specific individuals. However, it is known that ADAM proteins have diverse functions, which include the shedding of cell-surface proteins such as cytokines and cytokine receptors (Van Eerdewegh *et al.* 2002). Loss of function may lead to impaired processing of cytokines and growth factors (Holgate *et al.* 2003) with a possible implication in the metabolic pathway of asthma.

IL4-RP2 and *GSDML-236* pair of genes showed a significant interaction accounting for severe asthma in the Madeira population. Again, to date, no studies addressing the joint effect of both genes have been described, but a potential explanation could be postulated. *GSDML* gene lies within intron 1, in the proximities of *ORMDL3* (Moffatt *et al.* 2007). It has been hypothesized that ORMDL proteins given their ER-membrane localization, participate in correct protein folding and/or trafficking in the ER, or are involved in the cellular UPR (Hjelmqvist *et al.* 2002). As explained previously, the UPR can trigger the activation of NF-kB and JNK, key molecules in the onset of inflammation (Cantero-Recasens *et al.* 2010) and IL4, which activates STAT6, synergizes with activators of NF-kB to induce *IL4*-responsive genes (Shen & Stavnezer 1998).

Leung *et al.* (2009) suggests it would also be interesting to investigate the epigenetic interactions for asthma and atopy between genes on chromosome 17q21 and other known candidate genes.

Finally, *ADRB2-c.16* and *GSDML-236* pair showed significant differences between Madeira population reference set and mild asthma. *ADRB2-c.16*AA GSDML-236*CT* combined genotype, for example, was found at a higher frequency in mild asthma compared to Madeira population. Once more, the absence of bibliographic references with respect to the interaction of these two genes does not allow broader considerations.

When analyzing the eight SNP profile on asthma patients *versus* the Madeira population reference set, unique genetic profiles were identified in each group. In patients, the most frequent profile, *GGCC253253AACCGGCTCC*, differed from the Madeira's group *GGCC253253AGCCGGCTCT* by two SNPs: *ADRB2-c.16* and *STAT6-21*. Amongst the common profiles to both groups, *GGCC253253GGCCGGCCCT* appears more frequently among asthmatics, while *GGCC253253AACCGGTTCC* is more frequent within Madeira population reference group, differing in *ADRB2-c.16*, *GSDML-236* and *STAT6-21.* Although in asthma the synergy of genetic variants may cause an increase in disease risk (Vercelli 2008), the validity of these genetic profiles must be considered with caution. A larger sample size is probably necessary to contextualize the meaning of each profile in asthma prediction. In fact, the identification and characterization of gene-gene interactions have been limited by the lack of powerful statistical methods and large sample size (Chan *et al.* 2006; Vercelli 2008). However, the biological plausibility of the underlying hypothesis offers credibility to results (Vercelli *et al.* 2008).

As previously discussed (see sections 6.3.3, 6.3.5 and 6.3.6) *ADRB2-c.16*, *GSDML-236* and *STAT6-21*) neither of the SNPs *per se* is involved in asthma susceptibility for our Madeira population. However, gene–gene interactions, are likely to contribute to the complexity of genetic diseases, as each variant typically has modest effects in isolation, but

synergizes effectively with other variants to magnify the impact on disease risk (Vercelli 2008).

Recent developments in powerful analytical methods such as network analysis provide new opportunities to shift our understanding of diseases from a morphological to a molecular basis (Bhavnani *et al.* 2011). By using network visualizations and exploratory analysis it was possible to identify three clusters of asthma patients with three clusters of cytokines and the complexity of these interactions offering a perception in the classification of these patients. Interestingly, the clusters were different of those obtained by key pulmonary functions, suggesting that the classification of asthma based on the molecular level could bring improvement to the diagnosis and management of asthma (Bhavnani *et al.* 2011).

Besides, a meta-analysis consisting of 127 asthma-related genes and their corresponding proteins, revealed that 96 could be connected to a same gene-mRNA-protein and protein–protein interaction network, and were found to be enriched significantly with protein binding, signal transduction, and endopeptidase activities (Renkonen *et al.* 2010). By using computational means and databases the level of knowledge can be increased by performing systems level analyses of previously characterized genes carrying SNPs related to asthma (Renkonen *et al.* 2010).

Asthma candidate genes are thought, however, to contribute only 40–60% to the overall risk (Su *et al.* 2012), but in contrast with genetics, research into the environmental causes of asthma is in its infancy (von Mutius *et al.* 2009).

5.5. Gene-environment interactions

The recognition of the importance of environmental factors and its interaction with genetics in influencing the outcome of the asthma disease is a largely acccepted concept (Vercelli 2010; Ege *et al.* 2011; Kauffmann & Demenais 2012; Zervas *et al.* 2012).

The presence of birds as pets, ever, is inversely related to the presence of more severe forms of asthma, for individuals with *ADAM33-V4*CC* genotype or genotype *ADAM33-S1c.710*GG*. Despite asthma severity not having been assessed, owning a bird in the first two years of life was not found to be a risk factor for asthma (Carlsen *et al.* 2012). On the other hand, Hypersensitivity pneumonitis (HP), a form of allergic disease has been described to be caused by inhalation of proteins present on feather dust and birds excrements (Bogaert *et al*. 2009), where allergens are present (Plaut *et al.* 1996). However, in this case, the presence of birds is inversely related to asthma severity, for individuals presenting the above-mentioned genotypes. It could be argued that both polymorphisms could account for protection against severe forms of asthma caused by allergens present in the bird's feathers or excrements. Being *ADAM33* gene associated to bronchial hyperresponsiveness, i.e. the increase in the aiways sensitivity to a variety of stimuli (Kang *et al.* 2012a) these genotypes could reveal as protective against BHR for bird allergen.

The presence of cockroaches, however, increases the odds of asthma severity, within the subgroups *ADAM33-V4*CC*, *ADAM33-S1c.710*GG* or *IL13-c.144*GG*. It is know that

cockroach allergen exposure leads to increased bronchial permeability of the epithelium, causing airway sensitization and eliciting allergenic response, which may be responsible for the development of asthma (Antony *et al.* 2002). A study has referred to the *IL13-c.144* polymorphism, and its link to serum concentrations of specific IgEs to cockroaches (Leung *et al.* 2001). In addition, increased levels of IL13 were found in cells from allergic subjects to cockroaches (Gao 2012). There seems to be, to date, no study about the relationship between *ADAM33*, asthma severity and/ or sensizitaztion to cockroach allergen, however, given its role in BHR, as previously described, a possible involvement of the protease in cockroach allergen reaction and asthma severity cannot be excluded.

Individuals within the 85th-95th percentile (overweight) with the *ADAM33-S1c.710*GG* genotype also seem to significantly contribute to the prediction of asthma severity.
An analysis on childhood asthma and obesity and the commom genetic factors contributing for both diseases has been performed, however, *ADAM33* was not considered in the analysis (Melén *et al.* 2010). Nevertheless, it could be argued that, by playing a role in lung function, an unfavourable genotype or combination of genotypes, may condition physical activity, contributing to a situation of overweight, leading to more severe forms of asthma.

Similarly, the odds of the outcome occurring are increased by legumes intake only once or twice a week, either for subgroup *ADAM33-S1c.710*GG* or *IL13-c.144*GG*. As discussed previously (see section 6.2.2.2) a study in a Spanish pediatric population, showed that legumes such as lentils and chickpeas were responsible for most allergic reactions (San Ireneo *et al.* 2008). Though the role of legume intake associated to ADAM33 protease in allergy has not yet been described, an interaction between the peanut allergen peptide and the production of IL13 cytokine is known (de Leon *et al.* 2007). Therefore, as previously proposed, given the ADAM proteins function in the shedding of cytokines (Van Eerdewegh *et al.* 2002) it may indirectly implied in the metabolic pathway leading to asthma.
Pasta intake (either once or twice a week or three or more times per week), and fast-food (once or twice a week) each are also positively related to an increase in asthma severity, given the presence of genotype *ADAM33-S1c.710*GG*. Similarly, as previously explained (see section 6.2.2.2) there is evidence of increased risk of asthma related to the comsumption of pasta, noodles or fast food once or more a week, though in this study, severity was not taken into account (Awasthi *et al.* 2004). However, so far, no biological mechanism has been proposed that could explain the role of ADAM33 in food allergy, leading to asthma severity. However, a relationship was found between polymorphisms within *ADAM33* and Japanese cedar pollinosis, a common seasonal allergic rhinitis in Japan (Cheng *et al.* 2004). Therefore, possible yet non-studied relationships between *ADAM33* and food allergens might be a possibility. In fact, a role has been suggested for ADAM33 in stimulating cytokine network, similarly to ADAM10 and 17 (Raby *et al*. 2004).

Both butter consumption once or twice a week and margarine intake three or more times per week, for patients exhibiting *ADAM33-S1c.710*GG*, are inversely correlated with the display of severe forms of asthma. However, as earlier discussed, the formation of arachidonic acid–derived eicosanoids from omega-6 fatty acids leads to increased production of IgE, eliciting allergic sensitization (Bolte *et al.* 2001). Therefore, a role could be proposed for this genotype, in modifying the response to IgE production, derived from the metabolism of omega-6 fatty acids, and promoting a protective role against allergy and consequentely to asthma and/or asthma severity.

Positive skin prick tests for *Blomia sp.* house dust mite extract, are positively correlated with more severe forms of asthma, as well as fruit comsumption only once or twice a week, for subgroup *IL13-c.144*GG*. As discussed previously, a role for *IL13-c.144*GG* in the severity of asthma, given other factors, such as, in this case, a positive reaction to *Blomia sp.* and fruit consumption, might be proposed since this cytokine is involved in the Th2 response ocurring in asthma (Brightling *et al*. 2010).

It is important to note that the environmental factors may also produce epigenetic changes in the DNA since as early as *in utero* life (Relton *et al.* 2010; Thornburg *et al.* 2010; Durham *et al.* 2011) in interaction with genetic variants affecting the DNA susceptibility to methylation (Karmaus *et al.* 2013) and, as recently reported, modelling the risk of complex diseases, such as asthma (Liu *et al.* 2008; Reinius *et al.* 2013).. Therefore, and as an example, a methyl rich diet has been shown to affect the DNA methylation status and enhance allergic airway disease in mice offspring (Reinius *et al.* 2013). In addition, the current food intake may also affect methylation, though not to the same degree as intrauterine exposure (Reinius *et al.* 2013). Future work hypothesis in the Madeira population regarding asthma should therefore consider the possibility of exploring the potential epigenetic patterns, resulting from a combined action of both environment and genes affecting the disease phenotype.

Thus, the results above described, must be carefully considered and interpreted. Despite the fact that a biological explanation can be found to explain the different outcomes, limitations in the study, such as small sample size and multiple testing, amongst others, can constitute strong constraints to the viability of the explored hypothesis.

6. Limitations of the study

Throughout the previous chapters, possible limitations of this study were pointed out such as insufficient sample size, possible confounding factors such as the patient's genetic background and absence of data regarding some of asthma's phenotypes, such as the IgEs levels.

In fact, the Madeira population reference set used to establish comparisons with the patient's sample was not matched in age or sex to the case group. Unmatched control groups are likely to prove inappropriate (Cardon & Bell 2001), and a number of questions should be addressed, in particular whether control subjects are free of disease symptoms, associated intermediate phenotypes, whether they have been exposed to relevant environmental influences disease-related, while remaining unaffected and also whether they are matched for both demographics and environmental factors (Silverman & Palmer 2000). In the present study there was no specific data regarding the subject's exposure to the environmental variables assessed for patients, nor there was any thorough information regarding the absolute exclusion of disease-related symptoms, despite their classification as healthy.

Another possible confounding factor might be related to potential population stratification, defined by Cardon & Bell (2001) as the presence of multiple subgroups with different allele frequencies within a population. The different underlying allele frequencies in sampled subgroups might be independent of the disease within each group, leading to erroneous conclusions of LD or disease relevance (Cardon & Bell 2001). Despite the fact that, in the Madeira population reference set, the chosen subjects were from local origin such as their parents and grandparents, in the patient's group, the assessment criteria was fundamentally based on the disease symptoms of asthmatic individuals and their parents, but might have neglected aspects related to the origin of their grandparents, introducing possible confounding effects.

In order to reduce the putative impact of population stratification, the TDT was assessed. The TDT tests for distortion in transmission of alleles from heterozygous parents to affected offspring being robust against stratification (Lewis 2002). According to this test, both the *IL4-590* and *IL4-RP2* polymorphisms, previously identified as both susceptibility and severity *loci*, were not in linkage with the disease. Despite the subsequent justifications as possible causes for this outcome, the fact is that the TDT itself throws away some genotype information, owing to its reliance on heterozygous parents, which creates a loss of statistical power to detect genuine allelic association (Cardon & Bell 2001). Another possibility that might have affected the TDT lies in putative cases of false paternity. The percentage of paternal discrepancy measure for reasons of disputed paternity tests, for Portugal, was estimated in 29.84% (Geada *et al.* 2000).

Although too often neglected, genotyping errors affect most data and can markedly influence the biological conclusions of a study (Pompanon *et al.* 2005). In SNP studies, allele calling has been identified as a potential problem; other issues such as the DNA quality and/or quantity might favour allelic dropouts and false alleles (Pompanon *et al.* 2005).

Opposite to patients, whose DNA was extracted from blood collected at the immunoallergology consultation by qualified professionals, the families' DNA was extracted from saliva, and, despite the instructions for maximizing DNA yield and avoid contamination, some samples might have been contamined. Possible contaminants that cannot be excluded result from smoking and/or kissing and constitute PCR inhibitors (Anzai-Kanto *et al.* 2005).

Given the high number of variables, both environmental and genetic, and given the existence of patient's subgroups, such as with respect to disease severity, multiple tests were performed. Multiple testing generates subgroups that are small, providing less robust results, and also creates a substantial risk of associations being described by chance, i.e. a statistical Type I error (Cardon & Bell 2001). In fact, if the significance cut-off level is 0.05 then 1 out of 20 positive associations is likely to be false (Juran & Lazaridis 2007). Therefore, the introduction of a post hoc test, such as Bonferroni correction, could substancially reduce the probability of commiting a Type I error (Cardon & Bell 2001). However, the use of this test reduces statistical power, by increasing unnaceptably the possibility of incurring in Type 2 error (Perneger 1998; Moran 2003; Nakagawa 2004; Buchanan *et al.* 2006; Juran & Lazaridis 2007). The overcorrection for the false-positive rate eliminates valid information in the sample (Cardon & Bell 2001). The applicability of this test has been issue of discussion on the literature (Bailey-Wilson *et al.* 1995; Risch & Merikangas 1996). The Bonferroni test assumes that all comparisons are independent, which may not apply to genetic studies in which SNPs may be correlated by LD, making it too conservative (Johnson *et al.* 2010).

In addition, thousands of cases and controls may be needed if a study is to have sufficient statistical power to identify the alleles of interest (Vercelli 2008). However, the opposite might also create additional constraints since extremely large populations may also lead to heterogeneity in environmental exposures, a variable critical for the outcome of any genetic study (Vercelli 2008).

7. Conclusions and future perspectives

Globally, potential susceptibility and severity environmental and genetic markers were found to be associated to asthma in the Madeira population. However, these results must be cautiously interpreted since the sample size for the asthma subgroups lack robustness, implying that these observations require confirmation in future studies including a larger sample size. However, as initially proposed, both the environmental and particularly the genetic markers might be useful tools in the management of asthma. Thus the studied genetic markers may be of crucial importance in the clinic diagnosis, allowing a personalized intervention in patients with the disease. Primary prevention is thus an important goal that can be achieved by an early intervention aimed to define the risk factors and to better prepare and manage the disease burden. A summary of the significant environmental and genetic associations to asthma severity and/ or susceptibility found in this study follows.

In the present study, family factors namely the presence of rhinitis in the mother was found to account for mild and persistent asthma as well as for the presence of wheezing and rhinitis amongst the asthmatic offspring.

Social factors, such as living in a urban environment, was found to account for persistent asthma, whereas living in a humid house, having potable water and electricity and the house floor made of tiles was associated with the presence of intermittent asthma. The smoking habits in the family revelead that the father passive smoking was related to mild asthma.

As for the nutritional habits, the weekly intake of fish was inversely associated to persistent asthma, while legumes consumption was found related to moderate and severe forms of the disease. Pasta intake habits was further associated to moderate and persistent asthma.

The allergy profile analysis revelead that the *Lepidoglyphus sp*. mite wheal size was found associated to severe asthma, compared to mild, while the *Platanus sp.* pollen extract showed a higher frequency of sensitization in intermittent asthma. Dog allergen extract was positively correlated to persistent asthma and its mean wheal size significantly larger in severe compared to the mild form.

As for the genetic polymorphisms, the alleles *IL4-590*T* and *IL4-RP2*183* as well as the genotypes *IL4-590*CT/IL4-590*TT* and *IL4-RP2*253183/IL4-RP2*253183* clustered together were found associated to both asthma susceptibility and asthma severity, namely moderate, severe and persistent asthma when compared to the Madeira reference sample set. *GSDML-236*TT* was found associated only to severity, with the genotype presenting a lower frequency in moderate and severe asthma when compared to mild asthma. Allele *ADAM33-V4*C* was significantly over-transmitted to asthmatic offspring being linked with the disease by TDT.

Combinations between pairs of polymorphisms at the 5q31 region namely *IL13-c.144/ADRB2-c.16*, *IL4-RP2/ADRB2-c.16* were associated to asthma severity while *IL4-590* and *IL4-RP2* was associated both to susceptibility and severity. Other gene-gene interaction revelead that *IL4-590* or *IL4-RP2* combined with *STAT6-21*, *IL4-RP2* paired

with *ADAM33-S1 c.710*, *IL4-RP2* and *GSDML-236* and finally *ADRB2-c.16* and *GSDML-236* were all found to account for the severity of the disease.

As for gene-environment interactions, birds as pets, are inversely related to asthma severity, given the presence of genotypes *ADAM33-V4*CC* or *ADAM33-S1c.710*GG*. The presence of cockroaches is positively correlated to asthma severity, in the presence of *ADAM33-V4*CC*, *ADAM33-S1c.710*GG* or *IL13-c.144*GG*. Overweight and the *ADAM33-S1c.710*GG* genotype presence also increase the odds of severity. Weekly legume intake and *ADAM33-S1c.710*GG* or *IL13-c.144*GG* as well as weekly fast food or pasta intake with each genotype *ADAM33-S1c.710*GG* are associated to the severe form of the disease. Weekly butter consumption or margarine intake for patients exhibiting the ADAM33-*S1c.710*GG* genotype are inversely correlated to the display of severe forms of asthma, contrary to positive skin prick tests for *Blomia sp*. or weekly fruit consumption each for subgroup *IL13-c.144*GG*, found to predict severe asthma forms.

Again, these interactions must be interpreted carefully considering the initially exposed study constraints. In future studies, it is therefore important to reinforce the use of a larger sample size in order to validate these results. In addition to the expansion of the sample size, further genetic markers implied in the metabolic pathways of asthma should be a future object of study in this population with the purpose of broadening the understanding of the disease genetic grounds. Under these circumstances, the creation, for the first time, of a DNA biobank, has been an important contribution as part of this work, since it could be used as a reference in future studies addressing asthma genetics, in a population where the disease represents an important health issue. Through the increase of the disease knowledge in what it concerns its genetic foundations, new leads could arise, contributing to a better management of the asthma in the Madeira population.

8. Bibliography

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Appendix

Original Article: Distribution of polymorphisms *IL4 -590 C/T* and *IL4 RP2* in the human populations of Madeira, Azores, Portugal, Cape Verde and Guinea-Bissau.

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Original Article

Distribution of polymorphisms IL4 -590 C/T and IL4 RP2 in the human populations of Madeira, Azores, Portugal, Cape Verde and Guinea-Bissau

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Abstract: The IL4 gene is located on chromosome 5q23.3-31.2. Polymorphisms within this cytokine gene, like the derivative allele T of IL4-590, have been reported as being associated to elevated IgE serum levels and asthma. In the present work, the allelic and genotypic frequency of the IL4-590 and IL4 RP2 polymorphisms was carried out in 599 individuals from Madeira, Azores, Portugal mainland, Cape Verde and Guinea-Bissau and in a sample of 101 asthmatics from Madeira population. In all populations the polymorphisms were in LD and presented a significant dissimilar allelic and genotypic distribution ($p<0.05$) except between mainland Portugal and Madeira when compared to Azores. Significant differences regarding both loci were found between Madeira population and the group of asthmatics. Genotype 183183TT frequency is higher for African populations while 253253CC prevails in Caucasian populations. The existence of a Hardy-Weinberg Disequilibrium in Guinea-Bissau population not observed in neutral markers leads to the hypothesis of natural selection occurring in these loci probably associated to a rapid population growth an hypothesis strengthened by neutral STRs D5S818 and CSF1PO gene diversity.

Keywords: IL4-590, IL4 RP2, D5S818, CSF1PO, Asthma, Madeira, Azores, Portugal mainland, Cape Verde, Guinea-Bissau

Introduction

The Interleukin 4 (IL4) cytokine mediates a variety of interactions among components of the immune system. It induces immature effector T cells to assume a Th2 phenotype and also B-cells to undergo immunoglobulin type-switching and secretion of IgE [1].

The IL4 gene located on chromosome 5q23.3-31.2 presents the IL4-590 (C-590T) single nucleotide polymorphism (SNP) on the promoter region of the gene [2]. Phylogenetic studies indicate that this polymorphism belongs to a conserved region in all primates except for humans. The derivative allele T has been related to elevated serum levels of IgE and asthma, a complex disease affecting the worldwide population mainly on developed countries. High frequencies of this allele may be the result of positive selection [1] perhaps justified by an association between IL4 -590T allele and elevated anti-malarial IgG levels [3].

Polymorphism RP2 is a repetitive sequence located at the second intron of IL4 gene. This polymorphism can be classified as a VNTR as it consists of a 70 bp repetitive motif unit generally appearing in two or three copies sized 183 and 253 bp, respectively [2].

The Atlantic Islands of Madeira, Azores and Cape Verde were colonized by the Portuguese in the 15th and 16th centuries and received different levels of sub-saharan slave contribution from the African coast of Guinea [4]. Studies using mtDNA show that more than 20% of Madeira haplotypes belong to sub-Saharan haplogroups contrarily to the 8.7% found at Azores [5] but these results are completely different to Y chromosome where no sub-saharan lineages were found [6].

The data obtained for Cape Verde through chromosome Y studies indicate no more than a 15.9% influence from Guinea-Bissau (the putative place of origin of its sub-Saharan popula-

tion) while the European contribution through male lineages is about 53.5% [7], contrasting to mtDNA studies where 93% holds sub-Saharan haplogroups [8].

Considering the prevalence of asthma and atopy, the results from Cape Verde and Madeira presented a significant difference of atopy in the population: from 9% in Cape Verde to 54% in Madeira, and active asthma between 7% in Cape Verde and 14.6% in Madeira. The significant variation of asthma prevalence found in several populations remains in discussion, but although genetics linked to ethnicity seems to play a role, it will be strongly modulated by environmental variables and lifestyle [9]. In the last few years in Cape Verde the atopy prevalence raised threefold although the prevalence of atopic asthma increased with the growth of the tourism industry and the improvement of standards of living of the local population [9].

Given the importance of IL4 as an immune-regulatory gene it is of great interest to investigate the genetic background of populations for polymorphisms within the gene proved to influence its expression.

Association studies between mutations and STR allow favorable mutations to be detected. If the frequency of the selected allele will increase, the same is expected for the frequency of the STR allele that is on the same genomic region as the selected allele but this will depend on the number of alleles and overall heterozygosity of the locus [10].

This study aims to determinate the frequency for both IL4-590 and IL4 RP2 polymorphisms in the Atlantic Islands of Madeira, Azores and Cabo-Verde as well as mainland Portugal (Western Europe) and Guinea-Bissau (West African coast), to compare the populations by analysing the distribution of these two polymorphisms and observing if there is local selection on this genomic region and if these mutations behave favorably. Additionally by analyzing a group of asthmatics from Madeira Island we intent to assess the importance of both loci on detecting asthma predisposition in the Madeira population.

Material and methods

DNA samples from 599 unrelated male subjects from the following populations were used: Madeira (n=110), Azores (n=116), Portugal Mainland (n=106), Cape Verde (n=152) and Guinea-Bissau (n=115). A sample of asthmatics (n=101) from the immunoalergology consultation at Dr. Nélio Mendonça Hospital, Funchal, was also analyzed.

The IL4-590 SNP (rs 2243250) was analyzed by real-time PCR using the 7300 System SDS Software v1.4. (Applied Biosystems). The IL4 RP2 VNTR was analyzed according to Mout and colleagues [2].

Comparison between each pair of populations was done by Fisher's exact test using ARLEQUIN vs.3.01. Statistical significance was defined as $p < 0.05$.

The average gene diversity of both loci was assessed using previously published data for two STRs - D5S818 and CSF1PO in chromosome 5 - for Madeira [11], Azores [12], Cape Verde [13] and Guinea-Bissau [14] excluding the double heterozygous individuals in both IL4 and STRs polymorphisms, in a whole of 159 individual from all populations. Rst values were determined by FSTAT 2.9.3.2.

Results

Genotyping and allelic frequencies for all populations are shown in **Table 1**. All studied populations are in Hardy-Weinberg equilibrium for both loci except for Guinea-Bissau ($p=0.00764$) since it presents a significant lower frequency of heterozygous for the IL4-590 locus (0.261) than expected (0.361). For all populations IL4-590 and IL4 RP2 loci were found to be in linkage disequilibrium (LD). All pairs of populations present significant differences ($P<0.05$) for both polymorphisms except in case of Madeira and mainland Portugal when compared with Azores but only in the case of IL4-590.

Both -590T and 183 allelic frequencies are higher for Guinea-Bissau population (0.765/0.517), while Madeira presents the lowest frequencies (0.105/0.105). Genotypic TT frequency varies decreasingly from 0.635 in Guinea-Bissau to 0.009 in Madeira and Azores whereas genotype 183/183 varies from 0.252 in Guinea-Bissau to 0.027 in Madeira population.

When both IL4-590 and II4 RP2 polymorphism were considered, nine genotypes were found.

Table 1. Distribution of allelic and genotypic frequencies, p value for HWE and LD for IL4-590 and RP2 polymorphisms for five populations: Madeira Asthmatics (MA);Madeira (M); Azores (A); Portugal Mainland (P); Cabo-Verde(CV) and Guinea-Bissau(GB)

	MA	M	A	P	CV	GB
N	101	110	116	106	152	115
C	0,792	0,895	0,871	0,802	0,408	0,235
T	0,208	0,105	0,129	0,198	0,592	0,765
CC	0,624	0,800	0,750	0,651	0,191	0,104
CT	0,337	0,191	0,241	0,302	0,434	0,261
TT	0,039	0,009	0,009	0,047	0,375	0,635
HWE	1.00000	1.00000	0.68945	0.54906	0.24029	0.00764
253	0,817	0,895	0,871	0,722	0,572	0,483
183	0,183	0,105	0,129	0,278	0,428	0,517
253/253	0,673	0,818	0,776	0,557	0,335	0,217
183/253	0,287	0,155	0,190	0,330	0,474	0,530
183/183	0,040	0,027	0,034	0,113	0,191	0,252
HWE	0.73635	0.09135	0.09331	0.08841	0.74169	0.57814
253/253CC	0,624	0,791	0,741	0,547	0,184	0,070
253/253CT	0,050	0,027	0,034	0,009	0,105	0,061
253/253TT	-	-	-	-	0,046	0,087
183/253CC	-	-	0,009	0,085	0,007	0,035
183/253CT	0,287	0,145	0,181	0,226	0,296	0,191
183/253TT	-	0,009	-	0,019	0,171	0,304
183/183CC	-	0,009	-	0,019	-	-
183/183CT	-	0,018	0,017	0,066	0,033	0,009
183/183TT	0,040	-	0,017	0,028	0,158	0,243
LD (p)	0,000	0,000	0,000	0,000	0,000	0,000

Genotype 253253CC is mainly found in Madeira (0.791) and Azores (0.741) while at Guinea-Bissau 183183TT appears in a higher frequency (0.243). In Cape Verde the highest frequency is observed for genotype 183253CT (0.296).

When average gene diversity is considered for D5S818 and CSF1PO STRs from chromosome 5 a significant LD association between each pair of loci was found except between D5S818 and CSF1PO (0.27077). The allelic distribution for D5S818 and CSF1PO within 253253CC, 183183TT and 253253TT genotypes is shown in **Figure 1**. Average gene diversity for STRs D5S818 and CSF1PO is higher for 253253 TT (0.841667) followed by 183183 TT (0.783019) and 253253 CC (0.740385). Estimated value of Rst over all samples for both STR loci D5S818 and CSF1PO was 0.071 and 0.167, respectively.

When comparing the group of asthmatics with the Madeira population, significant differences were found for both IL4 -590 and IL4 RP2 loci regarding genotype (p=0.010 and p=0.048) and allele distribution (p=0.005 and p=0.025) respectively.

Discussion

In this study, significant inter-populational differences regarding both IL4-590 and IL4 RP2 polymorphisms indicate a clearly distinct genetic background amongst all studied populations. However, genotype 183183TT seems to prevail in African populations (Guinea-Bissau and Cape-Verde) while 253253CC appears in a higher frequency among typically Caucasian populations (Madeira, Azores and Portugal mainland).

Nevertheless significant differences were found within populations of Guinea-Bissau and Cape

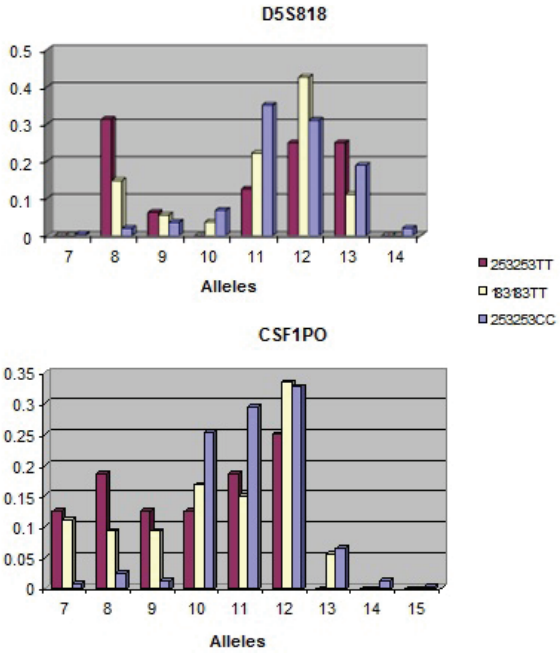

Figure 1. Allelic distribution for D5S818 and CSF1PO within 253253CC (n=248), 183183TT (n=54) and 253253TT (n=16) genotypes.

Verde. This last population presents the highest frequency of genotype 183253CT (0.296), maybe as the result of the high level of admixture in this population that can be explained by the extensive male Caucasian contribution to its genetic background [7].

Guinea-Bissau is in Hardy-Weinberg disequilibrium for the IL4 -590 locus possibly due to natural selection affecting the IL4 gene since the same population was found to be in Hardy-Weinberg equilibrium when neutral STRs were used [14].

It is known that malaria is a growing pathology affecting African populations [15]. Recent studies among the Fulani from Mali showed a higher prevalence of *Plasmodium falciparum* among the carriers of the T allele. It seems that the persistence of infection among T carriers may result in the production of anti-malarial antibodies [16]. Therefore the high frequency for TT genotype in Guinea-Bissau may come as a possible protective defence mechanism against malaria infection.

Phylogenetic studies indicate IL4-590 polymorphism belongs to a conserved region in all primates except for humans [1]. Therefore allele C is though to be the ancestral while T allele the derived. LD between both IL4-590 and IL4 RP2 suggests allele 253 is linked to C allele and therefore one should expect this to be the ancestral haplotype. Thus genotype 253253CC should present the highest average gene diversity for both studied STRs. However genotype 253253TT holds the highest value. A possible natural selective process of an advantageous allele (most likely T allele from IL4-590 locus) might have been responsible for this particular pattern. The relatively high Rst value for CSF1PO nearby STR locus (0.167) seems to strengthen this hypothesis since high Rst values are likely to mark genomic regions that have been subjected to selection [10].

Both IL4 -590 and IL4 RP2 can be useful genetics markers to detect asthma predisposition in Madeira Population. In addition, LD between both loci may lead to a possible synergic action contributing to the pathofisiology of asthma.

According to these results a new approach must be used for populations with African background, especially in the developing populations. A high frequency of the allele T associated with social and cultural development could determine changes in the atopy and asthma in a near future in these populations.

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Questionnaire B

(Adapted questionnaire from ISAAC-International Study of Asthma and Allergies in Childhood)

B. Genetic profile of a group of asthmatics

RUP Region: MAD AZO CAN GUY Family number

B1. Epidemiological evaluation of asthma in the family

B1.1. Father/Mother/Sibling/Asthmatic

1.1. Have you ever had sneeze attacks, runny nose or nasal congestion apart from having a cold or flu? (Yes/No)

1.2. In the last 12months did you experience sneeze attacks, runny nose or nasal congestion apart from having a cold or flu? (Yes/No)

1.3. In the last 12months was that nose problem accompanied by itchy and watery eyes? (Yes/No)

1.4. In which of the last 12 months did your nose problem occur?

1.5. In the last 12 months did that problem affect your daily activities? (Not at all/A little/Moderately/Very much)

1.6. Have you ever had rhinitis? (Yes/No)

2.1. Have you ever had wheezing or whistling in the chest? (Yes/No)

2.2. In the last 12 months have you ever had wheezing or whistling in the chest? (Yes/No)

2.3. How many wheezing attacks did you experience in the last 12 months?(None/ 1 to 3/ 4 to 12/ > 12)

2.4. In the last 12 months how often, on average, has your sleep been disturbed by wheezing? (Never/ Less than one night a week/ One or more nights a week)

2.5. In the last 12 months has wheezing been severe enough to limit your speech to just one or two words at a time between breaths? (Yes/No)

2.6. Have you ever had asthma? (Yes/No)

2.7. In the last 12months, have you experienced wheezing in the chest during or after exercise? (Yes/No)

2.8. In the last 12 months, did you have a dry cough at night apart from a cough associated with a cold or respiratory infection? (Yes/No)

B2. Inhabitancy Conditions

Urban /Suburban/Rural

Humid Area/Humid House

Basic Sanitation/Water /Electricity

Number of years of the house < 1; 1 to 3; 3 to 5; 5 to 10; > 10

Number of years in the house < 1; 1 to 3; 3 to 5; 5 to 10; > 10

Number of rooms:

Number of people living in the house:

Room Area (m^2):

Living Room area (m^2):

House Floor: Concrete/Tiles/Wood/Carpeted/Other:

Room Floor: Concrete/Tiles/Wood/Carpeted/Other:

Window (Yes/No)

Air conditioning (Yes/No)

Walls: Stucco/Paper/Oil-Based Paint/Water-Based Paint/Wood/Concrete

Curtains (Yes/No); Light /Heavy

Mattress: Sprung /Straw /Foam/ Feathers/ Anti-allergic Cover/Other

Matress (years):

Number of teddy bears < 5; 5 to 10; >10

Pillow: Foam/Feathers/Cotton/Kapok/Wool/Other

Pillow (years):

Blankets: Woollen/Cotton/Fiber/Duvet

How many people sleep in the room:

How many people sleep in the bed:

Bedroom Contiguity: WC/Kitchen

Pets/Animals: Dog/Cat/Birds/Cockroaches (Big /Small)/Others

Pets/Animals in the last 12 months: Dog/Cat/Birds/Cockroaches/Others

Fuel used for heating;

Fuel used in the kitchen;

Passive smoking (Yes/No);

Passive smoking: Father/Mother/Others;

Number of cigarette packs per year:

Number of cigarettes per day;

Active smoking (Yes/No);

Number of cigarette packs per year:

Number of cigarettes per day:

B3. Food habits and lifestyles

Weight (Kg):

Height (cm):

1. In the last 12 months how often, on average, has your son had the following?

Fish: Never or occasionally/Once or Twice a Week/Three times a Week or more

Fruit: Never or occasionally/Once or Twice a Week/Three times a Week or more

Vegetables (Green beans; Carrot, etc): Never or occasionally/Once or Twice a Week/Three times a Week or more

Legumes (peas, beans, lentils): Never or occasionally/Once or Twice a Week/Three times a Week or more

Cereals: Never or occasionally/Once or Twice a Week/Three times a Week or more

Pasta: Never or occasionally/Once or Twice a Week/Three times a Week or more

Rice: Never or occasionally/Once or Twice a Week/Three times a Week or more

Butter: Never or occasionally/Once or Twice a Week/Three times a Week or more

Margarine: Never or occasionally/Once or Twice a Week/Three times a Week or more

Dried fruits: Never or occasionally/Once or Twice a Week/Three times a Week or more

Potatoes: Never or occasionally/Once or Twice a Week/Three times a Week or more

Milk: Never or occasionally/Once or Twice a Week/Three times a Week or more

Egg: Never or occasionally/Once or Twice a Week/Three times a Week or more

Fast Food/ Hamburgers: Never or occasionally/Once or Twice a Week/Three times a Week or more

2.How many times a week does your son practice intense physical activity?

Never or occasionally/Once or Twice a Week/Three times a Week or more

3.During a normal week, how many hours a day does your son spend watching TV? <1h; 1 to 3h; 3 to 5 h; 5h or more.

B4. Allergy profile

Skin prick tests (Yes/No); Negative/Positive

Dpt (mm):

Df (mm):

Blomia sp. (mm):

Other House Dust Mites:

Grasses/Gramineae (mm):

Parietaria sp. (mm):

Urtica sp.(mm):

Dog (mm):

Cat (mm):

Cockroaches:

Periplaneta americana (mm);

Blattella germanica (mm);

Blatta orientalis (mm);

Mould:

Penicillium sp. (mm);

Aspergillus sp. (mm);

Alternaria sp. (mm);

Cladosporium sp. (mm);

Trees:

Pinus sp. (mm);

Others (mm);

Food: Others;

Parasitology: Negative/Positive;

Total IgE

Specific IgE (Yes/No); Negative/Positive;

Dpt (g/dl):

Df (g/dl):

Blomia sp. (g/dl):

Other House Dust Mites;

Grasses/Gramineae (g/dl);

Parietaria sp. (g/dl);

Urtica sp. (g/dl);

Dog (g/dl);

Cat (g/dl);

Cockroaches:

Periplaneta americana (g/dl);

Blattella germanica (g/dl);

Blatta orientalis (g/dl);

Mould:

Penicillium sp. (g/dl)

Aspergillus sp. (g/dl)

Alternaria sp. (g/dl)

Cladosporium sp. (g/dl);

Trees:

Pinus sp. (g/dl)/

Others (g/dl);

Food: Others.

Supplementary Tables

ST1. Frequency of asthma symptoms (in percentage, %), for overall asthma, asthmatics divided by age group and gender and for each family member (father, mother, sibling).

		Age group				Gender		Family members		
	Overall asthma	6-10	11-13	14-17	18-25	Male	Female	Father	Mother	Sibling
n	97	29	24	26	18	57	40	88	95	86
1.1. Sneeze attacks, runny nose or nasal congestion apart from having a cold or flu.										
	97.9	100	91.7	100	100	98.2	97.5	56.8	83.2	80.2
1.2. Sneeze attacks, runny nose or nasal congestion apart from having a cold or flu, in the last 12months.										
	94.8	96.6	87.5	96.2	100	94.7	95	51.1	76.8	74.4
1.3. Previous nose problem accompanied by itchy and watery eyes in the last 12months.										
	76.3	82.8	66.7	61.5	100	73.7	80	30.7	58.9	57.0
1.4. In which of the last 12 months did your nose problem occur.										
January	48.5	44.8	41.7	46.2	66.7	47.4	50	14.8	30.5	39.5
February	51.5	48.3	45.8	46.2	72.2	54.4	47.5	14.8	33.7	44.2
March	71.1	75.9	66.7	61.5	83.3	70.2	72.5	23.9	49.5	52.3
April	75.3	82.8	66.7	69.2	83.3	71.9	80	25.0	56.8	53.5
May	67	86.2	54.2	57.7	66.7	64.9	70	23.9	52.6	50.0
June	44.3	62.1	33.3	23.1	61.1	43.9	45	14.8	31.6	37.2
July	34.0	41.4	29.2	11.5	61.1	35.1	32.5	9.1	23.2	30.2
August	30.9	37.9	29.2	11.5	50	31.6	30	5.7	17.9	30.2
September	41.2	44.8	41.7	30.8	50	45.6	35	9.1	26.3	34.9
October	48.5	55.2	45.8	38.5	55.6	56.5	37.5	17.0	30.5	37.2
November	53.6	65.5	37.5	53.8	55.6	61.4	42.5	18.2	30.5	40.7
December	51.5	51.7	41.7	53.8	61.1	57.9	42.5	18.2	26.3	39.5
1.5. Daily activities affected by that problem.										
Not at all	20.2	0	28.6	41.7	11.1	22.6	16.7	40.5	21.4	34.4
A little	21.3	30.8	23.8	8.3	22.2	26.4	13.9	26.2	21.4	23.0
Moderately	51.7	57.7	47.6	45.8	55.6	47.2	58.3	31.0	44.3	32.8
Very much	6.7	11.5	-	4.2	11.1	3.8	11.1	2.4	12.9	9.8
1.6.Ever had rhinitis.										
	94.8	96.6	91.7	96.2	94.4	94.7	95	35.2	69.5	73.3
2.1. Ever had wheezing or whistling in the chest.										
	97.9	100	95.8	96.2	100	96.5	100	29.5	53.7	52.3
2.2. Ever had wheezing or whistling in the chest in the last 12 months.										
	74.2	82.8	70.8	69.2	72.2	75.4	72.5	15.9	33.7	31.4
2.3. How many wheezing attacks experienced in the last 12 months.										
None	2.6	3.8	-	-	7.1	2.3	3.1	21.4	9.1	3.6
1-3	57.9	34.6	70.6	68.4	71.4	54.5	62.5	57.1	66.7	53.6
4-12	34.2	53.8	29.4	21.1	21.4	34.1	34.4	14.3	18.2	42.9
>12	5.3	7.7	-	10.5	-	9.1	-	7.1	6.1	-
2.4. Sleep disturbed by wheezing in the last 12 months, on average.										
Never	19.7	11.5	11.8	31.6	28.6	18.2	21.9	28.6	30.3	21.4
(<1 night/week)	57.9	57.7	70.6	47.4	57.1	59.1	56.3	21.4	48.5	46.4
(≥ 1 night/week)	22.4	30.8	17.6	21.1	14.3	22.7	21.9	50	21.2	32.1
2.5. Wheezing severe enough to limit speech to just one or two words at a time between breathes in the last 12 months										
	30.9	44.8	37.5	19.2	16.7	35.1	25	5.7	12.6	15.1
2.6. Ever had asthma?										
	100	100	100	100	100	100	100	23.9	40	51.2
2.7. Wheezing in the chest during or after exercise in the last 12 months.										
	58.8	72.4	54.2	46.2	61.1	56.1	62.5	14.8	32.6	24.4
2.8. Dry cough at night apart from a cough associated with a cold or respiratory infection, in the last 12 months.										
	48.5	37.9	54.2	50	55.6	47.4	50	25.0	32.6	22.1

ST1.1. χ^2 p-values and odds ratio (95%CI) for comparisons of asthma symptoms by gender.

p-value, OR(95%CI)	Male *vs.* Female
1.1. Sneeze attacks, runny nose or nasal congestion apart from having a cold or flu.	
	0.799, 1.43(0.087-23.655)
1.2. Sneeze attacks, runny nose or nasal congestion apart from having a cold or flu, in the last 12months.	
	0.954, 0.947(0.151-5.945)
1.3. Previous nose problem accompanied by itchy and watery eyes in the last 12months.	
	0.472, 0.700(0.264-1.853)
1.4. In which of the last 12 months did your nose problem occur.	
January	0.863, 0.931(0.413-2.096)
February	0.504, 1.318(0.586-2.964)
March	0.804, 0.892(0.364-2.187)
April	0.365, 0.641(0.244-1.684)
May	0.600, 0.793(0.333-1.888)
June	0.911, 0.955(0.423-2.154)
July	0.791, 1.123(0.477-2.644)
August	0.868, 1.077(0.448-2.589)
September	0.296, 1.558(0.677-3.583)
October	0.071, 2.133(0.933-4.876)
November	0.066, 2.152(0.945-4.902)
December	0.135, 1.860(0.821-4.216)
1.5. Daily activities affected by that problem	
Not at all	0.491, 1.463(0.493-4.341)
A little	0.157, 2.226(0.723-6.854)
Moderately	0.301, 0.638(0.271-1.499)
Very much	0.175, 0.314(0.054-1.813)
1.6.Ever had rhinitis	
	0.954, 0.947(0.151-5.945)
2.1. Ever had wheezing or whistling in the chest.	
	0.231, 1.727(1.455-2.050)
2.2. Ever had wheezing or whistling in the chest in the last 12 months	
	0.745, 1.165(0.465-2.922)
2.3. How many wheezing attacks experienced in the last 12 months.	
None	0.819, 0.721(0.043-11.975)
1-3	0.488, 0.720(0.284-1.824)
4-12	0.979, 0.987(0.378-2.578)
>12	0.080, 0.556(0.452-0.863)
2.4. Sleep disturbed by wheezing in the last 12 months, on average.	
Never	0.690, 0.794(0.255-2.470)
(<1 night/week)	0.804, 1.123(0.447-2.823)
(≥ 1 night/week)	0.930, 1.050(0.351-3.141)
2.5. Wheezing severe enough to limit speech to just one or two words at a time between breathes in the last 12 months	
	0.290, 1.622(0.660-3.984)
2.6. Ever had asthma?	
	-
2.7. Wheezing in the chest during or after exercise in the last 12 months.	
	0.531, 0.768(0.336-1.755)
2.8. Dry cough at night apart from a cough associated with a cold or respiratory infection, in the last 12 months.	
	0.798, 0.900(0.401-2.021)

ST1.2 χ^2 p-values and OR (95%CI) for comparisons between overall asthmatics and each family member (father, mother and sibling), for each asthma symptom.

p-value, OR (95%CI)	Overall asthma *vs.* Father	Mother	Sibling
1.1. Sneeze attacks, runny nose or nasal congestion apart from having a cold or flu.			
	<0.05, 0.028(0.006-0.120	<0.05, 0.104 (0.023-0.466)	<0.05, 0.085(0.019-0.382)
1.2. Sneeze attacks, runny nose or nasal congestion apart from having a cold or flu, in the last 12months.			
	<0.05, 0.057(0.021-0.153)	<0.05, 0.180 (0.065-0.499)	<0.05, 0.158(0.057-0.439)
1.3. Previous nose problem accompanied by itchy and watery eyes in the last 12months.			
	<0.05, 0.138(0.072-0.264)	0.010, 0.446(0.240-0.831)	0.005, 0.412(0.218-0.775)
1.4. In which of the last 12 months did your nose problem occur.			
January	<0.05, 0.184(0.091-0.375)	0.011, 0.467(0.259-0.844)	0.225, 0.696(0.386-1.252)
February	<0.05, 0.163(0.080-0.332)	0.012, 0.477(0.267-0.855)	0.320, 0.744(0.415-1.333)
March	<0.05, 0.127(0.066-0.246)	0.002, 0.397(0.219-0.721)	0.009, 0.445(0.242-0.820)
April	<0.05, 0.110(0.056-0.214)	0.007, 0.433(0.234-0.800)	0.002, 0.378(0.202-0.707)
May	<0.05, 0.154(0.081-0.295)	0.042, 0.547(0.305-0.981)	0.020, 0.492(0.271-0.895)
June	<0.05, 0.218(0.107-0.444)	0.069, 0.580(0.321-1.045)	0.328, 0.744(0.411-1.347)
July	<0.05, 0.194(0.084-0.449)	0.096, 0.584(0.310-1.103)	0.584, 0.840(0.451-1.567)
August	<0.05, 0.135(0.049-0.366)	0.036, 0.487(0.247-0.960).	0.919, 0.968(0.515-1.817)
September	<0.05, 0.143(0.062-0.327)	0.029, 0.509(0.277-0.937)	0.377, 0.763(0.419-1.391)
October	<0.05, 0.219(0.110-0.433)	0.011, 0.467(0.259-0.844)	0.125, 0.630(0.349-1.139)
November	<0.05, 0.19(0.098-0.377)	0.001, 0.308(0.210-0.687)	0.081, 0.594(0.330-1.068)
December	<0.05, 0.209(0.107-0.409)	<0.05, 0.336(0.183-0.615)	0.104, 0.615(0.341-1.106)
1.5. Daily activities affected by that problem			
Not at all	0.895, 1.051(0.503-2.194)	0.747, 0.882(0.413-1.887)	0.334, 1.418(0.697-2.884)
A little	0.258, 0.627(0.278-1.414)	0.491, 0.770(0.365-1.622)	0.561, 0.798(0.373-1.709)
Moderately	<0.05, 0.192(0.094-0.391)	0.037, 0.537(0.299-0.964)	0.001, 0.336(0.177-0.637)
Very much	0.072, 0.174(0.021-1.478)	0.396, 1.587(0.542-4.647)	0.019, 0.514(0.446-0.593)
1.6.Ever had rhinitis			
	<0.05, 0.030(0.011-0.080)	<0.05, 0.124(0.045-0.336)	<0.05, 0.149(0.054-0.412)
2.1. Ever had wheezing or whistling in the chest.			
	<0.05, 0.009(0.002-0.039)	<0.05, 0.024(0.006-0.105)	<0.05, 0.023(0.005-0.100)
2.2. Ever had wheezing or whistling in the chest in the last 12 months			
	<0.05, 0.060(0.029-0.127)	<0.05, 0.176(0.095-0.329)	<0.05, 0.150(0.079-0.287)
2.3. How many wheezing attacks experienced in the last 12 months.			
None	0.573, 1.676(0.274-10.274)	0.634, 1.549(0.253-9.484)	0.633, 0.559(0.050-6.273)
1-3	<0.05, 0.120(0.053-0.276)	0.001, 0.342(0.182-0.641)	<0.05, 0.254(0.128-0.505)
4-12	<0.05, 0.064(0.015-0.277)	<0.05, 0.184(0.072-0.472)	0.032, 0.443(0.208-0.945)
>12	0.211, 0.267(0.029-	0.422, 0.500(0.089-	0.057, 0.520(0.451-

	2.438)	2.797)	0.598)
2.4. Sleep disturbed by wheezing in the last 12 months, on average.			
Never	0.015, 0.260(0.083-0.817)	0.309, 0.643(0.273-1.513)	0.072, 0.410(0.151-1.110)
(<1 night/week)	<0.05, 0.043(0.013-0.144)	<0.05, 0.244(0.125-0.477)	<0.05, 0.215(0.105-0.437)
(≥ 1 night/week)	0.053, 0.407(0.160-1.034)	0.052, 0.403(0.158-1.029)	0.172, 0.550(0.231-1.308)
2.5. Wheezing severe enough to limit speech to just one or two words at a time between breathes in the last 12 months			
	<0.05, 0.135(0.049-0.366)	0.002, 0.323(0.154-0.679)	0.012, 0.398(0.192-0.826)
2.6. Ever had asthma?			
	<0.05, 5.619(3.813-8.281)	<0.05, 3.553(2.173-4.652)	<0.05, 3.205(2.508-4.095)
2.7. Wheezing in the chest during or after exercise in the last 12 months.			
	<0.05, 0.122 (0.060-0.248)	<0.05, 0.340(0.189-0.613)	<0.05, 0.227(0.120-0.429)
2.8. Dry cough at night apart from a cough associated with a cold or respiratory infection, in the last 12 months.			
	0.001, 0.355(0.190-0.663)	0.026, 0.515(0.287-0.952)	<0.05, 0.302(0.158-0.576)

ST2. Frequency of asthma severity, wheezing and rhinitis (%) according to the family member's asthma status, presence of rhinitis and wheezing.

	Asthma severity categories						
	Mild	Moderate	Severe	Persistent	Intermittent	Wheezing ever	Rhinitis ever
Only mother with asthma							
%	33.3	25.0	25.0	30.0	25.0	28.2	28.0
Only father with asthma							
%	13.9	20.0	50.0	18.3	10.0	16.7	17.3
Both parents with asthma							
%	5.6	10.0	-	6.7	15.0	9.0	9.3
Both parents without asthma							
%	47.2	45.0	25.0	45.0	50.0	46.2	45.3
Sibling with asthma							
%	41.7	55.0	50.0	46.7	70.0	52.6	52.0
Sibling without asthma							
%	58.3	45.0	50.0	53.3	30.0	47.4	48.0
Only mother with rhinitis							
%	61.1	30.0	50.0	50.0	20.0	43.6	45.3
Only father with rhinitis							
%	11.1	10.0	0	10.0	5.0	9.0	8.0
Both parents with rhinitis							
%	16.7	35.0	50.0	25.0	30.0	26.9	26.7
Both parents without rhinitis							
%	11.1	25.0	0	15.0	45.0	20.5	20.0
Sibling with rhinitis							
%	77.8	70.0	100.0	76.7	70.0	75.6	77.3
Sibling without rhinitis							
%	22.2	30.0	0	23.3	30	24.4	22.7
Wheezing ever mother only							
%	36.1	25.0	25.0	33.3	35.0	32.1	33.3
Wheezing ever father only							
%	11.1	15.0	50.0	15.0	10.0	14.1	13.3
Wheezing ever both parents							
%	16.7	15.0	25.0	16.7	20.0	17.9	18.7
Both parents without wheezing							
%	36.1	40.0	0	35.0	35.0	35.9	34.7
Sibling with wheezing							
%	44.4	55.0	50.0	48.3	70.0	53.8	53.3
Sibling without wheezing							

%	55.6	45.0	50.0	51.7	30.0	46.2	46.7

ST3. Inhabitancy conditions (%), for overall asthma and by asthma severity.

		Asthma severity categories				
Inhabitancy conditions	Overall asthma	Mild asthma	Moderate asthma	Severe asthma	Persistent asthma	Intermittent asthma
Urban	30.9	39.5	26.9	75.0	37.0	13.0
Suburban	35.1	32.6	42.3	25.0	35.6	30.4
Rural	34.0	27.9	30.8	-	27.4	56.5
Humid area	64.9	62.8	69.2	50	64.4	69.6
Humid house	37.1	32.6	26.9	25	30.1	60.9
Basic sanitation	94.8	95.3	92.3	75	93.2	100
Potable water	68.0	62.8	53.8	50	58.9	100
Electricity	68.0	62.8	53.8	50	58.9	100
House (years)						
< 1	1.0	-	-	-	-	4.5
>10	64.6	79.1	46.2	25.0	64.4	63.6
1 to 3	3.1	-	11.5	-	4.1	-
3 to 5	9.4	7.0	23.1	-	12.3	-
5 to 10	21.9	14.0	19.2	75	19.2	31.8
Living in the house (years)						
< 1	1.1	-	-	-	-	4.3
>10	40.7	47.6	26.1	-	38.8	43.5
1 to 3	5.5	2.4	17.4	-	7.5	-
3 to 5-	14.3	16.7	26.1	-	19.4	-
5 to 10	38.5	33.3	30.4	100	34.3	52.2
Number of rooms (mean+-s.d.)	3.165+-0.965)	3.349+-0.752	2.885+-1.211	3.250+-1.258	3.178+-0.977	3.174+-0.937
Number of residents (mean +-s.d)	4.417+-1.139	4.405+-1.170	4.154+-0.834	4.250+-0.500	4.306+-1.030	4.783+-1.413
Room Area (mean +-s.d. m^2)	13.333+-3.041	13.818+-2.905	12.611+-2.810	12.000+-0.000	13.186+-2.805	15.250+-5.377
Living Room area (mean+-s.d. m^2)	24.915+-10.996	22.619+-11.048	26.111+-12.112	30.000+-8.660	24.643+-11.365	28.000+-8.641
House Floor						
Concrete	2.1	4.7	-	-	2.7	-
Tiles	48.5	46.5	34.6	25.0	41.1	69.6
Wood	69.1	72.1	69.2	75.0	71.2	65.2
Carpeted	1	-	-	-	-	4.3
Plastic	4.1	4.7	3.8	-	4.1	4.3
Room Floor						
Concrete	2.1	4.7	-	-	2.7	-
Tiles	22.7	20.9	23.1	25	21.9	21.7
Wood	71.1	69.8	69.2	75	69.9	73.9
Carpeted	-	-	-	-	-	-
Other	5.2		7.7	-	6.8	
Plastic	-	4.7	-	-	-	4.3
Carpet room	4.1	4.7	-	-	2.7	8.7
Window	97.9	97.7	100	75	97.3	100
Air conditioning	-	-	-	-	-	-
Walls						
Concrete	8.2	7.0	15.4	-	9.6	0
Stucco	13.4	18.6	15.4	-	16.4	4.3
Water-based paint	74.2	72.1	61.5	100	69.9	91.3

Oil-based paint	4.1	2.3	7.7	-	4.1	4.3
Curtains						
Light	82.5	81.4	88.5	75	83.6	78.3
Heavy	12.4	11.6	23.1	-	15.1	4.3
Mattress						
Anti-allergic cover	7.3	7	4.0	25	6.9	8.7
Foam	12.5	14	12.0	-	12.5	8.7
Sprung	80.2	79.1	84.0	75	80.6	82.6
Mattress (mean+-s.d. years)	4.323+-2.715	4.805+-3.311	4.125+-2.232	2.750+-0.957	4.449+-2.908	4.000+-2.111
Pillow						
Foam	76.3	79.1	76.9	50	76.7	73.9
Feathers	-	-	-	-	-	-
Cotton	9.3	7	11.5	-	8.2	13
Kapok	2.1	-	3.8	25	2.7	-
Wool	1	2.3	-	-	1.4	-
Pillow (mean+-s.d. years)	2.333+-2.080	2.313+-2.264	2.636+-1.965	2.750+-0.957	2.466+-2.071	1.636+-1.217
Teddy bears	32.0	25.6	30.8	25	27.4	43.5
Teddy bears (number of)						
<5	67.7	72.7	62.5	-	65.0	80.0
>10	12.9	18.2	-	100.0	15.0	-
5 to 10	19.4	9.1	37.5	-	20.0	20.0
Bed cloth covers/Blankets						
Woollen	5.2	7	3.8	-	5.5	4.3
Cotton	2.1	4.7	-	-	2.7	-
Fibre	3.1	7	-	-	4.1	-
Duvet	95.9	97.7	96,2	75	95.9	95.7
People sleeping in the room (mean+-s.d.)	1.558+-0.695	1.488+-0.768	1.583+-0.654	1.500+-0.577	1.521+-0.714	1.696+-0.635
People sleeping in bed (mean+-s.d.)	1.067+-0.328	1.024+-0.156	1.143+-0.478	1.000+-0.000	1.061+-0.298	1.087+-0.417
Bedroom Contiguity						
WC	44.3	46.5	42.3	25	43.8	43.5
Kitchen	10.3	9.3	11.5	25	11.0	8.7
Pets/Animals ever	62.9	67.4	61.5	75	65.8	52.2
Dog	45.4	48.8	46.2	50	47.9	34.8
Cat	18.6	23.3	19.2	-	20.5	8.7
Birds	15.5	18.6	7.7	25	15.1	17.4
Cockroaches	20.6	23.3	11.5	-	17.8	30.4
Big cockroaches	18.6	23.3	7.7	-	16.4	26.1
Small cockroaches	1	-	3.8	-	1.4	-
Other pets	5.2	4.7	3.8	25	5.5	4.3
Pets/Animals last 12 months	56.7	62.8	53.8	50	58.9	47.8
Dog	40.2	44.2	42.3	50	43.8	26.1
Cat	18.6	23.3	19.2	-	20.5	8.7
Birds	12.4	18.6	-	-	11.0	17.4
Cockroaches	15.5	14	11.5	-	12.3	26.1
Other pets	5.2	7.0	3.8	-	5.5	4.3
Fuel used for heating the room						
Electricity	2.1	4.7	-	-	2.7	-
Gas	9.3	16.3	3.8	-	11.0	4.3

No Response	88.7	79.1	96.2	-	86.3	-
Fuel used for heating the kitchen						
Electricity	2	-	-	25	1.4	4.3
Gas	69.1	72.1	57.7	50	65.8	82.6
No Response	28.9	27.9	42.3	25	32.9	13
Passive smoking	37.1	41.9	26.9	25	35.6	43.5
Father	69.4	88.9	57..1	-	76.9	50.0
Mother	2.8	-	14.3	-	3.8	-
Father and Mother	5.6	5.6	14.3	-	7.7	-
Sibling	8.3	5.6	-	-	3.8	20.0
Grandfather	2.8	-	-	-	-	10.0
Uncle	5.6	-	14.3	-	3.8	10.0
Others	5.6	-	-	100.0	3.8	10.0
Number of cigarettes per day (mean+-s.d.)	17.655+-11.258	17.692+-5.991	27.167+-16.618	5.000+-0.000	19.900+-11.271	12.667+-10.062
Active smoking	1.0	-	-	25	1.4	-
Number of cigarettes per day	8	-	-	8	8	-

ST4. Food habits and lifestyle for overall asthma and by asthma severity (%).

	Asthma severity categories					
Food habits and lifestyle	Overall asthma	Mild asthma	Moderate asthma	Severe asthma	Persistent asthma	Intermittent asthma
Male						
BMI for age and sex						
<85th percentile	80	81.8	73.3	75	78	92.3
85th–95th percentile	16.4	18.2	20	25	19.5	7.7
>95th percentile	3.6	-	6.7	-	2.4	-
Female						
BMI for age and sex						
<85th percentile	84.6	89.5	90.9	-	90	66.7
85th–95th percentile	12.8	5.3	9.1	-	6.7	33.3
>95th percentile	2.6	5.3	-	-	3.3	-
Fish						
Never or occasionally	3.2	2.4	4.0	-	2.8	4.3
Once or Twice a Week	49.5	45.2	32.0	50	40.8	73.9
Three times a Week or more	47.4	52.4	64.0	50	56.3	21.7
Fruit						
Never or occasionally	5.3	7.1	4.0	-	5.6	4.3
Once or Twice a Week	10.5	11.9	8.0	-	9.9	13.0
Three times a Week or more	84.2	81.0	88.0	100	84.5	82.6
Vegetables (green beans, carrot)						
Never or occasionally	8.4	9.5	4.0	-	7.0	13.0
Once or Twice a Week	17.9	11.9	20.0	25	15.5	26.1
Three times a Week or more	73.7	78.6	76.0	75	77.5	60.9
Legumes (peas, beans, lentils)						
Never or occasionally	17.0	22.0	12.0	-	17.1	17.4
Once or Twice a Week	29.8	17.1	48.0	75	31.4	21.7
Three times a Week or more	53.2	61.0	40.0	25	51.4	60.9
Cereals						
Never or occasionally	7.4	2.4	12.0		5.6	8.7
Once or Twice a Week	12.6	21.4	8.0		15.5	4.3
Three times a Week or more	80.0	76.2	80.0	100.0	78.9	87.0
Pasta						

Never or occasionally	-	-	-	-	-	-
Once or Twice a Week	26.3	26.2	8.0	50	21.1	43.5
Three times a Week or more	73.7	73.8	92.0	50	78.9	56.5
Rice						
Never or occasionally	1.1	2.4	-	-	1.4	-
Once or Twice a Week	21.1	19.0	12.0	25	16.9	34.8
Three times a Week or more	77.9	78.6	88.0	75	81.7	65.2
Butter						
Never or occasionally	27.4	21.4	36.0	75	29.6	21.7
Once or Twice a Week	26.3	23.8	24.0	-	22.5	39.1
Three times a Week or more	46.3	54.8	40.0	25	47.9	39.1
Margarine						
Never or occasionally	73.7	69.0	76.0	75	71.8	78.3
Once or Twice a Week	20.0	21.4	20.0	25	21.1	17.4
Three times a Week or more	6.3	9.5	4.0	-	7.0	4.3
Dried fruits						
Never or occasionally	72.6	66.7	80.0	50	70.4	78.3
Once or Twice a Week	24.2	31.0	12.0	50	25.4	21.7
Three times a Week or more	3.2	2.4	8.0	-	4.2	
Potatoes						
Never or occasionally	6.3	7.1	4.0	-	5.6	8.7
Once or Twice a Week	21.1	16.7	16.0	50	18.3	30.4
Three times a Week or more	72.6	76.2	80.0	50	76.1	60.9
Milk						
Never or occasionally	1.1	2.4	-	-	1.4	-
Once or Twice a Week	2.1	-	-	-	-	8.7
Three times a Week or more	96.8	97.6	100	100	98.6	91.3
Egg						
Never or occasionally	16.8	16.7	28.0	-	19.7	8.7
Once or Twice a Week	63.2	66.7	48.0	75	60.6	73.9
Three times a Week or more	20.0	16.7	24.0	25	19.7	17.4
Fast Food/Hamburgers						
Never or occasionally	80.6	85.7	82.6	75	84.1	73.9
Once or Twice a Week	15.1	9.5	13.0	25	11.6	21.7
Three times a Week or more	4.3	4.8	4.3	-	4.3	4.3
Intense physical activity						
Never or occasionally	4.2	4.8	8.0	-	5.6	-
Once or Twice a Week	53.7	57.1	44.0	50.0	52.1	56.5
Three times a Week or more	42.1	38.1	48.0	50.0	42.3	43.5
TV (Hours per day)						
<1h	15.1	12.2	8.3	50	13.0	21.7
1 to 3h	55.9	56.1	79.2	25	62.3	39.1
3 to 5 h	18.3	19.5	8.3	25	15.9	26.1
5h or more	10.8	12.2	4.2	-	8.7	13.0

ST5. Allergy profile for overall asthma and by asthma severity. Frequencies are given in percentage (%) of positive skin prick test, together with mean wheal size (mm).

		Asthma severity categories				
Allergy profile	Overall asthma	Mild asthma	Moderate asthma	Severe asthma	Persistent asthma	Intermittent asthma
Skin prick tests (+)	85.4	88.4	76.9	75	83.6	90.9
Dpt (+)	89.0	81.4	76.9	50.0	78.1	65.2
Wheal size (mm)	9.007	9.457	9.150	9.500	9.351	7.900
Df (+)	81.7	72.1	69.2	50.0	69.9	65.2
Df (mm)	7.940+-3.293	7.613+-2.977	9.028+-3.987	8.500+-6.364	8.147+-3.463	7.300+-2.763
Blomia sp. (+)	65.9	58.1	46.2	50.0	53.4	60.9
Blomia sp. (mm):	7.204+-3.152	7.140+-3.287	7.542+-3.50	11.500+-3.536	7.487+-3.410	6.500+-2.378
Lepidoglyphus sp. (+)	42.7	46.5	23.1	50.0	38.4	26.1
Lepidoglyphus sp. (mm)	6.514+-2.628	5.675+-1.894	7.917+-4.341	9.500+-3.536	6.429+-2.844	6.750+-1.725

Glycifagus sp (+)	31.7	27.9	26.9	25	27.4	26.1
Glycifagus sp (mm)	6.440+-3.015	6.167+-2.209	7.429+-4.276	6.000+-0	6.600+-2.998	5.800+-3.347
Tyrophagus sp. (+)	9.8	7.0	3.8	0	5.5	17.4
Tyrophagus sp. (mm)	5.125+-1.356	5.667+-1.528	6.000+-0	-	5.750+-1.258	4.500+-1.291
Euroglyphus sp. (+)	22.0	16.3	15.4	50.0	17.8	21.7
Euroglyphus sp. (mm)	7.333+-3.850	7.429+-3.445	8.125+-4.211	8.000+-4.243	7.731+-3.462	6.300+-5.020
Gramineae (+)	23.2	23.3	26.9	0	23.3	8.7
Gramineae (mm)	5.632+-3.059	6.500+-3.837	4.429+-1.718	-	5.647+-3.239	5.500+-0.707
Parietaria sp. (+)	13.4	20.9	3.8	0	13.7	4.3
Parietaria sp. (mm)	4.180+-1.401	4.110+-1.364	3.000+-0	-	4.000+-1.333	6.000+-0
Urtica sp. (+)	6.1	9.3	3.8	0	6.8	0
Urtica sp. (mm)	3.800+-0.837	3.750+-0.957	4.000+-0	-	3.800+-0.837	-
Artemisia sp. (+)	2.4	4.7	0	0	2.7	0
Artemisia sp. (mm)	5.000+-1.414	5.000+-1.414	-	-	5.000+-1.414	-
Aster sp. (Daisy) (+)	1.2	2.3	0	0	1.4	0
Aster sp. (Daisy) (mm)	7.000+-0	7.000+-0	-	-	7.000+-0	-
Taraxacum sp. (Dandelion)(+)	1.2	2.3	0	0	1.4	0
Taraxacum sp. (Dandelion) (mm)	5.000+-0	5.000+-0	-	-	5.000+-0	-
Chenopodium sp.(+)	2.4	4.7	0	0	2.7	0
Chenopodium sp.(mm)	6.500+-0.707	6.500+-0.707	-	-	6.500+-0.707	-
Pinus sp. (Pine tree) (+)	3.7	2.3	3.8	0	2.7	4.3
Pinus sp. (Pine tree) mm	4.667+-1.528	3.000+-0		-	4.500+-2.121	5.000+-0
Cupressus sp.(Cypress) (+)	3.7	4.7	0	0	2.7	4.3
Cupressus sp.(Cypress) (mm)	5.333+-1.155	5.000+-1.414	-	-	5.000+-1.414	6.000+-0
Juglans sp. (Walnut tree) (+)	2.4	4.7	0	0	2.7	0
Juglans sp. (Walnut) (mm)	3.500+-0.707	3.500+-0.707	-	-	3.500+-0.707	-
Quercus sp. (Oak) (+)	1.2	2.3	0	0	1.4	0
Quercus sp. (Oak) (mm)	3.000+-0	3.000+-0	-	-	3.000+-0	-
*Castanea sp.(*Chestnut tree) (+)	4.9	4.7	3.8	0	4.1	4.3
Castaneae sp. (Chestnut tree) (mm)	4.750+-1.258	4.500+-2.121	5.000+-0	-	4.667+-1.528	5.000+-0
Salix sp. (Willow) (+)	1.2	2.3	0	0	1.4	0
Salix sp. (Willow)(mm)	6.000+-0	6.000+-0	-	-	6.000+-0	-
Mimosa sp. (+)	2.4	2.3	3.8	0	2.7	0
Mimosa sp. (mm)	3.000+-0	3.000+-0	-	-	3.000+-0	-
Platanus sp. (+)	7.3	2.3	0	25.0	2.7	17.4
Platanus sp. (mm)	5.500+-1.049	6.000+-0	6.000+-0	7.000+-0	6.500+-0.707	5.000+-0.817
Tilia sp. (Lindens) (+)	1.2	2.3	0	0	1.4	0
Tilia sp. (Lindens) (mm)	6.000+-0	6.000+-0	-	-	6.000+-0	-
Penicillium sp. (+)	1.2	2.3	0	0	1.4	0
Penicillium sp. (mm)	5.000+-0	5.000+-0	-	-	5.000+-0	-
Aspergillus sp. (+)	13.4	11.6	11.5	25.0	12.3	8.7
Aspergillus sp. (mm)	4.045+-0.850	4.300+-0.975	3.667+-0.577	5.000+-0	4.167+-0.866	3.500+-0.707
Alternaria sp. (+)	20.7	14.0	15.4	25.0	15.1	26.1
Alternaria sp. (mm)	4.853+-1.599	4.917+-1.744	4.250+-1.500	8.000+-0	4.955+-1.823	4.667+-1.211
Cladosporium sp.(+)	8.5	4.7	11.5	0	6.8	8.7
Cladosporium sp.(mm)	3.929+-0.608	3.750+-0.354	4.000+-1.000	-	3.900+-0.742	4.000+-0
Mucor sp. (+)	6.1	7.0	3.8	0	5.5	4.3
Mucor sp. (mm)	5.800+-	6.667+-	5.000+-0	-	6.250+-	4.000+-

	2.388	2.887			2.500	
Candida sp. (+)	2.4	2.3	0	0	1.4	4.3
Candida sp. (mm)	5.000+-1.414	6.000+-0	-	-	6.000+-0	4.000+-0
Periplaneta americana (+)	14.6	16.3	7.7	0	12.3	13.0
Periplaneta americana (mm)	3.625+-1.069	3.786+-1.075	2.500+-0.707	-	3.500+-1.11	4.000+-1.00
Blattella germanica (+)	8.5	11.6	0	25.0	8.2	4.3
Blattella germanica (mm)	5.140+-1.773	5.000+-2.12	-	5.000+-0	5.000+-1.897	6.000+-0
Blatta orientalis (+).	4.9	7.0	3.8	0	5.5	0
Blatta orientalis (mm)	4.250+-1.258	3.667+-0.577	6.000+-0	-	4.250+-1.25	-
Dog (+)	23.2	25.6	23.1	25.0	24.7	4.3
Dog (mm)	4.895+-1.560	4.364+-1.027	5.333+-1.966	8.000+-0	4.889+-1.605	5.000+-0
Cat (+)	20.7	23.3	23.1	0	21.9	4.3
Cat (mm)	5.735+-2.001	5.100+-1.449	6.333+-2.503	-	5.563+-1.931	8.500+-0
Histamine (+)	31.7	32.6	11.5	25.0	24.7	30.4
Histamine (mm)	6.192+-2.040	6.000+-2.184	5.667+-1.528	8.000+-	6.056+-2.043	6.857+-2.035

ST6. LD tests between pairs of *loci* for the Madeira reference set and overall asthma. χ^2 p-values are shown.

Pairs of *loci*	Madeira reference (p- value)	Overall asthma (p-value)
IL4-RP2/ IL4-590	0.000	0.000
IL4-RP2 / ADRB2-c.16	0.070	0.560
IL4-RP2 /IL13-c.144	0.010	0.004
IL4-590 / ADRB2-c.16	0.010	0.360
IL4-590 / IL13-c.144	0.030	0.020
ADRB2-c.16/ IL13-c.144	0.010	0.930
ADAM33-V4 /ADAM33-S1 c.710	0.000	0.000

ST7. Genotype frequencies for the Madeira reference set, overall asthma, and category of asthma severity.

	Madeira reference	Overall asthma	Mild asthma	Moderate asthma	Severe asthma	Persistent asthma	Intermittent asthma
IL13-c.144							
GG	0.676	0.693	0.767	0.692	0.500	0.726	0.609
GA	0.305	0.287	0.209	0.308	0.500	0.260	0.348
AA	0.019	0.020	0.023	0	0	0.014	0.043
IL4-590							
CC	0.800	0.624	0.698	0.538	0.250	0.616	0.696
CT	0.191	0.337	0.302	0.423	0.500	0.356	0.261
TT	0.009	0.040	0	0.038	0.250	0.027	0.043
IL4-RP2							
253253	0.818	0.673	0.744	0.577	0.250	0.658	0.783
253183	0.155	0.287	0.256	0.385	0.500	0.315	0.174
183183	0.027	0.040	0	0.038	0.250	0.027	0.043
ADRB2-c.16							
AA	0.181	0.188	0.256	0.077	0.000	0.178	0.261
AG	0.457	0.495	0.349	0.731	0.250	0.479	0.435
GG	0.362	0.317	0.395	0.192	0.750	0.342	0.304
ADAM33-V4							
GG	0.029	0.010	0.023	0	0	0.014	0
CG	0.209	0.238	0.163	0.269	0.250	0.205	0.304
CC	0.762	0.752	0.814	0.731	0.750	0.781	0.696

ADAM33-S1 c.710							
GG	0.905	0.921	0.930	0.923	1.000	0.932	0.913
GA	0.095	0.079	0.070	0.077	0.000	0.068	0.087
AA	0	0	0	0	0.000	0	0
GSDML-236							
CC	0.171	0.158	0.140	0.231	0	0.164	0.174
CT	0.467	0.475	0.372	0.577	0.750	0.466	0.478
TT	0.362	0.366	0.488	0.192	0.250	0.370	0.348
STAT6-21							
CC	0.428	0.436	0.535	0.385	0.250	0.466	0.391
CT	0.486	0.436	0.279	0.577	0.500	0.397	0.478
TT	0.086	0.129	0.186	0.038	0.250	0.137	0.130

ST7.1. Genotype χ^2 p-values and OR (95%CI) between the Madeira reference set and overall asthma and also each of the asthma severity categories. Significant χ^2 p-values are highlighted in bold and only significant OR are shown.

	Madeira reference set *vs.*					
	Overall asthma	Mild asthma	Moderate asthma	Severe asthma	Persistent asthma	Intermittent asthma
IL13-c.144	0.962	0.444	1.000	0.626	0.835	0.484
IL4 -590	**0.010**, 2.413 (1.302-4.470)	0.338	**0.015**, 3.429 (1.392-8.446)	**0.009**, 12 (1.190-121.006)	**0.016**, 2.489 (1.281-4.835)	0.203
IL4-RP2	**0.048**, 2.184 (1.153-4.135)	0.237	**0.024**, 3.300 (1.320-8.252)	**0.015**, 13.500 (1.334-136.618	**0.033**, 2.344 (1.182-4.647)	0.688
ADRB2-c.16	0.787	0.392	-	0.388	0.978	0.659
ADAM33-V4	0.597	0.853	0.795	1.000	0.947	0.599
ADAM33-S1 c.710	0.684	0.755	1.000	1.000	0.591	1.000
GSDML-236	0.969	0.411	0.248	0.657	1.000	1.000
STAT6-21	0.555	-	0.752	0.399	0.384	0.793

ST8. Allele frequencies for the Madeira reference set, overall asthma and by category of asthma severity.

	Madeira reference	Overall asthma	Mild asthma	Moderate asthma	Severe asthma	Persistent asthma	Intermittent asthma
IL13-c.144 G/A	0.829/ 0.171	0.837/ 0.163	0.872/ 0.128	0.846/ 0.154	0.750/ 0.25	0.856/ 0.144	0.783/ 0.217
IL4-590 C/T	0.895/ 0.105	0.792/ 0.208	0.849/ 0.151	0.750/ 0.250	0.500/ 0.500	0.795/ 0.205	0.826/ 0.174
IL4-RP2 253183	0.895/ 0.105	0.817/ 0.183	0.872/ 0.128	0.769/ 0.231	0.500/ 0.500	0.815/ 0.185	0.870/ 0.130
ADRB2-c. 16 A/G	0.410/ 0.590	0.436/ 0.564	0.430/ 0.570	0.442/ 0.558	0.125/ 0.875	0.418/ 0.582	0.478/ 0.522
ADAM33-V4 G/C	0.133/ 0.867	0.129/ 0.871	0.105/ 0.895	0.135/ 0.865	0.125/ 0.875	0.116/ 0.884	0.152/ 0.848
ADAM33-S1 c.710 G/A	0.952/ 0.048	0.960/ 0.040	0.965/ 0.035	0.962/ 0.038	1.000/ 0	0.966/ 0.034	0.957/ 0.043
GSDML-236 C/T	0.405/ 0.595	0.396/ 0.604	0.326/ 0.674	0.519/ 0.481	0.375/ 0.625	0.397/ 0.603	0.413/ 0.587
STAT6-21 C/T	0.671/ 0.329	0.653/ 0.347	0.674/ 0.326	0.673/ 0.327	0.500/ 0.500	0.664/ 0.336	0.630/ 0.370

ST8.1. Allelic χ^2 p-values and OR (95%CI) between the Madeira reference set and overall asthma and also each of the asthma severity categories. Significant χ^2 p-values are highlighted in bold and only significant OR are shown.

	Overall asthma	Mild asthma	Moderate asthma	Severe asthma	Persistent asthma	Intermittent asthma
IL13-c.144	0.827	0.388	0.832	0.631	0.553	0.519
IL4 -590	**0.005**, 2.207 (1.301- 2.743)	0.318	**0.009**, 3.532 (1.450-8.604)	**0.009**, 43.439 (4.918 -383.717)	**0.009**, 2.254 (1.245 -4.082)	0.205
IL4-RP2	**0.025**, 1.902 (1.102- 3.285)	0.554	**0.024**, 3.070 (1.247- 7.558)	**0.008**, 43.439 (4.918 -383.717)	**0.031,** 1.973 (1.074- 3.626)	0.613
ADRB2-c.16	0.592	0.8	-	0.155	0.91	0.406
ADAM33-V4	0.889	0.565	1	1	0.749	0.811
ADAM33-S1 c.710	0.691	0.763	1	1	0.599	1
GSDML-236	0.857	0.237	0.158	1	0.911	1
STAT6-21	0.7	-	1	0.446	0.911	0.602

ST9. Genotype frequencies for the asthma patient's family members.

	Father	Mother	Sibling
IL13-c.144	n=88	n=95	n=86
GG	0.682	0.737	0.729
GA	0.307	0.242	0.271
AA	0.011	0.021	0
IL4-590			
CC	0.701	0.695	0.706
CT	0.276	0.263	0.271
TT	0.023	0.042	0.024
IL4-RP2			
253253	0.733	0.716	0.723
253183	0.233	0.253	0.241
183183	0.035	0.032	0.036
ADRB2-c.16			
AA	0.207	0.126	0.214
AG	0.517	0.558	0.476
GG	0.276	0.316	0.310
ADAM33-V4			
GG	0.023	0.032	0.012
CG	0.253	0.274	0.214
CC	0.724	0.695	0.774
ADAM33-S1 c.710			
GG	0.966	0.916	0.930
GA	0.023	0.084	0.070
AA	0.011	0	0
GSDML-236			
CC	0.205	0.116	0.128
CT	0.500	0.453	0.488
TT	0.295	0.432	0.384
STAT6-21			
CC	0.420	0.495	0.442
CT	0.409	0.442	0.453
TT	0.170	0.063	0.105

ST9.1. χ^2 p-value assessed to determined genotype differences between each family member and overall asthma.

	Overall asthma *vs.*		
	Father	Mother	Siblings
IL13-c.144	0.868	0.498	0.586
IL4 -590C/T	0.264	0.295	0.238
IL4-RP2	0.387	0.519	0.467
ADRB2-c.16	0.540	0.987	0.915
ADAM33-V4	0.659	0.366	0.734
ADAM33-S1 c.710	0.186	0.898	0.807
GSDML-236	0.410	0.387	0.554
STAT6-21	0.833	0.407	0.932

ST10. Allelic frequencies for the asthma patient's family members.

	Father	Mother	Sibling
IL13-c.144	0.835/0.165	0.858/0.142	0.865/0.135
IL4-590	0.839/0.161	0.826/0.174	0.841/0.159
IL4-RP2253183	0.849/0.151	0.842/0.158	0.843/0.157
ADRB2-c.16	0.466/0.534	0.405/0.595	0.452/0.548
ADAM33-V4G/C	0.149/0.851	0.168/0.832	0.119/0.881
ADAM33-S1 c.710	0.977/0.023	0.958/0.042	0.965/0.035
GSDML-236	0.455/0.545	0.342/0.658	0.372/0.628
STAT6-21	0.625/0.375	0.716/0.284	0.669/0.331

ST10.1. χ^2 p-value assessed to determined allele differences between each family member and overall asthma.

	Overall asthma *vs.*		
	Father	Mother	Siblings
IL13-c.144	0.971	0.559	0.451
IL4 -590C/T	0.243	0.389*	0.225
IL4-RP2	0.410	0.506	0.501
ADRB2-c.16	0.561	0.543	0.747
ADAM33-V4	0.562	0.268	0.779
ADAM33-S1 c.710	0.351	0.900	0.811
GSDML-236	0.251	0.269	0.635
STAT6-21	0.565	0.185	0.758

*Compared to Madeira reference set

ST11. Genetic profile frequencies for each pair of SNPs for overall asthma and by category of asthma severity.

SNP 1	SNP2	Profile	Overall Asthma	Madeira reference	Mild asthma	Moderate asthma	Severe asthma	Persistent asthma	Intermittent asthma
IL13-c.144	*IL4-590 - RP2253183*	GGCC	0.475	0.562	0.512	0.462	0.250	0.479	0.478
		GGCT	0.208	0.105	0.256	0.231	0.250	0.247	0.087
		GGTT	0.010	0.010	0	0	0	0	0.043
		GACC	0.149	0.190	0.186	0.077	0	0.137	0.217
		GACT	0.109	0.105	0.023	0.192	0.250	0.096	0.130
		GATT	0.030	0.010	0	0.038	0.250	0.027	0
		AACC	0	0.010	0	0	0	0	0
		AACT	0.020	0.010	0.023	0	0	0.014	0.043
		AATT	0	0	0	0	0	0	0
		GG253253	0.525	0.577	0.558	0.500	0.250	0.521	0.565

		GG183253	0.158	0	0.209	0.192	0.250	0.205	0
		GG183183	0.010	0	0	0	0	0	0.043
		GA253253	0.149	0.202	0.186	0.077	0	0.137	0.217
		GA183253	0.109	0.087	0.023	0.192	0.250	0.096	0.130
		GA183183	0.030	0.019	0	0.038	0.250	0.027	0
		AA253253	0	0.010	0	0	0	0	0
		AA183253	0.020	0.010	0.023	0	0	0.014	0.043
		AA183183	0	0	0	0	0	0	0
		GGAA	0.139	0.162	0.233	0.0385	0	0.151	0.130
		GGAG	0.307	0.314	0.209	0.500	0	0.301	0.261
		GGGG	0.248	0.200	0.326	0.154	0.500	0.274	0.217
		GAAA	0.050	0.019	0.023	0.038	0	0.027	0.130
		GAAG	0.188	0.133	0.140	0.231	0.250	0.178	0.174
		GAGG	0.050	0.152	0.047	0.038	0.250	0.055	0.043
	ADRB2-c.16	AAAA	0	0	0	0	0	0	0
		AAAG	0	0.010	0	0	0	0	0
		AAGG	0.020	0.010	0.023	0	0	0.014	0.043
		GGGG	0	0.019	0	0	0	0	0
		GGCG	0.129	0.162	0.093	0.154	0	0.110	0.174
		GGCC	0.564	0.495	0.674	0.538	0.500	0.616	0.435
		GAGG	0.010	0.010	0.023	0	0	0.014	0
		GACG	0.109	0.048	0.070	0.115	0.250	0.096	0.130
	ADAM33-V4	GACC	0.168	0.248	0.116	0.192	0.250	0.151	0.217
		AAGG	0	0	0	0	0	0	0
		AACG	0	0	0	0	0	0	0
		AACC	0.020	0.019	0.023	0	0	0.014	0.043
		GGGG	0.644	0.600	0.721	0.654	0.5	0.685	0.522
		GGGA	0.050	0.076	0.047	0.038	0	0.041	0.087
		GGAA	0	0	0	0	0	0	0
	ADAM33-S1 c.710	GAGG	0.257	0.286	0.186	0.269	0.5	0.233	0.348
		GAGA	0.030	0.019	0.023	0.038	0	0.027	0
		GAAA	0	0	0	0	0	0	0
		AAGG	0.020	0.019	0.023	0	0	0.014	0.043
		AAGA	0	0	0	0	0	0	0
		AAAA	0	0	0	0	0	0	0
		GGCC	0.129	0.105	0.116	0.231	0	0.151	0.087
		GGCT	0.297	0.333	0.279	0.308	0.5	0.301	0.304
		GGTT	0.267	0.238	0.372	0.154	0	0.274	0.217
		GACC	0.030	0.057	0.023	0	0	0.014	0.087
	GSDML-236	GACT	0.178	0.124	0.093	0.269	0.25	0.164	0.174
		GATT	0.079	0.124	0.093	0.038	0.25	0.082	0.087
		AACC	0	0.010	0	0	0	0	0
		AACT	0	0.010	0	0	0	0	0
		AATT	0.020	0	0.023	0	0	0.014	0.043
		GGCC	0.307	0.267	0.419	0.269	0	0.342	0.217
		GGCT	0.277	0.352	0.186	0.385	0.250	0.260	0.304
		GGTT	0.109	0.057	0.163	0.038	0.250	0.123	0.087
		GACC	0.109	0.152	0.093	0.115	0.250	0.110	0.130
		GACT	0.158	0.124	0.093	0.192	0.250	0.137	0.174
	STAT6-21	GATT	0.020	0.029	0.023	0	0	0.014	0.043
		AACC	0.020	0.010	0.023	0	0	0.014	0.043
		AACT	0	0.010	0	0	0	0	0
		AATT	0	0	0	0	0	0	0
		CC 253253	0.624	0.791	0.698	0.538	0.250	0.616	0.696
		CC 253183	0	0	0	0	0	0	0
		CC 183183	0	0.009	0	0	0	0	0
		CT 253253	0.05	0.027	0.047	0.038	0	0.041	0.087
		CT 253183	0.287	0.145	0.256	0.385	0.500	0.315	0.174
		CT 183183	0	0.018	0	0	0	0	0
	IL4-RP2	TT 253253	0	0	0	0	0	0	0
		TT 253183	0	0.009	0	0	0	0	0
		TT 183183	0.04	0	0	0.038	0.250	0.027	0.043
		CCAA	0.139	0.171	0.209	0	0	0.123	0.217
IL4-590	*ADRB2-c.16*	CCAG	0.297	0.333	0.256	0.423	0	0.301	0.261
		CCGG	0.188	0.257	0.233	0.115	0.25	0.192	0.217
		CTAA	0.050	0.010	0.047	0.077	0	0.055	0.043

		CTAG	0.168	0.124	0.093	0.269	0.25	0.164	0.130
		CTGG	0.119	0.086	0.163	0.077	0.25	0.137	0.087
		TTAA	0	0	0	0	0	0	0
		TTAG	0.030	0	0	0.038	0	0.014	0.043
		TTGG	0.010	0.019	0	0	0.25	0.014	0
		CCGG	0.010	0.029	0.023	0	0	0.014	0
		CCCG	0.168	0.181	0.140	0.154	0	0.137	0.261
		CCCC	0.446	0.552	0.535	0.385	0.25	0.466	0.435
		CTGG	0	0	0	0	0	0	0
		CTCG	0.059	0.029	0.023	0.115	0	0.055	0.043
	ADAM33-V4	CTCC	0.277	0.190	0.279	0.308	0.5	0.301	0.217
		TTGG	0	0	0	0	0	0	0
		TTCG	0.010	0	0	0	0.25	0.014	0
		TTCC	0.030	0.019	0	0.038	0	0.014	0.043
		CCGG	0.574	0.686	0.651	0.500	0.250	0.575	0.609
		CCGA	0.050	0.076	0.047	0.038	0	0.041	0.087
		CCAA	0	0	0	0	0	0	0
		CTGG	0.307	0.200	0.279	0.385	0.500	0.329	0.261
	ADAM33-S1 c.710	CTGA	0.030	0.019	0.023	0.038	0	0.027	0
		CTAA	0	0	0	0	0	0	0
		TTGG	0.040	0.019	0	0.038	0.250	0.027	0.043
		TTGA	0	0	0	0	0	0	0
		TTAA	0	0	0	0	0	0	0
		CCCC	0.109	0.143	0.116	0.154	0	0.123	0.087
		CCCT	0.297	0.371	0.302	0.269	0.250	0.288	0.348
		CCTT	0.218	0.248	0.279	0.115	0	0.205	0.261
		CTCC	0.050	0.029	0.023	0.077	0	0.041	0.087
		CTCT	0.149	0.086	0.070	0.269	0.250	0.151	0.130
	GSDML-236	CTTT	0.139	0.105	0.209	0.077	0.250	0.164	0.043
		TTCC	0	0	0	0	0	0	0
		TTCT	0.030	0.010	0	0.039	0.25	0.027	0
		TTTT	0.010	0.010	0	0	0	0	0.043
		CCCC	0.267	0.324	0.349	0.154	0	0.260	0.304
		CCCT	0.267	0.352	0.209	0.346	0	0.247	0.348
		CCTT	0.089	0.086	0.140	0.038	0.250	0.110	0.043
		CTCC	0.158	0.095	0.186	0.231	0	0.192	0.087
		CTCT	0.139	0.124	0.070	0.192	0.500	0.137	0.087
	STAT6-21	CTTT	0.040	0	0.047	0	0	0.027	0.087
		TTCC	0.010	0.010	0	0	0.250	0.014	0
		TTCT	0.030	0.010	0	0.038	0	0.014	0.043
		TTTT	0	0	0	0	0	0	0
		253253 AA	0.139	0.163	0.209	0	0	0.123	0.217
		253253 AG	0.327	0.356	0.279	0.462	0	0.329	0.304
		253253 GG	0.208	0.269	0.256	0.115	0.250	0.205	0.261
		183253 AA	0.050	0.010	0.047	0.077	0	0.055	0.043
	ADRB2-c.16	183253 AG	0.139	0.096	0.070	0.231	0.250	0.137	0.087
		183253 GG	0.099	0.087	0.140	0.077	0.250	0.123	0.043
		183183 AA	0	0	0	0	0	0	0
		183183 AG	0.030	0.010	0	0.038	0	0.014	0.043
		183183 GG	0.010	0.010	0	0	0.250	0.014	0
		253253 GG	0.010	0.029	0.023	0	0	0.014	0
		253253 CG	0.168	0.173	0.140	0.154	0	0.137	0.261
		253253 CC	0.495	0.587	0.581	0.423	0.25	0.507	0.522
		183253 GG	0	0	0	0	0	0	0
	ADAM33-V4	183253 CG	0.059	0.029	0.023	0.115	0	0.055	0.043
		183253 CC	0.228	0.163	0.233	0.269	0.5	0.260	0.130
		183183 GG	0	0	0	0	0	0	0
		183183 CG	0.010	0.010	0	0	0.25	0.014	0
		183183 CC	0.030	0.010	0	0.038	0	0.014	0.043
		253253 GG	0.624	0.712	0.698	0.538	0.25	0.616	0.696
		253253 GA	0.050	0.077	0.047	0.038	0	0.041	0.087
	ADAM33-S1 c.710	253253 AA	0	0	0	0	0	0	0
		183253 GG	0.257	0.173	0.233	0.346	0.5	0.288	0.174
		183253 GA	0.030	0.019	0.023	0.038	0	0.027	0
		183253 AA	0	0	0	0	0	0	0
IL4-RP2		183183 GG	0.040	0.019	0	0.038	0.25	0.027	0.043

		183183 GA	0	0	0	0	0	0	0
		183183 AA	0	0	0	0	0	0	0
		253253 CC	0.109	0.154	0.116	0.154	0	0.123	0.087
		253253 CT	0.327	0.375	0.302	0.308	0.250	0.301	0.435
		253253 TT	0.238	0.260	0.326	0.115	0	0.233	0.261
		183253 CC	0.050	0.019	0.023	0.077	0	0.041	0.087
		183253 CT	0.119	0.087	0.070	0.231	0.25	0.137	0.043
		183253 TT	0.119	0.087	0.163	0.077	0.25	0.137	0.043
	GSDML-236	183183 CC	0	0	0	0	0	0	0
		183183 CT	0.030	0	0	0.038	0.25	0.027	0
		183183 TT	0.010	0.019	0	0	0	0	0.043
		253253 CC	0.277	0.327	0.372	0.154	0	0.274	0.304
		253253 CT	0.287	0.375	0.209	0.385	0	0.260	0.391
		253253 TT	0.109	0.087	0.163	0.038	0.25	0.123	0.087
		183253 CC	0.149	0.096	0.163	0.231	0	0.178	0.087
		183253 CT	0.119	0.096	0.070	0.154	0.5	0.123	0.043
		183253 TT	0.020	0	0.023	0	0	0.014	0.043
	STAT6-21	183183 CC	0.010	0.010	0	0	0.25	0.014	0
		183183 CT	0.030	0.010	0	0.038	0	0.014	0.043
		183183 TT	0	0	0	0	0	0	0
		AAGG	0.010	0.010	0.023	0	0	0.014	0
		AACG	0.030	0.057	0.023	0	0	0.014	0.087
		AACC	0.149	0.114	0.209	0.077	0	0.151	0.174
		AGGG	0	0.010	0	0	0	0	0
		AGCG	0.149	0.105	0.093	0.231	0	0.137	0.130
	ADAM33-V4	AGCC	0.347	0.343	0.256	0.5	0.25	0.342	0.304
		GGGG	0	0.010	0	0	0	0	0
		GGCG	0.059	0.048	0.047	0.038	0.25	0.055	0.087
		GGCC	0.257	0.305	0.349	0.154	0.5	0.288	0.217
		AAGG	0.168	0.152	0.209	0.077	0	0.151	0.261
		AAGA	0.020	0.029	0.047	0	0	0.027	0
		AAAA	0	0	0	0	0	0	0
		AGGG	0.446	0.419	0.326	0.654	0.250	0.438	0.391
	ADAM33-S1 c.710	AGGA	0.050	0.038	0.023	0.077	0	0.041	0.043
		AGAA	0	0	0	0	0	0	0
		GGGG	0.307	0.333	0.395	0.192	0.750	0.342	0.261
		GGGA	0.010	0.029	0	0	0	0	0.043
		GGAA	0	0	0	0	0	0	0
		AACC	0.010	0.019	0	0	0	0	0.043
		AACT	0.119	0.076	0.140	0.077	0	0.110	0.174
		AATT	0.059	0.086	0.116	0	0	0.068	0.043
		AGCC	0.099	0.057	0.093	0.154	0	0.110	0.087
	GSDML-236	AGCT	0.178	0.238	0.023	0.423	0	0.164	0.130
		AGTT	0.218	0.162	0.233	0.154	0.250	0.205	0.217
		GGCC	0.050	0.095	0.047	0.077	0	0.055	0.043
		GGCT	0.178	0.152	0.209	0.077	0.750	0.192	0.174
		GGTT	0.089	0.114	0.140	0.038	0	0.096	0.087
		AACC	0.109	0.076	0.140	0.077	0	0.110	0.130
		AACT	0.059	0.095	0.070	0	0	0.041	0.130
		AATT	0.020	0.010	0.047	0	0	0.027	0
		AGCC	0.188	0.190	0.209	0.231	0	0.205	0.130
		AGCT	0.248	0.219	0.093	0.462	0.250	0.233	0.174
ADRB2-c.16	*STAT6-21*	AGTT	0.059	0.048	0.047	0.038	0	0.041	0.130
		GGCC	0.139	0.162	0.186	0.077	0.250	0.151	0.130
		GGCT	0.129	0.171	0.116	0.115	0.250	0.123	0.174
		GGTT	0.050	0.029	0.093	0	0.250	0.068	0
		GGGG	0.010	0.019	0.023	0	0	0.014	0
	ADAM33-S1 c.710	GGGA	0	0.010	0	0	0	0	0
		GGAA	0	0	0	0	0	0	0
ADAM33-V4		CGGG	0.168	0.124	0.116	0.192	0.25	0.151	0.217
		CGGA	0.069	0.086	0.047	0.077	0	0.055	0.087
		CGAA	0	0	0	0	0	0	0
		CCGG	0.743	0.762	0.791	0.731	0.75	0.767	0.696
		CCGA	0.010	0	0.023	0	0	0.014	0

		CCAA	0	0	0	0	0	0	0
		GGCC	0	0	0	0	0	0	0
		GGCT	0.010	0.029	0.023	0	0	0.014	0
		GGTT	0	0	0	0	0	0	0
		CGCC	0.010	0.048	0.023	0	0	0.014	0
		CGCT	0.129	0.086	0.047	0.231	0.250	0.123	0.130
	GSDML-236	CGTT	0.099	0.076	0.093	0.038	0	0.068	0.174
		CCCC	0.149	0.124	0.116	0.231	0	0.151	0.174
		CCCT	0.337	0.352	0.302	0.346	0.500	0.329	0.348
		CCTT	0.267	0.286	0.395	0.154	0.250	0.301	0.174
		GGCC	0	0	0	0	0	0	0
		GGCT	0.010	0.029	0.023	0	0	0.014	0
		GGTT	0	0	0	0	0	0	0
		CGCC	0.089	0.086	0.093	0.038	0.25	0.082	0.130
		CGCT	0.139	0.105	0.047	0.231	0	0.110	0.174
	STAT6-21	CGTT	0.010	0.019	0.023	0	0	0.014	0
		CCCC	0.347	0.343	0.442	0.346	0	0.384	0.261
		CCCT	0.287	0.352	0.209	0.346	0.5	0.274	0.304
		CCTT	0.119	0.067	0.163	0.038	0.25	0.123	0.130
		GGCC	0.149	0.162	0.116	0.231	0	0.151	0.174
		GGCT	0.436	0.429	0.349	0.500	0.75	0.425	0.478
		GGTT	0.337	0.314	0.465	0.192	0.25	0.356	0.261
		GACC	0.010	0.010	0.023	0	0	0.014	0
		GACT	0.040	0.038	0.023	0.077	0	0.041	0
	GSDML-236	GATT	0.030	0.048	0.023	0	0	0.014	0.087
		AACC	0	0	0	0	0	0	0
		AACT	0	0	0	0	0	0	0
		AATT	0	0	0	0	0	0	0
		GGCC	0.406	0.410	0.488	0.385	0.250	0.438	0.348
		GGCT	0.386	0.410	0.256	0.500	0.500	0.356	0.435
		GGTT	0.129	0.086	0.186	0.038	0.250	0.137	0.130
		GACC	0.030	0.019	0.047	0	0	0.027	0.043
		GACT	0.050	0.076	0.023	0.077	0	0.041	0.043
	STAT6-21	GATT	0	0	0	0	0	0	0
		AACC	0	0	0	0	0	0	0
		AACT	0	0	0	0	0	0	0
		AATT	0	0	0	0	0	0	0
ADAM33-S1 c.710		CCCC	0.069	0.086	0.070	0.115	0	0.082	0.043
		CCCT	0.079	0.076	0.070	0.115	0	0.082	0.087
		CCTT	0.010	0.010	0	0	0	0	0.043
		CTCC	0.188	0.181	0.140	0.231	0.250	0.178	0.217
		CTCT	0.218	0.238	0.140	0.308	0.250	0.205	0.217
	STAT6-21	CTTT	0.069	0.048	0.093	0.038	0.250	0.082	0.043
		TTCC	0.178	0.162	0.326	0.038	0	0.205	0.130
		TTCT	0.139	0.171	0.070	0.154	0.250	0.110	0.174
		TTTT	0.050	0.029	0.093	0	0	0.055	0.043
GSDML-236									

ST12. Haplotype frequencies for 5q31 (*IL13-c.144*; *IL4-590*, *IL4-RP2*; *ADRB2-c.16*) for overall asthma and by category of asthma severity (0) and Madeira reference set (1) determined by PHASE 2.

Overall asthma vs. Madeira reference set (p-value=0.290)							
5q31	Haplotype	E(freq)	S.E	E[Freq(0)]	S.E.(0)	E[Freq(1)]	S.E.(1)
1	GC 2 G	0.001	0.001	5×10^{-5}	4.94×10^{-4}	0.002	0.002
2	GC 2 A	0.001	0.001	0	4.9×10^{-5}	0.002	0.002
3	GC 3 G	0.395	0.01	0.386	0.015	0.404	0.012
4	GC 3 A	0.325	0.01	0.311	0.015	0.338	0.012
5	GT 2 G	0.056	0.006	0.068	0.01	0.044	0.007
6	GT 2 A	0.031	0.005	0.047	0.01	0.016	0.006
7	GT 3 G	0.017	0.004	0.018	0.005	0.016	0.005
8	GT 3 A	0.007	0.003	0.007	0.004	0.006	0.004
9	AC 2 G	0.001	0.001	0	4.6×10^{-5}	0.002	0.002
10	AC 2 A	0.001	0.001	4.9×10^{-5}	4.92×10^{-4}	0.002	0.002
11	AC 3 G	0.07	0.007	0.052	0.01	0.087	0.009
12	AC 3 A	0.038	0.007	0.043	0.01	0.032	0.008
13	AT 2 G	0.037	0.006	0.04	0.009	0.033	0.007
14	AT 2 A	0.02	0.005	0.028	0.008	0.012	0.006
15	AT 3 G	7.07×10^{-4}	0.001	0	2.2×10^{-5}	0.001	0.003
16	AT 3 A	4.84×10^{-4}	9.700×10^{-4}	0	6×10^{-6}	9.49×10^{-4}	0.002
Mild asthma Madeira reference set (p-value= 0.980)							
index	Haplotype	E(freq)	S.E	E[Freq(0)]	S.E.(0)	E[Freq(1)]	S.E.(1)
1	GC 2 A	0.002	0.002	3.45×10^{-4}	0.002	0.003	0.003
2	GC 2 G	0.002	0.002	1.21×10^{-4}	0.001	0.002	0.002
3	GC 3 A	0.335	0.009	0.336	0.018	0.335	0.01
4	GC 3 G	0.403	0.011	0.404	0.019	0.403	0.012
5	GT 2 A	0.024	0.005	0.041	0.009	0.017	0.006
6	GT 2 G	0.054	0.007	0.067	0.011	0.049	0.009
7	GT 3 A	0.006	0.004	0.006	0.006	0.007	0.004
8	GT 3 G	0.015	0.004	0.017	0.006	0.014	0.005
9	AC 2 A	0.001	0.002	0	0	0.002	0.002
10	AC 2 G	0.002	0.002	2×10^{-6}	1.63×10^{-4}	0.003	0.002
11	AC 3 A	0.038	0.007	0.046	0.014	0.034	0.008
12	AC 3 G	0.082	0.008	0.062	0.015	0.09	0.01
13	AT 2 A	0.008	0.004	0	0	0.011	0.006
14	AT 2 G	0.025	0.006	0.019	0.006	0.027	0.007
15	AT 3 A	7.1×10^{-4}	0.001	0	0	0.001	0.002
16	AT 3 G	0.002	0.002	2×10^{-6}	1.48×10^{-4}	0.003	0.003
Moderate asthma *vs.* Madeira reference set (p-value=0.730)							
index	haplotype	E(freq)	S.E	E[Freq(0)]	S.E.(0)	E[Freq(1)]	S.E.(1)
1	GC 2 G	0.002	0.002	1.910×10^{-4}	0.002	0.003	0.003
2	GC 2 A	0.002	0.002	3.760×10^{-4}	0.003	0.002	0.002
3	GC 3 G	0.406	0.012	0.408	0.03	0.405	0.011
4	GC 3 A	0.326	0.011	0.282	0.029	0.337	0.011

5	GT 2 G	0.049	0.008	0.065	0.025	0.045	0.007
6	GT 2 A	0.027	0.007	0.07	0.022	0.016	0.006
7	GT 3 G	0.013	0.004	0.009	0.01	0.014	0.005
8	GT 3 A	0.007	0.004	0.011	0.01	0.006	0.004
9	AC 2 G	0.002	0.002	$4x10^{-6}$	$2.72x10^{-4}$	0.002	0.002
10	AC 2 A	0.002	0.002	$4x10^{-6}$	$2.72x10^{-4}$	0.002	0.002
11	AC 3 G	0.076	0.009	0.03	0.019	0.087	0.009
12	AC 3 A	0.031	0.007	0.029	0.019	0.032	0.008
13	AT 2 G	0.034	0.008	0.045	0.021	0.032	0.007
14	AT 2 A	0.019	0.006	0.05	0.021	0.012	0.006
15	AT 3 G	0.003	0.003	$1x10^{-6}$	$1.1x10^{-4}$	0.003	0.004
16	AT 3 A	$8.880x10^{-4}$	0.002	$1x10^{-6}$	$1.11x10^{-4}$	0.001	0.002

Severe asthma *vs.* Madeira reference set (p-value=0.430)

index	haplotype	E(freq)	S.E	E[Freq(0)]	S.E.(0)	E[Freq(1)]	S.E.(1)
1	GC 2 G	0.003	0.002	0.001	0.012	0.003	0.002
2	GC 2 A	0.002	0.002	$5.200x10^{-5}$	0.003	0.003	0.002
3	GC 3 G	0.404	0.012	0.423	0.063	0.403	0.012
4	GC 3 A	0.325	0.009	0.033	0.055	0.336	0.01
5	GT 2 G	0.056	0.007	0.267	0.046	0.048	0.007
6	GT 2 A	0.018	0.006	0.023	0.048	0.017	0.006
7	GT 3 G	0.013	0.005	0.002	0.016	0.013	0.005
8	GT 3 A	0.006	0.003	$6.100x10^{-5}$	0.003	0.006	0.004
9	AC 2 G	0.002	0.002	$6.100x1^{-5}$	0.003	0.003	0.003
10	AC 2 A	0.002	0.002	$9.990x10^{-4}$	0.011	0.002	0.003
11	AC 3 G	0.087	0.01	0.023	0.048	0.089	0.01
12	AC 3 A	0.032	0.008	0.019	0.045	0.033	0.008
13	AT 2 G	0.033	0.008	0.158	0.055	0.029	0.008
14	AT 2 A	0.012	0.006	0.049	0.061	0.011	0.006
15	AT 3 G	0.004	0.003	$5.200x10^{-5}$	0.003	0.004	0.003
16	AT 3 A	0.001	0.002	$5.200x10^{-5}$	0.003	0.001	0.002

Intermittent asthma Madeira reference set (p-value=0.930)

index	haplotype	E(freq)	S.E	E[Freq(0)]	S.E.(0)	E[Freq(1)]	S.E.(1)
1	GC 2 A	0.002	0.002	$1x10^{-6}$	$1.710x10^{-4}$	0.002	0.002
2	GC 2 G	0.002	0.002	0	0	0.003	0.003
3	GC 3 A	0.335	0.009	0.33	0.026	0.336	0.01
4	GC 3 G	0.397	0.01	0.351	0.026	0.407	0.011
5	GT 2 A	0.02	0.006	0.031	0.014	0.018	0.006
6	GT 2 G	0.04	0.006	0.026	0.009	0.043	0.007
7	GT 3 A	0.007	0.004	0.009	0.011	0.006	0.004
8	GT 3 G	0.017	0.005	0.034	0.011	0.013	0.005
9	AC 2 A	0.002	0.002	$6x10^{-6}$	$3.49x10^{-4}$	0.003	0.002
10	AC 2 G	0.002	0.002	$2.150x10^{-4}$	0.002	0.002	0.002
11	AC 3 A	0.039	0.007	0.073	0.019	0.032	0.008
12	AC 3 G	0.084	0.008	0.071	0.02	0.087	0.009
13	AT 2 A	0.016	0.006	0.034	0.019	0.012	0.006
14	AT 2 G	0.033	0.006	0.038	0.016	0.032	0.007

15	AT 3 A	$8.980x10^{-4}$	0.002	$1x10^{-6}$	$1.710x10^{-4}$	0.001	0.002
16	AT 3 G	0.003	0.003	2.120x10-4	0.002	0.004	0.004

TD

Asthma Polymorphic *Loci* Genotyping in Madeira Population
Identification of potential genetic markers of disease susceptibility, severity and clinical relevance

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