
Genetic Aspects of Respiratory Allergy

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<http://dx.doi.org/10.5772/59097>

1. Introduction

Genetic and environmental factors contribute to the pathogenesis of asthma [44]. A clear familial incidence and genetic susceptibility was recognized in early XXth century and subsequent studies established high levels heritability [14, 42, 49, 67]. Analysis of genetic association to asthma susceptibility and disease traits has long been used to try and identify pathways involved in asthma pathogenesis and potential therapeutic targets.

In this chapter we review the recent advances in the understanding of the genetic factors contributing to asthma development. We also discuss some of our recent results on the association between allergic asthma and human leukocyte antigen (HLA) and cytokine loci. We present gene and protein expression analysis for some of these relevant disease markers in the context of allergen challenging of asthmatic patients.

1.1. Genetic studies

Asthma is a complex disease with heterogeneous presentations in different populations [44]. These variable presentations have been grouped into "endotypes" [41]. The high phenotypic diversity led others to propose that asthma is not a single disease but instead that the term "asthma" has been used to define a spectrum of related, overlapping syndromes [41].

At present, no single gene was found to be determinant for asthma susceptibility or, indeed, disease endotypes. Multiple molecular mechanisms seem to be involved in the pathogenesis of each disease feature and many genetic variations have been associated with risk and/or phenotypes. While some genes seem to contribute to asthma susceptibility, others might specifically influence disease features such as age of onset, atopy, progression (severity) or response to treatment. On the other hand, asthma is a multifactorial disease. In addition to

genetic predisposition, there are well-established environmental or developmental factors that interact and influence gene expression [16, 52].

The knowledge in asthma genetics has evolved with the advances in biotechnology. Three major technical approaches have been used to search for associations between genes and disease (or phenotypes): genome-wide linkage studies, hypothesis-driven candidate gene association studies and genome-wide association studies (GWAS).

Genome-wide linkage studies using microsatellite markers performed in families identify chromosome segments associated with disease. Cloning of the “linked” alleles might then lead to the identification of genes and, potentially, to novel disease pathways, as it allows unbiased analysis of the genome. A downside of these linkage followed by positional candidate studies is the difficult study design and their poor resolution, as they often identify broad chromosome regions that might include hundreds of genes.

The sequencing of the whole human genome enabled the study of associations between disease/traits and single nucleotide polymorphisms (SNPs) in particular genes of interest. The candidate genes might be selected for their presumptive functional involvement in disease pathology or for their chromosomal position in a linkage region, or near a previous association signal (positional candidate). Association studies present some advantages over family linkage studies: use of independent case-controls and non-related populations, detect genes with modest effects, have a simpler study design and their results are easier to interpret. On the other hand, the candidate gene approaches require previous knowledge or assumptions about disease pathogenesis and, therefore, do not search for novel genes and pathways.

Linkage and candidate studies have contributed for pivotal discoveries in many monogenic diseases. However, genetic association studies provide limited information as only a small number of genes can be studied at a time. Furthermore, in complex diseases, such as asthma, the contribution of many genes is hard to encompass. Recently, the publication of the Human Genome Project in 2003 (www.genome.gov/) and the International HapMap Project (<http://hapmap.ncbi.nlm.nih.gov>) in 2005 (The International HapMap Consortium 2003) allowed the development of genome-wide association studies (GWAS), [26, 43]. GWAS analyse hundreds of thousands of genetic variants (such as SNPs) across the whole human genome and search for their association to a disease characteristic by comparing the frequency of a genetic variant among the afflicted population versus a control population. Powerful computational statistics are then required to analyse the data and find significant differences in frequency that support association and suggest involvement in disease pathogenesis. Case-control studies are useful to determine disease susceptibility. For particular disease characteristics, however, the associations might be better explored using case-only studies, comparing phenotypes, severity or response to treatment among subgroups of the diseased population.

GWAS brought significant improvement to previous genetic studies of disease because genetic variants are researched across the whole genome and a great number of patients and controls can be included. Importantly, the search is unbiased and does not require previous knowledge of the disease or the biological pathways involved, allowing the finding of unsuspected genes.

This advantage has, sometimes, proved thorny, as some identified genes have little biological plausibility. One important limitation in GWAS is the low detection of uncommon risk variants, as genotyping platforms mostly include common gene variants.

GWAS have now been used in many diseases with important results [26, 78]. The National Center for Biotechnology Information (part of the National Library of Medicine, USA) compiles data from GWAS performed in many diseases and conditions. Data can be accessed at the Database of Genotype and Phenotype at <http://www.ncbi.nlm.nih.gov/gap> and at the National Human Genome Research Institute, National Institutes of Health, USA: <http://www.genome.gov/gwastudies/>.

1.2. Linkage and candidate studies on the susceptibility and phenotypes of asthma

The first genome-wide linkage screening for asthma susceptibility loci was published in 1989 [9] and, in 2002, *ADAM33*, located in chromosome 20, was the first gene associated with asthma by positional cloning [75]. This association has then been replicated numerous times in different populations [27]. Ironically, the function of the encoded metallopeptidase in asthma pathogenesis is still unclear [72]. Only 9 genes were identified by positional candidate studies in linked regions [4, 12, 52]: *ADAM33*, *DPP10*, *PHF11*, *NPSR1*, *HLA-G*, *CYFIP2*, *IRAK3*, *COL6A5* and *OPN3/CHML*.

Taking also into consideration the vast literature on candidate gene associations, more than 200 genes have been proposed as asthma related genes through human and murine studies [77]. In 2006, previous to the introduction of GWAS in asthma research, Ober and Hoffjan extensively reviewed the literature and identified a “top10” of genes more frequently associated to asthma [51]: *ADAM33*, *IL4*, *IL4RA*, *IL13*, *CD14*, *ADRB2*, *TNF*, *HLA-DRB1*, *HLA-DQB1* and *FCER1B*. Several of these genes are positioned on region 5q31-33, an early recognized and replicated association in linkage studies. This region includes genes for Th2 mediators [22, 51] thought to be involved in asthma development (e.g. *IL4* or *IL13*), and also other genes that might contribute for asthma pathogenesis, such as environment interaction genes (e.g. *CD14*) and drug-response genes (e.g. *ADRB2*). *ADRB2* encodes the β_2 adrenergic receptor expressed on bronchial smooth muscle cells and influences both the bronchial tonus and the response to adrenergic medications (e.g. albuterol). Further to asthma susceptibility, it has been associated also with several phenotypes, such as severity [8] or disease persistence into adulthood [24]. HLA genomic region (6p21) also has been repeatedly associated with asthma susceptibility, and in particular the *HLA-DRB1*, *HLA-DQB1* and *TNF* genes [45, 58, 60].

1.3. Genome-wide association studies in asthma

In 2007 the first GWAS on asthma susceptibility was published [46]. This study strongly associated gene *ORMDL3* in 17q21 with susceptibility to childhood asthma. This region includes also the gene *GSDBM*. Initially identified in populations of European ancestry [46, 61], many studies have now replicated the association of genes located in 17q21 with childhood-onset asthma and several asthma phenotypes [3, 4] also in Latin American [19, 69],

African American or African Caribbean [69, 19] European American [69], and Chinese [35] populations. *ORMDL3* gene encodes a transmembrane protein localized in the endoplasmic reticulum and may be involved in the endoplasmic reticulum response to stress and inflammation, increasing the unfolded-protein response [6]. The functional connections of *ORMDL3* and *GSDMB* to asthma development are not fully understood. Another GWAS on asthmatic children confirmed the relevance of 17q21 and found a novel association with the gene *DENND1B* (1q31) in children of European and African ancestry [62]. *DENND1B* is expressed by natural killer cells and dendritic cells and predicted to interact with TNF-alpha receptor [62].

So far, 43 GWAS were published on childhood and adult asthma using different populations – search at www.genome.gov, as of August 2014 – and a great deal of information is available about novel genes associations as well as confirmations of relevant genes previously described. This includes studies on asthma susceptibility as well as GWAS on several of asthma phenotypes, e.g., IgE [30], forced vital capacity [40], or response to glucocorticoid treatment [63]. Two important meta-analyses reviewed the published data.

In 2010, the **GABRIEL consortium** published a large-scale GWAS meta-analysis including European ancestry-matched 10365 asthmatic patients and 16110 controls [47]. Asthma was associated with *IL1RL1/IL18R1*, *HLA-DQB1*, *IL33*, *SMAD3*, *IL2R*, *GSDMB* and *GSDMA* genes, and using with a lower threshold to *SLC22A5*, *IL13* and *RORA*. A stronger association was found between these genes and childhood-onset asthma than adult onset asthma, except for *HLA-DQ* which associates more with adult-onset. *ORMDL3* and *GSDMB* were associated only to childhood-onset asthma. No significant association was found for severe or occupational asthma. Notably, genes previously associated to asthma in other studies, such as *PDE4D*, *CHI3L1*, *DPP10*, *GPR154* [*NPSR1*], *ADAM33*, *PHF11*, *OPN3*, *IRAK3*, *PCDH1*, *HLA-G*, and *DENND1B*, were not found to be significantly associated with asthma in this study. High IgE serum levels were associated with previously described *FCER1A*, *IL13*, *STAT6* and *IL4R/IL21R* genes and to a new locus within MHC II – *HLA-DRB1*. Only *IL13* showed some association with asthma, whereas the others genes associated with serum high IgE levels were not associated with asthma. This is somewhat unexpected and suggests that asthma development is not related to the levels of IgE. Only *HLA-DR* was found to associate with high serum IgE levels [47].

The **EVE Consortium** meta-analysis searched for susceptibility genes across ethnic groups in North America: European American, African American or African Caribbean, and Latino ancestries [69]. Notably, four associations with asthma risk were found in all three ethnic groups: 17q21 region, *IL1RL1*, *TSLP* and *IL33*. Remarkably, some of these were associated in the two large published meta-analyses, GABRIEL and EVE [47, 69]. A novel asthma susceptibility locus was described by the EVE consortium at *PYHIN1* specifically in individuals of African ancestry [69].

It seems now clear that some genes are replicated across different populations whereas others seem to be ancestry specific. In another study, 6q14.1 was found to be related to asthma susceptibility in European and African patients [70].

1.4. Biological significance of genetic studies

It is reassuring that many of the genes identified were related to immunological physiopathology, e.g. several cytokines and antigen presentation genes. Particularly, genes coding for cytokines and pathways associated with Th2 cells (e.g. *IL13*, *IL2R*) and regulatory cells (e.g. SMAD, TGF-beta) were among them. This agrees with the vast literature showing roles for these cells in asthma, atopy and immune regulation. The role of epithelial cells in asthma pathogenesis is underlined by the associations with *TSLP*, *IL33* and *IL1RL1* (that encodes for the receptor of IL33, expressed on mast cells, Th2 cells, T-regulatory cells, and macrophages) [47, 69].

Some genes, however, do not immediately relate to immunological pathways. This was the case for *RAD50* [38]. In this study, Li and colleagues found that SNPs on *HLA-DQB1*, *HLA-DQA2* and *RAD50* region were associated with severe or difficult-to-treat asthma. *RAD50* is a DNA repair gene and a connection with asthma pathogenesis is not obvious. Furthermore, the associated SNPs were found in an intron. Notably, previous research on Th2 cytokine expression in mice showed the presence of a locus control region (LCR) in this location that is an important regulator of the genes encoding the Th2 cytokines interleukins 4, 5 and 13 [33, 34]. Genetic ablation of this LCR did not affect the expression of a linked *RAD50* gene, but reduced long-range intrachromosomal interactions with the promoters of the Th2 cytokine genes. This example shows that some associations require further understanding of genetic or functional interactions of the genes. Nevertheless, some of the associated genes remain with no known immunological or genetic connection to asthma and more information is needed to understand their role in pathogenesis.

1.5. Limitations of GWAS results in asthma

The results obtained so far demonstrate the polygenic and multifactorial nature of asthma. Low levels of replication have been found between GWAS and individual study heritability estimates for the associated genes were found to be low [52] and, therefore, with little prognostic utility. These setbacks might be, at least in part, due to methodological or sampling differences between GWAS. A fundamental question is that some variants might increase risk only in specific individuals and not in others, for instance in the case of some genotype-environmental exposure interactions. Moreover, GWAS underestimate the role of rare genetic variants [65, 70] that might have an overall larger effect than common variants on the proportion of genetic risk for common diseases [15]. Epigenetic mechanisms [13] and epistatic effects might further difficult gene expression analysis [81].

The challenges with GWAS are discussed in further detail elsewhere [77]. Several methods have been proposed to improve the power of GWAS (De 2014). Utilizing a combination of approaches, in which GWAS-type studies are performed using selected SNPs of candidate genes, a strategy was devised and named “prioritized subset analysis” [37]. This strategy combines the strength of GWAS computational statistics with previous knowledge from the literature, allowing for power improvement [37]. Novel mathematical models have been proposed for data analysis in asthma [74].

In the future, the study of complex diseases might incorporate data from whole-genome sequencing [71] or exome sequencing to identify both common and rarer genetic variants in complex diseases [64]. These techniques and analysis are becoming more affordable and feasible on large numbers of individuals and therefore sufficient power will be possible for detecting associations with asthma. A recent study, for example, analysed the role of copy number variants asthma using whole genome sequencing [5].

Genetic approaches require replication and gene expression analysis. Ultimately, *in vitro/ex vivo/in vivo* studies are required to confirm the biological significance and to understand the precise functions of the associated genes in asthma pathogenesis. In this chapter, we analyse the expression of some candidate genes in the context of the allergen-specific response in allergy respiratory patients.

2. Experimental study

2.1. Cytokine single nucleotide polymorphism and HLA expression

2.1.1. Material and methods

2.1.1.1. Subjects

85 voluntary adult patients with respiratory allergy to *Dermatophagoides pteronyssinus* (Dp) under observation in outpatient Immunoallergy were enrolled for study (Group A). This group included patients of both sexes (female= 55; male=30; average age: 30.09 ± 9.22 years), wherein 36 of them had the diagnosis of allergic rhinitis and the others 49 patients had simultaneously allergic asthma and rhinitis diagnosis, according criteria from ARIA and GINA guidelines [2, 20]. At the moment of the study all the patients were under clinical stability, and never were submitted to specific immunotherapy in the past.

Pregnancy, systemic inflammatory illness or other chronic disease, any exacerbation of allergic disease or a respiratory tract infection in the previous month to the study, as well as nasal polyposis or previous nasal surgery in the past were considered exclusion criteria.

2.1.1.2. Methods

DNA was extracted from polymorphonuclear blood cells (PMBC) using MagAttract DNA Blood Mini M48 Kit on the BioRobot M48. After extraction, the quality and quantification of DNA was evaluated and PCR -RSSO Cytokine SNP Typing kit from LIFE CODES was used to investigate single cytokine polymorphism (SNP's) from 14 cytokines using bead array xMAPTM technology from LuminexTM.

The HLA genomic typing to class I (*locus* A and B) and class II (*locus* DR) were obtained from peripheral blood samples. The study of cytokine single nucleotide polymorphisms (SNP's) were also performed in the promoter region of IL-1 (α, β, R, RA), IL-2, IL-4, IL-6, IL-10, IL-12, IFN- γ , TGF- β and TNF- α .

2.1.1.3. Statistical analysis

Statistical analysis was performed using SPSS[®] Statistics 17.0 software. Were analyzed the frequency distribution for the different qualitative variables. For quantitative variables Average and Standard Deviation (SD) were calculated, according the two patients groups (A and C).

Differences between the two groups were analysed by means of χ^2 tests and Mann–Whitney test (two independent samples) for qualitative and quantitative variables respectively. A statistical significant difference was assumed with $p < 0.05$.

2.1.1.4. Results

In allergic group (A), it was observed a higher genotype frequency of A^*01 and A^*68 alleles, while A^*02 presented a lower frequency (Figure 1), compared to non-allergic healthy group (C). Concerning locus B (Figure 2), the most frequent allele was B^*51 , while B^*35 was less frequent in the allergic population. With respect to HLA class II (Figure 3), the alleles DRB1*03 and DRB1*11 were more frequent in group A, while for DRB1*04 and DRB1*13 it was the opposite. None of these differences was statistically significant. The $A^*01\ B^*08\ DRB1*03$ haplotype was the most frequent in both groups. Nevertheless in allergic patients the $A^*02\ B^*51\ DRB1*11$ haplotype was the second most frequent, but the fifth one in healthy individuals (5.8% vs 1.4%, $p=0.059$).

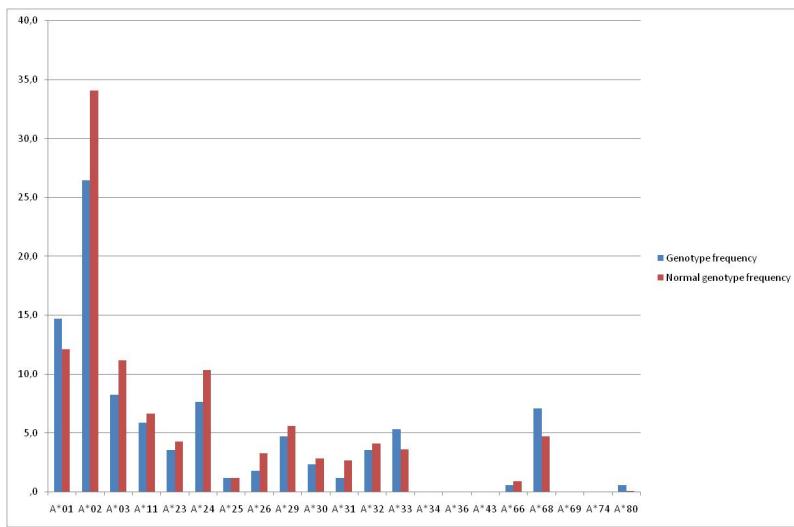


Figure 1. HLA class I, locus A. Frequency distribution of allergic patients (blue) compared to control group (red), not significant

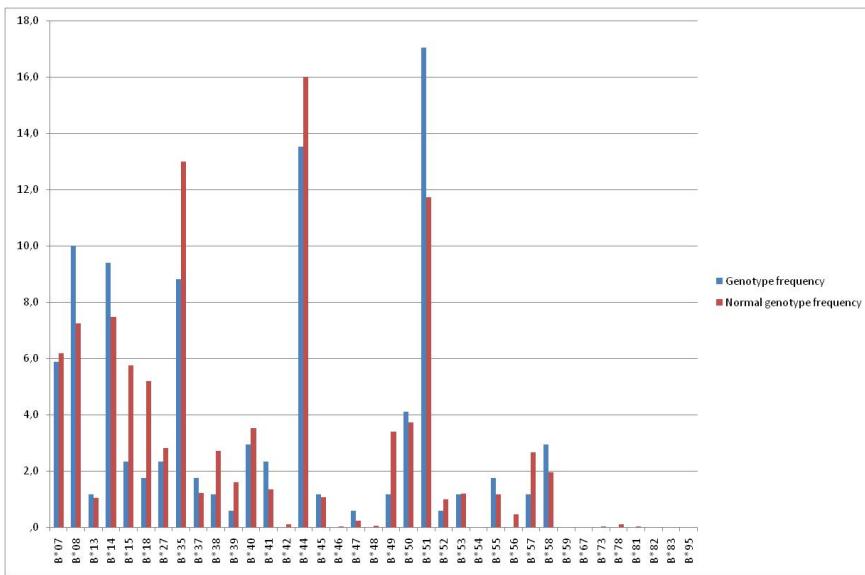


Figure 2. HLA class I, locus B. Frequency distribution of allergic patients (blue) compared to control group (red), not significant

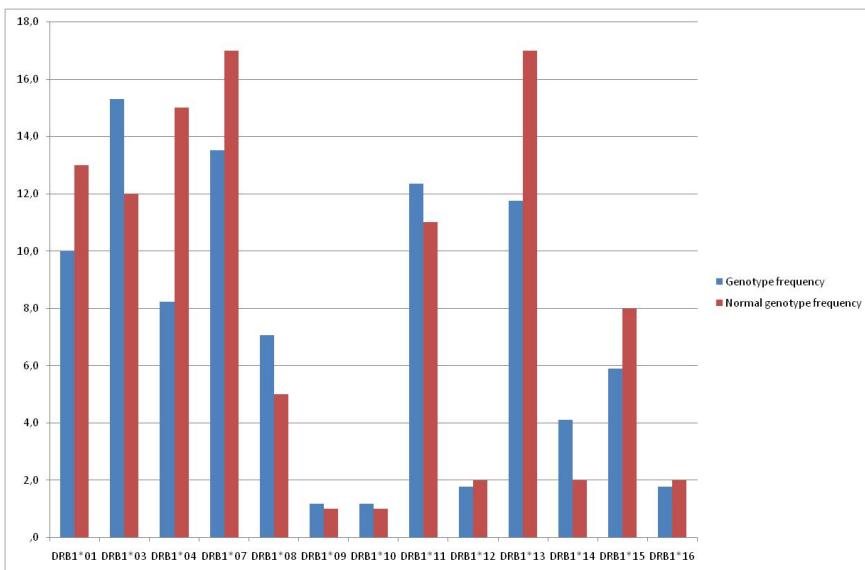


Figure 3. HLA class II, locus DR. Frequency distribution of allergic patients (blue) compared to control group (red), not significant

Results of phenotypic frequency of cytokine single nucleotide polymorphisms (SNP's) in the promoter region for subjects in the study the results are expressed in Figures 4 and 5.

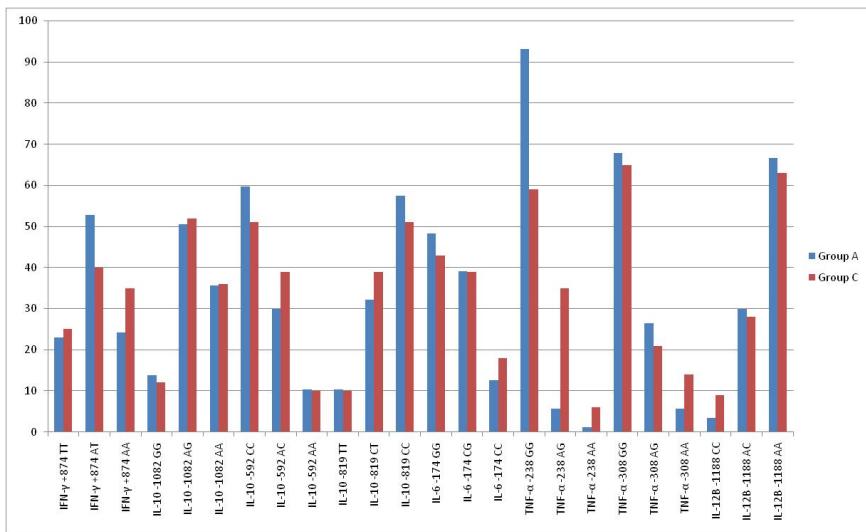


Figure 4. Cytokine single nucleotide polymorphisms (SNP's) : IFNg, IL-10, IL-6, TNFa, and IL-12b Frequency distribution of allergic patients (blue) compared to control group (red)

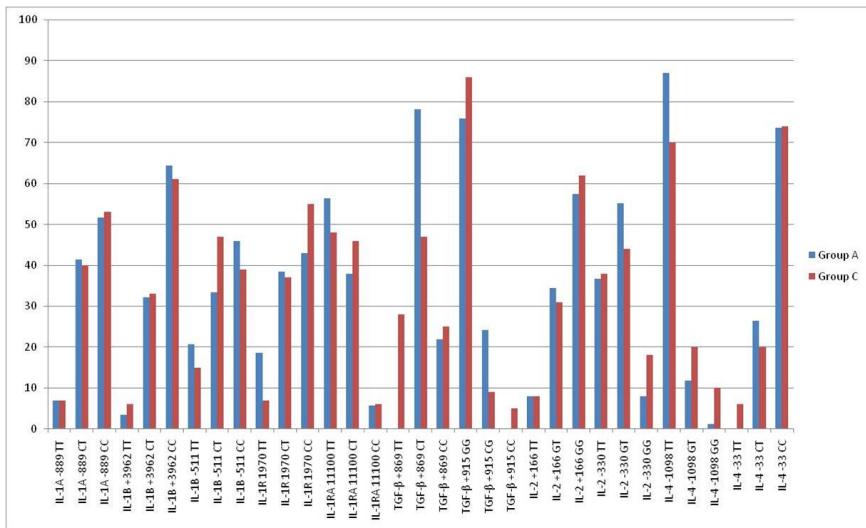


Figure 5. CCL4 gene expression in the patients submitted to CPT and NPT, before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

It was found a higher phenotypic frequency for the following cytokine SNP's: TNF α -238GG ($p<0.0001$), TGF β +869CT ($p<0.0001$) and IL4-1098TT ($p=0.003$). On the other hand, there were lower frequencies for TNF α -238AG ($p<0.0001$), TGF β +915CC ($p=0.024$) and IL4-1098GG ($p=0.005$). In the allergic group it was not found any homozygous individual for TGF β +869TT ($p<0.0001$), but conversely There was an increase of heterozygous individual compared with the control group ($p=0.004$).

No differences were observed between patients with concomitant asthma complaints and those with only rhinitis symptoms.

2.2. Gene expression and allergy challenge test

2.2.1. Subjects

From the previous mentioned 85 voluntary adult patients studied, with respiratory allergy to *Dermatophagoides pteronyssinus* (Dp) we selected a sample of 42 patients. These patients were enrolled to being submitted to an specific allergic challenge test, so H1-antihistamines and corticosteroids, either nasal or oral, were stopped 2 weeks and 4 weeks prior to the challenge test, respectively.

The specific allergic challenge test was performed using a standardized lyophilized Dp extract by nasal and ocular in 21 patients for each methodological procedure [21]. All patients were submitted to a previous respiratory function test (pletismography using *Master screen Body Jaeger®*) to assure the standard safe criteria ($FEV_1 \geq 80\%$ and $FEV_1/FVC \geq 80$).

Symptoms as dyspnoea, thoracic oppression, wheezing or cough were asked to being mentioned to all patients after challenge test.

The local ethics committee approved the study and all the participants gave written informed consent before entry.

2.2.2. Specific nasal and conjunctival challenge tests

All the patients performed skin prick tests with an aqueous extract of Dp with 5 mg/ml concentration (23 mg/ml of *Der p 1*, BialAristegui, Bilbao, Spain), with the dilutions of 1/1, 1/10, 1/100 and 1/1000 and negative and positive controls, according to standardized procedures [21, 54] (Dreborg 1993). The concentration used for specific provocation was the minimum that induced a prick test wheal at least equal to that induced by histamine.

All the specific challenge tests were performed after a period of adapting of room temperature for at least 30 minutes, and at the same period of the morning.

Nasal provocation test (NPT) were carry out by the administration of 160 μ l, corresponding a 2 consecutive puffs, of aerosolized spraying of Dp extract administered to the unilateral inferior nasal turbinate of the less congested nostril, and asking patients to perform apnea during the application of the allergen. Conjunctival provocation test (CPT) comprised on the unilateral

administration of 50µl, one drop, of allergen extract in the inferior and external quadrant of the bulbar conjunctiva.

Both ocular and nasal symptoms were recorded after challenge test and registered filled at the 1st and the 5th minutes, according specific clinical score system [21, 39].

2.2.3. Clinical score scaling

For evaluation the clinical responses an adaptation of the previously used a nasal clinical score, NCS, [39] and ocular clinical score, OCS, [48] were applied at the 1st and the 5th minutes [21, 54] as well as the total clinical score (TCS), representing the sum of NCS (range: 0-15) and OCS (range: 0-13) ranging from 0 to 28 points.

Although the late record symptoms occurred 5 minutes later after the challenge test all patients remained under strictly clinical observation for at least 4 additional hours. Clinical evaluation scores was interrupted after the 5th minute, but all the patients maintained medical observation during the next 4 hours.

2.2.4. Patient groups

Blood samples were collect to PAXgene blood RNA tubes to all patients before the allergic challenge test (NPT or CPT) and at 60 or 240 minutes, according to the following subgroups.

T0 and T60 minutes	T0 and T240 minutes
CPT	CPT
n=11 [F=8+M=3]	n=11 [F=7+M=4]
Age: 28.36±5.0 years [20-36]	Age: 27.90±6.8 years [17-39]
NPT	NPT
n=12 [F=6+M=6]	n=10 [F=6+M=4]
Age: 32.5±8.7 years [19-43]	Age: 25.3±7.5 years [18-43]

2.2.5. Methods

Peripheral blood was collected in a PAXGene Blood RNA tube (Qiagen) and total RNA extraction performed with the PAXgene Blood RNA kit (Qiagen) according to the supplier's instructions in a QIAcube BioRobot [59]. Total RNA quantification and RNA integrity evaluation was analyzed using a 6000 Nano Chip kit, in an Agilent 2100 bioanalyzer (Agilent Technologies) and 2100 expert software.

RNA was reverse transcribed with SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen), according to the manufacturer's instructions. Relative quantification of gene expression by real-time PCR was performed in the LightCycler 480 II (Roche Diagnostics).

Real-time PCR reactions were carried out using QuantiTect SYBR Green PCR Master Mix and QuantiTect Primer Assay (Qiagen) and 20 ng of cDNA sample, in a total volume of 10 µl. All samples were run in duplicate.

Real-time PCR results were analyzed with the LightCycler software (Roche Diagnostics). GeNorm Reference Gene Selection kit (PrimerDesign Ltd) in conjunction with the geNorm software (PrimerDesign Ltd.) were used to select the reference genes to normalize data. The normalized expression levels of the genes of interest were calculated by using the delta-Ct method.

2.2.6. Statistical analysis

Statistical analyses were performed using SPSS® Statistics 17.0 software. To determine the statistical significance of the differences observed, the non-parametric Mann–Whitney U-test and Wilcoxon paired-sample test were performed. A statistical significant difference was assumed with $p < 0.05$.

2.2.7. Results

All the patient groups demonstrated clinical positive response to a single dose of *Dp* extract [21], the symptoms were progressively decreasing, without need of therapeutic intervention. There were neither bronchial symptoms nor systemic reactions in any of the provocation tests.

It was observed in most of the patients, even at 60 minutes after challenge, differences in gene expression for cytokines, chemokines and nuclear transcription factors related to allergen exposure.

No differences were observed between patients with only nasal complaints compared to those that had associated clinical asthma.

We did not observe a similar pattern between the two routes of specific allergen challenge and in the two different periods of time in analysis.

Results of some chemokine gene expression after specific allergen challenge test are presented in figures 6 to 8.

The cytokine gene response to allergens did not show a homogeneous profile in the subgroups of patients studied, but it was evident that individually, there were changes in mRNA expression in many patients, as seen in Figures 9 and 12.

So we can distinguish two different groups of patients: one with very high values (also with high values of total serum IgE but without correlation with the clinical severity- data not shown) and the other with low expression of gene levels (Figures 13 and 14). However, this difference was not found for the cytokine receptor gene, IL-4R, Figure 15.

Figures 16-18 show the results of specific response to allergens in genes related to nuclear transcription factors on the four subgroups of patients.

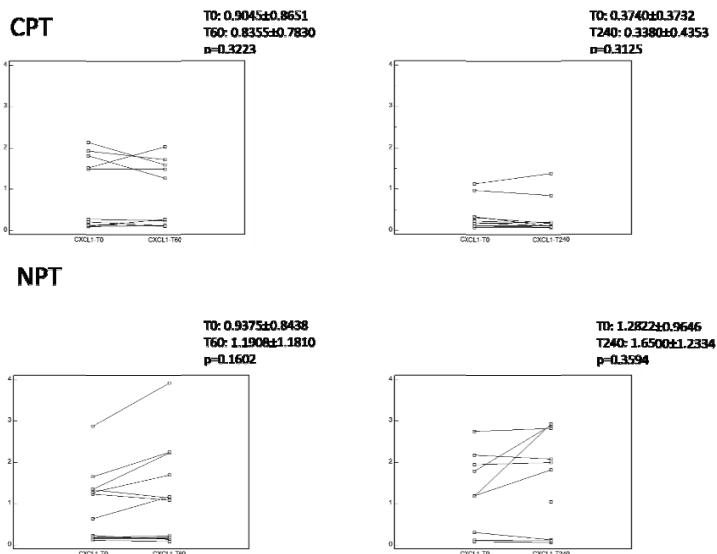


Figure 6. CXCL1 gene expression in the patients submitted to CPT and NPT, before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

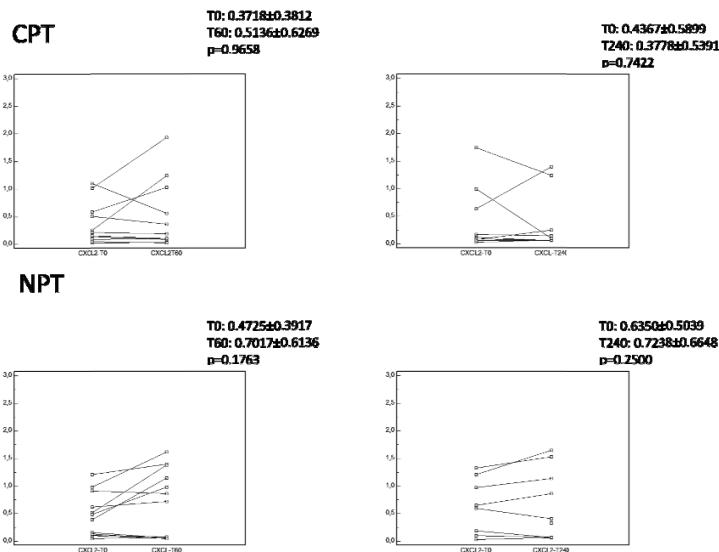


Figure 7. CXCL2 gene expression in the patients submitted to CPT and NPT, before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

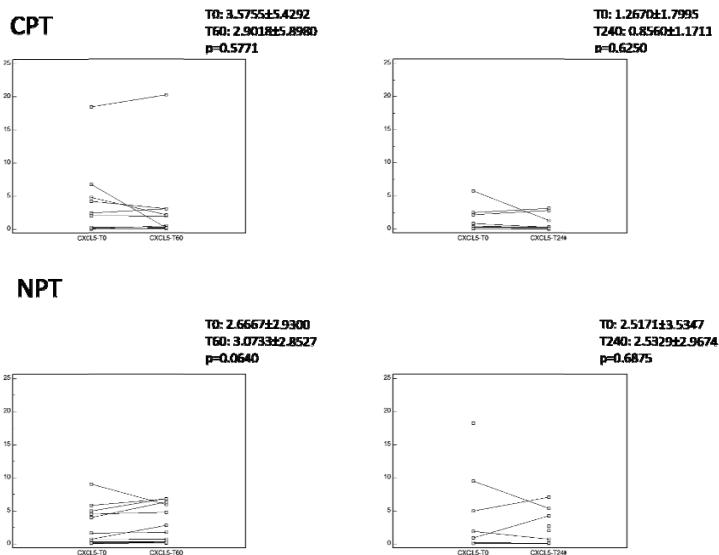


Figure 8. CXCL5 gene expression in the patients submitted to CPT and NPT, before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

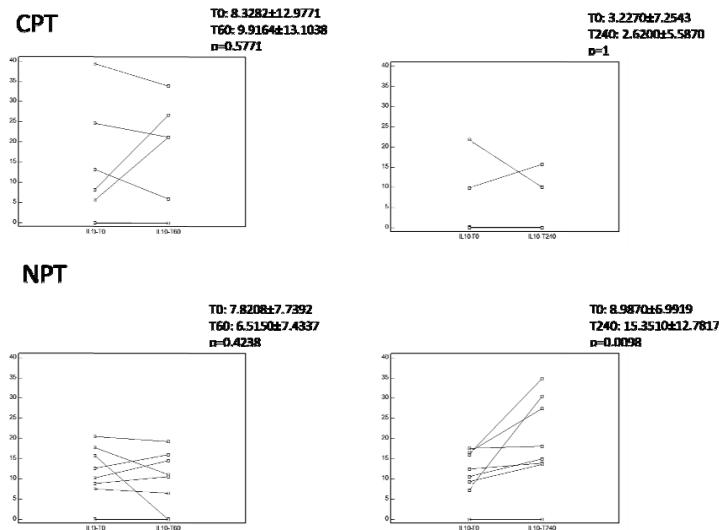


Figure 9. IL-10 gene expression in the patients submitted to CPT and NPT, before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

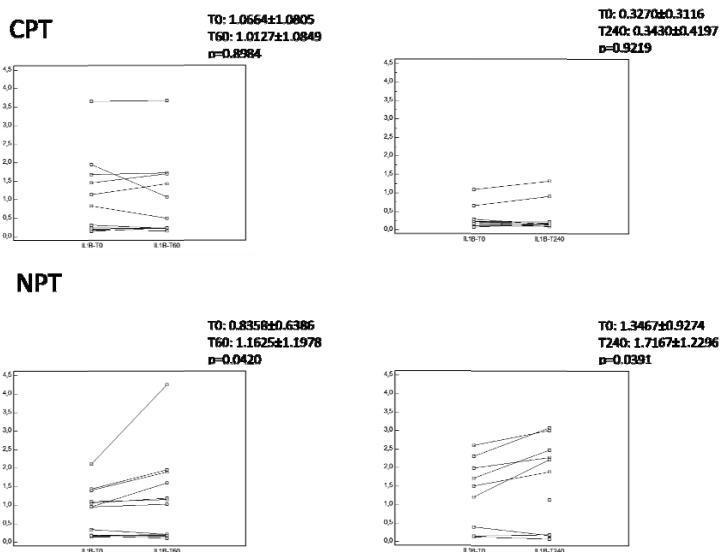


Figure 10. IL-1 β gene expression in the patients submitted to CPT and NPT, before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

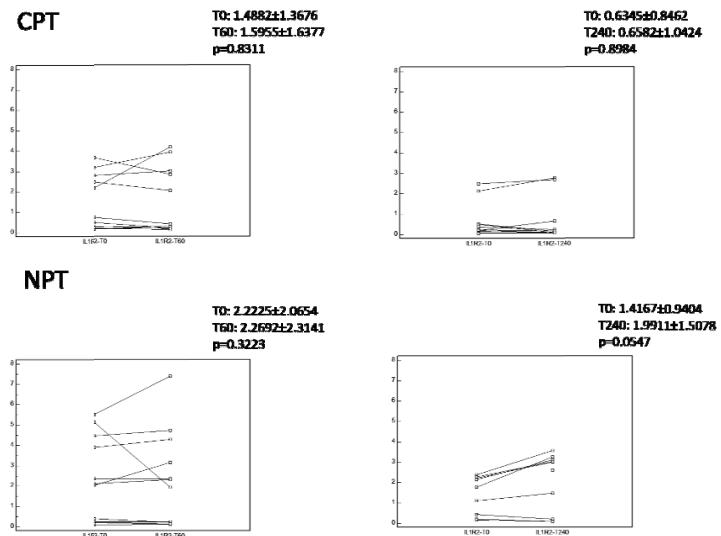


Figure 11. IL-1R2 gene expression in the patients submitted to CPT and NPT, before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

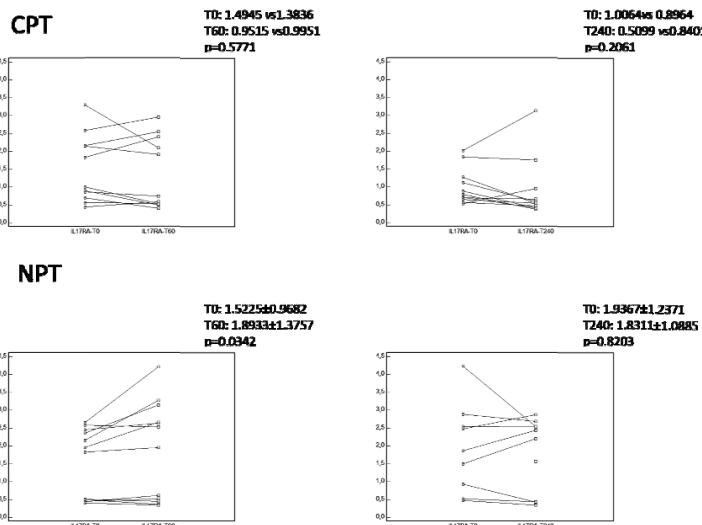


Figure 12. IL-17RA gene expression in the patients submitted to CPT and NPT, before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

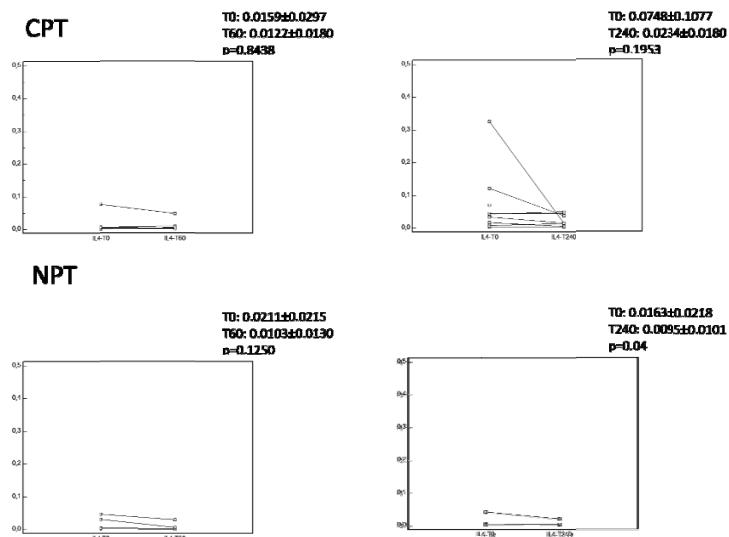


Figure 13. IL-4 (low levels) gene expression in the patients submitted to CPT and NPT before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

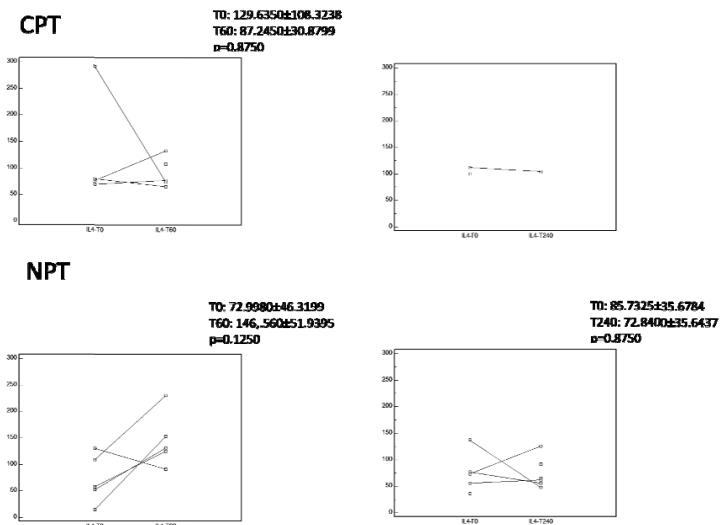


Figure 14. IL-4 (high levels) gene expression in the patients submitted to CPT and NPT, before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

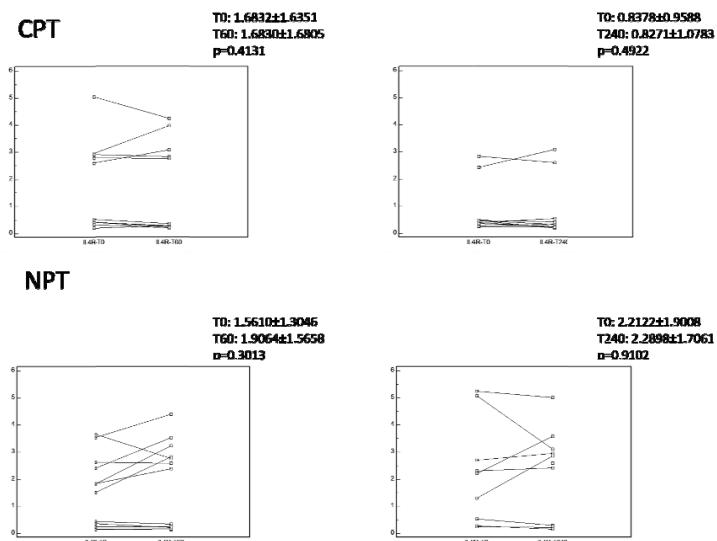


Figure 15. IL-4R gene expression in the patients submitted to CPT and NPT before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

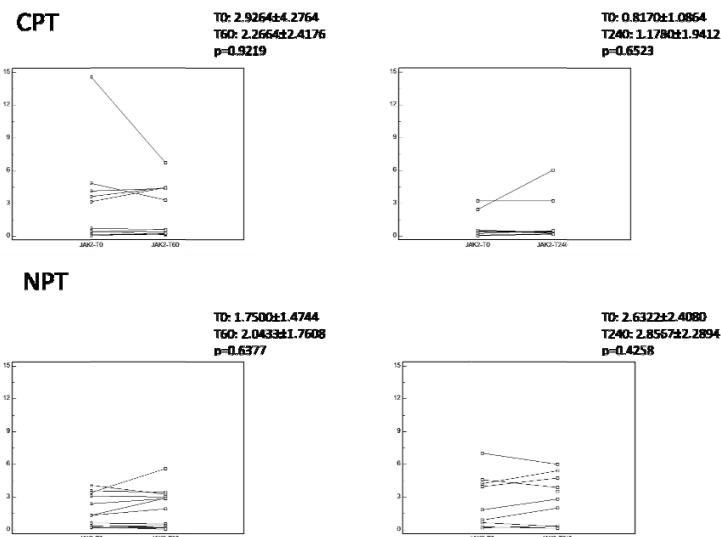


Figure 16. JAK2 gene expression in the patients submitted to CPT and NPT, before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

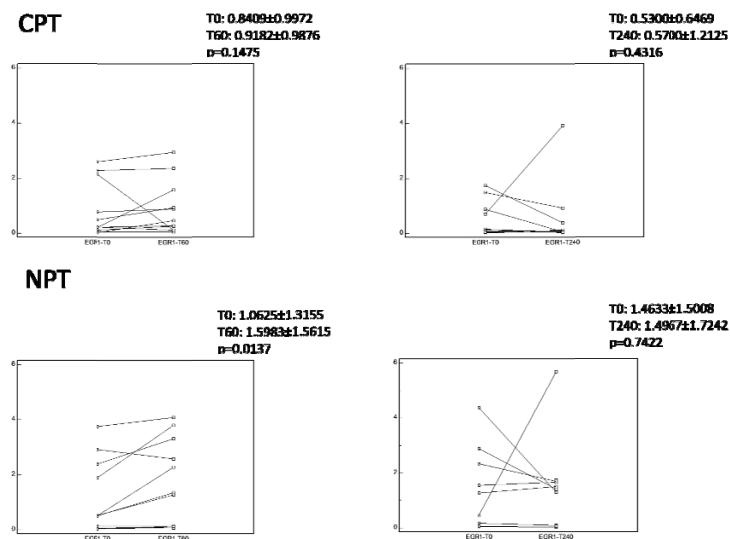


Figure 17. EGR1 gene expression in the patients submitted to CPT and NPT, before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

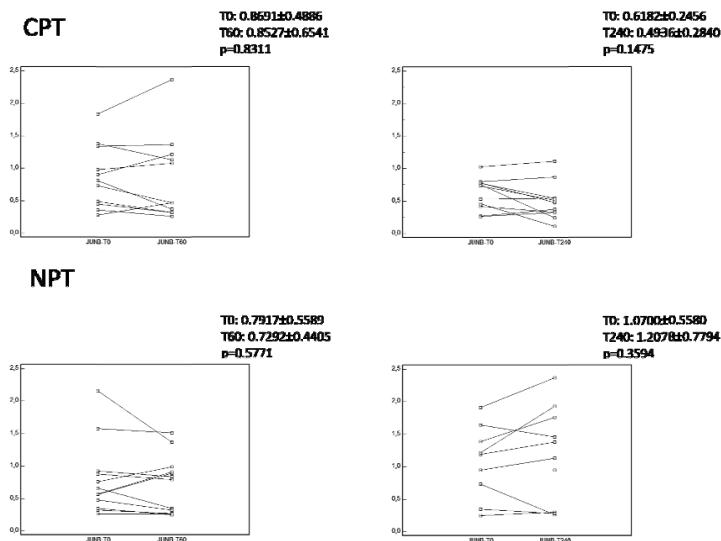


Figure 18. JUNB gene expression in the patients submitted to CPT and NPT, before challenge (T0) and at 60 and 240 minutes after challenge (individual response values, average and SD).

Overall, there seems that does not exist a specific profile of genes in response to cytokines, chemokines and nuclear transcription factors. However, even in first period of time after challenge (T60), many patients showed marked changes, since the beginning of the allergic reaction (Figures 19 to 21).

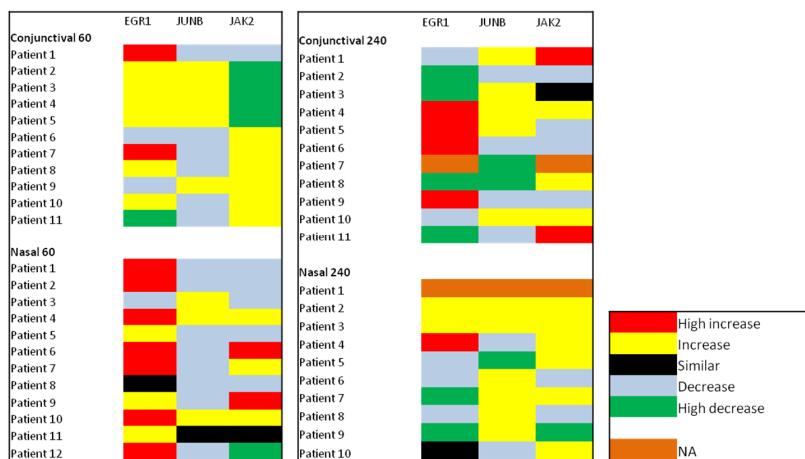


Figure 19. Global nuclear transcription factor gene responses in the patients submitted to CPT and NPT, according to the periods of time in study (A: 60 minutes; B: 240 minutes).

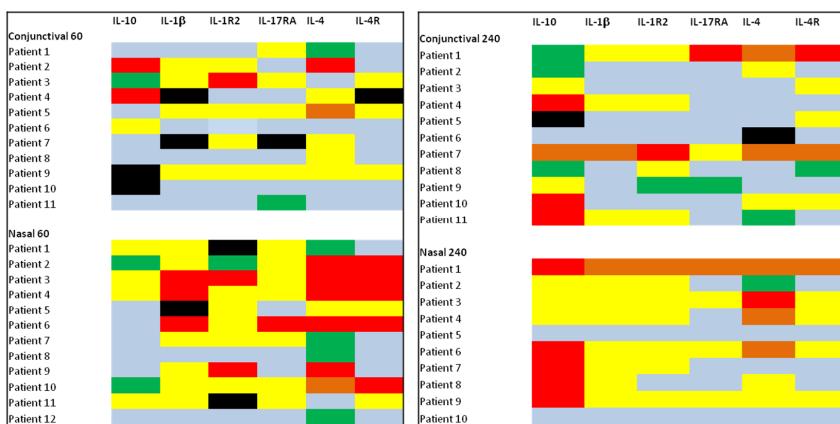


Figure 20. Global cytokine gene responses in the patients submitted to CPT and NPT, according to the periods of time (A: 60 minutes; B: 240 minutes).

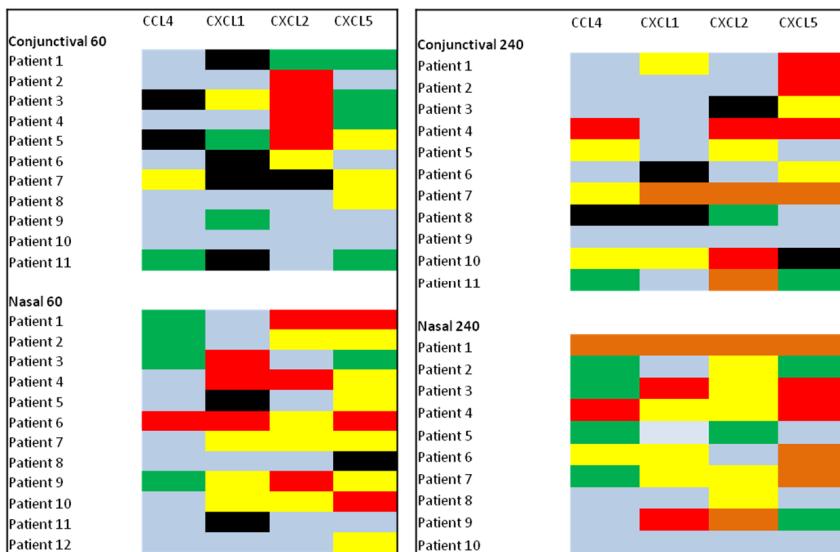


Figure 21. Global chemokine gene responses in the patients submitted to CPT and NPT, according to the periods of time (A: 60 minutes; B: 240 minutes).

2.2.8. Discussion

Allergic diseases and particularly respiratory allergy are complex disorders often present in the same family or closely related subjects. Genetic factors undoubtedly contribute to disease

susceptibility but the expression of the disease can be modulated by environmental exposures and the interactions between the two. In this context it is emphasized the role of infection, allergens, pollutants, oxidative stress and cytokine dysregulation [50]. In the case of allergy and asthma in particular because they constitute a complex and multifactorial disease, each of the individual factors by itself can determine distinct phenotypes that result in the polarization of distinct cellular pathways, resulting into the final clinical expression.

The last decade has been marked by the publication of a lot of genome-wide association studies (GWAS) in asthma or allergy phenotypes. GWAS have reported novel and interesting genes but have also confirmed the role of some functionally relevant genes previously described. However, heritability of allergic diseases has not been elucidated completely so far [1].

Considering that respiratory allergy, like epigenetic mechanisms, is heritable, nevertheless exists a strongly familial condition (36% to 79% heritability) with a non-Mendelian pattern of inheritance and polymorphisms in more than 100 genes, but these associations have infrequently been replicated, scientifically supported, and so the genetic has explained only a small portion of the cause of this disease [80, 81].

In the selection of the sample of our patients we were intended to be homogenous, in particular to the type of allergen sensitivity, ethnicity and area of residence and similarly of environmental surrounding.

Although we found some more frequent haplotypes in allergic patients we could not identify significant differences compared to healthy subjects. However, the second most frequent haplotype was *A*02 B*51 DRB1*11*, corresponding to the fifth most frequent in control group.

Ours results are according to others studies, namely in Greek allergic children, that also failed to demonstrate a positive correlation between *HLA* haplotypes and allergy to *Dermatophagoides* species [53]. So, as previously reported by a European study, it seems reasonable to assume that specific sensitization is influenced by genetic variants leading to its initiation, as well as to its enhancement [32].

HLA super-locus is a genomic region in the chromosomal position 6p21 [31]. It is highly promising for future research particularly since it carries on the correlation of *HLA-DRB1* in allergic asthma, *HLA-DQB1* in occupational asthma and *HLA-DPB1* in aspirin-sensitive asthma. However, it is difficult to study the role of class II genes *in vivo* because of the tremendous heterogeneity of human population, the strong linkage disequilibrium among different class II genes, and also the highly complexity of MHC.

An interesting issue in this subject is the “epistasis”, representing the action between 2-loci, in opposite side to Mendel’s classical law of heredity. Epistasis designation applies when two or more loci interact to create a new phenotype, and when an allele at one locus masks or modifies the effects of alleles at one or more other loci. The phenotypic effect of a specific gene mutation entirely depends on the overall genetic background affected by many other gene mutations. Furthermore, the combined effects of more than 2 loci on phenotypes are not simply additive, but are either synergistic or antagonistic [81].

A wide range of studies has demonstrated the enormous variability of clinical phenotypes in allergic disease and respiratory allergy, representing the tremendous variability and gene diversity.

An interesting study, focused on single nucleotide polymorphisms (SNPs) from 17q21 locus, demonstrated scattering different frequencies of SNPs between distinct ethnic populations [36]. Therefore, comparisons of results between different populations are often hazardous as explained earlier.

Regarding the results of SNPs in our patients with respiratory allergy we stress relevant differences concerning IL-4, TNF- α and TGF- β genes.

TNF- α and IL-4 are relevant cytokines interested in the allergic reaction and also promotes the development of Th2 cell response to aeroallergens [7]. In the population that we studied we found strong increment of TNF α -238GG and IL4-1098TT phenotypes compared to healthy individuals. Conversely a decrease in phenotypic expression was found to TNF α -238AG and IL4-1098GG.

TGF- β is a master regulator of the immune response and exerts important anti-inflammatory functions [68] and is deeply involved in allergy. In our allergic patients TGF β +869CT phenotype had a clearly increased frequency, and the genotype TGF β +869TT was completely absent compared to healthy population.

So far, as our knowledge, this phenotype was not previously involved in allergy or asthma. A study in cystic fibrosis demonstrated that higher plasma levels of TGF β 1 were observed in patients carrying the TGF β 1 +869TT genotype, intermediate levels for +869CT genotype and low levels to +869CC genotype. Together with these results it was also observed that the +869CC genotype had a more severe phenotype, and the +869TT genotype had the higher rate of decline of lung function, compared to +869CT genotype [17].

The IgE-mediated allergic reaction dictates a premature systemic effect, which will develop simultaneously to immuno-inflammatory mechanisms at the local of allergen exposure. In previous studies we have demonstrated that in parallel to the immediate response that results from an IgE-dependent allergenic mechanism, the cellular response occurs early in the process, and the involvement of the central immune organs is crucial since the beginning of the reaction [54, 55].

In this study we provide evidence that mRNA gene expression take place earlier on peripheral T cells after allergen exposure, at least 60 minutes later. This fact occurs independently of the technique of allergen provocation, in spite of the differences on the mechanisms and cell traffic ways involved, because they are distinct mucosae. Obviously, this modification in the production of mRNA does not imply that all of it will be completed in an efficient manner, particularly since there are strictly necessary requirements in cell regulation and biological skill conditions that allows the synthesis of the protein or its biological mediator.

Naturally, this modification in the production of mRNA does not imply that all of it will be completed in an efficient manner, particularly since in cell regulation there are specific

requirements and biological conditions allowing the synthesis of the corresponding protein or its biological mediator.

In this context, it is very important to analyse the individual results in the different subgroups, since the high individual variation does not allow an unequivocal evaluation of the average values of the sample. For this study we did not consider to be relevant the existence of a separate control group of allergic patients, since the time point T0 would be a better internal control [54]. It was also evident that the individual response of each patient is highly variable, most likely depending on the genetic endowment of each patient, although the clinical pattern and the allergic sensitization (D_p) was the same.

During the inflammatory mechanism a set of different cytokine and chemokine molecules are able to promote not only the appropriate regulation of leukocytes and other cells recruitment but also the efficient network that permitting an accurate intracellular signaling control [73].

The existence of 23 chemokine receptors and 48 chemokine ligands guarantees a tight control and fine-tuning of the immune system [83]. Conceptually, it is considered that these biological mediators require long periods of cell maturation and synthesis, as described in several papers using culture cells [25; 29].

However, our results support the involvement of genic cytokines and chemokines are earlier events, indeed consistent with the early cell trafficking between places of allergenic aggression and those dependent on the central and secondary immune system, as noted in previous work

However, our results support that the involvement of genic cytokines and chemokines are earlier events, indeed consistent with the quickly cell trafficking between the local site of allergenic aggression and those sites dependents on the central and secondary immune system, as noted in previous research from our group [55].

More than a common standard for the same respiratory allergic disease and for the same the most important allergic sensitization is the observation of the individual profile of each patient. Indeed, IgE-mediated allergy in spite of a pathogenic mechanism conductor has at clinical level, is extremely heterogeneous. The results shown in Figures 18 to 20 also showed a tremendous heterogeneity in gene expression after allergen provocation, between our patients.

The gene expression levels of IL-4 results are extremely interesting, because unquestionably two distinct profiles are observed (high and low levels), but without correspondence with those achieved for IL-4R. Also, the level of gene expression of IL-4 was correlated with total serum IgE levels, but without correlation with the clinical severity profile, as supported by clinical practice [18].

Unlike other diseases, allergy and asthma appear to be arguably multifactorial and multigenic disorders, and therefore it has not been possible identify till now one or a set of specific genes that may be genetic markers [57, 81], although recent paper identifies the involvement of *GSTO2* loci in asthma, and *GSTA1* as risk factor for asthma and allergies in Italian patients [56], while Genome-wide association studies (GWAS) represent the most powerful approach for asthma, have identified several distinct others genotypes from IL-18R1, IL-33, *SMAD3*, *ORMDL3*, *HLA-DQ* and IL-2RB loci [31].

As mentioned before, allergy and asthma are chronic inflammatory and polygenic disorders, and so any isolated specific gene could claim be an asthma gene. Therefore, recently in order to best understanding the intricate heterogeneity of this disease the designation of phenotype and endotype have been used to stress the complexity of the mechanisms and clinical patterns [10, 31].

In the pathogenesis of chronic inflammatory diseases such as asthma several transcription (signal transducer and activator) factors are known to play a crucial role, acting as agonists or antagonists [79]. A transcription factor is formed by a sequence-specific DNA-binding factor. It is a protein that specific binds to specific DNA sequences, so it is able to controlling the rate of transcription of genetic information from DNA to messenger RNA.

These factors perform this purpose alone or with other proteins in a complex, by promoting (activator), or blocking (repressor) the recruitment of RNA polymerase. These enzymes permit the efficient genetic transcription from DNA to RNA to a specific gene.

A defining feature of transcription factors is that they contain one or more DNA-binding domains, which linked to specific sequences of DNA adjacent to the genes that they regulate.

For an efficient transcription are needed additional proteins (not classified as transcription factors) such as coactivators, chromatin remodelers, histone acetylases, deacetylases, kinases, and methylases, having crucial roles in gene regulation, but lack DNA-binding domains.

So these factors are very important and therefore we also studied three of them.

EGR1, also knew as early growth response protein 1, have engaged in multi functions such as transcriptional regulator, mitogenesis, cell proliferation and differentiation regulation, apoptosis and tumor suppressor gene [28]. Previous report had highlighted the potential stimulatory and inhibitory effects on *EGR1* by *STAT6* (Th2 differentiation enhancer) and *STAT1* (Th1 differentiation enhancer), respectively.

Overall our patients, we have observed at 60 minutes later the allergen exposure an increase of expression of *EGR1*, favoring the allergic reaction, but at 240 minutes after provocation levels had decreased, possibly due to mechanisms of self-control of allergic reaction.

In opposite, the *JUNB* expression decreased at least after 60 minutes of the beginning of the allergic reaction, and going increasing later at 240 minutes. This factor is mainly a Th2 cell related transcription factor (IL-4), but also have role favoring other cytokines: IL-1, IL-2, and IL-10 [79]. So, we hypothesize that the earlier involvement could favouring the beginning of the allergic reaction, but also could be later a down-regulator factor.

JAK2 belongs to a tyrosine kinase family, and its activation engages the transcription factor *STAT3* to initiate *JAK-STAT* pathway [82]. It signalizes the interferon receptors, and GM-CSF receptor family (IL3R, IL5R, GM-CSF-R). In our patients we did not observed. In our patients, we did not observe relevant performance for this transcription factor.

Allergic diseases are complex and multifaceted, and asthma in particular was widely recognized as condition in which both genes and the environment play critical roles.

Currently, GWAS try to identifying a number of candidate genes evocative of biological relevance, but have failed to report for more than a reduced amount of phenotypic variance – a situation that has come to be known as the problem of the missing or hidden heritability [13, 76].

In our patients we could verify that the allergen challenge determines a very early cellular response with gene and protein expression.

At present, the environmental influence with direct effects on epigenetic plan offers an explanation for this extreme individual variability, one clinical disease but different phenotypes and endotypes, not yet fully characterized.

So, in spite of the same genetic heritage, each individual and each patient, with an equal number of chromosomes and genes, have a specificity and uniqueness: all equals but all different from one to each other.

In Figure 22 we present the results of 4 allergic patients with asthma and rhinitis.

		IL10	IL1B	IL1R2	IL17RA	IL4	IL4R
Severe	Conjunctival 240	↓↓	↓	↓↓	↓	↓	↓
Moderate	Nasal 240	↑	↑	↑	↓	↓↓↓	=
Mild	Nasal 60	↓↓↓	↑	↓↓	↑	↑↑↑	↑↑
Moderate	Conjunctival 60	↓	↓	↓↓	↓	↓	↓↓

		EGR1	JUNB	JAK2
Severe	Conjunctival 240	↓	↑	=
Moderate	Nasal 240	↑	↑↑	↓
Mild	Nasal 60	↑↑	↓	↓↓
Moderate	Conjunctival 60	↑	↓↓	↓

		CCL4	CXCL1	CXCL2	CXCL5
Severe	Conjunctival 240	↓	↓	↓	↓
Moderate	Nasal 240	↓	↓	↑	↓↓
Mild	Nasal 60	↓↓	↓	↑	↑
Moderate	Conjunctival 60	↓	↓	↓	↓

Figure 22. Overall results of 4 patients with allergic asthma and rhinitis

Gene expression of cytokines and chemokines seem to have a strictly correlation with the results obtained in the study of nuclear transcription factors, *EGR1* and *JUNB*, as discussed before.

The susceptibility to elicit an allergic response or in opposite side its own inhibition is under liability of a time-dependent dynamic response since the allergen exposure. It might be more appropriate to view genes as a frame surrounding a complex network of biological processes. The frame delimits and supports the network, but the connections within the network determine its functions.

Besides the identification of novel pathological mechanisms and therapeutics, the wealth of information leads to the vision of genes forecasting asthma risk and phenotypes, with genetic scores and eventual personalized medicine with more effective treatments. Gene-environment interactions might difficult the achievement of such knowledge. Thus, even after the completed sequence of the human genome, the scientific community maintains expectations that genetics may be a decisive tool in the allergic risk prediction and personalized tailor-made treatments. At present asthma epigenetics is still in its infancy and a promise to keep [13].

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