LEONARDO ARAUJO PINTO

INFLUÊNCIA DE POLIMORFISMOS GENÉTICOS NOS DIFERENTES FENÓTIPOS DE SIBILÂNCIA

CAMPINAS

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Tese de doutorado apresentada à Pós-graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutor em Saúde da Criança, área de concentração em Pediatria

Orientador: José Dirceu Ribeiro

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Este trabalho foi desenvolvido em duas fases que gostaria de descrever nestas primeiras páginas. A fase inicial desenvolveu-se na *Ludwig-Maximilians Uiversität*, em Munique, na Alemanha. Certamente foi a parte mais difícil, considerando a complexidade do tema e a dificuldade da língua. Este início de trabalho incluiu o aprendizado e treinamento em técnicas como sequenciamento, genotipagem, e epidemiologia genética. O primeiro projeto, denominado "*IRF1*", foi trabalhoso e longo, mas rendeu participações em congressos e uma das publicações mais relevantes da minha carreira de pesquisador. O projeto seguinte chamado "*STAT1*" foi mais divertido, pois além do trabalho no laboratório, tinha a missão de ensinar as técnicas que já conhecia a dois doutorandos do curso de medicina, tudo em alemão. O último projeto desta primeira fase, quando foi estudado o gene *MMP9*, é o único dos 3 que ainda não foi totalmente concluído, mas está em fase final. Este é o projeto que mais precisou de tempo e despertou interesse, afinal este foi o primeiro gene que eu mesmo pude escolher para estudar e analisar. Nesta primeira fase, gostaria de agradecer em especial aos professores Dr. Michael Kabesch e Dra. Erika von Mutius, que tornaram as minhas tarefas na Alemanha mais fácil com seu estilo exigente e compreensivo.

A segunda fase ocorreu simultaneamente em Porto Alegre (PUCRS) e Campinas (UNICAMP). Esta fase incluiu análise dos dados, redação, revisão, discussão, submissão de artigos e aulas do curso de pós-graduação. Este período foi super interessante considerando que as idéias de renomados pesquisadores brasileiros puderam influenciar a análise dos resultados obtidos na Alemanha. Neste período pude analisar os resultados considerando os diferentes fenótipos de sibilância, o que acabou contribuindo para os principais resultados da tese. Gostaria de agradecer em especial aos professores Paulo M. Pitrez, Marcus H. Jones e Renato T. Stein, que sempre incentivaram e colaboraram com a

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RESUMO

Introdução: O desenvolvimento da asma pode ser influenciado pela predisposição genética para respostas imunes específicas. A produção de citocinas com predomínio de resposta Th1 ou Th2 é controlada por diferentes fatores de transcrição, dos quais o Fator de Regulação do Interferon 1 (FRI-1) e o Transdutor de Sinal e Ativador da Transcrição 1 (TSAT-1) são de fundamental importância. A metaloproteinase 9 (MP-9) é uma outra proteína envolvida na degradação do colágeno da matriz extracelular e que pode influenciar o desenvolvimento de doenças pulmonares. O objetivo deste estudo incluiu a pesquisa de variações genéticas nestes genes e o estudo da associação entre polimorfismos e fenótipos relacionados a asma.

Métodos: Entre 1995-96, foi realizado um estudo transversal na Alemanha, como parte do protocolo ISAAC para determinar a prevalência de asma e atopia. A genotipagem de polimorfismos nos genes *FRI-1*, *TSAT-1* e *MP-9* foi realizada com o método MALDI-TOF. Foram realizadas revisões sistemáticas utilizando a base de dados *Genetic Association Database.*

Resultados: Além de genes indutores de resposta Th2 como interleucina (*IL*)-13*, IL4 e CD14,* os fatores de transcrição *FRI-1*, *TSAT-1* foram associados a fenótipos de atopia como IgE elevada e sensibilização a testes cutâneos. Por outro lado, os genes *MP-9* e *IL-8* estão fortemente associados à sibilância não atópica, e podem ser determinantes para o desenvolvimento de doenças respiratórias na infância.

Conclusão: Estes resultados sugerem que os diferentes fenótipos de asma na infância podem ser determinados por polimorfismos genéticos diversos. Pode-se chamar atenção para a necessidade de que os estudos de associação genética levem em consideração os diferentes desfechos e fenótipos em estudo. Além disso, uma análise estratificada para atopia deve ser realizada sempre que este dado estiver disponível.

ABSTRACT

Introduction: It has been speculated that the development of asthma may be influenced by genetic predisposition for specific immune responses. Th1/Th2 balance is influenced by several transcription factors, of which Interferon Regulatory Factor 1 (IRF-1) and Signal Transducer Activator of Transcription 1 (STAT-1) are of special importance. As matrix metalloproteinase 9 (MMP-9) plays an important role in airway wall thickening and airway remodelling, it may also influence the development of obstructive airway disease in children. To investigate the presence and role of genetic variations in these genes, we performed association studies with asthma-related phenotypes.

Methods: Genotyping of tagging SNPs in the *IRF-1, STAT-1* and *MMP-9* gene was performed using MALDI-TOF in independent cross-sectional study populations of German children phenotyped for asthma and atopic phenotypes according to ISAAC standard procedures in 1995-96. Additionally we performed systematic reviews using Genetic Association Database.

Results: Functional polymorphisms in Th2 genes as interleukin (*IL)-13, I-L4, CD14, IRF-1* and *STAT-1* were significantly associated with atopy, total or specific IgE levels. On the other hand, SNPs in *MMP-9* and *IL-8* genes significantly increased the risk for non-atopic wheezing and non-atopic asthma.

Conclusion: We have shown evidences that different wheezing disorders in childhood may be affected differently by genetic variations, considering their role on airway inflammation and atopy. Future genetic association studies should consider the different wheezing phenotypes in infancy. Moreover, the analyses stratified for atopy may be useful to clarify the mechanisms of the disease.

LISTA DE ABREVIATURAS

GAD	Genetic Association Database
NIH	National Institute of Health
MZ	monozygotic
DZ	dizygotic
ADRB2	beta-2-adrenergic receptor gene
MHCII	major histocompatibility complex class II
HLA	human leukocyte antigen
TNF	tumor necrosis factor
ADAM	a disintegrin and metalloprotease domain
GWA	Genome-wide Association Studies
IRF-1	interferon regulatory factor 1
ISAAC	International Study of Asthma and Allergy in Childhood
EMSA	electrophoretic mobility shift assay
IFNγ	Interferon γ
IL	interleukin
Th2	T helper 2
NK	natural killer cells
SPT	skin prick test
MALDI-TOF	matrix-assisted laser desorption/ionization time-of flight
EM	expectation-maximisation algorithm
UTR	untranslated region
MAF	minor allele frequency

LD	linkage disequilibrium
SNP	single nucleotide polymorphism
EGR1	Early Growth Response 1
dbSNP	public SNP databases
TF	transcription factors
STAT	signal transducer and activator of transcription
CNS	conserved non-coding sequences
HWE	Hardy-Weinberg Equilibrium
BAL	bronchoalveolar lavage
MMPs	matrix metalloproteinases
ECM	extra-cellular matrix
FEV1	forced expiratory volume in one second
MEF	maximum expiratory flows
MMEF	maximum mid-expiratory flow: the average expiratory flow
FVC	forced vital capacity
LRI	lower respiratory illnesses
RSV	respiratory syncytial virus

SUMÁRIO

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Leonardo Araujo Pinto reviewed the publications and wrote the first version of the manuscript; Renato T. Stein and Michael Kabesch supervised the review and participated in the preparation of the final version of the manuscript. The authors declare that they have no competing financial or personal interests.

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ABSTRACT

Objectives: to present the most important and recent results of researches on genetics of asthma. This data may help general physicians to understand the impact of genetics on this complex disorder and how genes and polymorphisms influence asthma and atopy.

Sources of data: data were collected from MEDLINE. Genetic association studies were selected from the Genetic Association Database (GAD), which is an archive of human genetic association studies of complex diseases and disorders, organized by the National Institute of Health (NIH).

Summary of findings: considering the data from several important twin-studies in the genetics of asthma, the heritability, that measures the contribution of genetic factors to the variance of asthma, may be estimated in 0.48 - 0.79. A huge number of genetic association studies have been trying to identify asthma susceptibility genes. The most replicated results in the genetic association studies involve the following 5 regions in human genome: 5q31-32, 6p21, 11q12-13, 16p11-12, 20p13. Only recently, a new asthma susceptibility gene (*ORMDL3*) has been identified by a whole genome association study, considered to be a major determinant for childhood asthma

Conclusions: genetic contribution to asthma may be estimated ranging from 48 to 79%. Several different loci seem to influence asthma susceptibility. Genes located on chromosome 5q (*ADRB2, IL13* and *IL4)* and the recently identified *ORMDL3*, in the chromosome 17, seem to be determinants for childhood asthma. Diagnostics and pharmacogenetics may be the first clinical implication of the extensive researches on the genetics of asthma.

Keywords:

Asthma, genetics, childhood, pharmacogenetics.

Introduction

It has been recognized that asthma is of hereditary nature, but that inheritance does not follow the classical Mendelian patterns in this disease. Several family studies showed evidence of a substantial familial aggregation pattern in asthma. (1, 2) However, the genetics of asthma is specially complicated by its polygenic nature and due to the interaction between genetic and environmental factors. (3) A better understanding of the genetic mechanisms of both asthma and allergy will enhance our knowledge of its pathophysiology, and represents an important advance for further efforts towards prevention and treatment.

Some aspects of this field of basic research may directly influence our clinical practice. The genetic determination of allergic responses to environmental stimuli and the role of pharmacogenetic in the management of asthma are highly regarded research topics. (4) The mapping of complex traits such as asthma is one of the most important areas of research in human genetics.

Although it is known that genetic susceptibility contributes to the risk of asthma, only few linkage or association genetic studies have been performed in non-European populations. Some studies have shown evidence of genetic control that differs among ethnic groups. (5, 6) However, this evidence is scarce and further studies on the genetics of asthma in non-European populations are necessary and may help to determine worldwide differences in the role of genetic variations in the development of asthma and allergies.

A classic design to study the impact of genetics on complex traits and to distinguish between genetic and environmental influences is the study of twin pairs, in which the concordance between monozygotic (MZ) and dizygotic (DZ) twins is compared. Monozygotic twins share 100% of their

genetic make-up and dizygotic twins share on average 50% of their genes. Using these studies it is possible to calculate asthma heritability (h^2). In genetics, heritability is the proportion of phenotypic variation in a population that is attributable to genetic variation. (7) It estimates the relative contribution of genetic and non-genetic factors to the total phenotypic expression in a population.

Twin-studies in the genetics of asthma

If genes influence a particular trait, monozygotic twins, due to their greater genetic similarity, should share that trait more than dizygotic twins. A Australian study investigated 3808 twin pairs, (8) and the correlations for asthma were greater for MZ twins than for DZ twins (0.48 for MZ, 0.09 for DZ, male; and 0.33 for MZ, 0.12 for DZ, female) implying that there is a significant proportion of the variance accounted by genetics in the pathogenesis of asthma ($h^2 = 0.60$). Duffy et al. (9) reanalyzed the 3808 Australian twin-pairs in 1990, and the correlation of self reported asthma was 0.65 among monozygotic twins and 0.24 among dizygotic twins. Heritability was estimated in 0.60 for females and 0.75 for males.

Nieminen et al (10) published a large population-based study of >13,000 adult Finnish twin pairs. The diagnosis of asthma was made by linking the twin registries with databases on hospital admissions and utilization of medication. Data was collected from a central office for epidemiology. A total of 4307 monozygotic and 9581 dizygotic twin pairs aged 18–70 were included. The heritability estimation was 0.68 for women and 0.48 for men (aged 28–59 years). In this study there was a marked gender difference in heritability and there was a difference in heritability between the various age groups, with heritability decreasing with age, which is in accordance with the clinical and epidemiological evidences.

Another twin study published in 1997 included 1,480 Swedish twin pairs aged 7–9 years (from the Swedish Twin Registry). (11) All twins born in Sweden between 1985 and 1986 were received a detailed asthma questionnaire. The correlation for parental-report of asthma was 0.79 for monozygotic male twin pairs and 0.64 for monozygotic female pairs, with correlations of 0.25 and 0.27 for dizygotic male and female pairs, respectively. The contribution of genetic factors to variance of asthma in this study was about 0.76 for boys and 0.64 for girls.

In a Norwegian study, all twins born between 1967 and 1974 (5,864 children) were identified through the Norwegian National Birth Registry. (12) The prevalence of self-reported asthma was ca. 5% and there were no significant sex differences. The concordance for asthma was 0.45 for monozygotic twins and 0.12 for dizygotic twins. This study showed that genetic effects explained 75% of the variation in both sexes. The remaining 25% was accounted for by environmental influences.

Another Finnish twin study in 16-year-old twins and their parents presented combined twin-family data on the genetics of asthma. The heritability of asthma was approximately 79% and the remaining 21% was due to environmental influences (13) If only the families with parental asthma were considered, genetic influences explained as much as 87% of the development of asthma in the offspring.

Another large-scale study on 11,688 twin pairs aged 12–41 years was published in 1999 and the heritability was estimated in 0.73 (Danish population sample). (14) A more recent twin study with an estimation of asthma heritability was published in 2001. (15) This last asthma twin study estimates a heritability of 0.68 in a population from UK.

All these twin studies have shown the importance of the genetics on asthma variance, with results of heritability estimation ranging from 48-79%. An important finding is that most of these twin studies in different parts of the world (especially in Northern Europe) showed similar and consistent results and stress the fact that especially childhood asthma has a strong genetic background. Table 1 presents the summary of these important population-based twin studies.

Although we can estimate to what extent genetic susceptibility contributes to the risk of asthma, the specific loci that influence the clinical phenotypes are yet far from being clearly identified. A significant number of genetic association studies have been describing asthma susceptibility genes, but these data demonstrates the extreme complexity of the disease, and the identification of these genes and polymorphisms may be still considered a difficult challenge.

Candidate-gene association studies.

A widely used approach for the identification of asthma susceptibility genes is the study of polymorphisms in candidate genes. Genetic association studies test whether a specific genetic variant is more common in asthmatics than in non-asthmatics. Controls for association studies should be recruited from a population that shares ethnic or geographic similarities with the cases. The advantages of association studies include their power to detect susceptibility genes and their applicability to the general populations. This approach is powerful if (and only if) the candidate selected for the study is clearly involved in the pathogenesis of the disease. (16)

However, because of the multitude of potential candidate genes for a complex trait, the work involved in a comprehensive candidate-gene approach might be overwhelming. In addition, as it is now recognized, results of individual polymorphism studies may be misleading (especially because of

linkage disequilibrium), thus the candidate-gene approach has to include multiple variants that are evaluated simultaneously. Considering this, correction for multiple comparisons or replication in different population samples and / or functional analysis have been required to define causality in these association studies. (17, 18)

Several candidate genes (> than 100 loci) have been proposed and studied in asthma. Several factors contribute to this abundance of candidates. Results from genome screens have provided evidence of linkage to multiple sites in the genome. Therefore, there are many positions including several candidate genes. In addition, immunological pathways associated to the asthmatic response involve a large array of inflammatory mediators such as cytokines and chemokines. However, the best replicated results in the genetic association studies involve the following 5 regions in the human genome: 5q31-32, 6p21, 11q12-13, 16p11-12, 20p13 (http://geneticassociationdb.nih.gov/). (19)

Genetic Association Database

The Genetic Association Database (GAD) is an archive of human genetic association studies of complex disorders, organized by the National Institute of Health (http://geneticassociationdb.nih.gov/). The objective of this database is to allow the researchers to rapidly identify medically relevant polymorphisms from the large volume of gene variations, in the context of a standardized nomenclature for genes and polymorphisms (rs numbers). The database includes selected published scientific papers. Study data is recorded with the official nomenclature used for the human genome. If a study investigates more than one gene for a particular disorder, there will be more than one record. The submitted records are reviewed before inclusion in the archive. (19)

Using GAD, we have selected 8 genes, located in the five above mentioned regions of the genome, for the current review that have been associated with asthma in more than 5 population based genetic association studies. These relevant asthma susceptibility genes are discussed below in detail (Table 2).

Chromosome 5: ADRB2, IL13 and IL4

The beta-2-adrenergic receptor gene (*ADRB2*) is a member of the G protein-coupled receptor superfamily. This receptor-channel complex contains a G protein, an adenylyl cyclase and the counterbalancing phosphatase. The assembly of the signaling complex provides a mechanism that ensures specific signaling by this G protein-coupled receptor. Different polymorphic loci of this gene have been associated with asthma diagnosis, nocturnal asthma, asthma exacerbations and response to beta-2 agonists in asthma treatment.

Turki found a higher frequency of glycine at position 16 (Gly16, SNP rs1042713), compared to Arg16, among individuals with nocturnal asthma. (20) Other evidence has suggested that the presence of Gly16 (amino-acid sequence) of *ADRB2* imparts enhanced agonist-promoted downregulation of the type that characterizes this form of asthma.

In a meta-analysis published recently, Contopoulos-Ioannidis et al confirmed the association between the Gly16 polymorphism and nocturnal asthma, but found no association between this variant and bronchial hyperresponsiveness. (21) Other studies found association between rs1042713 and different responses to beta-2 agonists (especially Albuterol and Salmeterol). (22)

IL13 encodes an immunoregulatory cytokine produced primarily by activated Th2 cells. This cytokine up-regulates major histocompatibility complex class II (MHCII) expression and promotes IgE isotype switching. IL13 inhibits the production of pro-inflammatory cytokines and chemokines. This cytokine is found to be critical to the pathogenesis of allergen-induced asthma but operates through mechanisms

independent of IgE. *IL3, IL5, IL4, IRF1* and *CSF2* form a cytokine gene cluster on chromosome 5q, with *IL13* located particularly close to *IL4*.

Howard et al. reported that the promoter variant (C-1112T, rs1800925) of the *IL13* gene contributes significantly to bronchial hyperresponsiveness and asthma susceptibility but not to total serum IgE levels. (23) Heinzmann (24) determined that a R130Q variant of *IL13* (rs20541) is associated with asthma in case-control populations from Britain and Japan (OR = 2.31, 95% confidence interval, 1.33 - 4.00); the variant also predicted asthma and higher serum IL13 levels in a Japanese pediatric population.

The protein encoded by the *IL4* gene is a Th2 cytokine produced by activated T cells that influence allergic immune response. The IL4 receptor also binds to IL13, which may contribute to many overlapping functions of IL4 and IL13. IL4, IL13 and IL5 are found to be regulated coordinately by several regulatory elements on the chromosome 5.

Kabesch (25) have demonstrated a possible involvement of SNPs in the *IL4* gene in the development of asthma and the regulation of total serum IgE. In addition, this group has shown in 2006 (26) that especially the combined analyses of genetic alterations in the IL-4/IL-13 pathway reveal its significance to the development of atopy and childhood asthma. Additionally, other genes harbored in the locus, as CD14 and IRF1, may also contribute to asthma and allergy.

Chromosome 6: *HLA-DQB1* and *TNF*

HLA-DQB1 belongs to the HLA (human leukocyte antigen) beta chains. This molecule is a heterodimer consisting of an alpha (DQA) and a beta chain (DQB), both anchored in the membrane. It plays a central role in the immune system by presenting peptides derived from extracellular proteins.

Within the DQ molecule both the alpha and the beta chains contain polymorphisms, leading to four different molecules. Several studies (27-29) have shown association between *HLA-DQB1* variations and asthma or aspirin induced asthma (Table 2).

The *TNF* gene encodes a multifunctional pro-inflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. This cytokine is mainly secreted by macrophages. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation and apoptosis.

Witte (30) evaluated the relation between the G-308A (rs1800629) promoter polymorphism of the *TNF* gene and risk of asthma in 236 cases and 275 non-asthmatic controls. This study indicated that having 1 or 2 copies of the -308A allele increased the risk of asthma (OR =1.58), the magnitude of which was increased when restricting the cases to those with acute asthma (OR =1.86, p =0.04) or further restricting the subjects to those with a family history of asthma and those of European American ancestry (OR = 3.16, p = 0.04).

Instead, Aoki et al. (31) did not find a significant association between this *TNF* G-308A polymorphism (rs1800629) and childhood atopic asthma in 2 independent Japanese populations; however, meta-analysis of a total of 2,477 asthma patients and 3,217 control individuals showed that the G-308A polymorphism was significantly associated with asthma. The combined OR was 1.46 for fixed or random effects ($p < 0.001$).

Chromosome 11: *SCGB1A1 (*or *UGB; CC16; CCSP)*

Clara cell secretory protein (CC16) is a protein primarily expressed in the respiratory tract by nonciliated bronchiolar secretory cells (32) and the immunomodulatory activity of CC16 has been well

documented. Mice deficient in CC16 expression exhibit a higher susceptibility to lung injury and an excessive inflammatory response.

The *CC16* gene was screened for mutations and a polymorphism (A38G, rs3741240) was identified and associated with an increased risk of physician-diagnosed asthma in a population of Australian children. (33) In a study with adults, a moderate risk of asthma was found to be associated with the CC16 38A allele. (34) Laing et al has shown that the 38A sequence was associated with reduced plasma CC16 levels and individuals with lower plasma CC16 levels were more likely to have asthma (34). However, studies with larger population samples are required to confirm this association.

Chromosome 16: *IL4R*

This gene encodes the interleukin-4 receptor, a transmembrane protein that can bind interleukin 4 and interleukin 13 to regulate IgE production. Binding of IL13 or IL4 to the IL4 receptor (IL4R) induces the initial response for Th2 lymphocyte polarization. Both IL13 and IL4 are produced by Th2 cells and are capable of inducing isotype class-switching of B cells to produce IgE after allergen exposure.

Allelic variations in this gene have been associated with atopy, a condition that can manifest itself as allergic rhinitis, asthma, or eczema. Howard et al investigated 5 *IL4RA* single-nucleotide polymorphisms in a population of Dutch families ascertained through a proband with asthma. (35) The authors observed significant associations of atopy and asthma-related phenotypes with several IL4RA polymorphisms, especially S503P (rs1805015). A significant gene-gene interaction between S503P in *IL4RA* and the C-1112T promoter variation in *IL13*, previously shown to be associated with bronchial hyperresponsiveness, was detected. Individuals with the risk genotype for both genes were at almost 5 times greater risk for the development of asthma compared to individuals with no-risk genotypes.

These data suggest that variations in *IL4RA* contribute to elevated total serum IgE levels, and interaction between *IL4RA* and *IL13* markedly increases a subject susceptibility to asthma

Chromosome 20: *ADAM33*

This gene encodes a member of the ADAM (a disintegrin and metalloprotease domain) family. Members of this family are membrane-anchored proteins and have been implicated in a variety of biological processes including muscle development and neurogenesis. This protein is a transmembrane protein implicated in asthma and bronchial hyperresponsiveness. Alternative splicing of this gene results in two transcript variants encoding different isoforms. This was the first published asthma candidate gene detected by positional cloning.

Van Eerdevegh (36) performed a genomewide scan on 460 Caucasian families and identified a locus on chromosome 20p13 that was linked to asthma and bronchial hyperresponsiveness. A survey of 135 polymorphisms in 23 genes identified the *ADAM33* gene as being significantly associated with asthma using case control, transmission disequilibrium, and haplotype analyses ($p = 0.04$-0.000003). However, these results were not replicated by several other ADAM33 association studies. Studies in Icelandic and UK populations revealed no association when taken in isolation.

Recently, a meta-analysis showed that the rs511898 and rs574174 variants (located on *ADAM33* gene) were significantly associated with asthma (37). The additional risk imparted by these variations would account for 50,000 excess asthma cases in the UK alone.

Genome-wide Association Studies (GWA)

With the completion of the Human Genome Project in 2003 and the International HapMap Project in 2005, researchers have now a set of advanced research tools that may allow identifying genetic contributions to common diseases more easily. These tools include computerized databases containing the reference human genome sequence and a map of human genetic variation. A GWA is an approach that involves scanning markers across complete sets of the human polymorphisms.

Recently, the first asthma GWA study (38) characterized more than 317,000 SNPs in DNA from 994 patients with childhood-onset asthma and 1,243 non-asthmatics, using both family and case-referent panels. The authors showed multiple markers on chromosome 17q21 to be strongly and consistently associated with childhood asthma with a combined p value of less than $10^{(-12)}$. In independent replication study showed that the 17q21 locus has a significant association with the diagnosis of childhood asthma in 2,320 subjects from a cohort of German children ($p = 0.0003$) and in 3,301 subjects from the UK's 1958 Birth Cohort ($p = 0.0005$). This study (38) has evaluated the relationship between markers of the 17q21 locus and transcript levels of genes. The SNP (rs7216389) associated with childhood asthma were consistently and strongly associated ($p = 10^{(-22)}$) with transcript levels of *ORMDL3*, a member of a gene family that encodes transmembrane proteins anchored in the endoplasmic reticulum. Moffat, Kabesch et al, (38)concluded that genetic variants regulating *ORMDL3* expression are determinants of susceptibility to childhood asthma. In the subset of individuals for whom expression data were available, the T allele of SNP rs7216389 was the marker most strongly associated with disease in the combined GWA (Figure 1).

Conclusions and future perspectives

Several different loci seem to influence asthma susceptibility. Genes located on chromosome 5q (*ADRB2, IL13* and *IL4)* and the recently identified *ORMDL3*, in the chromosome 17, seem to be major determinants for childhood asthma.

However, there are only a few studies of asthma genetics in Latin America and not many in underdeveloped areas of the world where asthma is highly prevalent. A recent study from the ISAAC-Phase II group (39) has shown that asthma in non-affluent communities is significantly less associated with allergy in comparison with more socially developed areas.

It is not clear if the results from genetic studies in European populations can easily be transferred to population of different ethnicities. Epidemiological genetic studies in Latin America, Asia and Africa are needed to determine the impact of genes and environment in these regions, which may differ dramatically from the findings in population samples from Europe and the US.

Improvements in diagnostics and pharmacogenetics may be the first clinical implications of these extensive researches on the genetics of asthma. In a randomized, placebo-controlled study involving 78 patients with mild asthma, 37 with the R/R genotype and 41 with the G/G genotype (rs1042713), Israel (22) found that there were significant genotype-related differences in response to albuterol compared with placebo. Patients with the R/R genotype improved when beta-agonist therapy was withdrawn and replaced with ipratropium bromide, whereas those with the G/G genotype did better with regular beta-agonist therapy than when it was withdrawn. Genotype at amino acid 16 of ADRB2 affects significantly the response to albuterol. Furthermore, bronchodilator treatments avoiding albuterol may be appropriate for patients with the R/R genotype (22). This is only an example how genetics may influence our clinical practice in the near future.

References

1. Schwartz M. Heredity in bronchial asthma; a clinical and genetic study of 191 asthma probands and 50 probands with Baker's asthma. Acta Allergol Suppl (Copenh) 1952;2:1-288.

2. Speizer FE, Rosner B, Tager I. Familial aggregation of chronic respiratory disease: use of National Health Interview Survey data for specific hypothesis testing. Int J Epidemiol 1976;5:167-72.

3. Bierbaum S, Heinzmann A. The genetics of bronchial asthma in children. Respir Med 2007;101:1369-75.

4. Lima JJ. Treatment heterogeneity in asthma: genetics of response to leukotriene modifiers. Mol Diagn Ther 2007;11:97-104.

5. Barnes KC, Grant AV, Hansel NN, Gao P, Dunston GM. African Americans with asthma: genetic insights. Proc Am Thorac Soc 2007;4:58-68.

6. Choudhry S, Ung N, Avila PC, et al. Pharmacogenetic differences in response to albuterol between Puerto Ricans and Mexicans with asthma. Am J Respir Crit Care Med 2005;171:563-70.

7. Koppelman GH, Los H, Postma DS. Genetic and environment in asthma: the answer of twin studies. Eur Respir J 1999;13:2-4.

8. Hopper JL, Hannah MC, Macaskill GT, Mathews JD. Twin concordance for a binary trait: III. A bivariate analysis of hay fever and asthma. Genet Epidemiol 1990;7:277-89.

9. Duffy DL, Martin NG, Battistutta D, Hopper JL, Mathews JD. Genetics of asthma and hay fever in Australian twins. Am Rev Respir Dis 1990;142:1351-8.

10. Nieminen MM, Kaprio J, Koskenvuo M. A population-based study of bronchial asthma in adult twin pairs. Chest 1991;100:70-5.

11. Lichtenstein P, Svartengren M. Genes, environments, and sex: factors of importance in atopic diseases in 7-9-year-old Swedish twins. Allergy 1997;52:1079-86.

12. Harris JR, Magnus P, Samuelsen SO, Tambs K. No evidence for effects of family environment on asthma. A retrospective study of Norwegian twins. Am J Respir Crit Care Med 1997;156:43-9.

13. Laitinen T, Rasanen M, Kaprio J, Koskenvuo M, Laitinen LA. Importance of genetic factors in adolescent asthma: a population-based twin-family study. Am J Respir Crit Care Med 1998;157:1073-8.

14. Skadhauge LR, Christensen K, Kyvik KO, Sigsgaard T. Genetic and environmental influence on asthma: a population-based study of 11,688 Danish twin pairs. Eur Respir J 1999;13:8-14.

15. Koeppen-Schomerus G, Stevenson J, Plomin R. Genes and environment in asthma: a study of 4 year old twins. Arch Dis Child 2001;85:398-400.

16. Ober C, Hoffjan S. Asthma genetics 2006: the long and winding road to gene discovery. Genes Immun 2006;7:95-100.

17. Cordell HJ, Clayton DG. Genetic association studies. Lancet 2005;366:1121-31.

18. Hattersley AT, McCarthy MI. What makes a good genetic association study? Lancet 2005;366:1315-23.

19. Lin BK, Clyne M, Walsh M, et al. Tracking the epidemiology of human genes in the literature: the HuGE Published Literature database. Am J Epidemiol 2006;164:1-4.

20. Turki J, Pak J, Green SA, Martin RJ, Liggett SB. Genetic polymorphisms of the beta 2-adrenergic receptor in nocturnal and nonnocturnal asthma. Evidence that Gly16 correlates with the nocturnal phenotype. J Clin Invest 1995;95:1635-41.

21. Contopoulos-Ioannidis DG, Manoli EN, Ioannidis JP. Meta-analysis of the association of beta2-adrenergic receptor polymorphisms with asthma phenotypes. J Allergy Clin Immunol 2005;115:963-72.

22. Israel E, Chinchilli VM, Ford JG, et al. Use of regularly scheduled albuterol treatment in asthma: genotype-stratified, randomised, placebo-controlled cross-over trial. Lancet 2004;364:1505-12.

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

24. Heinzmann A, Mao XQ, Akaiwa M, et al. Genetic variants of IL-13 signalling and human asthma and atopy. Hum Mol Genet 2000;9:549-59.

25. Kabesch M, Tzotcheva I, Carr D, et al. A complete screening of the IL4 gene: novel polymorphisms and their association with asthma and IgE in childhood. J Allergy Clin Immunol 2003;112:893-8.

26. Kabesch M, Schedel M, Carr D, et al. IL-4/IL-13 pathway genetics strongly influence serum IgE levels and childhood asthma. J Allergy Clin Immunol 2006;117:269-74.

27. Gao J, Lin Y, Qiu C, Liu Y, Ma Y. Association between HLA-DQA1, -DQB1 gene polymorphisms and susceptibility to asthma in northern Chinese subjects. Chin Med J (Engl) 2003;116:1078-82.

28. Torio A, Sanchez-Guerrero I, Muro M, et al. HLA class II genotypic frequencies in atopic asthma: association of DRB1*01-DQB1*0501 genotype with Artemisia vulgaris allergic asthma. Hum Immunol 2003;64:811-5.

29. Schubert MS, Hutcheson PS, Graff RJ, Santiago L, Slavin RG. HLA-DQB1 *03 in allergic fungal sinusitis and other chronic hypertrophic rhinosinusitis disorders. J Allergy Clin Immunol 2004;114:1376-83.

30. Witte JS, Palmer LJ, O'Connor RD, Hopkins PJ, Hall JM. Relation between tumour necrosis factor polymorphism TNFalpha-308 and risk of asthma. Eur J Hum Genet 2002;10:82-5.

31. Aoki T, Hirota T, Tamari M, et al. An association between asthma and TNF-308G/A polymorphism: meta-analysis. J Hum Genet 2006;51:677-85.

32. Hermans C, Lesur O, Weynand B, Pieters T, Lambert M, Bernard A. Clara cell protein (CC16) in pleural fluids: a marker of leakage through the visceral pleura. Am J Respir Crit Care Med 1998;157:962-9.

33. Laing IA, Goldblatt J, Eber E, et al. A polymorphism of the CC16 gene is associated with an increased risk of asthma. J Med Genet 1998;35:463-7.

34. Laing IA, Hermans C, Bernard A, Burton PR, Goldblatt J, Le Souef PN. Association between plasma CC16 levels, the A38G polymorphism, and asthma. Am J Respir Crit Care Med 2000;161:124-7.

35. Howard TD, Koppelman GH, Xu J, et al. Gene-gene interaction in asthma: IL4RA and IL13 in a Dutch population with asthma. Am J Hum Genet 2002;70:230-6.

36. Van Eerdewegh P, Little RD, Dupuis J, et al. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. Nature 2002;418:426-30.

37. Blakey J, Halapi E, Bjornsdottir US, et al. Contribution of ADAM33 polymorphisms to the population risk of asthma. Thorax 2005;60:274-6.

38. Moffatt MF, Kabesch M, Liang L, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. Nature 2007;448:470-3.

39. Weinmayr G, Weiland SK, Bjorksten B, et al. Atopic sensitization and the international variation of asthma symptom prevalence in children. Am J Respir Crit Care Med 2007;176:565-74.

Table 1 Revised twin-studies with the outcome asthma and the estimated heritability (h^2):

Study Population	N *	Age range	h^2	References
Australian	3808	18–88	**0.60 – 0.75**	*Duffy 1990.* [9]
Finnish	13888	18–59	**0.48 – 0.68**	*Niemienen 1991.* [10]
Swedish	1480	7–9	**0.64 – 0.76**	*Lichtenstein 1997.* [11]
Norwegian	5864	18–25	**0.75**	*Harris 1997.* [12]
Finnish	1713	16	**0.79**	*Laitinen 1998.* [13]
Danish	11668	12–41	**0.73**	*Skadhauge 1999.* [14]
English	4910	4	**0.68**	*Koeppen-Schomerus 2001.* [15]

* Number of twin-pairs included in the twin population study.

Table 2 Genes with more than 5 positive genetic association studies with the outcome asthma (data from NIH Genetic Association Database, http://geneticassociationdb.nih.gov/) [19]

Position	Gene	Number of positive studies	Polymorphisms	References
5q31-32	***ADRB2***	11	rs1042713	*Holloway JW 2000, Wang Z 2001, Ohe M 1995, Turki J1995, Barr RG 2001, Dai LM 2004, Gao JM 2002, Gao G 2000, Xu X 2002, Summerhill E 2000, Fu J 2002*
	IL13	13	rs1800925 rs20541	*van der Pouw Kraan TC 1999, Arima K 2002, Xi D 2004, Heinzmann A 2000, Kim HB 2006, Hosseini-Farahabadi S 2007, Hunninghake GM 2007, Kabesch M 2006, Battle NC 2007, Leung TF 2001, Howard TD 2001, Tsunemi Y 2002, Noguchi E 2001*
	IL4	11	rs2243250	*Zhu S 2000, Chouchane L 1999, Burchard EG 1999, Rosenwasser LJ1995, Noguchi E 2001, Kabesch M 2003, Gervaziev, YV 2006, Hosseini-Farahabadi S 2007, Kabesch M 2006, Kabesch M 2003, Suzuki I 2000*
6p21.3	***HLA-DQB1***	10	rs12722107 rs1049086 rs1049107 rs1130386 rs1049133	*Gao J 2003, Guo X 2001, Torio A 2003, Kim SH 2005, Schubert MS 2004, Lara-Marquez ML 1999, Lin YC 2002, Cho SH 2000, Kim YK 2002,*
	TNF	6	rs1800629	*Witte JS 2002, Albuquerque R 1998, Winterton DL 2001, Noguchi E 2002, Hong SJ 2006, Kim SH 2006*
11q12-13	***SCGB1A1***	6	rs3741240	*Choi M 2000, Sharma S 2004, Gui Q 2003, Sengler C 2003, Laing IA 1998, Candelaria PV 2005*
16p11-12	***IL4R***	9	rs1805015	*A-M Hytonen CEA 2004, Ober C 2000, Mitsuyasu H 1999, Cui T 2003, Risma KA 2002, Mitsuyasu H 1998, Zhang AM 2006, Loza MJ 2007, Battle NC 2007*
20p13	***ADAM33***	9	SNP *	*Werner M 2004, Howard TD 2003, Lee JH 2004, Jongepier H 2004, Noguchi E 2006, Kedda MA 2006, Hirota T 2006, Qiu YM 2007, Sakagami T 2006*

* more than 15 ADAM33 plymorphisms have been associated with asthma or asthma related phenotypes

Figure 1

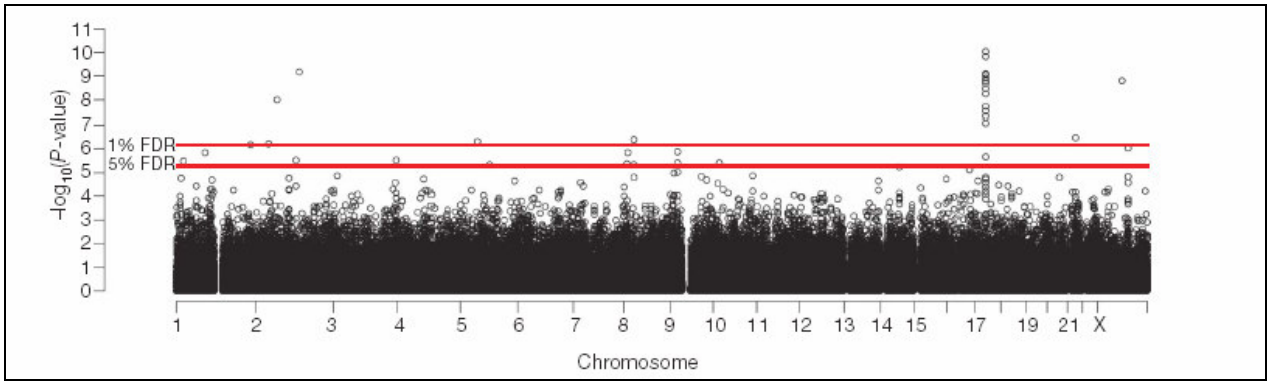

Figure 1 Genome-wide association of 317,447 SNPs and asthma in 994 asthmatic children and 1,243 non-asthmatic children (Figure from Moffat, Kabesch et al, Nature 2007). Position in the genome is divided by chromosome. Strength of association is shown on the y axis. The result for each individual marker is depicted as a black circle. The genome-wide thresholds for 1% and 5% false discovery rates (FDR) are shown as horizontal red lines. Numerous markers on chromosome 17q21 show association to asthma.

OBJETIVOS

OBJETIVO GERAL

Avaliar a associação de polimorfismos em genes ligados à resposta imune inata com fenótipos de asma e atopia.

OBJETIVOS ESPECÍFICOS DE CADA ARTIGO ORIGINAL

1. Descrever os polimorfismos do gene do Fator Regulador de Interferon 1 (FRI-1) e estudar a associação destas variações genéticas com desfechos de asma e atopia em duas amostras populacionais de origem caucasiana.
2. Estudar a associação de polimorfismos do gene Transdutor de Sinal e Ativador da Transcrição 1 (TSAT-1) com desfechos de asma e atopia em uma população caucasiana.
3. Estudar a associação entre polimorfismos do gene da Metaloproteinase de Matriz 9 (MP-9) e diferentes fenótipos de asma em uma população caucasiana.
4. Analisar as associações entre genes ligados à resposta imune inata e os diferentes fenótipos de sibilância.

CAPÍTULO 1

IRF-1 GENE VARIATIONS INFLUENCE IGE REGULATION AND ATOPY

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CAPÍTULO 1

IRF-1 GENE VARIATIONS INFLUENCE IGE REGULATION AND ATOPY

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Our results suggest that *IRF-1* polymorphisms influence atopy risk, making *IRF-1* an intriguing target for further studies. Polymorphisms in *IRF-1* may be important in different diseases where susceptibility to microbial exposure play a role.

Descriptor Number: 58 (Ashtma Genetics), word count: 2.826 words

ABSTRACT

Rationale: The development of atopic diseases is characterized by skewed immune responses to common allergens. Only recently, interferons have been identified to play a crucial role in these mechanisms.

Objective: As interferon regulatory factor 1 (IRF-1) is critical for interferon expression we tested the hypotheses that genetic changes in this essential transcription factor may have consequences for the development of atopy.

Methods: All exons, introns, adjacent and putatively regulatory regions of the *IRF-1* gene were re-sequenced in 80 human chromosomes. Association and haplotype analyses were performed in a cross sectional study population of German children (n=1,940). Results were replicated in a second population (n=1,159), both part of the International Study of Asthma and Allergy in Childhood (ISAAC phase II). Polymorphism function was studied using electrophoretic mobility shift assay (EMSA) and colorimetric binding assays. All statistical analyses were performed using SAS/Genetics.

Results: By re-sequencing 49 polymorphisms were identified within the *IRF-1* gene. Four blocks of polymorphisms containing a total of 11 polymorphisms were significantly associated with atopy, total or specific IgE levels in both populations ($p<0.05$). Functional analyses revealed that three of these SNPs lead to profound changes in transcription factor binding of NF-κB, EGR1 and SP1 to the *IRF-1* promoter.

Conclusion: Our results suggest that *IRF-1* polymorphisms influence atopy risk. Polymorphism induced changes in transcription factor binding may explain some of the observed effects and make *IRF-1* an intriguing target for further studies.

Abstract word count: 234

Key words: Asthma, Genes, Interferons, IRF-1 Transcription Factor, Genetic Polymorphism

INTRODUCTION

It has been speculated that the development of atopy may be influenced by genetic predisposition for specific immune responses and the exposure or absence of certain environmental stimuli. Predisposition and exposure may in turn lead to the activation of allergen associated immune cells and the expression of specific cytokine patterns. Type I and II interferons, inducible by environmental stimuli such as microbial exposure, are key players in the human immune system and may suppress atopy-associated skewed immunity (1). Interferon γ (IFNγ) production is controlled by several transcription factors of which interferon regulatory factor 1 (IRF-1) is of special importance. IRF-1 binds to the promoter region of genes critical for interferon expression and genes responsive to interferon (2). IRF-1 stimulates the production of interleukin 12 (IL-12) and IL-23 and increases the maturation of IL-18, which synergizes with IL-12 in the activation of *IFN-γ* expression. Additionally, IRF-1 binds to the *IL-4* promoter and represses *IL-4* transcription thereby inhibiting T helper 2 (Th2) responses (3). IRF-1 up-regulates the expression of *IL-15*, which induces the generation of IFNγ producing natural killer (NK) cells. As a consequence, mice deficient of *IRF-1* (*Irf1–/–*) show a strongly increased susceptibility to intracellular infections (4). Overall, IRF-1 has a complex role influencing many aspects of T-cell immunology.

The gene coding for IRF-1 is located in a cytokine gene cluster on chromosome 5q31, which is considered to play an important role in the development of allergic disorders *(*figure 1a) (5). To investigate the presence and role of genetic variations in the *IRF-1* gene in a Caucasian population, we re-sequenced the gene, performed association studies with allergy-related phenotypes in two independent populations of German children (n= 1,940 and n=1,159) and studied associated promoter polymorphisms for their putative functional role in gene regulation.

MATERIAL AND METHODS (word count 500)

Population description for association studies

Between 1995 and 1996, a cross sectional study was performed in Munich and in Dresden, Germany, as part of the International Study of Asthma and Allergy in Childhood (ISAAC phase II) to assess the prevalence of asthma and allergies in 5,629 schoolchildren at the age of 9 to 11 years. Of these, all children of German origin with DNA available (N=3,099) were included in this analysis (Table E1). Children whose parents reported a physician's diagnosis of asthma, spastic bronchitis, or recurrent asthmatic bronchitis in a self-administered questionnaire were classified as having asthma. Populations and methods have been described in detail before(6). The sensitivity to six common aeroallergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria tenuis*, cat dander, mixed grass and tree pollen, all from Alk Scherax, Germany) was assessed by skin prick test (SPT). A child was considered atopic if a wheal reaction ≥ 3 mm occurred to one or more allergens after subtraction of the negative control. Total serum IgE levels were measured using the Imulite System (DPC Biermann, Germany). Specific IgE antibodies (*Sx1* from Phadia, Germany) against inhalative allergens (local grass pollen, birch pollen, mugwort pollen, *dermatophagoides pteronyssinus*, cat dander, dog dander, *cladosporium herbarum*) were measured in a range between 0.35-100 IU/ml.

Mutation screening and genotyping

Using an ABI Prism 3730 sequencer (Applied Biosystems) 80 chromosomes from 40 unrelated randomly selected adult volunteers were sequenced for 12,985 bp in and around the *IRF-1* gene (exons, introns, 2,522 base pairs (bp) upstream and 2,820 bp downstream of the gene; except a 868bp Short Interspersed Nuclear Element (SINE) of highly repetitive sequence 232bp downstream of the gene).

Genomic DNA was extracted from whole blood by a standard salting out method (7). DNA samples were genotyped using matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) mass spectrometry (Sequenom Inc). Additional details on the sequencing and genotyping methods are provided in an online data supplement (Tables E2-E3).

Functional analyses of the promoter polymorphisms

The influence of genetic polymorphisms on binding of transcription factors in the proximal promoter of the *IRF-1* gene have been investigated by electrophoretic mobility shift assay (EMSA) and colorimetric nuclear factor κB (NF-κB) assay. Additional details on these methods are provided in an online data supplement (Tables E4).

Statistical analyses

Polymorphisms with $r^2 \geq 0.8$ were defined as a LD Block. Deviation from Hardy-Weinberg equilibrium was analysed with chi-square tests. Association between SNPs and dichotomous outcomes were evaluated using chi-square tests in a dominant model of the rare allele. All tests were two-sided and the differences were considered significant when $p<0.05$. For IgE, t test of log-transformed values were used in a dominant model. Haplotype frequencies were estimated with an EM (expectation-maximisation) algorithm (8) and associations of common haplotypes (frequency >0.03) with atopy and IgE levels were calculated with Haploview (9). For haplotype analysis, the 90th percentile of total IgE distribution within the study population was used as a dichotomous outcome variable as previously described (10). Calculations were carried out with the SAS software (version 9.1.3).

RESULTS

Mutation screening and polymorphism identification in the *IRF-1* gene

Upon re-sequencing of 40 adult volunteers of German origin, thirty-nine SNPs and one 16bp deletion with minor allele frequencies (MAF) of at least 10% were identified in the region of the *IRF-1* gene (table 1). Two polymorphisms (C-2606T and 3513del) were previously not described in public SNP databases (dbSNP). Twenty-three polymorphisms were located in intronic regions, nine in the promoter region, three in the 5' untranslated region (5'UTR), one in the 3'UTR and three SNPs in the 3' flanking region. One SNP (A3116G) found in exon 7 did not change the amino acid sequence. In addition, 24 mutations (MAF<3%) and 9 infrequent polymorphisms (MAF 0.03-0.10) were identified but not studied further (Table E5).

Associations between *IRF-1* SNPs, IgE levels and atopy in two independent populations

Based on re-sequencing results, the existence of polymorphisms in a white population was verified and linkage disequilibrium analysis could be performed. Analyzing polymorphisms with MAF ≥0.10 (figure 1a), five major LD blocks were identified with $r^2 \geq 0.8$ (table 1). SNP block one (A-1710C, G-1705A, and G-1595A), two (C-2606T, A-672C and G5250T) and four (C-1243G, C-1152T, C4174T) contained 3 SNPs each, while two SNPs were found in block three (A1244G and T8268G). SNP block five contained 26 polymorphisms in strong linkage disequilibrium. An additional 3 polymorphisms could not be assigned to any LD block. Thus, three single SNPs and one tagging SNP per LD block were genotyped in a cross sectional study population of 1,940 children from Dresden (table 1). All SNPs showing associations in the first sample were also genotyped in a second population from Munich (n=1,159) to avoid type I errors due to multiple testing.

In the Dresden population, four of the tested SNPs representing blocks 1, 2, 3 and 4 were associated with significant changes in total IgE levels (table 2). In addition, polymorphism C4174T (representing block 4), which was associated with decreased total serum IgE levels, also reduced the risk for atopic

sensitisation (measured by SPT). SNP A-672C (representing block 2) was not only associated with increased total serum IgE levels but also with an increased risk for specific sensitisation (table 2) in the Dresden population. Interestingly, the tagging SNP T8460G representing the largest LD block of 26 polymorphisms was not associated with any one of the investigated phenotypes. Next, genotyping was replicated in the second ISAAC phase II population (Munich) for all four tagging SNPs that had shown associations with elevated total serum IgE levels in the Dresden population. In the Munich population, changes in total IgE levels were significant for SNP C4174T and showed trends similar to the previous observations in the Dresden sample for all other SNPs. The associations with atopic sensitization observed in Dresden with SNPs A-672C and C4174T were replicated and extended to SNPs A-1710C and A1244G. In addition, C4174T was strongly protective against the development of asthma in the presence of atopic sensitisation (OR 0.35, 95%CI 0.19-0.65, $p<0.001$) while A-1710C (OR 1.96, 95%CI 1.02-3.76, $p=0.040$) and A1244G (OR 1.84, 95%CI 1.03-3.28, $p=0.036$) increased the risk for atopic asthma (data not shown). Haplotype analyses with the IRF-1 region were also performed and confirmed previous observations (table 3). H_a, the most common IRF-1 haplotype, was associated with a protective effect for atopy measured by RAST and SPT in Munich while H_b increased the risk for strongly elevated total serum IgE (above 90th percentile) in Dresden and atopy in Munich. The same trends for atopy were observed in Dresden.

Functional studies reveal SNP dependent variations in the binding of transcription factors in the proximal promoter of the *IRF-1* gene

Three of the eleven associated SNPs in blocks 1, 2, 3, and 4 were located in the previously defined proximal promoter (11) of the *IRF-1* gene (SNP A-1710C, A-1705G, A-1595G) and two in the 5' untranslated region (C-1152T, and C-1243G), potentially influencing *IRF-1* gene expression through changes in transcription factor binding (figure 2a). Thus, these regions were analysed for

polymorphism associated changes in transcription factor binding using EMSA with nuclear extracts from unstimulated or PMA/Ionomycin stimulated Jurkat T-cells. At the site harbouring SNPs A-1710C and A-1705G, NF-κB binding was significantly increased in the probe carrying the polymorphic C allele at position -1710 (figure 2b). Using a colorimetric NF-κB binding assay quantitative differences in NF-κB were confirmed (figure 3). Furthermore, the genotype at position -1705 does not seem to influence NFκB binding significantly (data not shown). At position -1595, EMSA experiments identified a DNA/protein complex that appeared in nuclear extract of Jurkat T-cell after stimulation (PMA/Ionomycin) only in the presence of the wildtype G allele. By supershift experiments this complex was identified as EGR1 (Early Growth Response 1) (figure 2c). Furthermore, differences in binding complexes were observed between probes containing the polymorphic site C-1152T. Supershift experiments demonstrated that the complex only present with the wildtype C allele contained SP1 (figure 3d) whereas no binding was detectable with the T allele (data not shown). For the site harbouring C-1243G, no significant change in transcription factor binding was observed (data not shown).

DISCUSSION

Chromosome 5q31-33 has been a hot spot of allergy genetics in recent years, due to repeated linkage of the locus with allergy in whole genome linkage studies and the pre-existing knowledge that many genes important for the immune system are clustered in this region. So far, the focus of 5q studies has been on Th2 related cytokines but our results may indicate that there is more to the 5q locus than that. Our association studies and functional analyses suggest that genetic variations in *IRF-1*, potentially acting through changes in transcription factor binding, influence the development of atopy and the regulation of total IgE levels. Even though the *IRF-1* gene is crucial for the regulation and expression of interferons, involved in many important and diverse immune responses, no systematic screening for

polymorphisms in a white population has so far been reported and previous association studies with atopic phenotypes were limited to the analysis of tandem repeat markers in Asian populations (12, 13). In our study, 49 polymorphisms were identified by re-sequencing 80 chromosomes. Based on this information LD patterns could be analyzed and eight tagging SNPs were selected for genotyping in a large and well phenotyped white population. Thus, it is unlikely that frequent SNPs or significant associations were missed. Replication and functional analyses were used to minimize the risk of a type I error, always present when multiple tests are performed. Therefore, it is also very unlikely that the observed associations in two independent populations and the differences in functional analyses would all have occurred by chance.

Four blocks of SNPs genotyped by tagging SNPs A-1710C, A-672C, A1244G, and C4174T were associated with total or specific IgE regulation as well as the development of atopy measured by SPT in the two populations. While in the Dresden population *IRF-1* SNPs had a stronger influence on total IgE levels, the effect on atopy was much more pronounced in the Munich sample, where, in addition, also associations with the development of atopic asthma were observed. Atopic asthma is the most common asthma phenotype in the developed world. While different asthma phenotypes (ex. atopic and non-atopic) show a very similar clinical picture, it is suspected that these forms may have different aetiologies and should be separately investigated(14). The gradient in *IRF-1* effects between Dresden (former East-Germany) and Munich (former West-Germany) is not surprising. Such effects have been observed with genetic variants in other immune-regulatory genes strongly susceptible to environmental stimuli. Thus, a similar mechanism as recently identified for a promoter SNP in the *CD14* gene, where the amount of endotoxin exposure determines the role of the *CD14* SNP in the development of atopy (either conferring a risk or even protection)(15), is suggested also for *IRF-1*. Children from Dresden, in contrast to children from Munich, had spent their first years of life in day

care centres exposed to infections from early on putatively stimulating the immune system with signals conferring tolerance to allergens (6). Thus, genetic changes in the *IRF-1* gene influencing IgE regulation may be less important for the development of atopy in such a protective environment as compared to Munich, where much less microbial or infectious stimuli were present early in life (smaller family size, less day care). In Munich, a much stronger effect of *IRF-1* polymorphisms for specific sensitization and subsequent disease was observed.

Functional studies of promoter SNPs as presented here give a first impression how these complex interactions between *IRF-1* polymorphisms, IgE regulation and atopy may develop. Of the 11 polymorphisms represented by the four tagging SNPs showing significant associations, five SNPs in the proximal promoter and 5'UTR of the *IRF-1* gene were studied and three SNPs were identified to lead to significant and profound changes in transcription factor binding in the proximal promoter of the IRF-1 gene: The presence of the C allele at position -1710 significantly increases binding of NF-κB in the proximal *IRF-1* promoter. In addition, the wildtype G allele at position -1595 leads to the binding of EGR1 while at position -1152, the wildtype C allele induces SP1 binding. Both transcription factors do no longer bind in the presence of the polymorphic allele. In independent previous studies cancer cell lines carrying polymorphic alleles at position -1710/-1705 and -1595 also showed increased type I interferon expression (IFN-α and IFN-β) (16). The combination of transcription factors altered by SNPs in the proximal promoter of *IRF-1* is intriguing. NF-κB is a key player in many inflammatory processes induced by microbial stimuli but is also necessary for IgE switching, expression and regulation, potentially acting through a feed back loop (17). EGR1 has been shown to play a role in lipopolysaccharide induced gene regulation, potentially also influencing Th2 suppression via SOCS pathways (18). Binding of transcription factors from the SP family (SP1-3) at a polymorphic *CD14* promoter site was shown to alter *CD14* expression influencing the development of

atopy (19). Thus, the three identified alterations in transcription factor binding may play a role in linking microbial exposure, *IRF-1* regulation and the development of atopy. However, also other tagged SNPs located in the distal promoter region (C-2606T), in intronic regions (A-672C, A1244G, C4174Tand G5250T) or the 3' UTR (T8268G) may potentially contribute to changes in IRF-1 function but have not yet been studied in detail. Also, the possibility that associations observed with SNPs in the *IRF-1* gene may have originated from extended linkage disequilibrium with SNPs in other candidate genes in the cytokine cluster on chromosome 5q31, was addressed. Thus, LD between *IRF-1* SNPs (A-1710C, C4174T, A-672C, A1244G) and SNPs in the proximal genes *IL-5* (C-746T), *IL-4* (C-589T) and *IL-13* (C-1112T and G2044A) previously associated with atopy was studied (20-22). However, LD between SNPs in the *IRF-1* gene and polymorphisms in *IL-5, IL-4* and *IL-13* was not relevant in our population and did not explain the observed associations (figure 1b).

Thus, we concluded that genetic variants in the *IRF-1* gene truly influence the regulation of specific and total IgE levels and the development of atopy, putatively by altered transcription factor binding in the proximal promoter of the *IRF-1* gene, changing *IRF-1* expression and consecutive interferon regulation. Considering the importance of IRF-1 in diverse immune responses, polymorphisms in *IRF-1* may be important in different diseases where susceptibility to microbial exposure play a role.

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collection; Stephan Weiland and Erika von Mutius contributed to the collection of data and manuscript preparation; Michael Lohoff supervised colorimetric assays, participated in the development of the study design, data analysis and manuscript preparation; Michael Kabesch supervised all other experiments, participated in the development of the study design, collection of data, data analysis and wrote the final version of the manuscript. Furthermore, we would like to thank Michael Kormann and Martin Depner for support and critical review of the paper. All authors declare that they have no competing financial or personal interests.

REFERENCES

1. Hohler T, Reuss E, Adams P, et al. A genetic basis for IFN-gamma production and T-bet expression in humans. J Immunol 2005;175(8):5457-62.

2. Lohoff M, Mak TW. Roles of interferon-regulatory factors in T-helper-cell differentiation. Nat Rev Immunol 2005;5(2):125-35.

3. Elser B, Lohoff M, Kock S, et al. IFN-gamma represses IL-4 expression via IRF-1 and IRF-2. Immunity 2002;17(6):703-12.

4. Lohoff M, Ferrick D, Mittrucker HW, et al. Interferon regulatory factor-1 is required for a T helper 1 immune response in vivo. Immunity 1997;6(6):681-9.

5. Xu J, Postma DS, Howard TD, et al. Major genes regulating total serum immunoglobulin E levels in families with asthma. Am J Hum Genet 2000;67(5):1163-73.

6. Weiland SK, von Mutius E, Hirsch T, et al. Prevalence of respiratory and atopic disorders among children in the East and West of Germany five years after unification. Eur Respir J 1999;14(4):862-70.

7. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16(3):1215.

8. Excoffier L, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. Mol Biol Evol 1995;12(5):921-7.

9. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21(2):263-5.

10. Schedel M, Carr D, Klopp N, et al. A signal transducer and activator of transcription 6 haplotype influences the regulation of serum IgE levels. J Allergy Clin Immunol 2004;114(5):1100-5.

11. Harada H, Takahashi E, Itoh S, Harada K, Hori TA, Taniguchi T. Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: implications for a gene network in the interferon system. Mol Cell Biol 1994;14(2):1500-9.

12. Nakao F, Ihara K, Kusuhara K, et al. Association of IFN-gamma and IFN regulatory factor 1 polymorphisms with childhood atopic asthma. J Allergy Clin Immunol 2001;107(3):499-504.

13. Noguchi E, Shibasaki M, Arinami T, et al. Mutation screening of interferon regulatory factor 1 gene (IRF-1) as a candidate gene for atopy/asthma. Clin Exp Allergy 2000;30(11):1562-7.

14. Stein RT, Martinez FD. Asthma phenotypes in childhood: lessons from an epidemiological approach. Paediatr Respir Rev 2004;5(2):155-61.

15. Vercelli D. Learning from discrepancies: CD14 polymorphisms, atopy and the endotoxin switch. Clin Exp Allergy 2003;33(2):153-5.

16. Saito H, Tada S, Ebinuma H, et al. Interferon regulatory factor 1 promoter polymorphism and response to type 1 interferon. J Cell Biochem Suppl 2001;Suppl 36:191-200.

17. Stutz AM, Woisetschlager M. Functional synergism of STAT6 with either NF-kappa B or PU.1 to mediate IL-4-induced activation of IgE germline gene transcription. J Immunol 1999;163(8):4383-91.

18. Mostecki J, Showalter BM, Rothman PB. Early growth response-1 regulates lipopolysaccharide-induced suppressor of cytokine signaling-1 transcription. J Biol Chem 2005;280(4):2596-605.

19. LeVan TD, Bloom JW, Bailey TJ, et al. A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of Sp protein binding and enhances transcriptional activity. J Immunol 2001;167(10):5838-44.

20. Rosenwasser LJ, Klemm DJ, Dresback JK, et al. Promoter polymorphisms in the chromosome 5 gene cluster in asthma and atopy. Clin Exp Allergy 1995;25 Suppl 2:74-8.

21. Kabesch M, Tzotcheva I, Carr D, et al. A complete screening of the IL4 gene: novel polymorphisms and their association with asthma and IgE in childhood. J Allergy Clin Immunol 2003;112(5):893-8.

22. Graves PE, Kabesch M, Halonen M, et al. A cluster of seven tightly linked polymorphisms in the IL-13 gene is associated with total serum IgE levels in three populations of white children. J Allergy Clin Immunol 2000;105(3):506-13.

23. Renard P, Ernest I, Houbion A, et al. Development of a sensitive multi-well colorimetric assay for active NFkappaB. Nucleic Acids Res 2001;29(4):E21.

24. Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res 1995;23(23):4878-84.

25. Grabe N. AliBaba2: context specific identification of transcription factor binding sites. In Silico Biol 2002;2(1):S1-15.

Table 1 **Description of *IRF-1* polymorphisms and their respective position within the gene, allele frequencies, rs numbers, linkage disequilibrium and genotyped tagging SNPs**

SNP	Position (to ATG)*	Position (to transcription start)*	Position in the gene structure	Minor allele frequencies†	rs number	LD (r^2) with tagging SNP	Tagging SNP	Tag SNPs (Block)
1	C-2655G	-1360	5'	0,29	rs2549004	1	40	(5)
2	C-2606T	-1310	CNS	0,38	-- §	0,96	13	(2)
3	-2485del	-1189	CNS	0,29	rs3840527	1	40	(5)
4	G-2021A	-726	5'	0,28	rs2549005	1	40	(5)
5	C-1895T	-600	Promoter	0,28	rs2549006	0,91	40	(5)
6	A-1710C	-415	Promoter	0,51‡	rs2706384	1	6	A-1710C (1)
7	G-1705A	-410	Promoter	0,53 ‡	rs2549007	0,94	6	(1)
8	C-1683T	-388	Promoter	0,21	rs2549008	1	8	C-1683T
9	G-1595A	-300	Promoter	0,54 ‡	rs2549009	0,89	6	(1)
10	C-1243G	53	5'UTR	0,35	rs11242115	0,82	30	(4)
11	C-1152T	144	5'UTR	0,38	rs10900809	0,96	30	(4)
12	C-1141T	156	5'UTR	0,29	rs960757	1	40	(5)
13	A-672C	624	Intron 1	0,39	rs2070721	1	13	A-672C (2)
14	T685G	1980	Intron 2	0,38	rs2070722	1	14	T685G
15	A1107T	2402	Intron 2	0,28	rs10213701	0,92	40	(5)
16	A1244G	2539	Intron 2	0,19	rs10035166	1	16	A1244G (3)
17	T1652C	2947	Intron 3	0,29	rs13170412	0,80	40	(5)
18	T1984C	3279	Intron 3	0,31	rs2070723	1	18	T1984C
19	A2947G	4242	Intron 6	0,29	rs9282761	1	40	(5)
20	A3038G	4333	Intron 6	0,29	rs9282763	1	40	(5)
21	T3098C	4393	Intron 6	0,3	rs2070724	1	40	(5)
22	A3116G	4411	Exon 7	0,3	rs9282762	1	40	(5)
23	A3305C	4600	Intron 7	0,3	rs10214312	1	40	(5)
24	G3383A	4678	Intron 7	0,28	rs2070725	0,93	40	(5)
25	G3433T	4728	Intron 7	0,3	rs2070726	1	40	(5)
26	3513del	4808	Intron 7	0,3	-- §	1	40	(5)
27	G3538T	4833	Intron 7	0,3	rs10068266	1	40	(5)
28	T3613C	4908	Intron 7	0,28	rs10053046	0,93	40	(5)
29	C3925T	5220	Intron 8	0,29	rs10068129	1	40	(5)
30	C4174T	5469	Intron 8	0,41	rs17622656	1	30	C4174T (4)
31	G4896T	6191	Intron 8	0,29	rs2070727	0,87	40	(5)
32	G5194A	6489	Intron 9	0,3	rs2070728	0,81	40	(5)
33	A5201G	6496	Intron 9	0,29	rs7701588	0,87	40	(5)
34	G5250T	6545	Intron 9	0,41	rs2070729	0,98	13	(2)
35	C5371T	6666	Intron 9	0,24	rs2070730	0,81	40	(5)
36	A5373G	6668	Intron 9	0,31	rs2070731	0,87	40	(5)
37	G6045A	7340	3'UTR	0,26	rs839	0,92	40	(5)
38	T8268G	9563	3'	0,25	rs10072700	0,98	16	(3)
39	A8455G	9750	3'	0,31	rs10065633	1	40	(5)
40	T8460G	9755	3'	0,31	rs10072571	1	40	T8460G (5)

* Based on NCBI sequence database, accession number NT034772 (Gene Bank) nucleotide 131.856.700-131.844.000. † MAF in the screening population (n=40); only polymorphisms with MAF > 0.1 are shown and were considered for further analyses. ‡ Minor allele frequencies differed between screening and genotyping populations and thus, MAF from the larger genotyping population (Dresden) was considered to define major and minor allele. § SNP not described in NCBI database (dbSNP) in progress.

Table 2 **Geometric means (and 95% confidence intervals) of total serum IgE (IU/ml) and odds ratios (and 95% confidence intervals) for associations with elevated specific serum IgE to inhalative allergens (measured by *SX1*, >0.35 IU/ml) and a positive skin prick test (SPT ≥3mm) comparing homozygote carriers of the wild type allele with carriers of at least one minor allele**

tested SNPs (tagged SNPs) *Block*		DRESDEN				MUNICH		
		total serum IgE geom. mean (CI) *	specific serum IgE (RAST ≥1) OR (CI)	Atopy SPT ≥3mm OR (CI)		total IgE geom. mean (CI) *	specific serum IgE (RAST ≥1) OR (CI)	Atopy SPT ≥3mm OR (CI)
A-1710C (G-1705A, G-1595A) *Block 1*	AA AC+CC p value	**66.2 (60.3 - 72.7)** **75.2 (69.0 - 82.0)** **p=0.050**	1.17 (0.97 - 1.41) p=0.105	1.17 (0.95 - 1.44) p=0.151	AA AC+CC p value	65.9 (57.7 - 75.2) 66.9 (58.9 - 76.1) p=0.871	**1.32 (1.02 - 1.71)** **p=0.037**	**1.46 (1.08 - 1.97)** **p=0.014**
C-1683T	CC CT+TT p value	69.4 (64.2 - 75.2) 74.4 (66.9 - 82.8) p=0.303	1.02 (0.84 - 1.24) p=0.848	1.02 (0.82 - 1.27) p=0.848				
A-672C (C-2606T, G5250T) *Block 2*	AA AC+CC p value	**63.4 (56.7 - 70.9)** **74.4 (68.9 - 80.4)** **p=0.020**	**1.25 (1.02 - 1.53)** **p=0.028**	1.21 (0.97 - 1.52) p=0.093	AA AC+CC p value	62.3 (53.4 - 72.7) 66.4 (59.1 - 74.6) p=0.530	**1.33 (1.01 - 1.75)** **p=0.041**	**1.41 (1.03 - 1.95)** **p=0.034**
T685G	TT TG+GG p value	66.4 (60.4 - 72.9) 74.1 (67.8 – 81.0) p=0.093	1.15 (0.95 - 1.39) p=0.152	1.13 (0.91 - 1.39) p=0.269				
A1244G (T8268G) *Block 3*	AA AG+GG p value	**65.9 (60.4 - 71.8)** **78.6 (71.7 - 86.3)** **p=0.006**	1.19 (0.99 - 1.44) p=0.062	1.18 (0.96 - 1.45) p=0.126	AA AG+GG p value	63.0 (55.7 - 71.2) 66.2 (57.8 - 75.7) p=0.593	**1.34 (1.04 - 1.73)** **p=0.022**	**1.40 (1.05 - 1.87)** **p=0.021**
T1984C	TT TC+CC p value	67.2 (61.2 - 73.9) 74.9 (68.7 - 81.7) p=0.095	1.17 (0.97 - 1.41) p=0.110	1.16 (0.94 - 1.43) p=0.176				
C4174T (C-1152T, A-1243G) *Block 4*	CC CT+TT p value	**76.7 (69.6 - 84.6)** **67.0 (61.6 - 72.8)** **p=0.038**	0.83 (0.69 - 1.01) p=0.061	**0.80 (0.65 - 0.98)** **p=0.034**	CC CT+TT p value	**74.2 (64.3 - 85.6)** **58.6 (52.0 - 66.0)** **p=0.013**	0.78 (0.60 - 1.01) p=0.059	**0.67 (0.50 - 0.89)** **p=0.007**
T8460G (25 SNPs – Table 1) *Block 5*	TT TG+ GG p value	66.8 (60.8 - 73.3) 74.7 (68.5 - 81.4) p=0.083	1.17 (0.97 - 1.41) p=0.099	1.15 (0.93 - 1.42) p=0.194				

* Considering that IgE levels are not normally distributed in the population, total serum IgE concentrations were log transformed. Differences between IgE levels were tested by t-test in a dominant model and values of log IgE were retransformed and presented as international units per ml

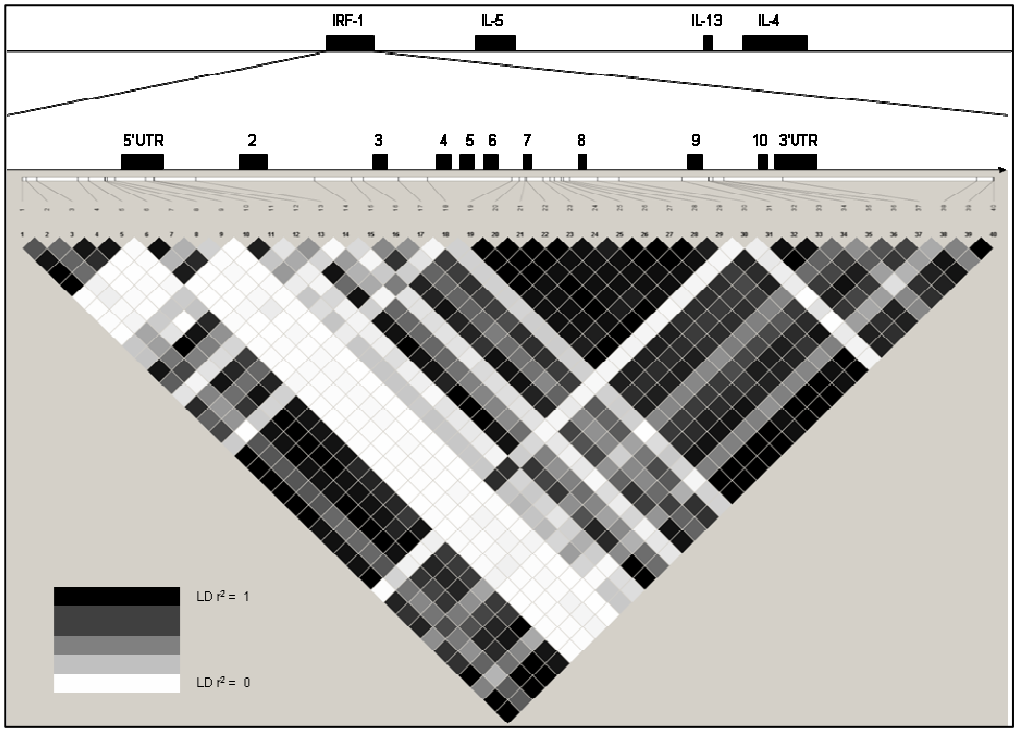
Table 3 **Odds ratios (and 95% confidence intervals) for haplotype associations between *IRF-1* haplotypes, 90th percentile of total serum IgE (>457 IU/ml), specific serum IgE (>0.35 IU/ml) and atopy measured by skin prick test (≥3mm)**

		total serum IgE (90th percentile)		specific serum IgE (RAST ≥1)		Atopy (SPT ≥3mm)	
DRESDEN	**Haplotype ***	**Freq% (controls vs cases)**	**OR (CI) † p value**	**Freq% (controls vs cases)**	**OR (CI) † p value**	**Freq% (controls vs cases)**	**OR (CI) † p value**
H_a	AAAT	35.81 31.42	0.82 (0.65 - 1.03) 0.094	36.52 33.48	0.87 (0.76 - 1.01) 0.066	36.20 33.15	0.87 (0.74 - 1.03) 0.099
H_b	**CCGC**	**25.19** **30.65**	**1.31 (1.04 - 1.66)** **0.023**	24.82 27.33	1.14 (0.98 - 1.33) 0.097	25.10 27.10	1.11 (0.93 - 1.32) 0.236
H_c	AAAC	22.33 23.68	1.08 (0.84 - 1.39) 0.554	22.82 21.87	0.95 (0.80 - 1.11) 0.510	22.74 22.27	0.97 (0.81 - 1.17) 0.771
H_d	ACAC	12.29 9.92	0.79 (0.55 - 1.12) 0.474	10.41 11.94	1.17 (0.94 - 1.45) 0.157	10.60 12.02	1.15 (0.91 - 1.46) 0.237
H_e	CCAC	5.37 4.30	0.79 (0.47 - 1.34) 0.381	5.22 5.35	1.03 (0.76 - 1.39) 0.871	5.18 5.42	1.05 (0.75 - 1.47) 0.777
MUNICH							
H_a	**AAAT**	34.99 33.79	0.95 (0.69 - 1.31) 0.751	**37.01** **32.26**	**0.81 (0.66 - 0.99)** **0.039**	**36.56** **31.05**	**0.78 (0.62 - 0.98)** **0.036**
H_b	**CCGC**	25.71 25.77	1.00 (0.71 - 1.43) 0.954	**23.61** **28.32**	**1.28 (1.03 - 1.59)** **0.025**	**24.02** **30.16**	**1.37 (1.01 - 1.74)** **0.011**
H_c	AAAC	21.30 20.99	0.98 (0.67 - 1.43) 0.927	21.24 21.12	0.99 (0.79 - 1.25) 0.957	21.38 20.27	0.93 (0.72 - 1.22) 0.620
H_d	ACAC	9.76 11.04	1.15 (0.70 - 1.88) 0.590	10.16 9.27	0.90 (0.65 - 1.25) 0.534	10.12 8.61	0.84 (0.57 - 1.22) 0.353
H_e	CCAC	6.79 7.09	1.05 (0.57 - 1.91) 0.869	6.66 7.20	1.09 (0.75 - 1.58) 0.662	6.60 7.73	1.19 (0.78 - 1.79) 0.421

* Haplotype frequencies were determined by EM algorithm based on all eight SNPs genotyped in the Dresden population (A-1710C, C-1683T, A-672C, T685G, A1244G, T1984C, C4174T, and T8460G). Five common haplotypes were identified using the EM algorithm but haplotype combinations and frequencies did not change significantly when the number of haplotype tagging SNPs was reduced to those four SNPs representing SNP blocks 1, 2, 3 and 4, genotyped in both populations

† Odds ratios were calculated comparing one haplotype vs. all others

Figure 1 (a)


IRF-1
IL-5
IL-13
IL-4
5'UTR
2
3
4
5
6
7
8
9
10
3'UTR
LD r² = 1
LD r² = 0

(b)

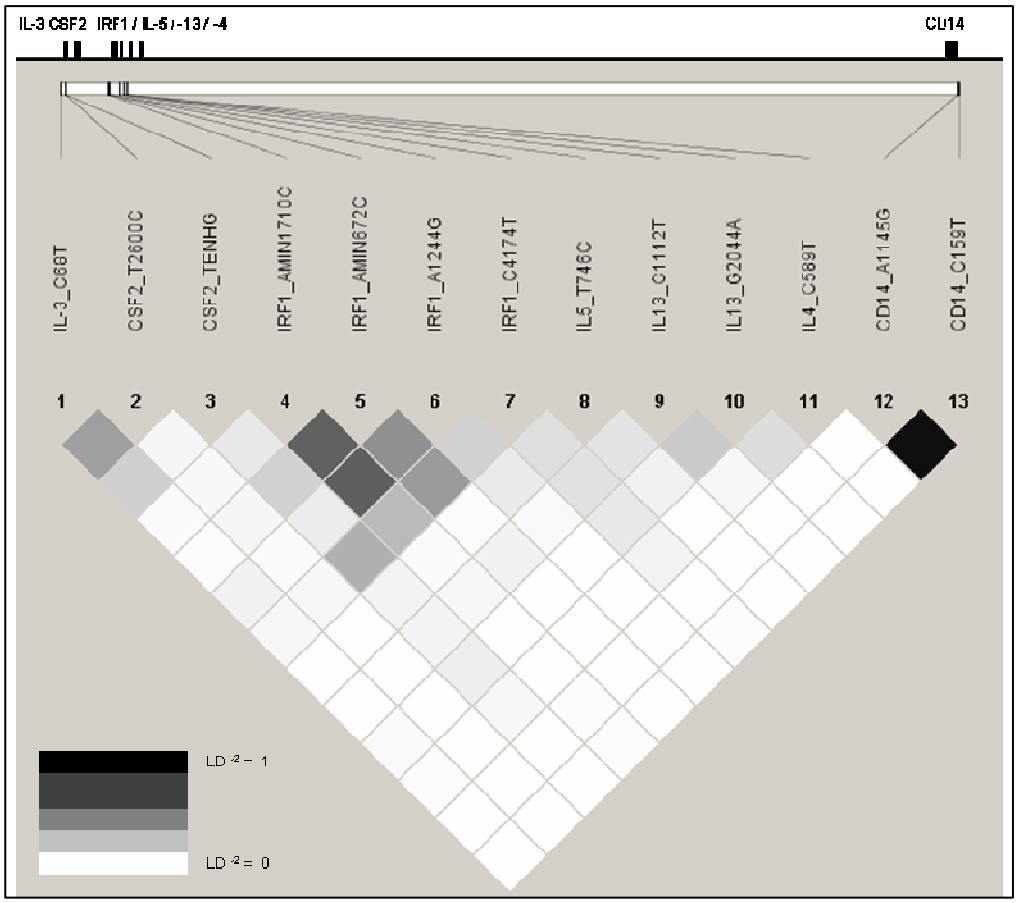

IL-3 CSF2
IRF1 / IL-5 / -13 / -4
CD14
IL-3_C68T
CSF2_T2600C
CSF2_TENHG
IRF1_AMIN1710C
IRF1_AMIN672C
IRF1_A1244G
IRF1_C4174T
IL5_T746C
IL13_C1112T
IL13_G2044A
IL4_C589T
CD14_A1145G
CD14_C159T
1
2
3
4
5
6
7
8
9
10
11
12
13
LD r² = 1
LD r² = 0

Figure 2 (a)


A-1710C
G-1705A
-442 GAGAGGGCGGCAAGTGCCCGGGCGATC[A/C]CCTC[G/A]CCTGCGTTCGGGAGATATACC[C/T]CCGCCCCCGCCCCGCCAGGAGGGTGAAAAGATGGCCCCAGGA
CTCTCCCGCCGTTCACGGGCCCGCTAG[T/G]GGAG[C/T]GGACGCAAGCCCTCTATATGG[G/A]GGCGGGGGCGGGGCGGTCCTCCCACTTTTCTACCGGGGTCCT
NF-κB
G-1595A
-342 GCCAGCCGGCTGGGACAAGGCGGAGTGAGAGGACAGGCTGGGGCC[G/A]GGGGCGCTGGGCTGTCCCGGGCAGCCCTCCTCCGGGCAAGCCGGAGCAGGGGTGGAT
CGGTCGGCCGACCCTGTTCCGCCTCACTCTCCTGTCCGACCCCGG[C/T]CCCCGCGACCCGACAGGGCCCGTCGGGAGGAGGCCCGTTCGGCCTCGTCCCCACCTA
EGR1
-242 TGGGAGCGCTCGGGGCGGGCCCGCGGTGGCCCCGGGGCGGTGGCGCCCGGCCGGAGAGGGTGGGGCGGAGCAGCCGCCCTGTACTTCCCCTTCGCCGCTA
ACCCTCGCGAGCCCCGCCCGGGCGCCACCGGGGCCCCGCCACCGCGGGCCGGCCTCTCCCACCCCGCCTCGTCGGCGGGACATGAAGGGGAAGCGGCGAT
-142 GCTCTACAACAGCCTGATTTCCCCGAAATGACGGCACGCAGCCGGCCAATGGGCGCCCGCGCGGCTGTCCGGGGGCGGGGCCGGCCAGGGCTGGGGAATC
CGAGATGTTGTCGGACTAAAGGGGCTTTACTGCCGTGCGTCGGCCGGTTACCCGCGGGCGCGCCGACAGGCCCCCGCCCCGGCCGGTCCCGACCCCTTAG
transcription start
C-1243G
-42 CCGCTAAGTGTTTGGATTGCTCGGTGGCGCCGCTGCCCTGGCAGAGCTCGCCACTCCTTAGTCGAGGCAAGACGTGCGCCCGAGCCCCGCCGAA[C/G]CGAGG
GGCGATTCACAAACCTAACGAGCCACCGCGGCGACGGGACCGTCTCGAGCGGTGAGGAATCAGCTCCGTTCTGCACGCGGGCTCGGGGCGGCTT[G/C]GCTCC
C-1152T
58 CCACCCGGAGCCGTGCCCAGTCCACGCCGGCCGTGCCCGGCGGCCTTAAGAACCCGGCAACCTCTGCCTTCTTC[C/T]CTCTTCCACTCGGAGTCGCGCTCCG
GGTGGGCCTCGGCACGGGTCAGGTGCGGCCGGCACGGGCCGCCGGAATTCTTGGGCCGTTGGAGACGGAAGAAG[G/A]GAGAAGGTGAGCCTCAGCGCGAGGC
SP1

Figure 2 (b, c, d)

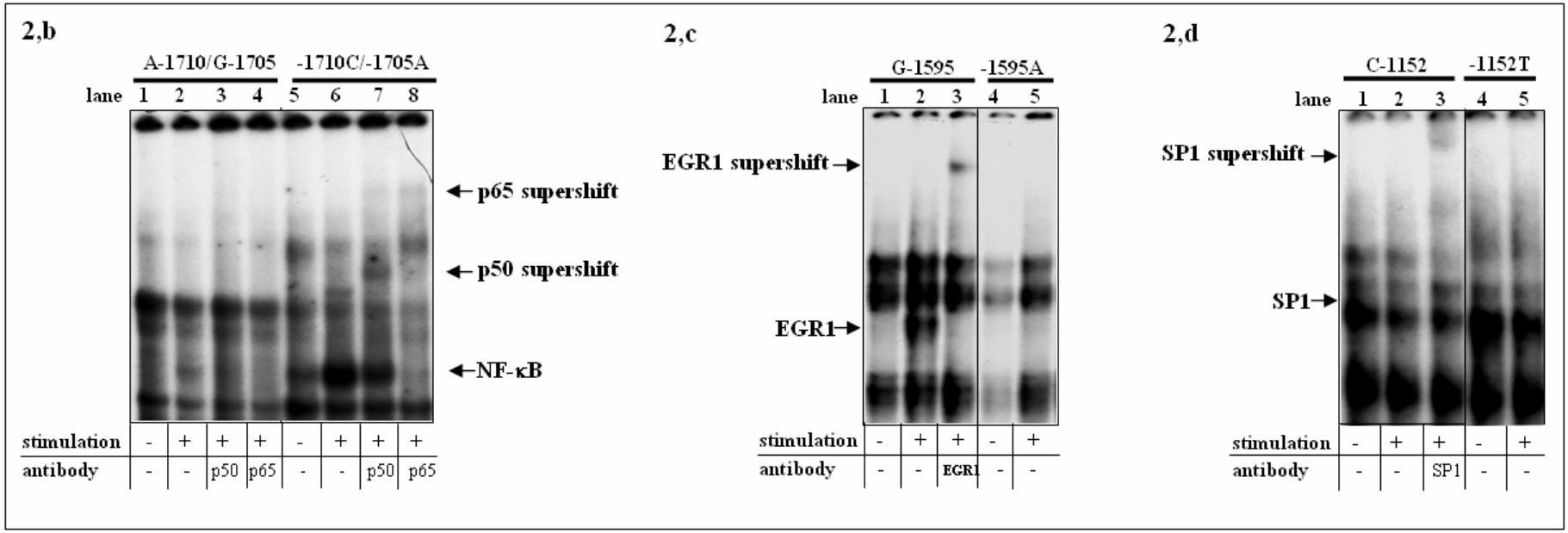

2,b
A-1710/G-1705
-1710C/-1705A
lane 1 2 3 4 5 6 7 8
p65 supershift
p50 supershift
NF-κB
stimulation - + + + - + + +
antibody - - p50 p65 - - p50 p65
2,c
G-1595
-1595A
lane 1 2 3 4 5
EGR1 supershift
EGR1
stimulation - + + - +
antibody - - EGR1 - -
2,d
C-1152
-1152T
lane 1 2 3 4 5
SP1 supershift
SP1
stimulation - + + - +
antibody - - SP1 - -

Figure 3

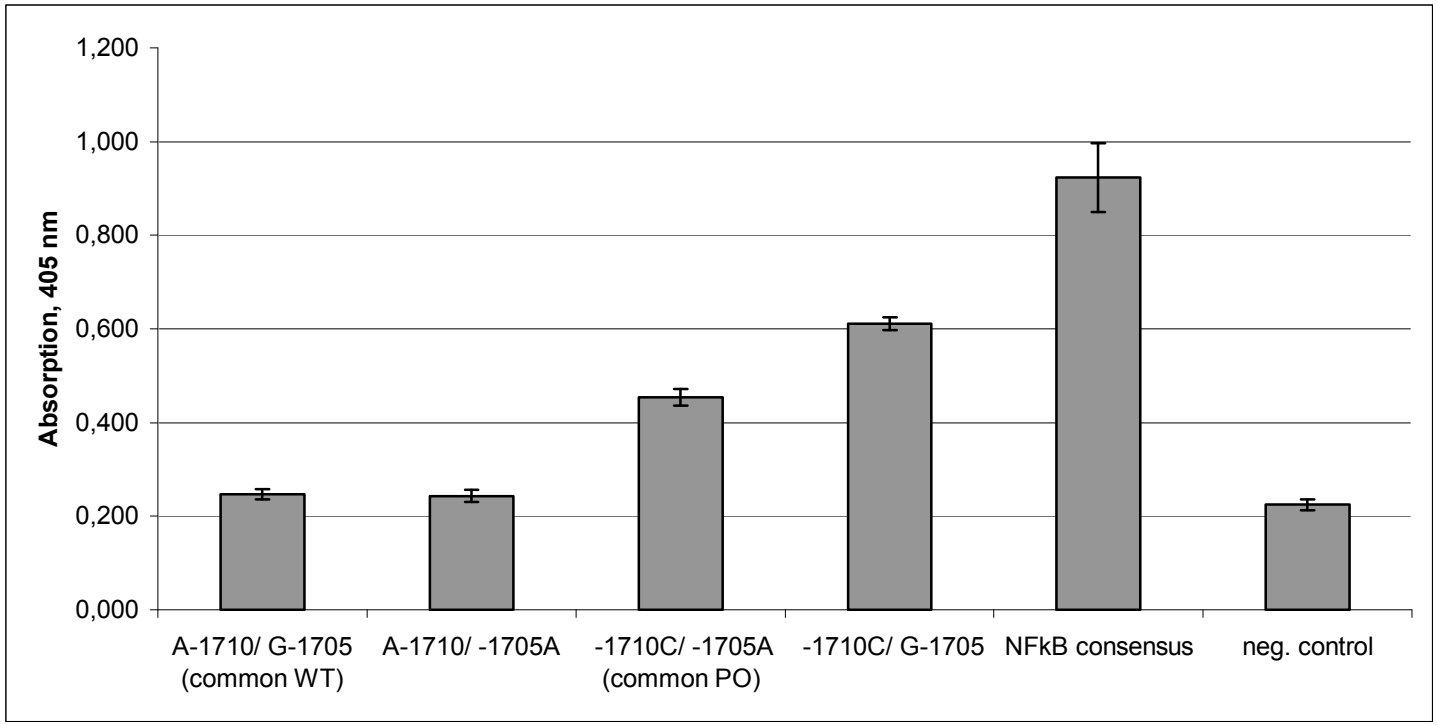

1,200
1,000
0,800
0,600
0,400
0,200
0,000
Absorption, 405 nm
A-1710/ G-1705 (common WT)
A-1710/ -1705A
-1710C/ -1705A (common PO)
-1710C/ G-1705
NFkB consensus
neg. control

FIGURE LEGENDS

Figure 1 (a and b)

Localisation of *IRF-1* on chromosome 5q31 and description of intragenetic (a) and intergenetic (b) linkage disequilibrium patterns (r^2 plots)

(a) Position of the *IRF-1* gene on chromosome 5q31 in relation to adjacent genes (upper panel), gene structure showing exons (middle panel) and position of frequent *IRF-1* SNPs (MAF > 0.1) and linkage disequilibrium (r^2 plot) in the re-sequenced population sample (n=40). **(b)** Linkage disequilibrium (r^2 plot) between *IRF-1* tagging SNPs representing blocks 1, 2, 3 and 4 and neighbouring SNPs in the genes coding for IL-3, CSF2, IL-5, IL-13, IL-4 and CD14. Colours in the LD Plot from Haploview: white ($r^2 = 0$), shades of grey ($0 < r^2 < 1$) and black ($r^2 = 1$)

Figure 2 (a, b, c, d)

Proximal promoter region of *IRF-1*, location of IgE/atopy associated promoter SNPs and genotype dependent changes in transcription factor binding revealed by EMSA

(a) IgE/atopy associated promoter SNPs are shown with their relative position to the ATG while also the transcription start site is depicted. Sequences used as EMSA probes are marked in bold letters. Transcription factors specific to a certain allele at a polymorphic site are shown and underlayed with grey as well as the respective allele the transcription factor binds to. Transcription factor binding was determined by EMSA using nuclear extracts from unstimulated or stimulated Jurkat T-cells (b, c, d). **(b)** For the site harbouring SNP A-1710C and A-1705G, EMSA probes either carrying a combination of wildtype alleles (A-1710 and G-1705) or both polymorphic alleles (-1710C and -1705A) were used, due to high LD of these two polymorphisms and their close vicinity. Comparing the patterns of DNA protein interaction between wildtype and polymorphic alleles, an extra complex was only detectable with the polymorphic probe (lane 3). By supershift experiments using NF-κB p50 (lane 5) or p65 (lane 6) antibodies, this complex was mainly identified as NF-κB p65. **(c)** Comparing G-1595 probe with -

1595A probe in stimulated nuclear extract, an extra complex was identified only in the presence of the G allele (lanes 2 and 5), which was identified as EGR1 by antibody supershift experiments (lane 3). **(d)** C-1152 probe was compared with a -1152T probe in unstimulated (lanes 1 and 4) or stimulated (lanes 2 and 5) nuclear extract. Hence, an extra complex was identified in the presence of the C allele, which was identified as SP1 by antibody supershift experiments (lane 3).

Figure 3

Colorimetric NF-κB p50 assay quantifies increased NF-κB binding in the presence of the polymorphic -1710C allele and identifies A-1710C as major influence on NF-κB binding.

Oligonucleotides representing common wildtype alleles (lane 1), and common polymorphic allele combination (lane 3) were tested for NF-κB (p50/p50) binding in comparison to rarely occurring combinations of SNP alleles at positions -1710 and -1705 (lanes 2 and 4), NF-κB consensus (lane 5) and non-sense probes (lane 6). The measured absorption (presented as means of five independent experiments +/- standard deviations) indicated the relative affinity of the respective sequence to NF-κB. As indicated, NF-κB binding occurs only in the presence of the C allele at position -1710 while the allelic state at position -1705 may only increase NF-κB binding in the presence of the -1710C allele.

CAPÍTULO 2

STAT1 GENE VARIATIONS, IGE REGULATION AND ATOPY

Artigo publicado na revista *Allergy* em 2007

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CAPÍTULO 2

STAT1 GENE VARIATIONS, IGE REGULATION AND ATOPY

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ABSTRACT

Background STAT1, an intracellular signal transducer and activator of transcription centrally involved in many inflammatory pathways, was recently suggested to play an important role in allergy related immune responses.

Aim Thus, we investigated the effect of polymorphisms in the *STAT1* gene on the development of atopic sensitisation and allergic diseases.

Methods Haplotype tagging SNPs previously described in the *STAT1* gene were genotyped by MALDI-TOF MS technology in a cross sectional study population of 3,099 German children recruited and phenotyped by the International Study of Asthma and Allergy in Childhood, phase II (ISAAC II). Effects of single SNPs and haplotypes were studied using SAS/Genetics and Haploview.

Results The polymorphism C39134A (rs3771300), located in a potentially *cis* acting regulatory element in STAT1 intron 24, was inversely related to atopy measured by skin prick test, total and specific serum IgE levels while no effect on atopic disease risk was observed.

Conclusion Our results indicate that *STAT1* SNP C39134A may protect from atopic sensitisation. Due to its location in a highly conserved non-coding sequence near a putative GATA binding site, this polymorphism represents an interesting target for further studies.

INTRODUCTION

Numerous mediators, such as cytokines, chemokines, and adhesion molecules contribute to the development of allergic inflammation. The expression of these very diverse mediators involved in allergic disease mechanisms is determined by a limited number of redundant intracellular transcription factors (TF). The STAT (signal transducer and activator of transcription) family represent a very prominent group of transcription factors regulating various genes important for cellular and humoral immunity (1). It has been known for some time that STAT4 and STAT6 are centrally involved in regulating T cell differentiation (2) and STAT6 is necessary for IgE switching in B-cells (3). Only recently, the role of other STAT molecules in allergy has been determined. Thus, it was shown that the blockade of STAT1 signalling inhibits the development of allergen-induced eosinophilic airway inflammation in a mouse model and decreases *in vivo* airway reactivity (4).

Previous studies indicated that genetic variations in *STAT4* and *STAT6* significantly affect IgE regulation and the development of atopy and asthma (3, 5). Thus, we investigate if polymorphisms in the *STAT1* gene may also influence atopic diseases and studied *STAT1* haplotype tagging SNPs in 3,099 German children.

METHODS

Population description

Between 1995 and 1996, a cross sectional study was performed in Munich and Dresden, Germany, as part of the International Study of Asthma and Allergies in Childhood (ISAAC phase II), to assess the prevalence of asthma and allergies in schoolchildren, age 9 to 11 years (n=5,629). All children of German origin with DNA available were included in this analysis (n=3,099). Informed written consent was obtained from parents and the study methods were approved by the respective ethics committees. The population and phenotyping methods have been described in detail before (6). Self-administered

questionnaires included the ISAAC core questions. Children whose parents reported a physician's diagnosis of asthma, spastic bronchitis or recurrent asthmatic bronchitis were classified as asthmatics. Children's classification of hay fever or atopic eczema was also based on parental report of a physician's diagnosis of the respective diseases. A child was considered atopic if a wheal reaction of ≥3 mm occurred in a skin prick test (Alk Scherax GmbH, Wedel, Germany) to one or more allergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria tenuis*, cat dander, mixed grass and tree pollen) after subtraction of the negative control. Total serum IgE levels were measured using the Imulite system (DPC Biermann GmbH, Bad Nauheim, Germany). Specific IgE against inhalative allergens (local grass pollen, birch pollen, mugwort pollen, *Dermatophagoides pteronyssinus*, cat dander, dog dander, *Cladosporium herbarum*) were measured in a range between 0.35-100 IU/ml, with specific IgE measurements considered positive when values were > 0.35 IU/ml.(*Sx1* from Phadia AB, Uppsala, Sweden).

Polymorphisms selection and genotyping

The *STAT1* gene was screened for mutations by the Innate Immunity Programs for Genomic Applications (https://innateimmunity.net/). Using *Haploview* software, eighteen haplotype tagging SNPs were selected for genotyping from all 28 *STAT1* polymorphisms with a minor allele frequency >0.10. In addition, two less frequent polymorphisms, however located in the coding region, were also genotyped were also genotyped. Genomic DNA was extracted from whole blood (7) and pre-amplified as described before (8). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom Inc., San Diego, USA) was used for genotpying. PCR assays and associated extension reactions were designed with the SpectroDESIGNER software (Sequenom Inc.). All amplification and extension reaction conditions have been previously described (8) and specific primers are given in table 1. Deviations from Hardy-Weinberg Equilibrium (HWE) were assessed for quality control of genotyping procedures.

Bioinformatics and statistical analysis

Association between polymorphisms and dichotomous outcomes were tested using chi-square tests in an allelic (9). Total serum IgE concentrations were log transformed and the differences between IgE levels were tested by t test. All tests were two-sided and the differences were considered significant with $p<0.05$. Calculations were carried out with the SAS software (version 9.1.3). Haplotype frequencies were estimated with an EM algorithm and associations of the common haplotypes with atopic sensitization were calculated with Haploview. Significant associations were replicated and validated in a second independent population (Munich and Dresden) to control for spurious associations. Transcription factor (TF) binding analyses and phylogenetic comparisons were performed using *Matinspector* (10) and the *VISTA* Genome Browser (11), respectively. Conserved non-coding sequences (CNS) were defined as a sequence of more than 100bp showing more than 70% homology with mouse sequence.

RESULTS

Initially, twenty haplotype tagging polymorphisms were genotyped in the ISAAC II study population from Dresden (n=1,940). All polymorphisms showed call rates of at least 93% and no significant deviation from Hardy-Weinberg equilibrium (table 1). No associations with asthma, hay fever, atopic dermatitis were found. However, the minor allele of single nucleotide polymorphism (SNP) G-306A and C39134A were inversely related to atopy measured by skin prick test and elevated total serum IgE levels (table 2). When these two polymorphisms were genotyped in the Munich population (n=1,159), SNP 39134A showed a protective effect against atopy (table 2). In the combined dataset, associations with SNP C39134A were inversely related to elevated total and specific serum IgE levels and atopy measured by SPT (table 2). Haplotype analyses did not contribute additional information to the single gene analysis (data not shown).

The gene variation C39134A, located in the last intron of *STAT1* (figure 1), was further analyzed for indications of putative role in STAT1 regulation. *In silico* bioinformatic tools indicated that SNP C39134A is located within a highly conserved non-coding sequence (figure 2) and transcription factor binding analysis predicted that the C39134A polymorphism is located three base pairs from the core sequence of a GATA motif.

DISCUSSION

STAT molecules represent a bottleneck in the signaling of many pathways contributing to immune responses as they carry the signal from specific ligand receptor interactions to the cell nucleus, activating transcription of numerous immune genes. While genetic variants in *STAT4* and *STAT6* had previously been identified and their effect on the genesis of atopic disease was investigated (3, 5), this study contributes with new knowledge to the field by systematically and extensively studying the role of *STAT1* polymorphisms in atopic diseases. In our large population sample, SNP C39134A showed a small but significant inverse relation with atopic sensitisation, which may be due to its location in a highly conserved *cis* regulatory element in intron 24, which was identified by *in silico* analyses. However, no significant effect of *STAT1* polymorphisms on atopic diseases was observed.

The region harbouring the *STAT1* gene on chromosome 2q32-q33 (figure 1), had previously been linked to elevated total serum IgE (12). In addition to *STAT1*, a number of potential allergy candidate genes map to the same region, such as *STAT4 (15Kb from STAT1), CD28 (12.6Mb)* and *CTLA4 (12.8Mb)*. However, association signals from neighbouring genes (*CTLA4* and STAT4) with total IgE levels (13) and specific sensitization to house dust mites (5) do not influence our finding. Using HapMap data, LD between C39134A and these SNPs in other candidate genes could also be excluded.

The location of polymorphism C39134A, which showed associations with atopic sensitisation in our study, is interesting. It is positioned at the boarder of a highly conserved non-coding sequence in intron 24, as indicated by inter-species sequence comparisons performed with the Vista Genome Browser (figure 2). These comparisons have proven to be a useful tool for identifying functionally important regions in the human genome, since these regions tend to stay conserved throughout evolution. Based on the location of the CNS element, a *cis* regulatory effect on *STAT1* is suggested. Within the CNS element, polymorphism C39134A is located three base pairs from the core sequence of a GATA motif (CAG**GATA**ATAC**ATAG**TAC[**C/A**]TGC), potentially capable to bind GATA-3, the master regulatory transcription factor for the differentiation and perpetuation of human Th2 cells.

However, it is not yet clear how polymorphism C39134A affect the role of *STAT1* in allergy as STAT1 may not only have pro-allergic effects (4) but is also involved in the IL-15 induced STAT1 binding to *IFN*-γ regulatory sites (14). Slight discrepancies in the effect of STAT1 polymorphism C39134A on total IgE regulation and specific sensitisation were observed between both study populations, which may be explained by modifications of the genetic effects by different environmental factors. Notably, children from Dresden spent their first 3 years of live in the former German Democratic Republic, exposed to very different life style factors (e.g. higher number of siblings, early day care, different outdoor pollutants, pet keeping) which were previously associated with the development of or the protection against atopy (6). Clearly, further studies are needed to better understand the role of STAT1 in these mechanisms.

In conclusion, our results suggested that the minor allele of SNP C39134A in the *STAT1* gene is inversely related to total IgE levels and atopic sensitization in German children. We suggest that this association may be due to C39134A altering a *cis* regulatory element in *STAT1*, making C39134A an interesting polymorphism for further genetic and functional studies.

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Leonardo Araujo Pinto performed genotyping, data analysis and drafted the first version of the manuscript; Lena Steudemann, Norman Klopp and Thomas Illig participated in genotyping; Martin Depner contributed to data analysis; Stephan Weiland and Erika von Mutius contributed to the collection of data and manuscript preparation; Michael Kabesch supervised all experiments, participated in the development of the study design, collection of data, data analysis and wrote the final version of the manuscript. All authors declare that they have no competing financial or personal interests.

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TABLES

Table 1

***STAT1* polymorphisms and their respective position within the gene, rs numbers, primers used for genotyping, minor allele frequency (MAF), successful genotyping call rate (Call R in %) and the p value for deviation from Hardy-Weinberg Equilibrium (pHWE).**

[1)] Based on the NCBI GenBank sequence (accession number NM_139266), chr2:191665770-191704442 [2)] The tagging SNPs representing a SNP block are marked in bold letters [3)] Infrequent SNPs located in coding sequences [4)] N/A: not available in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/)

TABLES

Table 1

STAT1 **Variations** [1]	***Location***	***rs number***	1st PCR Primer 2nd PCR Primer Extension Primer	**MAF**	**Call R%**	**pHWE**
1_T-5741C	Promotor	1467198	ACGTTGGATGCCCATGGATATGCCTATATG ACGTTGGATGTGTGAGGGTGCATTTGTGAC TGTGCAAAGAAAAAAAACTCA	0.31	97.99	p=0.190
2_G-4831A [2] **(G-5773C / G-3925C / AG-642del)**	Promotor	10195683	ACGTTGGATGAGAGGTGTGGACGGGATAAG ACGTTGGATGTTCTCCTAAACGCTGTGCTG AACGCTGTGCTGTGATGACCT	0.20	97.99	p=0.233
3_T-4410C	Promotor	N/A [4]	ACGTTGGATGTTCTGCAAACACAGCACGTC ACGTTGGATGTGTTGCCACGCTGGGAACTG TGGGAACTGGCGTTCTGTTTAC	0.13	96.86	p=0.912
4_A-3758G	Intron1	N/A [4]	ACGTTGGATGAGTGACGGTAAATGGGAAGG ACGTTGGATGATTGTCCTTGCCAGTCGTGC ccACTTGTGAAATATAATTTTCCTCT	0.10	93.45	p=0.630
5_G-306A	Intron 2	N/A [4]	ACGTTGGATGAGGTCAGATGGTGGTGTAAG ACGTTGGATGTTAAACTGCAACCACCTCCC TCCCACCCTGGAAACATG	0.12	98.20	p=0.774
6_T63C [3]	Exon 3	2066802	ACGTTGGATGTCAGTGGTACGAACTTCAGC ACGTTGGATGGCCAGGTACTGTCTGATTTC ATGGGAAAACTGTCATCATA	0.06	98.50	p=0.094
7_del569ATT [2] **(G-4688A / C-3387del)**	Intron 3	N/A [4]	ACGTTGGATGTAACTGATAGGTGTTCTAC ACGTTGGATGGGGACCCTTCACTTTCTATG GGACGTTTTTAAATTAGCAAATAA	0.42	98.86	p=0.313
8_T1177A	Intron 4	10199181	ACGTTGGATGCTTATGCTATATTTACTGATGC ACGTTGGATGATTGAGCACACACTTATTGG AATAGAGACTAACTCCATTTATGTT	0.32	97.53	p=0.766
9_G19209A	Intron 11	7562024	ACGTTGGATGCTAGGCCATTTACTGTGGTG ACGTTGGATGCTGATGGATGGATTTATAGC TATAGCTTTCATCAGTTTCTCA	0.36	98.20	p=0.628
10_T19856C	Intron 11	11887698	ACGTTGGATGAGCGTCCCATCTATAGTCAC ACGTTGGATGCTTCTCAAAGTCTCCAGCTC ggGCCCTCCATCTTTCCA	0.13	97.01	p=0.486
11_T23964G	Intron 14	2280232	ACGTTGGATGTTTCCCTCAGCAGAGTCCTC ACGTTGGATGTGACAGCTCAGAGATTGACG GTCCACTTGAGGGCAG	0.24	98.40	p=0.090
12_G24164A	Intron 14	2280233	ACGTTGGATGTGATTCCAAGAGATCCCTCC ACGTTGGATGACAAATTGGCCCTCGTTCAG GAGCAGAAGCACGTTTCC	0.48	98.76	p=0.693
13_C24631T	Intron 15	2280234	ACGTTGGATGGACACTCATGGACAACTCAG ACGTTGGATGTTCACCCGTAAGATGCGAAG AAGAATACAAAAGCCCACA	0.35	98.71	p=0.624
14_del28944G	Intron 18	5837215	ACGTTGGATGAAGGCAAAGTCAGCTGATGG ACGTTGGATGCTCCTCTAAAGAGAGGACAG GGA CAG TCC TTT TAA TGC C	0.35	98.45	p=0.756
15_G29005C	Intron 18	1547550	ACGTTGGATGAAGGACTGTCCTCTCTTTAG ACGTTGGATGAGGGAAAGCGGAAACATCAC AAACATCACAAAGCCTTACCATCTGCT	0.32	98.61	p=0.527
16_G34754A [2] **(T30900C / T32851C / C32971T)**	Intron 23	1914408	ACGTTGGATGTCTCTGGTGCTTTTCTGTCC ACGTTGGATGTA AGCCTAGGTGTGAGCATG TCACTACCCTGAGATGACAATGCCT	0.20	97.84	p=0.941
17_C35266T [2] **(T28593G / C31285T)**	Intron 24	2066793	ACGTTGGATGTGAGCACTGCACTCTCCTTG ACGTTGGATGTAAGTGTGTGTGCTCAGAGG ATCCCGGGCCTGTCTG	0.12	98.14	p=0.763
18_G38812A	Intron 24	7575823	ACGTTGGATGTAAGTTGTCCTGGTCAAGGC ACGTTGGATGGCAGAATTACTAAGAACCCC GCAGAATTACTAAGAACCCCTTCACC	0.11	99.02	p=0.768
19_C39134A	Intron 24	3771300	ACGTTGGATGAAGGTCTTGATTAGCCCAGG ACGTTGGATGATACCGTGGAATAAGAGGGC TGGAATAAGAGGGCCTTCAGCA	0.46	97.94	p=0.587
20_T39455C [3]	3'UTR	N/A [4]	ACGTTGGATGCAGTAAGATGCATGATGCCC ACGTTGGATGAGCAAATTCGCTGCAACCTG TGTTGATAGCAAGTGAATTTTTC	0.03	98.55	p=0.735

Table 2

Geometric means (IU/ml), log means, standard error and p values for total serum IgE. Odds ratios (and 95% confidence intervals) for associations with specific IgE and atopy (SPT ≥3mm) comparing major and minor alleles for all polymorphisms genotyped in Dresden (significant associations in bold)

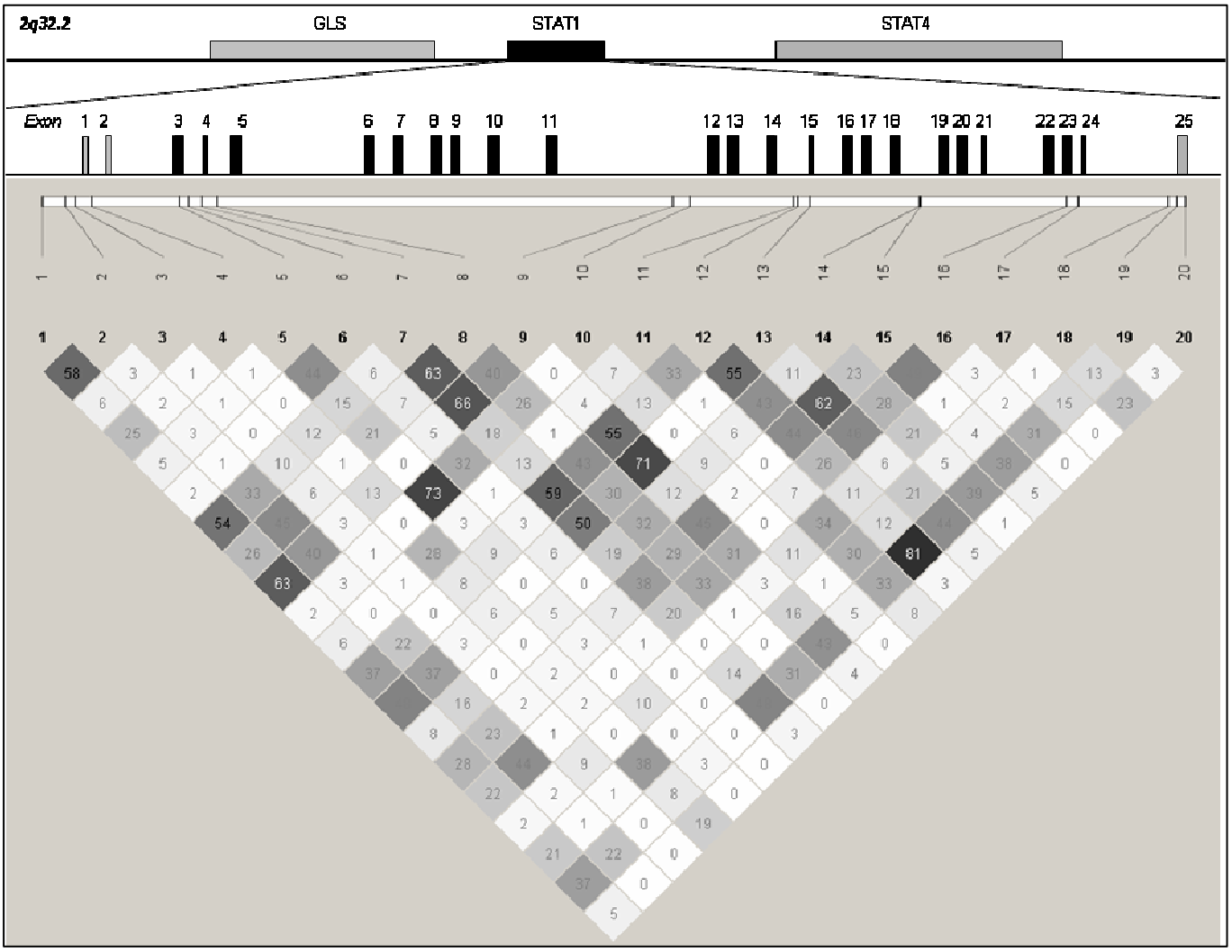
STAT1	**total serum IgE levels** [1]			**specific serum IgE** [2]	**atopy measured by SPT**
polymorphisms	**major Allele** geometric mean mean log IgE±SEM	**minor Allele** geometric mean mean log IgE±SEM	**p value** t test	**OR (CI)** p value	**OR (CI)** p value
	DRESDEN, N = 1,940			**n = 1,217 / 721**	**n = 1,366 / 493**
G-306A	72.24 4.28±0.024	66.69 4.20±0.066	0.295	0.93 (0.76 - 1.14) 0.494	**0.76 (0.59 - 0.96)** **0.022**
C39134A	**73.70** **4.30±0.031**	**67.35** **4.21±0.033**	**0.039**	0.88 (0.77 - 1.01) 0.066	0.93 (0.80 - 1.08) 0.335
	MUNICH, N = 1,159			**n = 696 / 463**	**n = 859 / 284**
G-306A	64.07 4.16±0.033	73.70 4.30±0.092	0.137	1.16 (0.90 - 1.49) 0.264	1.01 (0.75 - 1.35) 0.948
C39134A	65.37 4.18±0.045	63.43 4.15±0.044	0.618	0.87 (0.73 - 1.03) 0.101	**0.80 (0.65 – 0.97)** **0.022**
	POOLED DATA, N = 3,099			**n = 1,913 / 1,184**	**n = 2,225 / 777**
G-306A	68.72 4.23±0.020	68.72 4.23±0.054	0.940	1.01 (0.87 - 1.19) 0.855	0.85 (0.70 -1.02) 0.075
C39134A	**70.28** **4.26±0.026**	**66.02** **4.19±0.026**	**0.043**	**0.88 (0.79 - 0.98)** **0.017**	**0.88 (0.78 - 0.99)** **0.027**

[1)] An allelic model has been used for all comparisons. IgE levels represent the geometric means for the major allele vs. the minor allele. Considering that IgE levels are not normally distributed in the population, total serum IgE concentrations were log transformed. Differences between geometric means of IgE levels were tested by t-test in an allelic model comparing polymorphic alleles to the wild type alleles and values of log IgE were retransformed and presented as international units per ml.

[2)] Specific serum IgE against inhalative allergens (local grass pollen, birch pollen, mugwort pollen, *Dermatophagoides pteronyssinus*, cat dander, dog dander, *Cladosporium herbarum*)

FIGURES

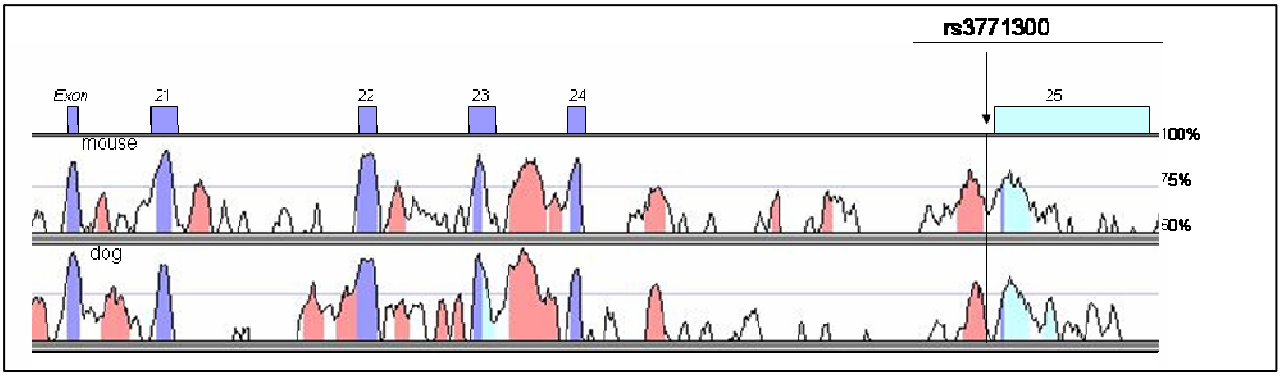
Figure 1



Position of *STAT1* on chromosome 2q32 and description of genetic linkage disequilibrium pattern (r^2 plots).

Position of the *STAT1* gene on chromosome 2q32 in relation to adjacent genes (upper panel), gene structure showing exons (middle panel) and LD (r^2) between genotyped *STAT1* polymorphisms.

Figure 2



Position of C39134A in the *STAT1* gene and the degree of conservation in this region.

VISTA plot displaying evolutionarily conserved sequences identified by comparison of *STAT1* human sequence and *STAT1* sequences from mouse and dog. The height of the peaks on the vertical axis indicates the level of conservation in percent identity. Conserved sequences (defined as regions >100bp and showing > 70% conservation) in coding exons (dark blue), UTR (light blue) and non-coding (red) are shown. Five conserved non-coding sequences (CNS) with both mouse and dog were identified in this region. SNP C39134A is located in an intronic CNS.

REFERENCES

1. Durbin JE, Hackenmiller R, Simon MC, Levy DE: Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell 1996, 84(3):443-450.
2. Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, Nakanishi K, Yoshida N, Kishimoto T, Akira S: Essential role of Stat6 in IL-4 signalling. Nature 1996, 380(6575):627-630.
3. Schedel M, Carr D, Klopp N, Woitsch B, Illig T, Stachel D, Schmid I, Fritzsch C, Weiland SK, von Mutius E et al: A signal transducer and activator of transcription 6 haplotype influences the regulation of serum IgE levels. J Allergy Clin Immunol 2004, 114(5):1100-1105.
4. Quarcoo D, Weixler S, Groneberg D, Joachim R, Ahrens B, Wagner AH, Hecker M, Hamelmann E: Inhibition of signal transducer and activator of transcription 1 attenuates allergen-induced airway inflammation and hyperreactivity. J Allergy Clin Immunol 2004, 114(2):288-295.
5. Park BL, Cheong HS, Kim LH, Choi YH, Namgoong S, Park HS, Hong SJ, Choi BW, Lee JH, Park CS et al: Association analysis of signal transducer and activator of transcription 4 (STAT4) polymorphisms with asthma. J Hum Genet 2005, 50(3):133-138.
6. Weiland SK, von Mutius E, Hirsch T, Duhme H, Fritzsch C, Werner B, Husing A, Stender M, Renz H, Leupold W et al: Prevalence of respiratory and atopic disorders among children in the East and West of Germany five years after unification. Eur Respir J 1999, 14(4):862-870.
7. Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988, 16(3):1215.
8. Kormann MS, Carr D, Klopp N, Illig T, Leupold W, Fritzsch C, Weiland SK, von Mutius E, Kabesch M: G-Protein-coupled receptor polymorphisms are associated with asthma in a large German population. Am J Respir Crit Care Med 2005, 171(12):1358-1362.

9. Sasieni PD: From genotypes to genes: doubling the sample size. Biometrics 1997, 53(4):1253-1261.

10. Quandt K, Frech K, Karas H, Wingender E, Werner T: MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res 1995, 23(23):4878-4884.

11. Frazer KA, Pachter L, Poliakov A, Rubin EM, Dubchak I: VISTA: computational tools for comparative genomics. Nucleic Acids Res 2004, 32(Web Server issue):W273-279.

12. Koppelman GH, Stine OC, Xu J, Howard TD, Zheng SL, Kauffman HF, Bleecker ER, Meyers DA, Postma DS: Genome-wide search for atopy susceptibility genes in Dutch families with asthma. J Allergy Clin Immunol 2002, 109(3):498-506.

13. Howard TD, Postma DS, Hawkins GA, Koppelman GH, Zheng SL, Wysong AK, Xu J, Meyers DA, Bleecker ER: Fine mapping of an IgE-controlling gene on chromosome 2q: Analysis of CTLA4 and CD28. J Allergy Clin Immunol 2002, 110(5):743-751.

14. Strengell M, Matikainen S, Siren J, Lehtonen A, Foster D, Julkunen I, Sareneva T: IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells. J Immunol 2003, 170(11):5464-5469.

CAPÍTULO 3

MMP-9 GENE VARIANTS INCREASE THE RISK FOR NON-ATOPIC ASTHMA IN CHILDREN

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CAPÍTULO 3

MMP-9 GENE VARIANTS INCREASE THE RISK FOR NON-ATOPIC ASTHMA IN CHILDREN

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ABSTRACT

As matrix metalloproteinase 9 (MMP-9) plays an important role in airway wall thickening and airway remodelling, it may also influence the development of obstructive airway disease in children. In the present study we investigated whether genetic variations in *MMP-9* influence the development of different forms of childhood asthma.

Genotyping of four HapMap derived tagging SNPs in the *MMP-9* gene was performed using MALDI-TOF MS in three cross sectional study populations of German children (age 9-11; N=4,264) phenotyped for asthma and atopic diseases according to ISAAC standard procedures.

SNP rs2664538 significantly increased the risk for non-atopic wheezing (OR 2.12, 95%CI 1.40-3.21, p<0.001) and non-atopic asthma (OR 1.66, 95%CI 1.12-2.46, p=0.011). Furthermore, the minor allele of rs3918241 may be associated with decreased expiratory flow measurements.

Our results suggest that homozygocity for *MMP-9* variants increase the risk to develop non-atopic forms of asthma and wheezing, which may be explained by a functional role of MMP-9 in airway remodelling.

Abstract word count: 155

Key words: Asthma, children, genetics, matrix metalloproteinase, polymorphism, wheezing

INTRODUCTION

Wheezing affects app. 50% of children up to the age of six, and many but not all of these children develop persistent wheezing or asthma later in life (1). Diverse wheezing phenotypes can be identified in hindsight based on differences in natural histories, trigger and risk factors (2, 3). Atopic and non-atopic wheezing can easily be discriminated in children by the presence or absence of sensitisation to allergens. Atopic and non-atopic wheezing show contrasting natural histories and may be caused by different aetiologies: while eosinophils are most prevalent in bronchoalveolar lavage (BAL) of older atopic asthmatic wheezers (4), neutrophils are predominantly found in BAL samples of young children with severe wheezing (5).

When severe airway inflammation with the involvement of neutrophilic inflammation is ongoing (6, 7), repair processes may contribute significantly to airway remodelling and irreversibility of lung injury. For repair and remodelling, matrix metalloproteinases (MMPs), a family of proteases that degrade components of the extracellular matrix, seem to be of central importance. Accordingly, matrix metalloproteinase 9 (MMP-9) acts as a pro-inflammatory molecule perpetuating immune responses (8) but it is also involved in repair processes after tissue injury and may down regulate remodelling during inflammatory reactions. As a consequence of its ambivalent nature, MMP-9 levels in sputum have been directly related to airway inflammation and inversely associated with airway thickening at the same time (7).

Based on the relevance of MMP-9 in both inflammation and airway remodelling (7, 9-13), it was hypothesized that genetic variations in the MMP-9 gene could be involved in different forms of wheezing and asthma. A number of polymorphisms have previously been described for the *MMP-9* gene, including variations in the promoter (rs3918241=A-1831T) and coding region (rs2664538=A2659G) resulting in decreased MMP-9 expression and activity (14). Thus, the

association between four haplotype tagging SNPs capturing all essential genetic information of the *MMP-9* gene locus (rs3918241, rs2664538, rs3918256 and rs3787268) and wheezing phenotypes was studied in three cross sectional population samples of German children.

METHODS

Population description

Between 1995 and 1996, a cross sectional study was performed in Munich and Dresden as part of the International Study of Asthma and Allergy in Childhood phase II (ISAAC II) to assess the prevalence of asthma and allergies in 5,629 schoolchildren, age 9 to 11 years. All children of German origin with DNA available were included in this analysis (N =3,099 / n= 1,159 in Munich and n=1,940 in Dresden). Additionally, 1,165 fourth graders from Leipzig, phenotyped with a very similar protocol were also analyzed. As described in detail before (15), self-administered questionnaires included the ISAAC core questions on symptoms and diagnoses of asthma, hay fever, and atopic eczema. Informed written consent was obtained from all parents of children included in these studies and all study methods were approved by the local ethics committees.

Children whose parents reported a physician's diagnosis of asthma once, and recurrent, spastic or asthmatic bronchitis more than once were classified as having asthma. Children were categorized as having current wheezing if the parents had reported wheeze in the past 12 months in Munich and Dresden or persistent wheezing in combination with a confirmative answer to the question "had your child ever had wheezing" in Leipzig.

The sensitivity to six common aeroallergens (Dresden and Munich: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria tenuis*, cat dander, and mixed grass and tree pollen; Leipzig: *Dermatophagoides pteronyssinus*, grass, birch and hazel pollen, cat and dog cander)

was assessed by skin prick test (SPT). A child was considered atopic if a wheal reaction ≥3 mm occurred to one or more allergens after subtraction of the negative control.

Atopic asthma was defined as asthma and the concomitant presence of a positive SPT, while non-atopic asthma was defined as asthma in the absence of a positive skin prick test. As controls for atopic or non-atopic asthma children without asthma and without atopy were used. Atopic/non-atopic wheeze was defined accordingly (current wheeze and/without concomitant presence of a positive SPT). Total serum IgE levels were measured using the Imulite System (DPC Biermann, Germany). Specific IgE antibodies (*Sx1* from Phadia, Germany) against inhalative allergens (local grass pollen, birch pollen, mugwort pollen, *dermatophagoides pteronyssinus*, cat dander, dog dander, *cladosporium herbarum*) were measured in a range between 0.35-100 IU/ml.

In 9-11 yr old children from Munich and Dresden, lung function was measured by MasterScope Version 4.1 (Jäger, Würzburg, Germany). A minimum of two baseline spirograms was performed and the highest of two reproducible measurements of forced expiratory volume in one second (FEV1) was recorded as baseline FEV1 (16). Reproducible measurements of maximum expiratory flows (MEF) at 25, 50 and 75% of vital capacity, MMEF (maximum mid-expiratory flow: the average expiratory flow over the middle half of the forced vital capacity, FVC) were determined.

SNPs selection and genotyping

The *MMP-9 gene* had previously been screened for polymorphisms (17). Based on all 11 SNPs genotyped by HapMap (www.hapmap.org) (18) with a minor allele frequency (MAF) >0.10 linkage disequilibrium was assessed using the software Haploview (19) and four haplotype tagging SNP capturing the genetic information of all common SNPs at the locus were selected for genotyping. Of note, reference SNP id rs2664538 was recently changed into rs17576, dbSNP but for better comparability with previous publications we continued to refer to rs2664538 in the text.

Genomic DNA was extracted from whole blood by a standard salting out method (20) and pre-amplified as described before (21). DNA samples were genotyped using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom Inc., San Diego, California). Additional details on genotyping are available in the online supplement.

Statistical Analysis

SNPs were tested for deviation from Hardy-Weinberg equilibrium (HWE) using chi-square tests, with expected frequencies derived from allele frequencies. Association between SNPs and dichotomous outcomes were tested using chi-square in a recessive model. Additionally odds ratio and 95% confidence intervals are given.

Lung function parameters were calculated as percentage of reference values. To test for differences in lung function parameters between genotypes t-tests in a recessive model were used. All tests were two-sided and the differences were considered significant with $p<0.05$. To correct for multiple testing we used a Bonferroni correction limited to the groups of tests for one phenotype in a population. Calculations were carried out with the SAS software (version 9.1.3). Haplotype frequencies were estimated with EM algorithm and common haplotypes (frequency >0.03) were analyzed with Haploview.

RESULTS

Using HapMap data, all 11 SNPs in the *MMP-9* gene could be allocated to four blocks of correlated SNPs showing high levels of linkage disequilibrium ($r^2 > 0.8$) with each other (figure 1) as described in the methods section. One tagging SNP per LD block was selected for genotyping in a cross sectional study population of German children from Munich, Dresden and Leipzig (n=4,264).

Genotyping success rates (call rates) ranged from 91.7% to 96.0% and no significant deviation from Hardy-Weinberg equilibrium was observed (table E1).

First, associations of the four tagging SNPs with main phenotypes asthma and current wheezing were assessed (table 1). SNP rs2664538 showed a trend for an increased risk to develop current wheezing. When analyses were performed for atopic and non-atopic wheezing, association effects were identified especially for non-atopic wheeze. In children homozygous for the polymorphic allele at rs2664538, odds ratios (OR) for non-atopic wheezing was 2.12 (95%CI 1.40-3.21, $p<0.001$). Additionally, SNP rs2664538 showed a significant association with non-atopic asthma (OR 1.66, 95%CI 1.12-2.46, $p=0.011$). SNPs rs3918241, rs 3918256 and rs3787268 showed also an increased risk for the carriers of the homozygous genotype in non-atopic wheeze or non-atopic asthma. When specific IgE of more than 0.35 IU/ml was used to define atopy instead of prick test results, very similar results were observed. In homozygous for the polymorphic allele at rs2664538, odds ratios (OR) for non-atopic wheezing would be 1.92 (95%CI 1.20-3.07, $p=0.006$).

When the same 4 SNPs were analyzed separately in the Dresden, Munich or Leipzig population, associations were stronger in Dresden and the same trends for non-atopic asthma were also replicated in the other two populations for SNP rs2664538 (table E2). In contrast, a significant protective effect with atopic wheeze was observed for the minor allele of rs3787268 (table 1).

Also, lung function parameters had been recorded in a random sample of approximately half of the study population from Munich and Dresden and thus, the effects of *MMP-9* SNPs on lung function at age 9-11 could be analyzed (table 2).

In non-atopic children, homozygote polymorphic individuals for SNP rs3918241 showed a trend for lower expiratory flows (MMEF% and MEF 25%). However, these effects do not remain significant after correction for multiple testing, and no effect was observed for other lung function measurements such as FEV1, FVC or BHR (data not shown).

SNPs rs2664538, rs3918256 and rs3787268 did not influence lung function parameters significantly. No significant effects on the development of atopy or total serum IgE levels were observed. Haplotype analyses did not contribute additional information to the analysis not observed in the single gene analysis and thus, data are not shown here.

DISCUSSION

Our systematic study of *MMP-9* variations suggests that polymorphisms in the *MMP-9* gene have significant effects on non-atopic forms of asthma. Moreover, promoter SNP rs3918241 may be associated with lower lung function parameters. The very specific effects of genetic variations in the *MMP-9* gene may help to dissect different forms of asthma and elucidate the diversity in the mechanisms leading to airway diseases such as non-atopic asthma and other common, non-atopic forms of childhood wheezing.

Based on the location of promoter SNP rs3918241, a *cis* regulatory effect on *MMP-9* may be suggested. Polymorphism rs3918241 is located exactly in the core sequence of a GATA motif (GTAAAGGAA***G(T/A)TA***ATTATCTC), which may be capable to bind GATA factors, master regulatory transcription factors for the differentiation and perpetuation of human Th2 cells. The variation rs2664538 (Q279R) is located in the MMP-9 fibronectin II domain (figure 2), which presumably enhances the binding of MMP-9 to its substrate, the extra-cellular matrix (ECM) (22). Using FASTSNP sofware (23) to predict functional effects of the amino acid change from glutamine

to arginine induced by rs2664538, this gene variation seems highly likely to change protein structure and exonic splicing.

Previous *in vivo* studies had also shown that rs2664538 is associated with lower MMP-9 levels (14) and decreased substrate binding (22). Consistent with our findings, lower MMP-9 levels in sputum have also been associated with airway wall thickening (7) and airflow obstruction. MMP-9 down regulation leads to airway wall thickening, presumably by insufficient degradation and clearance of extra-cellular matrix. In the airways of adult patients with asthma, ECM deposition is not only present in the basal membrane but also around smooth muscle cells and in the adventitial layer, which may gather volume and contribute significantly to the airway wall thickening (24). By decreasing MMP-9 activity, rs2664538 may directly affect these mechanisms by a decrease in ECM clearance and the subsequent increase in airway wall thickening.

While the evidence for a direct biological effect of SNP rs2664538 is rather convincing, it may still be argued that rs2664538 may only be a proxy for another SNP within the MMP-9 gene or a neighbouring gene, which is responsible and causal for the observed effects. Indeed, rs2664538 is in strong LD (r^2 =1.00) with C570T, a further SNP in intron 1 of the MMP-9 gene. However, by performing extensive *in silico* analysis of the region and the C570T SNP itself using phylogenetic comparisons and transcription factor bind prediction as described in the online supplement, no evidence for a functional role in regulation or transcription factor binding could be allocated to the intron 1 region or the polymorphism (figure E1).

Promoter SNP rs3918241, which was associated with lung function changes in non-atopic children, is in LD with other *MMP-9* coding variants with putative function. Of the 5 SNPs in LD with rs3918241, two are located in intronic and non-conserved regions, two are exonic variations leading to amino acid

changes and one is located in the 3' flanking region. SNPs rs2274756 (P574R) and rs 3918261 (R668Q) are located in the hemopexin domain, which may down-regulate the bioavailability of active MMP-9. Furthermore, the interactions with receptors are proposed to be the original function of hemopexin domains in MMPs. Considering LD, this study cannot discriminate which of the linked polymorphisms is responsible for the effect observed between the tagging SNP and lung function parameters.

LD assessments between MMP-9 and neighbouring genes did not reveal a significant LD pattern in the region harbouring MMP-9 on chromosome 20 (18) (data not shown). Thus, it seems very likely that rs2664538 could indeed be responsible for some of the observed effects, potentially due to its direct influences on MMP-9 activity as previously suggested.

It is not obvious why genetic variants in the *MMP-9* gene are almost exclusively associated with non-atopic forms of wheezing. Without analyzing atopic as well as non-atopic forms of asthma or wheeze, the effects would not have been detected in our population. Thus, it is not surprising that a previous study was unable to identify an effect of *MMP-9* variations in unstratified asthma (25), as in that case-control study atopic and non-atopic asthma were not analysed separately.

It may be hypothesized that different effects on atopic and non-atopic forms of wheezing may be due to the in part contrasting effects of MMP-9 in inflammation and remodelling. While ECM degradation protects from airway remodelling, degradation of ECM also facilitates the influx of inflammatory cells to the airways in allergic inflammation. On the other hand, MMP-9 (and MMP-2) have previously been shown to be essential factors in the clearance of lung inflammatory cells from the airways (10, 13). Considering these data and our findings, one may hypothesise that lower levels of MMP-9 are associated with decreased influx of eosinophils in atopic inflammation, outbalancing the increase in

airway remodelling also due to lower MMP-9 levels and neutralizing the effect on a population level. However, this cannot explain why *MMP-9* is acting differently in atopic and non-atopic inflammation and why the negative effect of increased remodelling due to changes in a matrix metalloproteinase becomes more important in the non-atopic asthma.

These results suggest that different wheezing disorders in childhood are affected differently by genetic alterations in the *MMP9* gene and show the need to better study the role of metalloproteinases in airway inflammation and wheezing.

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Leonardo Araujo Pinto performed genotyping, data analysis and drafted the first version of the manuscript; Martin Depner participated in data analysis and manuscript preparation; Norman Klopp and Thomas Illig participated in genotyping; Christian Vogelberg was involved in data collection; Stephan Weiland and Erika von Mutius contributed to the collection of data and manuscript preparation; Michael Kabesch supervised all experiments, developed the study design, participated in the collection of data and data analysis and wrote the final version of the manuscript. All authors declare that they have no competing financial or personal interests. This work was funded by the German Ministry of Education and Research (BMBF) / national genome research network (NGFN) research grant NGFN 01GS 0429. L. A. Pinto has been also supported by the German academic exchange service (DAAD).

REFERENCES

1. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. N Engl J Med 1995;332(3):133-8.

2. Stein RT, Martinez FD. Asthma phenotypes in childhood: lessons from an epidemiological approach. Paediatr Respir Rev 2004;5(2):155-61.

3. Illi S, von Mutius E, Lau S, Niggemann B, Gruber C, Wahn U. Perennial allergen sensitisation early in life and chronic asthma in children: a birth cohort study. Lancet 2006;368(9537):763-70.

4. Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, Venge P, Ahlstedt S, Simony-Lafontaine J, Godard P, et al. Eosinophilic inflammation in asthma. N Engl J Med 1990;323(15):1033-9.

5. Le Bourgeois M, Goncalves M, Le Clainche L, Benoist MR, Fournet JC, Scheinmann P, de Blic J. Bronchoalveolar cells in children < 3 years old with severe recurrent wheezing. Chest 2002;122(3):791-7.

6. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. Am J Respir Crit Care Med 1999;160(5 Pt 1):1532-9.

7. Matsumoto H, Niimi A, Takemura M, Ueda T, Minakuchi M, Tabuena R, Chin K, Mio T, Ito Y, Muro S, Hirai T, Morita S, Fukuhara S, Mishima M. Relationship of airway wall thickening to an imbalance between matrix metalloproteinase-9 and its inhibitor in asthma. Thorax 2005;60(4):277-81.

8. Renckens R, Roelofs JJ, Florquin S, de Vos AF, Lijnen HR, van't Veer C, van der Poll T. Matrix metalloproteinase-9 deficiency impairs host defense against abdominal sepsis. J Immunol 2006;176(6):3735-41.

9. Atkinson JJ, Senior RM. Matrix metalloproteinase-9 in lung remodeling. Am J Respir Cell Mol Biol 2003;28(1):12-24.

10. Corry DB, Kiss A, Song LZ, Song L, Xu J, Lee SH, Werb Z, Kheradmand F. Overlapping and independent contributions of MMP2 and MMP9 to lung allergic inflammatory cell egression through decreased CC chemokines. Faseb J 2004;18(9):995-7.

11. Lee YC, Lee HB, Rhee YK, Song CH. The involvement of matrix metalloproteinase-9 in airway inflammation of patients with acute asthma. Clin Exp Allergy 2001;31(10):1623-30.

12. Mattos W, Lim S, Russell R, Jatakanon A, Chung KF, Barnes PJ. Matrix metalloproteinase-9 expression in asthma: effect of asthma severity, allergen challenge, and inhaled corticosteroids. Chest 2002;122(5):1543-52.

13. McMillan SJ, Kearley J, Campbell JD, Zhu XW, Larbi KY, Shipley JM, Senior RM, Nourshargh S, Lloyd CM. Matrix metalloproteinase-9 deficiency results in enhanced allergen-induced airway inflammation. J Immunol 2004;172(4):2586-94.

14. Yasmin, McEniery CM, O'Shaughnessy KM, Harnett P, Arshad A, Wallace S, Maki-Petaja K, McDonnell B, Ashby MJ, Brown J, Cockcroft JR, Wilkinson IB. Variation in the human matrix metalloproteinase-9 gene is associated with arterial stiffness in healthy individuals. Arterioscler Thromb Vasc Biol 2006;26(8):1799-805.

15. Weiland SK, von Mutius E, Hirsch T, Duhme H, Fritzsch C, Werner B, Husing A, Stender M, Renz H, Leupold W, Keil U. Prevalence of respiratory and atopic disorders among children in the East and West of Germany five years after unification. Eur Respir J 1999;14(4):862-70.

16. Gardner RM, Hankinson JL. Standardization of spirometry--1987 ATS update (American Thoracic Society). J Occup Med 1988;30(3):272-3.

17. Zhang B, Henney A, Eriksson P, Hamsten A, Watkins H, Ye S. Genetic variation at the matrix metalloproteinase-9 locus on chromosome 20q12.2-13.1. Hum Genet 1999;105(5):418-23.

18. The International HapMap Project. Nature 2003;426(6968):789-96.

19. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21(2):263-5.

20. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16(3):1215.

21. Kormann MS, Carr D, Klopp N, Illig T, Leupold W, Fritzsch C, Weiland SK, von Mutius E, Kabesch M. G-Protein-coupled receptor polymorphisms are associated with asthma in a large German population. Am J Respir Crit Care Med 2005;171(12):1358-62.

22. Hu Z, Huo X, Lu D, Qian J, Zhou J, Chen Y, Xu L, Ma H, Zhu J, Wei Q, Shen H. Functional polymorphisms of matrix metalloproteinase-9 are associated with risk of occurrence and metastasis of lung cancer. Clin Cancer Res 2005;11(15):5433-9.

23. Yuan HY, Chiou JJ, Tseng WH, Liu CH, Liu CK, Lin YJ, Wang HH, Yao A, Chen YT, Hsu CN. FASTSNP: an always up-to-date and extendable service for SNP function analysis and prioritization. Nucleic Acids Res 2006;34(Web Server issue):W635-41.

24. Bai TR, Cooper J, Koelmeyer T, Pare PD, Weir TD. The effect of age and duration of disease on airway structure in fatal asthma. Am J Respir Crit Care Med 2000;162(2 Pt 1):663-9.

25. Ganter K, Deichmann KA, Heinzmann A. Association study of polymorphisms within matrix metalloproteinase 9 with bronchial asthma. Int J Immunogenet 2005;32(4):233-6.

TABLES

Table 1 Odds ratio (and 95% confidence intervals) for associations with asthma and current wheezing in the ISAAC population sample and the effect modification in the strata for atopy (N =4,264)

Tagging SNPs	Asthma (AS) Current wheeze (CW)	Atopic asthma (AA) Atopic wheeze (AW)	Non-atopic asthma (NA) Non-atopic wheeze (NW)
	AS N=**350/3835** **CW** N=**317/3776**	**AA** N=**164/2830** **AW** N=**162/2800**	**NA** N=**167/2830** **NW** N=**138/2800**
rs3918241 TT/TA/AA N=2856/964/88	AS 1.10 (0.53 - 2.29) p=0.806 CW 1.09 (0.50 - 2.38) p=0.832	AA 0.61 (0.15 - 2.52) p=0.488 AW 0.94 (0.29 - 3.05) p=0.920	NA 1.80 (0.76 - 4.25) p=0.173 NW 1.53 (0.54-4.28) P=0.418
rs2664538 (Q279R) * AA/AG/GG N=1655/1821/559	AS 1.27 (0.94 - 1.72) p=0.121 CW 1.33 (0.97 - 1.82) p=0.077	AA 0.92 (0.57 - 1.50) p=0.749 AW 0.81 (0.48 - 1.35) p=0.415	**NA 1.66 (1.12 - 2.46)** **p=0.011*** **NW 2.12 (1.40 - 3.20)** **p<0.001***
rs3918256 AA/AG/GG N=1278/2008/806	AS 1.20 (0.92 - 1.57) p=0.175 CW 1.17 (0.88 - 1.55) p=0.285	AA 1.08 (0.72 - 1.61) p=0.706 AW 0.93 (0.61 - 1.42) 0.750	NA 1.42 (0.98 - 2.05) p=0.061 **NW 1.60 (1.08 - 2.38)** **p=0.018**
rs3787268 * GG/GA/AA N=2419/1310/187	AS 0.97 (0.56 - 1.66) p=0.906 CW 1.01 (0.58 - 1.77) p=0.968	AA 0.27 (0.07 - 1.11) p=0.052 **AW 0.14 (0.02 - 0.99)** **p=0.021**	NA 1.50 (0.79 - 2.83) p=0.213 **NW 2.18 (1.17 - 4.06)** **p=0.012***

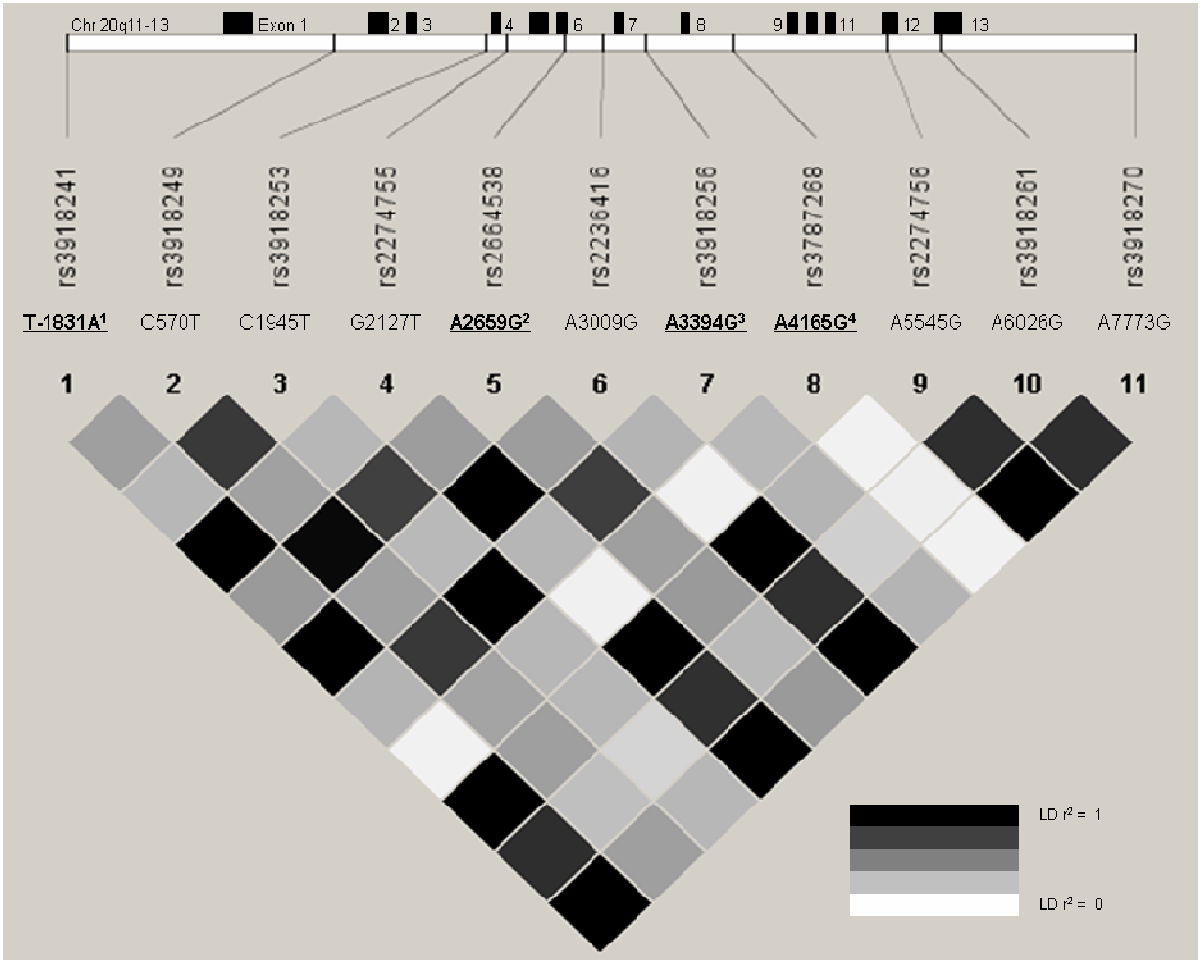
* Nominal significant differences ($p < 0.05$) are printed in bold letters, asterisks indicate significance after correction for multiple testing;

Table 2 Lung function parameters per genotype for non-atopic children with lung function available (population sub-sample from Munich and Dresden)

rs3918241	**FEV1 % (Mean ±SD)**	**MEF25 % (Mean ±SD)**	**MEF50 % (Mean ±SD)**	**MEF75 % (Mean ±SD)**	**MMEF % (Mean ±SD)**	**FVC % (Mean ±SD)**	**N**
TT	100.17 ±10.12	**99.08 ±30.67**	99.33 ±21.57	100.21 ±18.57	99.44 ±22.16	100.30 ±10.38	771
TA	100.15 ±10.22	**100.15 ±31.74**	100.33 ±23.29	99.81 ±19.07	99.26 ±21.89	100.61 ±11.05	247
AA*	98.18 ±10.11	**80.64 ±29.50**	89.85 ±24.46	99.08 ±21.07	88.82 ±24.57	100.29 ±9.17	14
p-value (t-test)*	0.468	**0.025**	0.101	0.837	0.087	0.976	
rs2664538	**FEV1 % (Mean ±SD)**	**MEF25 % (Mean ±SD)**	**MEF50 % (Mean ±SD)**	**MEF75 % (Mean ±SD)**	**MMEF % (Mean ±SD)**	**FVC % (Mean ±SD)**	**N**
AA	99.63 ±10.58	97.73 ±31.47	98.67 ±21.18	99.12 ±18.76	98.55 ±22.70	99.82 ±10.76	445
AG	100.35 ±9.70	100.71 ±30.15	100.03 ±21.52	100.45 ±18.42	99.58 ±21.24	100.25 ±10.31	499
GG	99.82 ±10.60	100.04 ±32.92	100.11 ±25.31	102.29 ±19.88	101.27 ±23.54	100.47 ±11.40	148
p-value (t-test) *	0.832	0.792	0.709	0.138	0.297	0.655	
rs3918256	**FEV1 % (Mean ±SD)**	**MEF25 % (Mean ±SD)**	**MEF50 % (Mean ±SD)**	**MEF75 % (Mean ±SD)**	**MMEF % (Mean ±SD)**	**FVC % (Mean ±SD)**	**N**
AA	99.91 ±10.75	99.40 ±31.79	99.21 ±21.61	**99.43 ±19.22**	99.72 ±23.65	99.92 ±10.66	354
AG	100.07 ±9.71	99.87 ±29.97	99.32 ±21.32	**99.93 ±18.14**	99.00 ±20.55	100.05 ±10.40	555
GG	100.03 ±10.46	99.51 ±32.84	100.79 ±24.33	**102.91 ±19.85**	100.98 ±23.50	100.58 ±11.08	206
p-value (t-test) *	0.983	0.941	0.371	**0.029**	0.353	0.478	
rs3787268	**FEV1 % (Mean ±SD)**	**MEF25 % (Mean ±SD)**	**MEF50 % (Mean ±SD)**	**MEF75 % (Mean ±SD)**	**MMEF % (Mean ±SD)**	**FVC % (Mean ±SD)**	**N**
GG	99.85 ±10.34	98.32 ±31.60	99.00 ±22.14	99.26 ±19.17	98.67 ±22.68	100.12 ±10.67	637
GA	100.31 ±9.35	99.64 ±28.68	100.02 ±20.53	101.47 ±17.29	99.71 ±20.97	100.55 ±9.84	341
AA	101.98 ±11.61	105.31 ±36.01	100.09 ±28.76	101.71 ±21.39	103.50 ±23.16	101.91 ±12.33	58
p-value (t-test) *	0.150	0.125	0.806	0.507	0.170	0.249	

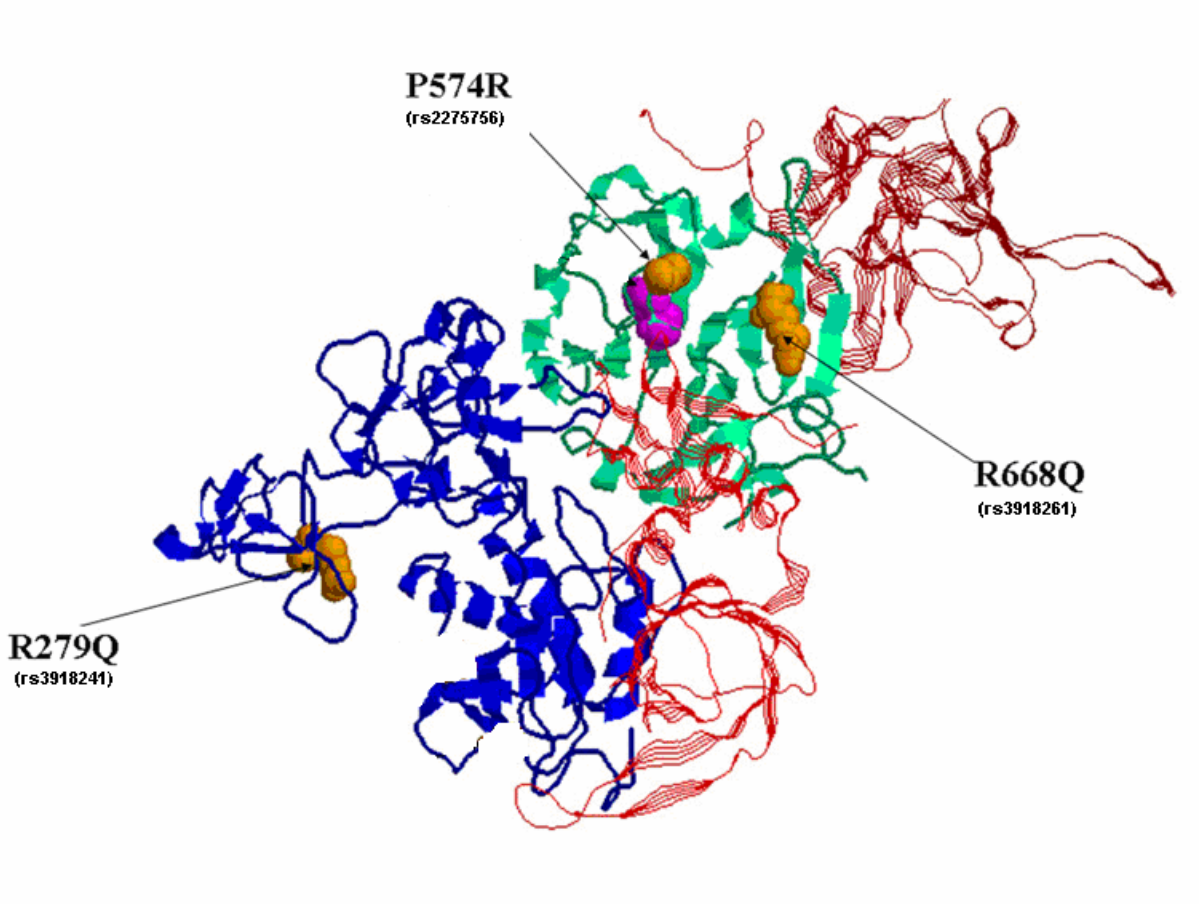
* t test using a recessive model, significant association ($p < 0.05$) are given in bold; the effects do not remain significant after correction for multiple testing; FEV1: forced expiratory volume in the first second; MEF: maximum expiratory flows (MEF) at 25, 50 and 75% of vital capacity; MMEF (maximum mid-expiratory flow) is the average expiratory flow over the middle half of the FVC; FVC: forced vital capacity.

Figure 1



MMP-9 **gene structure indicating all 13 exons, the position of frequent SNPs (MAF>0.1) and linkage disequilibrium (r^2 plot) based on HapMap data (CEPH population, n=90).** [1)] SNP rs3918241 is the tagging SNP (R^2 >0.8) for rs2274755, rs2236416, rs2274756, rs3918261 and rs3818270. [2)] rs2664538 is the tagging SNP for rs3918249. [3)] rs3918256 is the tagging SNP for C1945T. [4)] rs3787268 was not in LD with other polymorphisms. Positions based on NCBI sequence database, accession number AL162458.

Figure 2



Localization of coding SNPs on a model structure of MMP-9. The studied MMP-9 variations **rs3918241 (Q279R), rs2275756 (P574R), rs 3918261 (R668Q)** are shown in context of MMP-9 structure. Q279 is located in the fibronectin type II domain (blue), and P574 and R668 are located in the hemopexin domain (green). Figure adapted from Cotignola J et al., BMC Medical Genetics 2007 [26]

TITLE: GENETIC INFLUENCE ON THE CHILDHOOD WHEEZING PHENOTYPES

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INFLUENCE OF GENETIC POLYMORPHISMS ON THE CHILDHOOD WHEEZING PHENOTYPES

(INFLUÊNCIA DE POLIMORFISMOS GENÉTICOS NOS DIFERENTES FENÓTIPOS DE SIBILÂNCIA)

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ABSTRACT

Introduction: Diverse wheezing phenotypes can be identified based on differences in natural histories, risk factors and responses to the treatment. Atopic asthma or virus-induced wheezing may be discriminated in epidemiologic sturdies by the presence or absence of sensitisation to allergens. Children with atopic asthma have been shown to present lower levels of lung function by 3 years of age. On the other side, viral respiratory illnesses in the first years of life start out with normal lung function but show enhanced airway reactivity usually associated with viral infections.

Methods: Using the Genetic Association Database (GAD/NCBI), we systematically investigated genes and polymorphisms that have been associated with either viral-induced wheezing or atopic asthma.

Results: We identified that different genes and loci have been associated with viral-induced wheezing or atopic asthma. While viral induced wheezing has been frequently associated with polymorphisms in *IL-8*, SNPs located on Th2 genes positioned on chormossome 5 (as *CD14* and *IL-13*) have been frequently associated with atopic asthma or atopy.

Conclusions: This review has shown evidences that different wheezing disorders in childhood may be affected differently by genetic variations, considering their role on airway inflammation and atopy. Future genetic association studies should consider the different wheezing phenotypes in infancy. The analyses stratified for atopy may be useful to clear the mechanisms of the disease.

RESUMO

Introdução: Diversos fenótipos de sibilância têm sido identificados com base em diferenças na história natural, fatores de risco e resposta ao tratamento. A asma atópica ou sibilância induzida por vírus podem ser discriminadas em estudos epidemiológicos pela presença ou ausência de sensibilização a alergenos. As crianças com asma atópica apresentam medidas de função pulmonar reduzidas já aos 3 anos de idade. Por outro lado, a sibilância induzida por vírus nos primeiros anos apresenta-se inicialmente com função pulmonar normal, mas podem evoluir com hiper-reatividade da via aérea, que é usualmente induzida por infecções virais.

Métodos: Utilizando a base de dados *Genetic Association Database* (GAD/NCBI), foi realizada uma investigação sistemática sobre genes e polimorfismos associados a sibilância induzida por virus ou asma atópica.

Resultados: Com esta revisão, foi possível identificar que diferentes loci e polimorfismos estão associados à sibilância induzida por vírus ou asma atópica. Enquanto a sibilância não atópica foi mais frequentemente associada a *IL-8*, SNPs em genes localizados no cromossomo 5 e ligados a uma resposta Th2 (CD14 and IL-13) foram frequentemente associados a atopia ou asma atópica.

Conclusões: Estes resultados sugerem que os diferentes fenótipos de asma na infância podem ser determinados por polimorfismos genéticos diversos. Pode-se chamar atenção para a necessidade de que os estudos de associação genética levem em consideração os diferentes desfechos e fenótipos em estudo. Além disso, uma análise estratificada para atopia deve ser realizada sempre que este dado estiver disponível.

Introduction

Wheezing is highly prevalent in infants and children in the first six years of life, but only a group of these children develop persistent atopic asthma later in life (1). Diverse wheezing phenotypes can be identified based on differences in risk factors, natural histories and responses to the treatment (2-3). International guidelines, which are based on the efficacy of systemic corticosteroids in reducing hospitalization in children with classic atopic asthma, recommend the use of oral steroids for children with virus-induced wheezing who present to a hospital. However, the results of trials that have addressed the question of eficacy of systemic corticosteroids in young children with acute wheezing are contradictory. Atopic and non-atopic wheezing show contrasting natural histories and may be caused by different aetiologies. While atopic asthma have been associated with an allergic or eosinophilic response in older asthmatics (4), neutrophils are predominantly found in brochoalveolar samples of young children with viral induced wheezing (5).

In different epidemiologic studies (6-7), children with atopic asthma present with positive skin prick tests and increased airway responsiveness as major associated risk factors. There is a significant association between an early onset of wheezing symptoms and severity of disease among these children. Children with atopy have been shown to present lower levels of lung function by 3 years of age (8). For pre-school children with wheezing, early allergic sensitization increases the prevalence of respiratory symptoms, airway inflammation and the risk of asthma diagnosis later in life.

Several studies have shown that asthma during childhood is strongly associated with elevated serum IgE and positive skin prick tests (9-11). Early sensitization to allergens is associated with increased risk for the development of bronchial hyper-responsiveness (12). Elevated IgE levels at 9 months of age directly correlated with the risk of persistent wheezing, suggesting a form of IgE-mediated sensitisation during the first years of life (13) Children who had asthma by 7 years of age were

sensitized very early in life and had persistent sensitization when compared with children who did not have asthma (14). These findings may indicate that a genetic pre-disposition for atopy is associated with asthma symptoms that start early in life and persist into adulthood.

Viral lower respiratory illnesses (LRI) in infants and pre-school children may be also associated with persistent wheezing. Non-atopic wheezers start out with normal lung function but present a slightly lower lung function and enhanced airway reactivity later in childhood. Stein et al have previously (15) examined the relationship between LRIs in infants and the subsequent development of wheeze during the first decade of life. Most wheezing episodes are viral respiratory infections, with respiratory syncytial virus (RSV) being detected in the majority of these episodes. Analyses demonstrated that RSV infections in infants were associated with an increased risk of wheezing during the first 10 years of life, independently of other known risk factors for asthma or asthma-related symptoms, such as family history of asthma or atopy. RSV-induced wheezing has not been associated with an increased risk for atopy or higher serum IgE levels.

Children who had virus-induced wheezing early in life were more likely to have lower levels of lung function at 11 years of age compared with controls. One may suggests that many schoolchildren with a history of RSV and lower levels of pulmonary function were born with this reduced function, but we cannot exclude the possibility that in some children, virus infection led to a specific inflammatory response that caused this persistent airway obstruction. Therefore, a significant number of children who wheeze during the first decade of life do so in association with viral respiratory agents independently of atopy. This wheezing phenotype seems to be associated with less severe wheezing. Among school-aged children, this phenotype is probably less prevalent than the atopic phenotype in developed populations, but this may not hold true in different environments. Findings from developing countries (16) have led to the hypothesis that different risk factors, as recurrent or severe viral

agressions, may be associated with increased expression of this wheezing phenotype that is not associated with atopy.

Influence of genetics on different wheezing phenotypes

Genetic variants in the genes associated with the innate immune response may be associated with either non-atopic forms of wheezing or atopic asthma. Without analyzing atopic as well as non-atopic forms of wheeze separately, the effects may not be detected. The Genetic Association Database (GAD) is an archive of human genetic association studies of complex disorders, organized by the National Institute of Health (http://geneticassociationdb.nih.gov/). The objective of this database is to allow the researchers to identify relevant polymorphisms from the large volume of gene variations, in the context of a standardized nomenclature for genes and polymorphisms. The database includes selected published scientific papers. Study data is recorded with the official nomenclature used for the human genome. The submitted records are reviewed before inclusion in the database (17).

Using GAD in a systematic review, we identified that different genes and different loci have been associated with viral-induced wheezing or atopic asthma. While viral or specifically RSV induced wheezing has been frequently associated with polymorphisms in *IL-8* (Table 1), SNPs located on Th2 genes positioned on chormossome 5 (as *CD14* and *IL-13*) and 16 (*IL-4R*) have been frequently associated with atopy or atopic asthma (Table 2).

IL-4 protein is a pleiotropic cytokine produced by activated T cells. It binds to IL13, which may contribute for many overlapping functions of this cytokine. These genes are found to be regulated coordinately by several regulatory elements as interferon regulatory factors and may be considered key factors in the development of Th2 response and atopic asthma.

On the other side, IL-8 protein is a chemokine that has been associated preferentially to viral induced inflammation. This chemokine is one of the major mediators of the inflammatory response. It is secreted by several cell types, and functions as a chemoattractant factor especially for neutrophils. *IL-8* gene is believed to play a role in the pathogenesis of bronchiolitis, a common respiratory tract disease in infants caused by viral infection. Considering these data, this gene and other members of the chemokine gene family, may be considered relevant candidate genes for non-atopic forms of wheezing in childhood.

Genetics of atopic asthma

Twin studies have shown the importance of the genetics on asthma variance, with results of heritability estimation ranging from 48-79%. An important finding is that most of these twin studies in different parts of the developed world showed similar and consistent results and suggest that especially atopic asthma has a strong genetic background.

Although we can estimate to what extent genetic susceptibility contributes to the risk of asthma and atopy, all specific loci that influence this clinical phenotype are yet far from being clearly determined. A significant number of genetic association studies have been describing atopy susceptibility genes, but these data demonstrates the extreme complexity of this trait, and the identification of these polymorphisms may be considered a difficult challenge.

Several candidate genes have been studied in atopic disorders and different factors contribute to this abundance of candidates. Results from genome screens have provided evidence of linkage to multiple sites in the genome. Therefore, there are many positions including candidate genes. In addition, immunological pathways associated to the allergic response involve a large array of inflammatory

mediators. However, the best replicated results in the genetic association studies for atopic asthma involve the following 2 regions in the human genome: 5q31-32 and 16p11-12 (Table 2).

Th2 genes located on chromosome 5q (*IL13* and *IL4)* seem to be major determinants for atopic asthma. *IL13* encodes an immunoregulatory cytokine produced primarily by activated Th2 cells. This cytokine promotes IgE isotype switching. IL13 inhibits the production of pro-inflammatory chemokines. This cytokine is found to be critical to the pathogenesis of allergen-induced asthma. *IL13* and *IL4* form a cytokine gene cluster on chromosome 5q.

It has been reported that the promoter SNP rs1800925 of the *IL13* gene contributes significantly to bronchial hyperresponsiveness and susceptibility to atopic asthma (18). Heinzmann (19) determined that a coding SNP of *IL13* (rs20541) is associated with asthma in case-control populations; the variant also predicted asthma and higher serum IL13 levels in a Japanese population.

The protein encoded by the *IL4* gene is a Th2 cytokine produced by activated T cells that influence allergic immune response. The IL4 receptor also binds to IL13, which may contribute to overlapping functions of IL4 and IL13.

Moreover, it has been demonstrated a possible involvement of SNPs in the *IL-4* gene in the development of asthma and the regulation of total serum IgE (20). This group has shown (21) that especially the combined analyses of genetic alterations in the IL-4/IL-13 pathway reveal its significance to the development of atopy and childhood asthma. Additionally, other genes harbored in the loci, as *CD14* and *IRF1*, may also contribute to asthma and allergy.

Genetics and mechanisms of viral-induced asthma

The important role of IL-8 in the pathophysiology of bronchial inflammation has been confirmed by studies in humans and animals. Administration of IL-8 into the airways induces bronchial hyperreactivity in pigs (22, 23) and increased levels of IL-8 in sputum precede wheezing exacerbation in humans (24). Besides, IL-8 might be especially important in nonatopic wheezing, because IL-8 producing cells are more frequently found in this subgroup of patients with asthma (25). Also, IL-8 selectively inhibits IgE production in atopic patients by inhibiting IL-4 and thus might even protect against the development of atopy (26, 27)

RSV is involved in at least 70% of cases of bronchiolitis and has been repetitively linked to wheezing. It has been hypothesized that severe RSV infection in infancy might is associated with the development of recurrent wheezing or bronchitis (28, 29). According to the current evidence, genetic and environmental factors determine the type of immune response to RSV infection. Furthermore, this response may affect the development of control mechanisms in the regulation of airway diseases.

Increased concentrations of IL-8 have been described in the bronchoalveolar fluid and sputum of patients with recurrent wheezing (30). In addition, genetic association of IL-8 has been described with both persistent wheezing (31) and RSV bronchiolitis (32, 33).

Heinzmann et al have (31) demonstrated an association of polymorphisms in IL-8 with bronchial asthma. Furthermore, the findings suggested that RSV bronchiolitis and asthma have at least some different genetic factors: the same promotor polymorphism in IL-8 that causes susceptibility to RSV bronchiolitis might protect against asthma. The results may suggest a distinct and even opposite role of IL-8 in atopic and non-atopic wheezing.

Conclusions

This review demonstrated different effects of genetic variations on atopic and non-atopic wheezing. These differences should be interpreted considering the role of these genes on airway inflammation and atopy. Although these phenotypes may have different etiologies, no clinical index or test for the diferenciation between atopic asthma and viral wheezing has proved to be sufficiently accurate to be useful in young children. However, future genetic association studies should systematically investigate the wheezing phenotypes separately. These studies may identify clinical relevant genetic markers of viral-induced wheezing or atopic asthma.

TABLES

Table 1: Gene(s) with more than three genetic studies associated with virus induced wheezing.

Gene	Phenotype	Chr	Ch-Band	Reference
IL8	RSV induced wheezing	4	4q13-q21	Hull J 2000 (32)
IL8	RSV induced wheezing	4	4q13-q21	Puthothu, B. et al. 2006 (34)
IL8	RSV induced wheezing	4	4q13-q21	Heinzmann, A. et al. 2004 (31)
IL8	RSV infenction	4	4q13-q21	Lu, A. Z. et al. 2007 (35)

Table 2: Genes with more than three genetic studies associated with atopic asthma or atopy.

Gene	Phenotype	Chr	Ch-Band	Reference
CD14	Atopy (total IgE))	5	5q22-q32	Leung, T. F. et al. 2003 (36)
CD14	Atopy	5	5q22-q32	Buckova, D. et al. 2003 (37)
CD14	Atopy	5	5q22-q32	Kabesch, M. et al. 2004 (38)
CD14	Atopy	5	5q22-q32	Leynaert, B. et al. 2006 (39)
CD14	Atopy	5	5q22-q32	Koppleman GH 2001 (40)
IL13	Atopy	5	5q31	Liu X 2003 (41)
IL13	Atopy (specific IgE)	5	5q31	Leung TF 2001 (42)
IL13	Asthma and atopy	5	5q31	Howard, T. D. et al. 2001 (43)
IL13	Atopy	5	5q31	Nieters, A. et al. 2004 (44)
IL4R	Atopy	16	16p11-12	Ober C 2000 (45)
IL4R	Atopy	16	16p11	Liu X 2004 (46)
IL4R	Atopy (IgE)	16	16p11	Heinzmann A 2000 (19)
IL4R	Atopy	16	16p11	Nieters, A. et al. 2004 (44)
IL4R	Atopy	16	16p11	Deichmann KA et al. 1998 (47)
IL4R	Atopic asthma	16	16p11	Isidoro-Garcia, M. et al. 2005 (48)
IL4R	Asthma and atopy	16	16p11	Kruse S et al. 1999 (49)

REFERENCES

1. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. N Engl J Med. 1995; 332(3):133-8

2. Stein RT, Martinez FD. Asthma phenotypes in childhood: lessons from an epidemiological approach. Paediatr Respir Rev. 2004; 5(2):155-61

3. Illi S, von Mutius E, Lau S, Niggemann B, Gruber C, Wahn U. Perennial allergen sensitisation early in life and chronic asthma in children: a birth cohort study. Lancet. 2006; 368(9537):763-70

4. Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I et al. Eosinophilic inflammation in asthma. N Engl J Med. 1990; 323(15):1033-9

5. Le Bourgeois M, Goncalves M, Le Clainche L, Benoist MR, Fournet JC, Scheinmann P et al. Bronchoalveolar cells in children < 3 years old with severe recurrent wheezing. Chest. 2002; 122(3):791-7

6. Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM et al. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. N Engl J Med. 2003; 349(15): 1414-22

7. Morgan WJ, Stern DA, Sherrill DL, Guerra S, Holberg CJ, Guilbert TW et al. Outcome of asthma and wheezing in the first 6 years of life: follow-up through adolescence. Am J Respir Crit Care Med. 2005; 172(10):1253-8

8. Lowe L, Murray CS, Custovic A, Simpson BM, Kissen PM, Woodcock A. Specific airway resistance in 3-year-old children: a prospective cohort study. Lancet. 2002; 359(9321): 1904-8

9. Freidhoff LR, Marsh DG. Relationship among asthma, serum IgE and skin test reactivity to inhaled allergens. Int Arch Allergy Immunol. 1993; 100(4):355-61

10. Sears MR, Burrows B, Flannery EM, Herbison GP, Hewitt CJ, Holdaway MD. Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. N Engl J Med. 1991; 325(15):1067-71

11. Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. N Engl J Med. 1989; 320(5):271-7

12. Peat JK, Salome CM, Woolcock AJ. Longitudinal changes in atopy during a 4-year period, relation to bronchial hyperresponsiveness and respiratory symptoms in a population sample of Australian school children. J Allergy Clin Immunol. 1990; 85(1):65-74

13. Sherrill DL, Stein R, Halonen M, Holberg CJ, Wright A, Martinez FD. Total serum IgE and its association with asthma symptoms and allergic sensitization among children. J Allergy Clin Immunol. 1999; 104(1):28-36

14. Illi S, von Mutius E, Lau S, Nickel R, Niggemann B, Sommerfeld C et al. The pattern of atopic sensitization is associated with the development of asthma in childhood. J Allergy Clin Immunol. 2001; 108(5):709-14

15. Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM et al. Respiratory syncitial virus in early life and risk of wheeze and allergy by age 13 years. Lancet. 1999; 354(9178):541-5

16. Pereira MU, Sly PD, Pitrez PM, Jones MH, Escouto D, Dias AC et al. Nonatopic asthma is associated with helminth infections and bronchiolitis in poor children. Eur Respir J. 2007; 29(6):1154-60

17. Lin BK, Clyne M, Walsh M, Gomez O, Yu W, Gwinn M, et al. Tracking the epidemiology of human genes in the literature: the HuGE Published Literature database. Am J Epidemiol. 2006; 164(1):1-4

18. Howard TD, Koppelman GH, Xu J, Zheng SL, Postma DS, Meyers DA, et al. Gene-gene interaction in asthma: IL4RA and IL13 in a Dutch population with asthma. Am J Hum Genet. 2002; 70(1):230-6

19. Heinzmann A, Mao XQ, Akaiwa M, Kreomer RT, Gao PS, Ohshima K, et al. Genetic variants of IL-13 signalling and human asthma and atopy. Hum Mol Genet. 2000; 9(4):549-59

20. Kabesch M, Tzotcheva I, Carr D, Hofler C, Weiland SK, Fritzsch C, et al. A complete screening of the IL4 gene: novel polymorphisms and their association with asthma and IgE in childhood. J Allergy Clin Immunol. 2003;112(5):893-8

21. Kabesch M, Schedel M, Carr D, Woitsch B, Fritzsch C, Weiland SK, et al. IL-4/IL-13 pathway genetics strongly influence serum IgE levels and childhood asthma. J Allergy Clin Immunol. 2006; 117(2):269-74

22. Fujimura M, Xiu Q, Tsujiura M, Tachibana H, Myou S, Matsuda T, et al. Role of leukotriene B4 in bronchial hyperresponsiveness induced by interleukin-8. Eur Respir J. 1998; 11:306-11

23. Xiu Q, Fujimura M, Nomura M, Saito M, Matsuda T, Akao N, et al. Bronchial hyperresponsiveness and airway neutrophil accumulation induced by interleukin-8 and the effect of the thromboxane A2 antagonist S-1452 in guinea-pigs. Clin Exp Allergy. 1995; 25:51-9

24. Kurashima K, Mukaida N, Fujimura M, Schroder JM, Matsuda T, Matsushima K. Increase of chemokine levels in sputum precedes exacerbation of acute asthma attacks. J Leukoc Biol. 1996; 59:313-6

25. Amin K, Ludviksdottir D, Janson C, Nettelbladt O, Bjornsson E, Roomans GM, et al. Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. BHR Group. Am J Respir Crit Care Med. 2000; 162:2295-301

26. Kimata H, Lindley I, Furusho K. Selective inhibition of spontaneous IgE and IgG4 production by interleukin-8 in atopic patients. Blood 1995; 85: 3191-8

27. Kimata H, Yoshida A, Ishioka C, Lindley I, Mikawa H. Interleukin 8 (IL-8) selectively inhibits immunoglobulin E production induced by IL-4 in human B cells. J Exp Med. 1992; 176:1227-31

28. Sigurs N, Gustafsson PM, Bjarnason R, Lundberg F, Schmidt S, Sigurbergsson F et al. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. Am J Respir Crit Care Med. 2005; 171:137-41

29. Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. Am J Respir Crit Care Med. 2000; 161:1501-7

30. Norzila MZ, Fakes K, Henry RL, Simpson J, Gibson PG: Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. Am J Respir Crit Care Med. 2000; 161:769-74

31. Heinzmann A, Ahlert I, Kurz T, Berner R, Deichmann KA. Association study suggests opposite effects of polymorphisms within IL8 on bronchial asthma and respiratory syncytial virus bronchiolitis. J Allergy Clin Immunol. 2004; 114(3):671-6

32. Hull J, Ackerman H, Isles K, Usen S, Pinder M, Thomson A et al. Unusual haplotypic structure of IL8, a susceptibility locus for a common respiratory virus. Am J Hum Genet. 2001; 69:413-9

33. Hull J, Thomson A, Kwiatkowski D. Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families. Thorax. 2000; 55:1023-7

34. Puthothu B, Krueger M, Heinze J, Forster J, Heinzmann A. Impact of IL8 and IL8-receptor alpha polymorphisms on the genetics of bronchial asthma and severe RSV infections. Clin Mol Allergy. 2006; 17; 4:2

35. Lu AZ, Wang LB, Zhang MZ, Zhang XB. Association of interleukin 8 single nucleotide polymorphisms with the susceptibility to respiratory syncytial virus infection. Zhonghua Er Ke Za Zhi. 2007; 45(2):100-4

36. Leung TF, Tang NL, Sung YM, Li AM, Wong GW, Chan IH, Lam CW. The C-159T polymorphism in the CD14 promoter is associated with serum total IgE concentration in atopic Chinese children. Pediatr Allergy Immunol. 2003; 14(4):255-60

37. Bucková D, Hollá LI, Schüller M, Znojil V, Vácha J. Two CD14 promoter polymorphisms and atopic phenotypes in Czech patients with IgE-mediated allergy. Allergy. 2003; 58(10):1023-6

38. Kabesch M, Hasemann K, Schickinger V, Tzotcheva I, Bohnert A, Carr D et al. A promoter polymorphism in the CD14 gene is associated with elevated levels of soluble CD14 but not with IgE or atopic diseases. Allergy. 2004; 59(5):520-5

39. Leynaert B, Guilloud-Bataille M, Soussan D, Benessiano J, Guénégou A, Pin I et al. Association between farm exposure and atopy, according to the CD14 C-159T polymorphism. J Allergy Clin Immunol. 2006; 118(3):658-65

40. Koppelman GH, Reijmerink NE, Colin Stine O, Howard TD, Whittaker PA, Meyers DA et al. Association of a promoter polymorphism of the CD14 gene and atopy. Am J Respir Crit Care Med. 2001; 163(4):965-9

41. Liu X, Beaty TH, Deindl P, Huang SK, Lau S, Sommerfeld C et al. Associations between total serum IgE levels and the 6 potentially functional variants within the genes IL4, IL13, and IL4RA in German children: the German Multicenter Atopy Study. J Allergy Clin Immunol. 2003; 112(2):382-8

42. Leung TF, Tang NL, Chan IH, Li AM, Ha G, Lam CW. A polymorphism in the coding region of interleukin-13 gene is associated with atopy but not asthma in Chinese children. Clin Exp Allergy. 2001; 31(10):1515-21

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

44. Nieters A, Linseisen J, Becker N. Association of polymorphisms in Th1, Th2 cytokine genes with hayfever and atopy in a subsample of EPIC-Heidelberg. Clin Exp Allergy. 2004; 34(3):346-53

45. Ober C, Leavitt SA, Tsalenko A, Howard TD, Hoki DM, Daniel R et al. Variation in the interleukin 4-receptor alpha gene confers susceptibility to asthma and atopy in ethnically diverse populations. Am J Hum Genet. 2000; 66(2):517-26

46. Deichmann KA, Heinzmann A, Forster J, Dischinger S, Mehl C, Brueggenolte E et al. Linkage and allelic association of atopy and markers flanking the IL4-receptor gene. Clin Exp Allergy. 1998; 28(2):151-5

47. Isidoro-García M, Dávila I, Laffond E, Moreno E, Lorente F, González-Sarmiento R. Interleukin-4 (IL4) and Interleukin-4 receptor (IL4RA) polymorphisms in asthma: a case control study. Clin Mol Allergy. 2005; 29(3):15

48. Kruse S, Japha T, Tedner M, Sparholt SH, Forster J, Kuehr J et al. The polymorphisms S503P and Q576R in the interleukin-4 receptor alpha gene are associated with atopy and influence the signal transduction. Immunology. 1999; 96(3):365-71

CONCLUSÕES DA TESE DE DOUTORADO

1. Existe uma associação entre diferentes polimorfismos em genes ligados à resposta imune inata e fenótipos de asma e atopia.
2. Foram descritos 40 polimorfismos freqüentes do gene do Fator Regulador de Interferon 1 (FRI-1). Dentre as variações genéticas estudadas, 4 polimorfismos (rs2706384, rs2070721, rs10035166, rs17622656) demonstraram uma associação com os níveis de IgE total, IgE específica e a presença de atopia medida por testes cutâneos. Não foi encontrada nenhuma associação significativa com o desfecho asma.
3. O polimorfismo rs3771300 do gene Transdutor de Sinal e Ativador da Transcrição 1 (TSAT-1), localizado em uma região funcionalmente relevante do gene, foi associado a desfechos de atopia em uma população caucasiana. Não foi encontrada nenhuma associação significativa com o desfecho asma.
4. O polimorfismos rs2664538 do gene codificador Metaloproteinase de Matriz 9 (MP-9) foi associado a asma não-atópica e sibilância não-atópica.
5. Os resultados sugerem que os fenótipos de asma atópica e sibilância não-atópica podem ser determinados por polimorfismos genéticos diversos.

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

As pesquisas sobre etiologia da asma têm demonstrado sua face mais complexa através dos estudos de associação genética. Existem mais de 100 loci associados aos desfechos que médicos e/ou pesquisadores definiram como asma. Entretanto, a maioria destes genes demonstra associações irrelevantes (ex. OR =1,10 (IC =1,01 – 1,15)) ou inconsistentes (achados diferentes em diferentes populações). É crescente o consenso de que um grande número de genes interage com o ambiente e, desta forma, influencia muitos desfechos associados à asma (atopia, função pulmonar, risco de infecções virais, etc.). Os pesquisadores que buscam "o gene da asma" têm sido constantemente desenganados, e tudo indica que todos os que mantiverem os métodos mais utilizados atualmente vão continuar colecionando decepções (1).

Considerando estas reflexões recentes, muitos autores têm sugerido mudanças radicais nos objetivos, hipóteses e métodos em pesquisas relacionadas à genética da asma (2-3). A modificação mais importante a ser considerada é a mudança de objetivos. O foco principal não estaria mais na investigação etiológica, pois a busca incessante pelo "gene da asma" está cada vez mais cara e ineficiente. Os novos objetivos incluem especialmente o diagnóstico genético e a farmacogenética (4-5).

Os fenótipos de sibilância podem ser facilmente identificados em estudos retrospectivos. Entretanto, nenhum teste ou exame consegue definir com precisão se um lactente de poucos meses apresenta sibilância induzida por vírus ou asma atópica. Esta talvez seja uma das grandes contribuições que a genética pode trazer para a prática clínica nesta área. As pesquisas com diagnóstico genético devem incluir uma fenotipagem detalhada e minuciosa. Isto pode trazer um novo problema aos pesquisadores

na fase de inclusão dos casos e controles. Entretanto, a aplicação e relevância dos achados para a prática clínica também podem tornar estes estudos custo-efetivos.

Uma outra linha de pesquisa crescente e promissora é a farmacogenética. Crianças com asma recebem, em geral, tratamentos semelhantes, seguindo protocolos ou consensos internacionais (6). Porém, as respostas a esta terapêutica parece ser muito variável e clinicamente ainda existe muita dificuldade de determinar respostas individuis ou diversas em determinados subgrupos (6). A farmacogenética estuda exatamente as diferenças entre respostas terapêuticas dependentes de variações genéticas (polimorfismos). Estes estudos podem auxiliar na identificação de pacientes resistentes a corticosteróides, por exemplo, ou subgrupos com boa resposta a anti-leucotrienos (4-5).

Em conclusão, é fundamental que o investimento na genética de doenças complexas tenha foco especialmente em trabalhos de investigação sobre diagnóstico genético e farmacogenética. Estas pesquisas podem trazer novas rotinas no tratamento dos pacientes e mehorar a resposta terapêutica das crianças com doenças pulmonares com obstrução da via aérea.

REFERENCIAS BIBLIOGRÁFICAS

1. Bush A.J. Genes in their environment: how can we read the riddles? Pediatr (Rio J). 2008; 84(3):185-8.
2. Postma DS, Koppelman GH. Genetics of asthma: where are we and where do we go? Proc Am Thorac Soc. 2009; 6(3):283-7.
3. Willis-Owen SA, Valdar W. Deciphering gene-environment interactions through mouse models of allergic asthma. J Allergy Clin Immunol. 2009;123(1):14-23.
4. Lima JJ, Blake KV, Tantisira KG, Weiss ST. Pharmacogenetics of asthma. Curr Opin Pulm Med. 2009; 15(1):57-62.
5. Yu IW, Bukaveckas BL. Pharmacogenetic tests in asthma therapy. Clin Lab Med. 2008; 28(4):645-65.
6. van Weel C, Bateman ED, Bousquet J, Reid J, Grouse L, Schermer T, Valovirta E, Zhong N. Asthma management pocket reference 2008. Allergy. 2008 Aug;63(8):997-1004

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