



Biomarkers associated with disease severity in allergic and nonallergic asthma



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ARTICLE INFO

Article history:

Received 26 October 2016

Received in revised form 2 December 2016

Accepted 8 December 2016

Keywords:

Asthma

Allergy

Biomarkers

Gene expression

Severity

ABSTRACT

Asthma is a complex, chronic respiratory disease with a wide clinical spectrum. Use of high-throughput technologies has generated a great deal of data that require validation. In this work the objective was to validate molecular biomarkers related to asthmatic disease types in peripheral blood samples and define their relationship with disease severity. With this purpose, ninety-four previously described genes were analyzed by qRT-PCR in 30 healthy control (HC) subjects, 30 patients with nonallergic asthma (NA), 30 with allergic asthma (AA), and 14 patients with allergy (rhinitis) but without asthma (AR). RNA was extracted from peripheral blood mononuclear cells (PBMCs) using the TRIzol method. After data normalization, principal component analysis (PCA) was performed, and multiple approaches were used to test for differential gene expression. Relevance was defined by RQ (relative quantification) and corrected *P* value (<0.05). Protein levels of IL-8 and MSR1 were determined by ELISA and Western blot, respectively.

PCA showed 4 gene expression clusters that correlated with the 4 clinical phenotypes. Analysis of differential gene expression between clinical groups and HCs revealed 26 statistically relevant genes in NA and 69 in AA. Protein interaction analysis revealed IL-8 to be a central protein. Average levels of IL-8 were higher in the asthma patients' sera (NA: 452.28 ± 357.72 , AA: 327.46 ± 377 pg/ml) than in HCs (286.09 ± 179.10), but without reaching statistical significance. Nine genes, especially MSR1, were strongly associated with severe NA.

In conclusion, several molecular biomarkers of asthma have been defined, some of which could be useful for the diagnosis or prognosis of disease severity.

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1. Introduction

Asthma is an inflammatory disorder of the airways characterized by chronic inflammation and hyperresponsiveness (AHR) as well as symptoms such as recurrent wheezing, cough, and shortness of breath. According to the World Health Organization, asthma is the most common chronic disease in children, affecting more

than 300 million children and adults (Mukherjee and Zhang, 2011). Many different clinical phenotypes of asthma have been identified. There are 10%–33% of individuals with nonallergic asthma (NA) or subjects with asthma in whom allergic sensitization cannot be demonstrated. However, allergic mechanisms have been implicated in 50%–80% of asthmatic patients and in approximately 50% of individuals with severe asthma (D'Amato et al., 2014; Holgate, 2012). For this reason, asthma has been associated with type 2 airway inflammation, which is characterized by elevated levels of immunoglobulin E, eosinophils, and several interleukins (IL) such as IL-4, IL-5, IL-13, and IL-9.

Asthma severity reflects the intensity of the disease. The Spanish Guidelines for the Management of Asthma divide asthma into the following categories: intermittent, mild, moderate, and severe

Abbreviations: GEMA, Spanish guide for asthma management; PBMCs, peripheral blood mononuclear cells; qRT-PCR, quantitative real-time polymerase chain reaction; PCA, principal component analysis.

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persistent. These categories are assigned according to day and night symptoms, frequency of medication intake, activity limitation, pulmonary function, and exacerbations (GEMA., 2009).

It is well-known that reaction to treatment varies between different asthma patients. Given this clinical heterogeneity, many authors have begun classifying asthma into “endotypes” (Fajt et al., 2015; George et al., 2015; Wenzel., 2012; Anderson, 2008). An endotype consists of several mechanisms that cannot be clearly separated. Their description may rely on biomarkers, which can be the signature of a complex underlying pathway or a key molecule associated with or directly playing a role in a particular disease endotype (Agache et al., 2015). New strategies for discovery and validation of biomarkers such as the –omics approaches have been used to explore the mechanisms responsible for the asthma endotypes present in different tissues; these approaches have been used in airway epithelial cells (Woodruff et al., 2009; Woodruff et al., 2007) and in sputum (Baines et al., 2010), but fewer studies have been done in human peripheral blood and, in particular, in peripheral blood mononuclear cells (PBMCs). The studies that have been done have helped to confirm previous results and have identified new genes that need to be investigated (Faiz and Burgess, 2012). Several biomarkers have been described for the Th2 (T-helper 2) endotype, such as exhaled NO, sputum or blood eosinophils, total serum IgE or specific IgE, serum periostin, sputum or epithelial-cell (bronchial or nasal) gene signature, or salivary inflammatory mediator profile. Recently, the first results of molecularly targeted therapies have been reviewed (Fajt et al., 2015). In asthmatic patients with Th2 inflammation, consistent efficacy was observed in therapies targeting the Th2 cytokines, while in patients lacking Th2 biomarkers, biologically targeted therapies are still in their infancy.

Given all this information, our aim was to validate and to define molecular biomarkers that differentiate asthma from allergic diseases and predict disease severity in non-invasive samples obtained from a clinically well-characterized population.

2. Materials and methods

2.1. Subjects

The study population comprised 104 unrelated subjects, 30 healthy control (HC) subjects, 30 patients with nonallergic asthma (NA), 30 patients with allergic asthma (AA) (allergic to airborne allergens), and 14 patients with allergy (rhinitis) but without asthma (AR) (allergic to airborne allergens). The selection criteria appear in Baos et al., submitted.

Informed consent was obtained from each subject. Ethical approval for the study was obtained from the ethical and research committees of the participating hospitals.

2.2. PBMC isolation and RNA extraction

PBMCs were isolated from heparin-containing peripheral blood samples by gradient centrifugation using Lymphoprep (Comercial Rafer, Zaragoza, Spain) following the manufacturer's instructions. Total RNA was isolated from PBMCs (10^6 cells) using the TRIzol method (Invitrogen, Carlsbad, CA, USA). RNA was quantified and its purity was checked by spectrophotometry with the nanodrop (ND-1000 Spectrophotometer) system.

2.3. Gene selection criteria

Ninety-four genes (Table 1, Baos et al., submitted) were selected from previous studies according to the criteria described in Baos et al., submitted.

2.4. Differential gene expression by qRT-PCR

Three hundred nanograms of RNA from each subject was analyzed in the Scientific Park of Cantoblanco (Madrid, Spain) by quantitative real-time PCR using the TaqMan Gene Expression System (Applied Biosystems, Foster City, CA, USA) in 384-well microfluidic cards (Applied Biosystems) that included the 94 selected genes and 18S as a reference gene. Briefly, reverse transcription was performed using “High capacity RNA to cDNA kit” (Applied Biosystems). Real time PCR was performed using Taqman Gene Expression and the HT7900 System (Applied Biosystems) with 40 amplification cycles. The results were analyzed with the SDS software (Applied Biosystems).

Specific mRNA expression from each gene was analyzed in triplicate, normalized into 18S rRNA gene, and calculated according to the cycle threshold (Ct) method. After data normalization, the global gene expression of the samples was checked by an unsupervised statistical procedure called principal component assay (PCA). This method reduces multidimensional data to fewer dimensions to facilitate analysis and visualization. That is, the method groups the data according to a distance without using any external information to organize the groups. PCA is very useful for identifying the most significant patterns of data.

2.5. Measurement of soluble cytokine levels

Levels of IL-8 in the subjects' serum were analyzed using the human IL-8 ELISA kit by Diaclone (Besançon Cedex, France) following the manufacturer's protocol.

2.6. MSR1 protein analysis

MSR1 determination was analyzed in 9 HCs and 18 NA patients (8 severe NA patients and 10 with moderate-mild diagnosis) by Western blot. Specific details are summarized in Baos et al., submitted.

2.7. Statistical analysis

Multiple comparisons were used to test for differential gene expression by using the StatMiner program (<http://www.integromics.com/StatMiner>). This program follows a simple, step-by-step analysis workflow guide that includes parametric, non-parametric, and paired tests for relative quantification of gene expression, as well as 2-way ANOVA for two-factor differential expression analysis. Significance was defined by RQ (relative quantification) <-2 or >2 and corrected *P* value (<0.05) adjusting the *P* value with the Benjamini-Hochberg FDR method. DAVID software v6.7 was used for functional gene analyses (<http://david.abcc.ncifcrf.gov/home.jsp>) and the STRING v10 free program (<http://string-db.org/>) was used for the analysis of protein interactions.

3. Results

3.1. Subjects

The demographic and clinical parameters of the population studied are summarized in Table 1. Mean age was slightly different among the groups: the NA subjects were significantly older than the other groups, and the AR subjects were the youngest, with statistically significant differences when compared to the HC and NA groups.

The mean levels of total IgE were increased in the 2 groups of allergic subjects ($P<0.05$) compared with both the HC and NA groups.

Table 1
Characteristics of the study population.

	N	Age	Smoking	Clinical diagnosis	Total IgE	% FVC	% FEV ₁
Healthy control (HC) subjects	30	45.66 ± 12.39	92.86% non-smokers 7.14% smokers 0% former smokers	Not applicable	75.02 ± 128.21	–	–
Nonallergic asthmatic (NA) subjects	30	58.03 ± 13.14*	73.33% non-smokers 10% smokers 16.67% former smokers	50% severe asthma 30% moderate asthma 20% mild asthma	82.04 ± 80.63	81.11 ± 22.69	75.18 ± 22.82
Asthmatic allergic (AA) subjects	30	42.37 ± 15.44#	68.97% non-smokers 6.90% smokers 24.14% former smokers	50% severe asthma 26.7% moderate asthma 23.3% mild asthma	371.64 ± 437.69*#	75.53 ± 16.58	73.33 ± 16.28
Nonasthmatic allergic (AR) subjects	14	34 ± 8.86*#	85.71% non-smokers 14.29% smokers 0% former smokers	No asthmatic symptoms	250.65 ± 249.07*#	–	–

% FVC: percentage of predicted value of forced vital capacity; % FEV₁: percentage of predicted value of forced expiratory volume in 1 s.

* Statistically significant comparison ($P < 0.05$) between HC and patients group.

Statistically significant comparison ($P < 0.05$) between NA and selected group.

Percentages of FEV₁ and FVC were similar between the 2 asthma groups; however, severe NA vs moderate-mild NA patients showed statistically significant differences (66.33 ± 16.62 vs 85.38 ± 21.03 , $P = 0.0127$ in % FEV₁; 69.93 ± 19.94 vs 94 ± 19.52 , $P = 0.0031$ in % FVC) (data not shown).

The number and percentage of eosinophils were higher in severe AA patients (739.5 ± 1180 , 5.88 ± 3.69) than in severe NA patients (264 ± 152 , 3.72 ± 2.47), but without reaching statistical significance (data not shown).

3.2. Gene expression clustering according to clinical group

Global gene expression analysis of the 94 genes in the 104 RNA samples was performed. After qRT-PCR data normalization, the unsupervised analysis by PCA showed 4 clusters that correlated with the 4 clinical groups (Fig. 1A). Using an unsupervised hierarchical model by which samples are compared two by two, we were able to cluster the samples according to gene expression, with a 100% specificity and sensitivity for NA and AA vs HC subjects (Fig. 1B and C). The sensitivity and specificity were slightly lower—92.86% and 96.3%, respectively—for discrimination of AR vs HC. Distinguishing between NA and AA was the most difficult according to gene expression, with a sensitivity of 66.6% and a specificity of 61.5%. Asthmatic (AA and NA) samples vs AR samples showed clustering exhibiting a sensitivity of 78.57%, and the specificity was 88.4% for AA and of 90% for NA.

To define specific gene profiles related with each clinical condition, several types of statistical analyses were performed. Ten genes (*CLCA1*, *IL17A*, *IL-25*, *IL-33*, *IL-5*, *IL-9*, *MUC5AC*, *POSTN*, *SERPINB4*, and *TCF21*) were found to be indeterminate in all the samples, and 10 genes (*ADAM33*, *CCL17*, *IL-13*, *MUC5B*, *MUC2*, *NOS2*, *TSLP*, *CCL11*, *DRB1*, and *IL-4*) were unable to be assessed because they were detected only in some conditions or had inconsistent data. Firstly, differential gene-expression comparisons of the 3 clinical groups against the HC group were carried out. Seventy-two genes (76.6% of the total) were differential between HC and at least one of the clinical conditions. The 2 allergic groups (AA and AR) showed a higher number of statistically different genes relative to the HC group and the NA group: 69 (1 upregulated *MSR1* and 68 downregulated) in the AA group, 66 in the AR group (all downregulated), and 26 (16 upregulated and 10 downregulated) in the NA group. Of these differentially expressed genes, 22 were common to the 3 clinical groups, 42 were common to both groups of allergic subjects, and one (*MSR1*) was common to the 2 groups with asthma (allergic and nonallergic). On the other hand, *SERPINB2* was significantly overexpressed only in the NA group. In contrast, *ADRB1*, *ALOX15*, *CTSG*, and *CX3CR1* were significantly underexpressed only in the

AA group. None of the analyzed genes was differentially expressed in only the AR group.

Functional analysis of the differential genes in each clinical condition compared to the HC group was carried out using the DAVID program, which analyses gene-set enrichment according to functional pathways and biological processes. The main functional annotation clusterings for each clinical condition are shown in Tables 2, 3, and 4. The main cluster is the one related with inflammation in allergic (asthmatic and nonasthmatic) conditions and with extracellular compounds in NA patients. The enrichment score was higher in allergic conditions.

This cluster analysis correlated well with the results of the functional protein analysis by the STRING program (Fig. 2), which shows the complex and strong interactions in allergic clinical phenotypes and a clearer network in the NA clinical phenotype, where there is a central nucleus composed of IL-8 and TNF.

In order to find genes specifically related with asthma and/or allergy, the next step was the analysis of differences in gene expression among the 3 clinical groups. A comparison between NA and AA subjects showed 74 genes overexpressed in the NA group. Five genes showed highest differentiation ($RQ > 10$): *CCL5*, *CHI3L1*, *CTSG*, *GZMH*, and *IL1-R2*. Comparison of NA and AR subjects showed 66 genes as differentially expressed, 64 of which were upregulated and only 2, *CHI3L1* and *PI3*, were downregulated in the NA group. Gene selection using a more strict criterion ($RQ > 10$) revealed that 10 genes were highly overexpressed in asthmatic subjects: *CCL5*, *CRTAP*, *GPX3*, *HLA-DQB1*, *IL-10*, *IL2RB*, *MSR1*, *NLRP3*, *PHLDA1*, and *SERPINB2*. *PI3* was the only downregulated gene. Finally, as seen in a comparison between allergic subjects (i.e., AA vs AR), 14 genes were differentially expressed, 4 of which were upregulated (*IL-10*, *MSR1*, *PHLDA1*, and *SERPINB2*) and 10 downregulated (*ALOX15*, *CHI3L1*, *CPA3*, *CTSG*, *IL1R2*, *IL8*, *NFKB1Z*, *PI3*, *SVIL*, and *TNF*) in AA subjects. *CHI3L1*, *CPA3*, *CTSG*, and *PI3* were extremely downregulated in AA subjects ($RQ > 10$) (Table 2, Baos et al., submitted).

3.3. Gene expression analyses according to severity

In the groups containing asthmatic subjects (allergic and non-allergic), which were composed of 50% severe asthmatic patients and 50% moderate-mild asthmatic subjects, we analyzed the relationship between severity and gene expression.

Firstly, the 2 subgroups classified according to severity within the same clinical group (NA or AA) did not show any statistically significant results. However, the gene-expression comparisons of these severity subgroups against the HC group revealed interesting data (Table 5). Five genes (*IL10*, *MSR1*, *PHLDA1*, *SERPINB2*, and *CD86*) were overexpressed and 4 genes (*CHI3L1*, *CPA3*, *IL8*, and *PI3*)

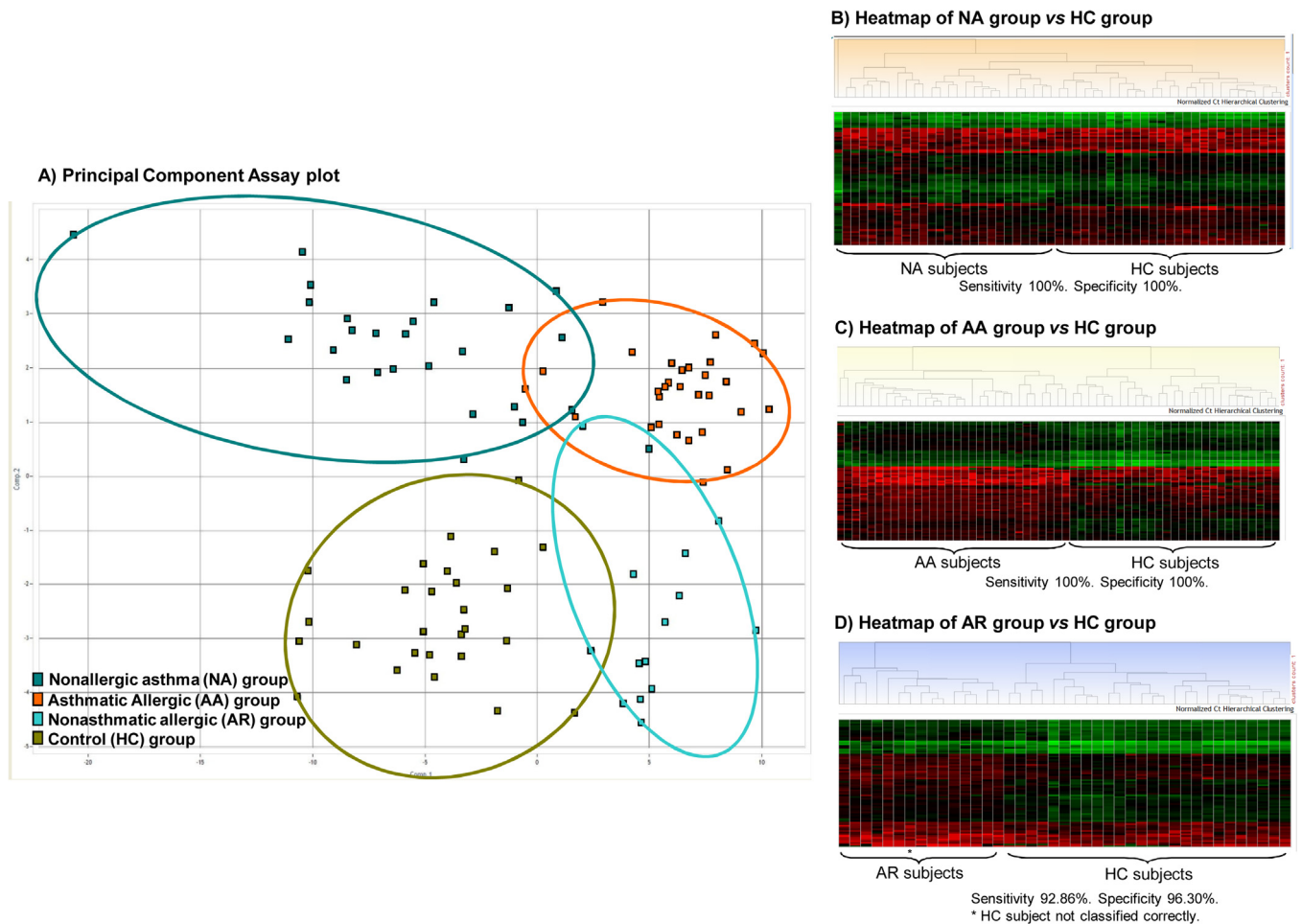


Fig. 1. Principal component assay plot and heatmaps of the four studied groups. a) Principal component assay plot; b) Heatmap of NA group vs HC group. Sensitivity 100%, specificity 100%; c) Heatmap of AA group vs HC group. Sensitivity 100%, specificity 100%; and d) Heatmap of AR group vs HC group. Sensitivity 92.86%, specificity 96.30%. * Control subject not classified correctly.

Table 2

Functional annotation clustering of the 26 significant genes of the nonallergic asthma (NA) group.

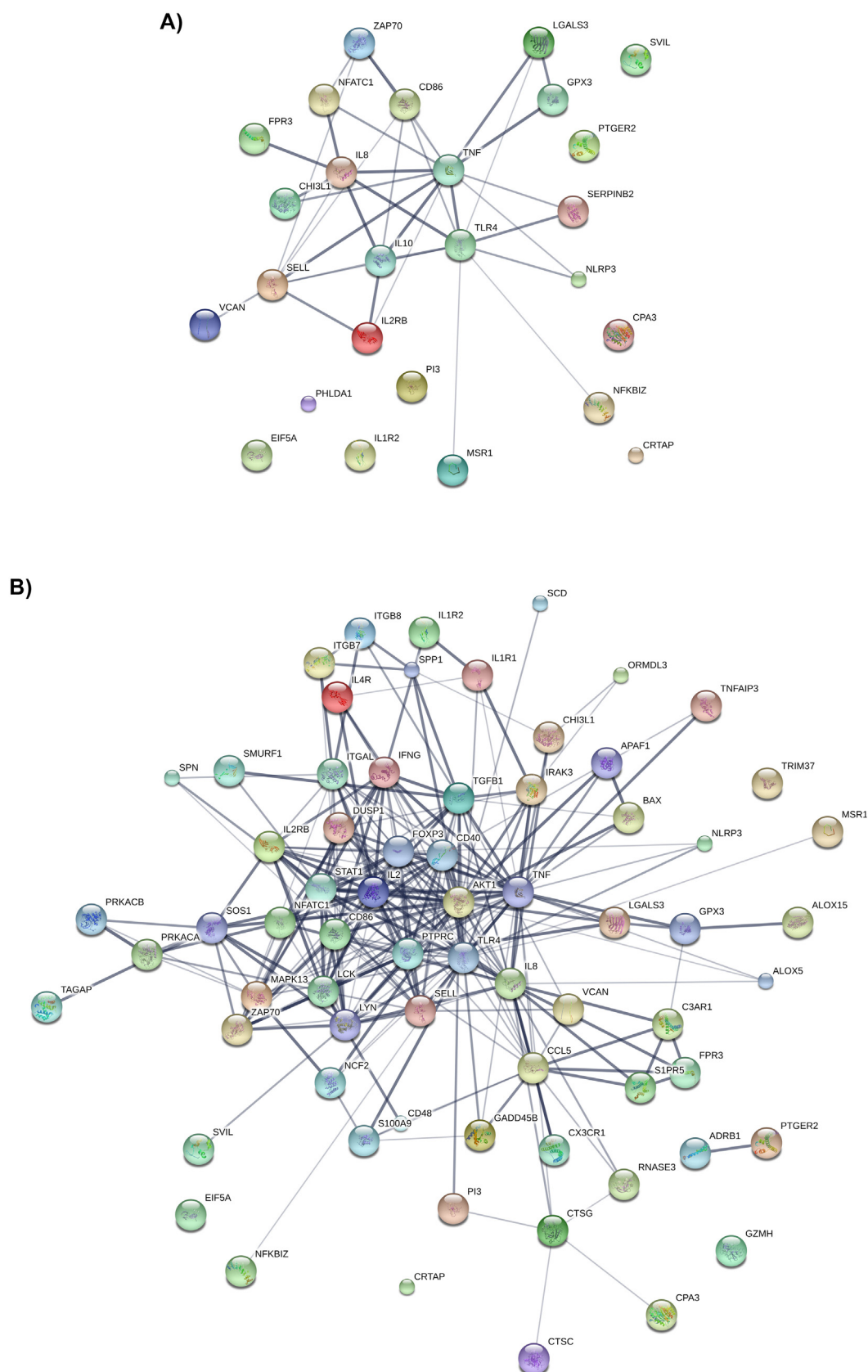
EXTRACELLULAR REGION	E.S: 3.73	CRATP, CHI3L1 , GPX3, IL10 , IL8 , LGALS3, MSR1 , PI3 , SERPINB2 , TNF, VCAN
EXTRACELLULAR MATRIX E.S: 2.64		CRTAP, CHI3L1 , LGALS3, PI3 , VCAN.
CELL SURFACE E.S: 2.31		CD86 , IL2RB, SELL, TLR4, TNF.
INFLAMMATORY PROCESSES	Cell activation	CD86 , IL10 , IL8 , TNF, ZAP70, TLR4.
E.S: 2.10	Inflammatory response	NLRP3, IL10 , IL8 , NFKBIZ, TLR4, TNF
	Extracellular space	CHI3L1 , GPX3, IL10 , IL8 , MSR1 , SERPINB2 , TNF
	Leukocyte activation	CD86 , IL10 , IL8 , ZAP70, TLR4
	Defense response	NLRP3, IL10 , IL8 , NFKBIZ, TNF, TLR4
	Leukocyte migration	IL10 , IL8 , TNF
	Cytokine-cytokine receptor interaction	IL1R2, IL10 , IL2RB, IL8 , TNF
IMMUNITY PROCESSES	Response to wounding	NLRP3, IL10 , IL8 , NFKBIZ, SERPINB2 , TNF, VCAN, TLR4
E.S: 1.94	Immune response	CD86 , NLRP3, IL1R2, IL10 , IL8 , TNF, ZAP70, TLR4
	Regulation of cytokine production	CD86 , NLRP3, IL10 , TNF, TLR4
	Regulation of apoptosis	NLRP3, EIF5A, IL10 , IL2RB, PHLDA1 , SERPINB2 , TNF, TLR4
OTHERS		CPA3 , FPR3, NFATC1, PTGR2, SVIL

E.S: Enrichment score. Genes associated with asthma severity appear in bold.

were underexpressed in severe NA patients compared with controls (Table 5A). Thirty-three genes were underexpressed in severe AA patients compared with controls (Table 5B), and the same 4 underexpressed genes in severe NA patients were highly underexpressed in severe AA patients. *MSR1* and *SERPINB2* were the only genes with statistically significant overexpression, though their increase was lower than the strict criteria established. It should

be noted the high number of differential genes underexpressed in severe AA patients. This data should be analyzed in depth.

The unsupervised hierarchical model comparing samples classified according to severity vs HCs was able to cluster the samples according to gene expression, doing so with 100% specificity and sensitivity for the severe NA group. The sensitivity and specificity for the discrimination of severe AA vs HC samples were slightly lower, at 86.67% and 92.5%, respectively (data not shown).



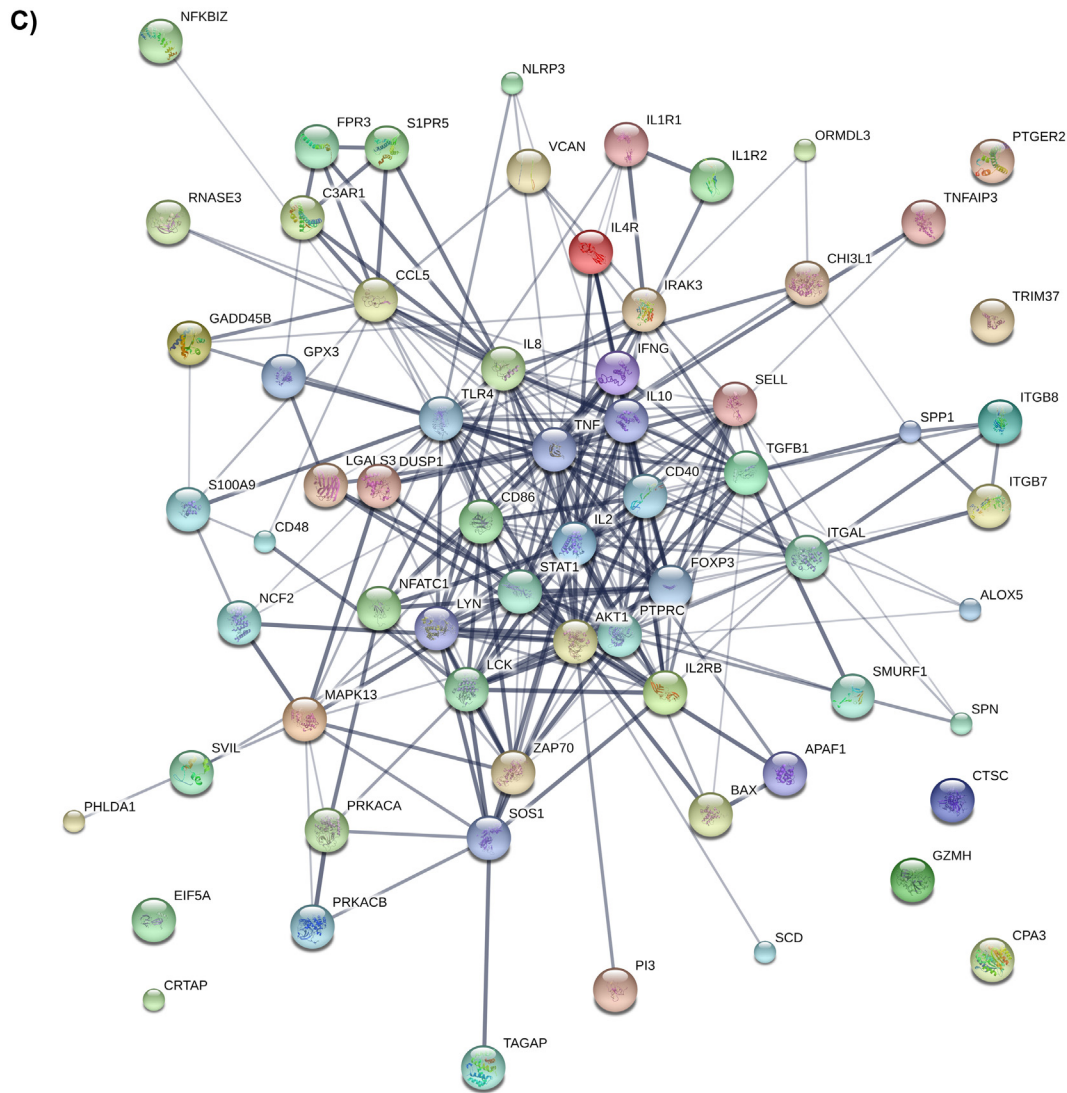


Fig. 2. (Continued)

3.4. Highest levels of IL-8 in nonallergic asthmatic subjects

As the IL-8 cytokine seems to be one of the central differential proteins, levels of this cytokine were measured in the sera of HC, NA, and AA subjects. Mean sera levels expressed as pg/ml are summarized in Fig. 3A. The highest mean sera levels were found in NA patients (452.28 ± 357) followed by AA patients (327.46 ± 377), and the lowest levels were detected in HCs (286.09 ± 179), but these differences were not statistically significant. There was a high percentage of subjects with undetectable levels of IL-8 in the 3 groups (53.3%, 61.9%, and 71.4% in NA, AA, and HC, respectively). Analyses of IL-8 levels according to severity showed slight but non-significant differences between severe NA patients (505.49 ± 387.8) and moderate-mild NA patients (377.7 ± 338.27) (Fig. 3A). Though not significant, the highest differences were observed in IL-8 levels of severe AA patients (468.63 ± 423) compared with moderate-mild AA patients (92.5 ± 79.6).

3.5. MSR1 protein quantification

MSR1, macrophage scavenger receptor type 1, was the most highly overexpressed gene in NA subjects, mainly in severe asthmatic patients. For this reason and in order to correlate gene

expression with MSR1 protein expression, Western blot was used in the total protein extracted from PBMCs from 9 control subjects and 18 NA patients (8 severe NA and 10 moderate-mild NA patients). Fig. 3B shows a representative example of the results of Western blotting, revealing the presence of 2 main bands—one upper band (approx. 100 kDa) with a molecular weight (MW) near 113 kDa, and a lower band (approx. 70 kDa) near 72.7 kDa. The relative quantification of these 2 bands with respect to β -actin showed a distinct distribution by groups; these differences were statistically significant in the lower protein band, which was more highly expressed in HCs than in NA subjects, but without differences according to severity.

4. Discussion

Chronic inflammatory diseases, including allergies and asthma, are the result of complex gene-environment interactions. A growing number of investigational therapeutics for asthma are biologic therapies that specifically target mediators of Th2 inflammation (Arron et al., 2013). However, mounting evidence has shown the high heterogeneity of asthma, which is why endotype-driven strategies are emerging of late. To become clinically relevant, the endotype should aim to validate biomarkers that correspond to

Table 3
Functional annotation clustering of the 69 significant genes of the allergic asthma (AA) group.

INFLAMMATORY PROCESSES E.S: 11.38	Defense response	CD40, CD48, NLRP3, S100A9 , APAF1 , ALOX15 , ALOX5 , CTSG , CCL5 , CX3CR1, C3AR1, ITGAL, IFNG , IL1R1, IL8 , NCF2 , NFKBIZ , PTPRC , RNASE3, SPP1, SPN , TLR4 , TGFB1 , TNF , AKT1, LYN
	Inflammatory response	CD40, NLRP3, S100A9 , ALOX15 , ALOX5 , CCL5 , C3AR1, ITGAL, IL8 , NFKBIZ , SPP1, TLR4 , TGFB1 , TNF , AKT1, LYN
	Response to wounding	BAX, CD40, NLRP3, S100A9 , ALOX15 , ALOX5 , CCL5 , CX3CR1, C3AR1, ITGAL, IL8 , NFKBIZ , SPP1, TLR4 , TGFB1 , TNF , AKT1, LYN , VCAN
UPREGULATION OF MACROMOLECULE METABOLIC PROCESSES E.S: 5.56 UPREGULATION OF IMMUNE SYSTEM PROCESS E.S: 4.93 REGULATION OF APOPTOSIS E.S: 4.65 PROCESSES OF RESPONSE E.S: 4.47	Upregulation of multicellular organismal process	BAX, CD40, NLRP3, ADRB1, ALOX5 , CCL5 , IFNG , SPP1, TLR4 , TGFB1 , TNF , AKT1
	Response to organic substance	CD40, CD86, SMURF1, EIF5A, FOXP3, IFNG , IL2, IL2RB, PTPRC , SPN , TLR4 , TGFB1 , TNF , AKT1, LYN
	Response to hormone stimulus Phosphorus metabolism	CD40, CD86, C3AR1, IFNG , IL2, IL4R , IRAK3, LCK, PTPRC , SPN , TLR4 , TGFB1 , LYN , ZAP70 BAX, NLRP3, APAF1 , DUSP1 , EIF5A, IFNG , IL2, IL2RB, LCK, PTPRC , SPN , STAT1 , SOS1 , TLR4 , TGFB1 , TNF , TNFAIP3 , AKT1 CD48, CTSC, CCL5 , DUSP1 , IFNG , IL1R1, IRAK3, PRKACA , PRKACB , SPP1, SELL , STAT1 , TLR4 , TGFB1 , TNF , AKT1, LYN CCL5 , DUSP1 , PRKACA , PRKACB , SPP1, STAT1 , TGFB1 , TNF , AKT1, LYN DUSP1 , GADD45B , IFNG , IRAK3, LCK, MAPK13, PRKACA , PRKACB , PTPRC , STAT1 , TGFB1 , TNF , AKT1, LYN , ZAP70
REGULATION OF PROTEIN PROCESSES E.S: 4.34	Regulation of production of molecular mediator of immune response	CD40, FOXP3, IFNG , IL2, IRAK3, PTPRC , TGFB1 , TNF
	Regulation of protein modification processes	BAX, SMURF1, FOXP3, IFNG , IL2, PTPRC , TLR4 , TGFB1 , TNF , LYN
	Protein regulation of transport	BAX, NLRP3, IFNG , IL2, PRKACA , TGFB1 , TNF , AKT1 FOXP3, IL2, IRAK3, PTPRC , SPP1, SPN , TGFB1 , AKT1
NEGATIVE REGULATION OF RESPONSE TO STIMULUS E.S: 3.32 CHEMOTAXIS PROCESSES E.S: 3.22 IMMUNE PROCESSES E.S: 3.14	Chemotaxis	S100A9 , CCL5 , CX3CR1, C3AR1, FPR3, IFNG , IL8 , SPN
	Leukocyte migration	S100A9 , CCL5 , IFNG , IL8 , TNF
	Leukocyte chemotaxis	S100A9 , CCL5 , IFNG , IL8
	Leukocyte activation	BAX, CD40, CD48, CD86, FOXP3, ITGAL, IL2, IL8 , LCK, PTPRC , SPN , TLR4 , TGFB1 , LYN , ZAP70
	Regulation of adaptive immune responses	CD40, CD86, FOXP3, IFNG , IL2, IL4R , PTPRC , SPN , TGFB1
	T-cell activation	BAX, CD48, CD86, FOXP3, ITGAL, IL2, LCK, PTPRC , SPN , TGFB1 , ZAP70
	Regulation of immune effector processes	CD40, FOXP3, IFNG , IL2, IRAK3, PTPRC , SPN , TGFB1 , TNF
	Upregulation of isotype switching	CD40, IFNG , IL2, PTPRC , TGFB1
	Regulation of immunoglobulin production	CD40, IFNG , IL2, PTPRC , TGFB1 , TNF
	Regulation of leukocyte proliferation	CD40, FOXP3, IFNG , IL2, PTPRC , SPN , TGFB1 , ZAP70
	Regulation of cytokine production	CD40, CD86, NLRP3, FOXP3, IFNG , IRAK3, SPN , TLR4 , TGFB1 , TNF
	Response to bacterium	CTSG , CCL5 , IFNG , IRAK3, RNASE3, SPN , STAT1 , TLR4 , TNF
	Response to virus	NLRP3, CCL5 , IFNG , IRAK3, PTPRC , STAT1 , TNF
	Immune system development	BAX, IL2, LCK, PTPRC , SPN , TGFB1 , TNF , LYN , ZAP70
	Regulation of B cell activation	CD40, IFNG , IL2, PTPRC , TGFB1
REGULATION OF RESPONSE TO EXTERNAL STIMULUS E.S: 2.64 CELL ADHESION E.S: 2.29 EXTRACELLULAR MATRIX E.S: 1.91 OTHERS		CCL5 , IL2, IL8 , SPP1, SPN , TLR4 , TGFB1
		CCL5 , CX3CR1, ITGAL, ITGB7, ITGB8, IL2, PTPRC , SPP1, SELL , TNF , VCAN
		CRTAP , CHI3L1 , LGALS3, PI3 , SPN , TGFB1 , VCAN
		CPA3 , GPX3, GZMH , IL1R2 , MSR1, NFATC1, ORM DL3, PTGER2, S1PR5, SCD, SVIL , TAGAP , TRIM37

E.S: Enrichment score. Genes associated with asthma severity appear in bold.

the underlying mechanism (Agache et al., 2015). This information needs to be contrasted and validated in samples that can be easily processed. Interestingly, common patterns of gene expression have recently been described in the sputum and blood of children and adults that were associated with near-fatal, severe, and milder asthma (Yan et al., 2015). Seeking to validate some of these previous results in samples obtained using non-invasive methods; we selected 94 genes (Table 1, Baos et al., submitted) from possible biomarkers previously described in different tissues and cell types, studying these genes in PBMCs from subjects with 4 clinical phenotypes (Table 1). The final purpose of this work was to introduce new diagnostic or prognostic tools for asthma and allergic phenotypes that can be easily analyzed.

Global gene-expression analysis carried out using PCA (unsupervised method) (Fig. 1A) showed the clustering of the 4 clinical groups according to the 94 genes. More specifically, two-by-two comparisons by hierarchical unsupervised analyses were able to differentiate asthmatic samples (allergic or nonallergic) from HCs (Fig. 1B and C) in all cases. These results indicate that gene selection was appropriate and raise the possibility of finding specific gene biomarkers for the different clinical phenotypes at a peripheral level.

Despite the limited number of samples, specific differential gene expression and functional analyses comparing clinical groups revealed many statistically significant results. Functional analyses of these genes and the network of protein interactions

Table 4

Functional annotation clustering of the 66 significant genes of the nonasthmatic allergy (AR) group.

INFLAMMATORY PROCESSES E.S: 10.65	Defense response	CD40, CD48, NLRP3, S100A9, APAF1, ALOX5, CCL5, C3AR1, ITGAL, IFNG, IL1R1, IL10, IL8, NCF2, NFKBIZ, PTPRC, RNASE3, SPP1, SPN, TLR4, TGFB1, TNF, AKT1, LYN
	Inflammatory response	CD40, NLRP3, S100A9, ALOX5, CCL5, C3AR1, ITGAL, IL10, IL8, NFKBIZ, SPP1, TLR4, TGFB1, TNF, AKT1, LYN
	Response to wounding	BAX, CD40, NLRP3, S100A9, ALOX5, CCL5, C3AR1, ITGAL, IL10, IL8, NFKBIZ, SPP1, TLR4, TGFB1, TNF, AKT1, LYN, VCAN
	Up-regulation of multicellular organismal process	BAX, CD40, NLRP3, ALOX5, CCL5, IFNG, SPP1, TLR4, TGFB1, TNF, AKT1
PROCESSES OF RESPONSE E.S: 5.98	Response to organic substance	CD48, CTSC, CCL5, DUSP1, IFNG, IL1R1, IL10, IRAK3, PRKACA, PRKACB, SPP1, SELL, STAT1, TLR4, TGB1, TNF, AKT1, LYN
	Response to hormone stimulus	CCL5, DUSP1, IL10, PRKACA, PRKACB, SPP1, STAT1, TGB1, TNF, AKT1, LYN
REGULATION OF APOPTOSIS E.S: 5.25		BAX, NLRP3, APAF1, DUSP1, EIF5A, IFNG, IL10, IL2, IL2RB, LCK, PHLDA1, PTPRC, SPN, STAT1, SOS1, TLR4, TGFB1, TNF, TNFAIP3, AKT1
		CD40, FOXP3, IFNG, IL10, IL2, IL2RB, PTPRC, SPN, TGFB1
REGULATION OF IMMUNE PROCESSES I E.S: 4.95	Regulation of leukocyte mediated immunity	FOXP3, IFNG, IL10, IL2, PTPRC, SPN, TGFB1, ZAP70
	Regulation of T-cell proliferation	FOXP3, IL10, IL2, IL4R, IRAK3, PTPRC, SPN, TGFB1
	Down-regulation of the immune system process	
	Positive regulation of molecular function	BAX, NLRP3, APAF1, GADD45B, IFNG, IL10, IL2, IRAK3, LCK, PRKACA, PRKACB, PTPRC, STAT1, TLR4, TGFB1, TNF, AKT1
SIGNALING I E.S: 4.42	Positive regulation of catalytic activity	BAX, NLRP3, APAF1, GADD45B, IFNG, IL2, LCK, PRKACA, PRKACB, PTPRC, STAT1, TLR4, TGFB1, TNF, AKT1
	Protein kinase cascade	GADD45B, IFNG, IL10, MAPK13, PRKACA, PRKACB, PTPRC, STAT1, TLR4, TNF, AKT1, ZAP70
		BAX, GADD45B, IFNG, IL2, IRAK13, PRKACA, PRKACB, PTPRC, TLR4, TGFB1, TNF, AKT1, LYN
	Regulation of phosphorylation	CD40, C3AR1, IRAK3, PTPRC, TLR4, LYN, ZAP70
SIGNALING II E.S: 4.38	Immune response-regulating signal transduction	

(Tables 2, 3, and 4 and Fig. 2) showed the high complexity of protein interactions and a higher enrichment score in allergic conditions (asthmatic and nonasthmatic) than in NA subjects, where IL-8 and TNF seem to be a core of these interactions.

Taking into account all the comparisons, there was a group of specific genes that seemed to be in especially close relation with each clinical condition. (Table 2, Baos et al., submitted).

Analyses according to disease severity in asthmatic subject groups showed that *IL10*, *MSR1*, *PHLDA1*, *SERPINB2*, and *CD86* were overexpressed, and *CHI3L1*, *CPA3*, *IL8*, and *PI3* (Table 5A) were underexpressed in severe NA patients compared with HC subjects. In severe AA patients, 33 genes were underexpressed compared with HC subjects (Table 5B), sharing the same 4 underexpressed genes as in severe NA. *MSR1* and *SERPINB2* were the only statistically significant overexpressed genes, but their increase was lower than the strict criteria established ($RQ < 4$).

Recently, genomic studies of asthma have been reviewed (Sircar et al., 2014), and several genes such as chemokine ligands (*CCL8*, *CCL5*, *CCL11*, and *CCL24*), SERPINS (*SERPINB2*, *SERPINB4*, and *SERPINA1*), and carboxypeptidase A3 (*CPA3*) have been defined as potential asthma biomarkers. In this study, *CCL5*, *CCL11*, *SERPINB2*, *SERPINB4*, and *CPA3* were some of the genes analyzed, although *CCL11* and *SERPINB4* were unable to be assessed. However, *CCL5* has been confirmed as a possible biomarker capable of differentiating allergy conditions from NA (Table 2, Baos et al., submitted). *CCL5* or *RANTES*, is a member of the CC subfamily of chemokine genes. It functions as a chemoattractant for blood monocytes, memory T-helper cells, and eosinophils, and it causes the release of histamine from basophils and activates eosinophils. In accordance with our results, individuals with NA were described as having higher expression of *RANTES* in mucosa and bronchoalveolar lavage fluid (Humbert et al., 1997). Also, several *CCL5* polymorphisms have recently been reviewed and associated with asthma risk (Huang et al., 2015).

SERPINS are members of the serine protease inhibitor family; these inhibit neutrophil protease cathepsin G and mast-cell

chymase and protect the lower respiratory tract from damage by proteolytic enzymes. *SERPINB2* has been detected in different kinds of cells, playing a role in inflammation and remodeling (Swartz et al., 2004). Indeed, the regulation of Th1 responses was described as a physiological function of inflammation associated with *SERPINB2* (Schroder et al., 2010). It was later reported that *SERPINB2* was specifically upregulated by IL-13 in airway epithelial cells (Woodruff et al., 2009; Peters et al., 2014) and their expression profile (together with periostin or *POSTN* and *CLCA1*) allowed the classification of asthma as Th2-high or

–low (Woodruff et al., 2009). However, a recent study failed to confirm *SERPINB2* gene expression as being differentially expressed in sputum cells from asthmatic patients (Peters et al., 2014). Here, *POSTN* and *CLCA1* were undetectable, but *SERPINB2* was strongly increased and associated with the asthma condition, mainly in severe NA, confirming it is a good biomarker candidate for severe asthma in PBMCs.

Carboxypeptidase A3 (*CPA3*) is a protease predominantly found in the mast-cell granule that was found to be upregulated in lung epithelium and in sputum cell pellets from patients with Th2-high asthma (Woodruff et al., 2009; Peters et al., 2014). Interestingly, this gene was highly underexpressed at the peripheral level in our study, and was associated with asthma severity in AA and NA and was one of the most differential genes between the AA and AR groups.

Another 3 genes that were positively associated with asthma severity in NA patients were *PHLDA1*, *CD86*, and *IL-10*. *PHLDA1* (pleckstrin homology-like domain, family A, member 1) is a gene that encodes a proline-histidine-rich nuclear protein and has been described as a putative epithelial stem-cell marker in the human small and large intestine (Sakthianandeswaren et al., 2011). *CD86* is a member of the immunoglobulin superfamily and encodes a type I membrane protein. It is expressed by antigen-presenting cells, and it is the ligand for 2 T-cell surface proteins, the CD28 antigen (costimulatory signal for activation) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (negative regulator of the immune

Table 5

Genes differentially expressed according to severity in asthma groups. A. Nonallergic asthma (NA) group. B. Allergic asthma (AA) group.

Gene	Expression	NA vs Control		Severe NA vs Control		Moderate-Mild NA vs Control	
		RQ	adjusted <i>P</i>	RQ	adjusted <i>P</i>	RQ	adjusted <i>P</i>
<i>CD86</i>	Higher	3.197	6.80E-05	4.853	4.12E-06	2.106	3.10E-02
<i>IL10</i>	Higher	6.142	1.62E-06	7.865	1.07E-06	4.796	1.74E-03
<i>MSR1</i>	Higher	12.891	1.44E-06	25.719	4.66E-07	6.461	3.41E-03
<i>PHLDA1</i>	Higher	6.899	1.86E-06	10.531	1.38E-07	4.520	1.72E-03
<i>SERPINE2</i>	Higher	8.948	7.61E-05	10.929	5.53E-04	7.326	3.46E-03
<i>CHI3L1</i>	Lower	0.027	3.20E-14	0.021	9.73E-12	0.034	2.39E-10
<i>CPA3</i>	Lower	0.222	1.07E-03	0.304	2.72E-02	0.162	2.61E-03
<i>IL8</i>	Lower	0.128	3.22E-08	0.151	7.90E-05	0.108	1.37E-06
<i>PI3</i>	Lower	0.006	4.61E-16	0.005	4.79E-12	0.007	9.47E-12

Gene	Expression	AA vs Control		Severe AA vs Control		Moderate-Mild AA vs Control	
		RQ	adjusted <i>P</i>	RQ	adjusted <i>P</i>	RQ	adjusted <i>P</i>
<i>ALOX15</i>	Lower	0.050	4.00E-09	0.040	4.57E-07	0.064	1.49E-05
<i>ALOX5</i>	Lower	0.235	6.14E-08	0.226	1.81E-05	0.244	7.63E-06
<i>APAF1</i>	Lower	0.141	6.31E-13	0.150	9.65E-09	0.132	2.22E-09
<i>C3AR1</i>	Lower	0.162	8.65E-09	0.149	4.31E-06	0.175	7.03E-08
<i>CCL5</i>	Lower	0.141	3.18E-12	0.139	1.62E-08	0.144	5.51E-11
<i>CHI3L1</i>	Lower	0.003	6.18E-22	0.003	3.39E-17	0.002	1.01E-17
<i>CPA3</i>	Lower	0.028	8.47E-13	0.033	4.28E-09	0.023	8.20E-10
<i>CRTAP</i>	Lower	0.193	1.16E-13	0.208	3.48E-09	0.180	8.00E-12
<i>CTSG</i>	Lower	0.043	1.53E-10	0.040	4.88E-08	0.046	8.30E-08
<i>DUSP1</i>	Lower	0.127	9.91E-16	0.126	1.22E-10	0.127	1.79E-10
<i>GADD45B</i>	Lower	0.116	9.55E-17	0.118	3.71E-11	0.113	1.15E-11
<i>GZMH</i>	Lower	0.151	1.46E-12	0.131	4.87E-09	0.174	2.04E-09
<i>IFNG</i>	Lower	0.089	1.37E-12	0.086	1.43E-10	0.093	6.06E-09
<i>IL1R2</i>	Lower	0.021	6.23E-18	0.030	4.28E-13	0.015	3.36E-14
<i>IL4R</i>	Lower	0.189	2.41E-13	0.189	1.34E-08	0.189	1.05E-10
<i>IL8</i>	Lower	0.026	3.38E-19	0.031	5.72E-12	0.022	1.33E-14
<i>LYN</i>	Lower	0.126	2.04E-18	0.135	4.51E-12	0.117	5.67E-13
<i>NCF2</i>	Lower	0.132	2.20E-11	0.135	2.45E-07	0.129	6.12E-10
<i>NFKBIZ</i>	Lower	0.106	6.98E-19	0.100	1.74E-13	0.112	4.05E-12
<i>PI3</i>	Lower	0.001	2.71E-24	0.001	7.17E-19	0.001	1.01E-17
<i>PRKACA</i>	Lower	0.202	4.23E-13	0.217	1.10E-08	0.188	2.49E-10
<i>PTPRC</i>	Lower	0.136	1.06E-09	0.104	2.37E-07	0.179	3.16E-07
<i>S100A9</i>	Lower	0.087	1.52E-15	0.085	1.22E-10	0.090	1.38E-11
<i>SELL</i>	Lower	0.110	3.24E-15	0.088	5.26E-11	0.137	8.72E-11
<i>SOS1</i>	Lower	0.166	9.76E-10	0.198	8.84E-06	0.139	1.67E-07
<i>SPN</i>	Lower	0.191	1.30E-13	0.170	1.09E-09	0.214	5.77E-10
<i>STAT1</i>	Lower	0.147	7.39E-16	0.154	1.43E-10	0.141	5.51E-11
<i>SVIL</i>	Lower	0.079	7.83E-19	0.089	4.43E-13	0.069	8.21E-13
<i>TAGAP</i>	Lower	0.092	3.38E-19	0.110	4.62E-12	0.077	1.18E-14
<i>TGFB1</i>	Lower	0.181	4.71E-20	0.199	4.28E-13	0.164	1.18E-14
<i>TLR4</i>	Lower	0.087	1.16E-13	0.113	2.30E-08	0.068	1.38E-11
<i>TNF</i>	Lower	0.067	3.47E-21	0.069	1.52E-15	0.065	3.97E-14
<i>TNFAIP3</i>	Lower	0.100	2.80E-19	0.103	1.39E-13	0.098	5.96E-13

Genes with a RQ (relative quantification) lower than 0.25 in the three comparisons performed. Genes shared with NA group appear in bold.

response) (Lichtenegger et al., 2012). IL-10 is a cytokine produced primarily by monocytes and, to a lesser extent, by lymphocytes. It has pleiotropic effects and, together with TGF- β , is considered to be the main regulatory cytokine. It has been extensively related with asthma and allergy diseases (Palomares et al., 2014). Recently, the role of these 2 cytokines in different diseases has been reviewed (Mingomataj and Bakiri, 2016) and they have been described as a potential switch of immunity response, indicating that IL-10 is mainly produced during transient immune conditions. In the present study, *IL-10* was highly overexpressed in NA compared to the HC group, mainly in severe patients but also in asthmatic (allergic or nonallergic) compared with allergic nonasthmatic subjects. *IL-10* seems to be an interesting potential biomarker of asthma severity.

MSR1 was found to be the most overexpressed gene in severe asthma conditions. This gene encodes the class A macrophage scavenger receptors, also called SR-A or CD204. These receptors are macrophage-specific trimeric integral membrane glycoproteins and have been implicated in many macrophage-associated physiological and pathological processes. Although *MSR1* was considered

a macrophage-specific receptor because it was predominantly found on macrophages (Naito et al., 1991), it has been further described in several other types of cells such as vascular smooth muscle cells, endothelial cells (EC), human lung epithelial cells, etc. (Tomokiyo et al., 2002). This fact increases the pathophysiologic importance of this receptor, pointing to a pivotal role in health and disease (Kelley et al., 2014). Interestingly, several studies have associated high *MSR1* expression with COPD (Thomsen et al., 2011; Ohar et al., 2010), and mechanisms leading to accumulation of macrophages with this marker in the lung could contribute to severe emphysema and COPD (Kaku et al., 2014). This protein has rarely been described in peripheral samples (Nakayama et al., 2008; Piechota et al., 2012).

In this study, *MSR1* was selected to validate our previous results (Aguerrri et al., 2013) of overexpression in the PBMCs of AA patients compared with HCs. Interestingly, this gene is one of the most differential in asthma disease and here we have also found evidence of its relevance in the severity of NA (Table 5A). The *MSR1* protein expression in the total of protein extracted from PBMCs (Fig. 3B) showed that the human antibody against *MSR1* recognized a dif-

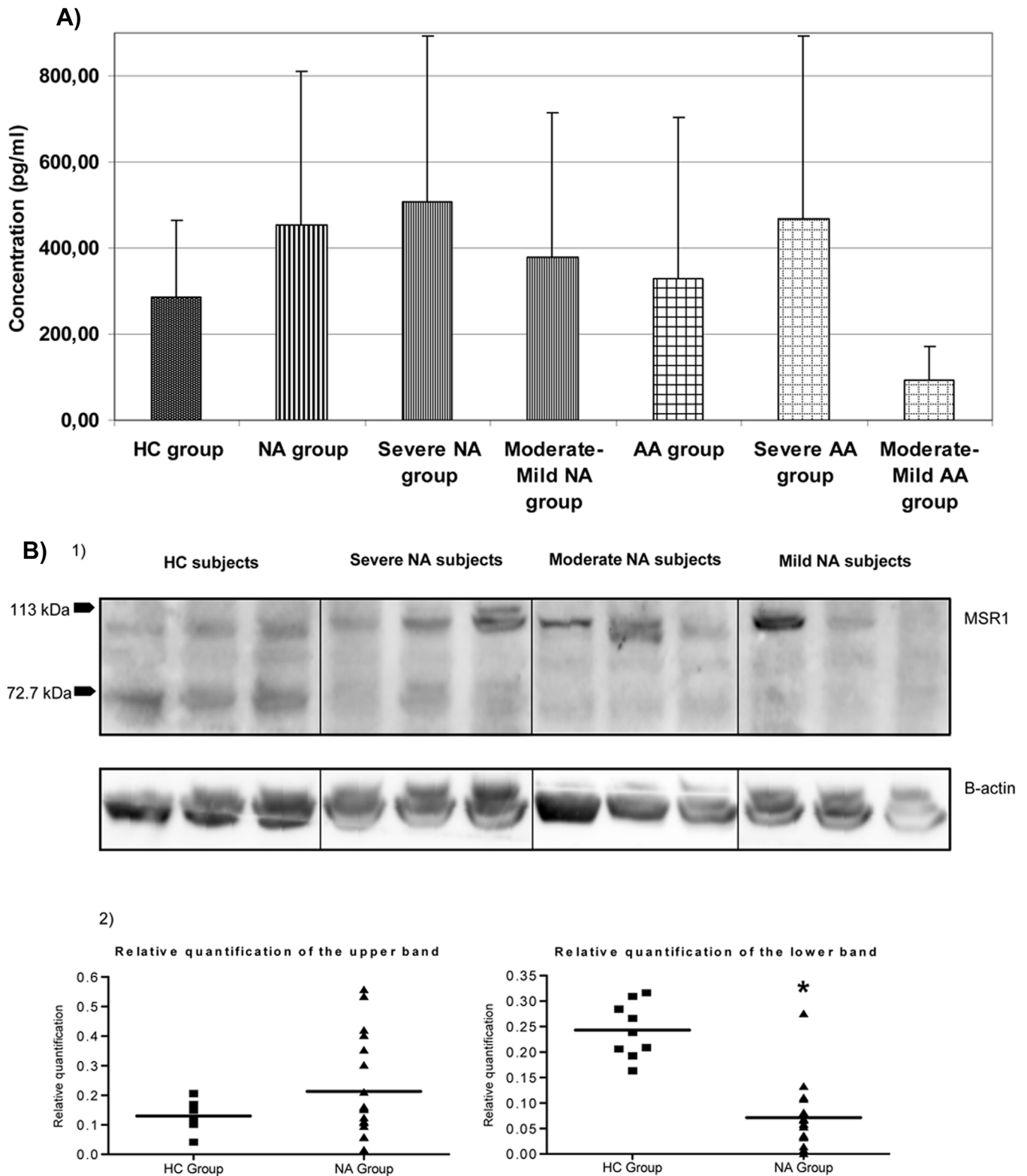


Fig. 3. A Mean levels of IL-8 in serum of the HC, NA and AA groups, and 3B: MSR1 determination. 1) Representative example of MSR1 expression by Western blot in 3 HCs, 3 severe, 3 moderate, and 3 mild NA subjects; and 2) Relative quantification of the 2 bands detected by the MSR1 antibody of all the subjects studied (9 HC, 8 severe NA, 10 moderate-mild NA). *Statistically significant comparison ($P < 0.001$) between NA and HC group.

ferent protein profile in HCs than in NA patients (Fig. 3B). These data confirm the proteic expression of MSR1 in PBMCs and point to interesting differences between healthy and asthmatic subjects that should be researched in depth.

Although a more exhaustive analysis of the peripheral expression of MSR1 could be interesting, taking these data together with data previously published for COPD (Thomsen et al., 2011; Ohar et al., 2010; Kaku et al., 2014) makes MSR1 a very good biomarker candidate for severity in several respiratory diseases.

Another 3 genes were found to be extremely downregulated in severe NA subjects: *CHI3L1*, *PI3*, and *IL8*. *CHI3L1* (chitinase 3-like 1) encodes a glycoprotein member of the glycosyl hydrolase 18 family or YKL-40. This protein lacks chitinase activity and is secreted by activated macrophages, chondrocytes, neutrophils, and synovial cells. It is thought to play a role in the process of inflammation and tissue remodeling (Lee et al., 2011), and its role as a versatile biomarker has been reviewed in YKL-40-regulated signaling mechanisms (Prakash et al., 2013). Its increase in the serum of asthmatic patients has been demonstrated (Chupp et al., 2007), and its levels have been correlated with disease severity (Ober et al., 2008; Konradsen et al., 2013). Specifically, it has been suggested that chitinase family members may be involved in the development of fibrosis and airway remodeling (Ober et al., 2008).

PI3 (peptidase inhibitor 3 skin-derived) encodes an elastase-specific inhibitor (Trappin-2 or Elafin) that functions as an antimicrobial peptide. This protein is a potent serine protease inhibitor that prevents excessive damage in different inflammatory states. It is locally expressed by epithelial cells and immune cells such as macrophages and γ T cells. Further, it has been proven to modulate a wide range of parameters that are critical for the inflammation process but having pleiotropic effects (Verrier et al., 2012).

The *IL8* gene encodes interleukin 8, a member of the CXC chemokine family and one of the major mediators of the inflammatory response and very important for many neutrophil functions, including chemotaxis and survival. It is secreted by several cell types. This gene is believed to play a role in the pathogenesis of bronchiolitis, a common respiratory disease caused by viral infection and which plays a significant role in the development of acute lung injury by, among other possible mechanisms, effecting neutrophil apoptosis (Allen and Kurdowska, 2014). Circulating levels of IL-8 can also stimulate the release of neutrophils into circulation, and it has been described that in noneosinophilic asthma, blood neutrophils release significantly higher levels of interleukin-8 than eosinophilic asthma and HCs (Baines et al., 2010). Interestingly, by investigating the interaction of airway epithelium and smooth muscles, it was described that YKL-40 could be involved in the inflammation of asthma by induction of IL-8 from epithelium (Tang et al., 2013).

All of these data—together with the fact that the IL-8 cytokine seems to be one of the central differential proteins in our study (Fig. 2), and that it is associated with severity in the asthma group (Table 5A)—led us to analyze its protein expression level. Fig. 3A shows the IL-8 mean sera levels in the HC, NA, and AA groups. Although there were no statistically significant differences—likely due to the small size of the population—the highest IL-8 levels were found in NA. These data are in agreement with the idea that many of the NA subjects and some of the most severe forms of asthma could be due to an increase of neutrophils, which could explain the high level of IL-8. We observed a higher number and percentage of eosinophils in severe AA than in severe NA (data not shown), although we lack data on neutrophils. According with this idea, in this study we found high levels of IL8 protein in sera of severe NA. The fact that there is an inverse correlation between gene expression and protein levels could be due to the different samples used to measure them (PBMCs and sera).

Another gene related with neutrophils was extremely downregulated in AA patients, namely, *CTGS* (Table 2, Baos et al., submitted). This gene encodes cathepsin G, a member of the peptidase S1 protein family that is found in azurophilic granules of neutrophils. It has been studied and described as being differentially expressed in the neutrophilic asthma phenotype relative to eosinophilic asthma (Baines et al., 2011).

5. Conclusions

In conclusion, using peripheral samples, we have confirmed several gene profiles that could be very useful for characterizing different asthma-allergic phenotypes. Additionally, we have described new potential biomarkers of severity in asthma. These data could be useful as biomarkers as well as new therapeutic and/or prognostic tools.

Author contribution statement

S. Baos, D. Calzada, and B. Cárda have worked on all project steps: design of the study, experimental work, discussion of results, and manuscript drafting.

L. Cremades collaborated in drafting the manuscript.

J. Sastre, J. Quirarte, and F. Florido performed patient selection and collaborated in the design of the study.

C. Lahoz has collaborated in the design of the study, results discussion, and manuscript drafting.

Conflict of interest statement

The authors declare that they have no conflicts of interest

Acknowledgments

This work was supported in part by research grant PI13/01730, co-supported by FEDER, CIBERES (ISCIII, 0013) and Biobank (PT13/0010/0012 from the Fund for Health Research (Spanish Ministry of Economy and Competitiveness)). S. Baos was supported by CIBERES (ISCIII, 0013) and *Conchita Rábago* Foundation. D. Calzada by *Conchita Rábago* Foundation, Madrid, Spain. L. Cremades was supported by a contract from MINECO (PEJ-2014-A-31609, *Sistema de Garantía Juvenil*), cofinanced by the European Social Fund (ESF) and the Youth Employment Initiative (YEI). We are also grateful to Oliver Shaw for revising the manuscript for English usage and style.

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