Promoter genotyping and mRNA expression analysis of PTGDR gene in allergy

Short Title: Expression of PTGDR in allergic diseases

San Segundo-Val I[1], García-Sánchez A[2,3], Sanz C[2,4], Cornejo-García JA[5], Isidoro-García M[1,2,6], Dávila I[2,3,7]

[1]Department of Clinical Biochemistry, University Hospital of Salamanca, Spain

[2]Institute for Biomedical Research of Salamanca, Spain

[3]Department of Biomedical Sciences and Diagnostics, University of Salamanca, Spain

[4]Department of Microbiology and Genetics, University of Salamanca, Spain

[5]Research Laboratory, IBIMA, Regional University Hospital of Malaga, UMA, Malaga, Spain

[6]Department of Medicine, University of Salamanca, Spain

[7]Department of Allergy, University Hospital of Salamanca, Spain

Corresponding Author

Catalina Sanz

Department of Microbiology and Genetics, University of Salamanca

Edificio Departamental, lab 302

Campus Unamuno, 37007, Salamanca, Spain

E-mail: catsof@usal.es

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ABSTRACT

Background: Prostaglandin D2 receptors are acquiring a relevant role as potential therapeutic targets in allergy. *PTGDR* has been described, as a candidate gene in allergic disease however there is a lack of functional studies on this gene.

Objective: the aim of this case-control study was to interrogate the potential role of *PTGDR* in allergy.

Methods: 195 allergic patients and 112 healthy controls were included. The *PTGDR* promoter polymorphic positions -1289G>A, -1122T>C, -881C>T, -834C>T, -613C>T, -549T>C, -441C>T, -197T>C and -95G>T were amplified by polymerase chain reaction and sequenced. *PTGDR* expression levels were analyzed by q-PCR and normalized to GAPDH and TBP mRNA levels. All procedures were performed following MIQE guidelines.

Results: *PTGDR* expression levels were significantly higher in allergic patients than in controls (P<0.001). ROC analysis for *PTGDR* expression showed a sensitivity of 81.4% compared to 67% for IgE levels. In addition, differences in the genotypic distribution of -1289G>A and -1122T>C polymorphisms were found in allergic patients (P = 0.009).

Conclusions: The results indicate that *PTGDR* overexpression is associated with allergy. In addition, polymorphisms -1289G>A and -1122T>C contribute to explain some of the observed expression variation. *PTGDR* expression could have a potential role as biomarker and pharmacogenetic factor in Allergy.

Key words: Allergy. Asthma. mRNA Expression. Polymorphisms. *PTGDR*. Rhinitis.

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RESUMEN

Antecedentes: los receptores de la prostaglandina D2 están adquiriendo un papel relevante como posibles dianas terapéuticas en la alergia. El gen *PTGDR* ha sido descrito como un gen candidato en una enfermedad alérgica, sin embargo, faltan estudios funcionales sobre este gen.

Objetivo: el objetivo de este estudio de casos y controles fue analizar el posible papel del gen *PTGDR* en la alergia.

Métodos: se incluyeron 195 pacientes alérgicos y 112 controles sanos. Un fragmento de la región promotora de *PTGDR* que comprendía las posiciones polimórficas -1289G>A, -1122T>C, -881C>T, -834C>T, -613C>T, -549T>C, -441C>T, -197T>C y -95G>T fue amplificado mediante la reacción en cadena de la polimerasa y secuenciado. Los niveles de expresión de *PTGDR* se analizaron mediante q-PCR y se normalizaron a los niveles de ARNm de GAPDH y TBP. Todos los procedimientos se realizaron siguiendo la guía MIQE.

Resultados: los niveles de expresión de *PTGDR* fueron significativamente más altos en pacientes alérgicos que en los controles (p<0,001). El análisis ROC para la expresión de *PTGDR* mostró una sensibilidad del 81,4% en comparación con el 67% para los niveles de IgE. Además, se encontraron diferencias en la distribución genotípica de los polimorfismos -1289G>A y -1122T>C en pacientes alérgicos (p=0,009).

Conclusiones: los resultados indican que la sobreexpresión de *PTGDR* se asocia con la alergia. Además, los polimorfismos -1289G>A y -1122T>C contribuyen a explicar parte de la variación de expresión observada. La expresión de *PTGDR* podría tener un papel potencial como biomarcador y factor farmacogenético en la alergia.

Palabras clave: Alergia. Asma. Expresión génica. Polimorfismos. *PTGDR*. Rinitis.

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INTRODUCTION

Allergy does not follow a classical Mendelian inheritance pattern, typical of monogenic disorders, but a multifactorial pattern, involving interactions between different genetic and environmental factors [1]. Genome-Wide Association Studies (GWASs) performed in the field of allergy have indicated that a large number of genes can influence the disease [2]. Among them, *PTGDR*, a prostaglandin D2 receptor, has been proposed among asthma (including allergic asthma) candidate genes [3, 4]. Prostaglandin D2 (PGD2) is increased after exposure to allergens [5]. The production of PGD2 has been linked to allergic asthma, atopic dermatitis and allergic rhinitis and conjunctivitis, and PGD2 has been detected in skin, tears and nasal secretion of allergic patients [6]. Locally, PGD2 acts through two transmembrane receptors, PTGDR (Prostaglandin D2 Receptor or DP1) and CRTH2 (chemokine receptor-homologous molecule expressed on Th2 lymphocytes or DP2), belonging to the superfamily of heterotrimeric G protein-coupled receptors [7].

Regarding *PTGDR*, linkage studies found a region located on chromosome 14 as a candidate to host genetic markers linked to asthma or related phenotypes [8, 9]. Association studies revealed an association between *PTGDR* and allergic diseases [2, 3, 10, 11]. Nevertheless, replication of association studies of *PTGDR* has generated some controversy, related to ethnicity and other factors. Thus, Single Nucleotide Polymorphisms (SNPs) of *PTGDR* have been associated with asthma in Caucasian populations [3, 4] and in an African American population [4], but not in Australian [12], Mexican, Puerto Rican, another African-American [13] and China-Han populations [14]. In addition, chronic rhinosinusitis with nasal polyposis has been associated with

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different prostaglandin receptors, including PTGDR, and also with the prostaglandin D2 synthase (PTGDS) [15]. Despite ethnic differences, *PTGDR* is one of the genes whose association with asthma and atopic asthma has been replicated [16], specifically in the case of polymorphisms of the promoter region, such as -549C/T, -441C/T and -197C/T [3, 4, 10]. These polymorphisms are located in binding sites of transcription factors, which could modify transcription levels [4]. Some haplotypic combinations of these variants have been associated with an increased transcriptional activity of *PTGDR* [3, 4, 10, 11].

In this study we decided to investigate the role of PTGDR in allergic diseases, including allergic asthma. The objectives of this study were: Firstly, to determine the *PTGDR* expression levels in allergic patients compared to controls; secondly, to compare allelic, genotypic and haplotypic frequencies of 9 *PTGDR* promoter polymorphisms in these two populations; lastly, to examine the putative relationship between *PTGDR* expression levels and the presence of *PTGDR* promoter polymorphisms.

METHODS

Study population

For this observational, analytical, case-control study of 314 Caucasian individuals (age > 16 yr), potentially eligible were selected over a period of 4 years, with 307 finally included. Seven samples were excluded because they did not fulfill the quality criteria for molecular analysis. The statistical power of the sample size was analyzed. The study was approved by the Ethics Committee of the University Hospital of Salamanca and an

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informed written consent from study subjects was obtained. One hundred and twelve individuals were enrolled as controls, provided they met the following criteria: (i) no symptoms or history of asthma or other pulmonary diseases; (ii) no symptoms or history of rhinitis; (iii) no symptoms or history of allergic diseases; (iv) negative skin prick tests to a battery of common aeroallergens; (v) absence of family history of asthma, rhinitis or atopy. In addition, 195 patients were recruited if they met these criteria: (i) physician-diagnosis of respiratory allergy (allergic asthma and/or allergic rhinitis); (ii) at least one positive skin prick test to a battery of previously described common aeroallergens [3]. A patient was considered to have allergic asthma if he/she had asthmatic symptoms restated to the exposure to the aeroallergens he/she was sensitized to and a positive bronchodilator or methacholine test. Allergic rhinitis was diagnosed with symptoms suggesting allergic rhinitis were present and correlated to patient's sensitizations. Skin prick tests were performed following the European Academy of Allergy and Clinical Immunology (EAACI) allergen standardization and skin test subcommittee recommendations [17]. Skin tests were considered positive if there were at least one wheal reaction of >3mm of diameter greater than the negative control. Patients should have positive skin prick test to at least one allergen. Concerning skin tests, patients were considered monosensitized if they had positive skin prick test to only one group of aeroallergens (house dust mites, molds, pollens or epithelia) and polysensitized if they had positive skin tests to two or more groups.

Total serum IgE levels were measured by a fluoroenzyme immunoassay (Themo Fisher Scientific, Waltham, USA).

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Genotype analysis

DNA extraction from total blood was performed using the automated system MagNA Pure Compact (Roche Applied Science, USA). A 1416 bp fragment comprising the nine single nucleotide polymorphism (SNP) positions -1289G>A, -1122T>C, -881C>T, -834C>T, -613C>T, -549T>C, -441C>T, -197T>C and -95G>T of the *PTGDR* promoter region was amplified by polymerase chain reaction. Forward and reverse primers employed in these amplifications were 5´ GGCATGAGGCCTAAAAATGAG 3´ and 5´ GAAGAAGGCGAAGGCTTGG 3´, respectively. Reaction mixtures and cycling conditions were performed as previously described [10]. To purify amplified fragments, ExoSAP-IT (USB, Cleveland, USA) was used. Amplicons were sequenced in an ABI PRISM 377 DNA Sequencer (Thermo Fisher Scientific, Waltham, USA). Chromas 2.3 (Technelysium, Tewantin, Australia) were employed for the analysis. Specific quality measures were taken in all procedures following EMQN guidelines [18].

RNA Extraction and cDNA Preparation

Total RNA was isolated from peripheral blood with RiboPure-Blood kit (Thermo Fisher Scientific, Waltham, USA). DNase treatment was performed using Turbo DNAse (Thermo Fisher Scientific, Waltham, USA). RNA quantification was performed in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA). RIN (RNA integrity number) algorithm was employed to determine RNA quality in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA).To ensure the viability of gene expression experiments only RNA samples with a RIN value above 7 were used. cDNA was generated using Superscript III First-Strand Synthesis System for

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RT-PCR (Thermo Fisher Scientific, Waltham, USA). Samples were stored at -20ºC until use.

Selection of constitutive expression genes

The selection of constitutive genes was carried out using *The Real-Time ready Human Reference Gene* panel (Roche Applied Science, Indianapolis, USA). On each plate, samples of patients and controls were added in duplicate, obtaining the Ct (Cycle threshold) of all housekeeping genes. Ct average and standard deviation for controls and patients for each of the 19 housekeeping genes was calculated, in order to evaluate its variability. The Ct average of housekeeping genes between controls and patients was estimated by determining the coefficient of variation from the total Ct average. Finally, ANOVA tests were performed after checking normality with the Kolmogorov-Smirnov test and homogeneity of variances with the Levene test.

Primers Efficiency Study

PTGDR primers were designed using the Primer 3Plus software. An 82bp *PTGDR* fragment was amplified with forward and reverse primers 5´-GGCATGAGGCCTAAAAATGAG-3´ and 5´-CCTTGACATCCTTAAATGCTCC-3´, respectively. It was checked that the primers sequence did not contain any known polymorphism, in order to avoid specificity variations. The efficiency study was carried out for *PTGDR* qPCR primers as well as for *GAPDH* and *TBP* primers of *The Real-Time ready Human Reference Gene* panel (Roche Applied Science, Indianapolis, IN, USA) [19]**.** *GAPDH* forward and reverse primers were 5´- CTCTGCTCCTCCTGTTCGAC-3´and 5´-ACGACCAAATCCGTTGACTC-3´, respectively. *TBP* forward and reverse primers were 5´-

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GAACATCATGGATCAGAACAACA-3´ and 5´-ATAGGGATTCCGGGAGTCAT-3´, respectively. Serial dilutions of a cDNA sample of known concentration were used and the efficiency was calculated according to the equation: E = 10 [-1 / slope] [20] in the cDNA concentration range from 0.39 to 100 ng, with a high linearity (Pearson correlation coefficient>0.95) [21].

Real-time Quantitative PCR

Expression analyses of the 3 genes were performed in a LightCycler 480 system (Roche Applied System, Manheim, Germany). Each well contained a final PCR reaction volume of 15 µL: 7.5µl Master Mix *SYBR Green I* (2X) (Roche Applied Science, Indianapolis, USA), 600 nM of each forward and reverse primer and 25 ng of cDNA template. PCR conditions included 10 min at 95ºC followed by 45 cycles of 10 s at 95ºC for denaturation, 10 s at 60ºC for annealing and 10 s at 72ºC for polymerization. Finally, melting curve analyses were conducted to check the specificity of the qPCR obtained products. All reactions were performed in triplicate and in each experiment non-template controls were included to detect any contamination. In order to detect and correct any potential inter-assay variations a known concentration sample (calibrator) was included. Control and patient samples were included in each experiment. The threshold cycle (Ct) was automatically determined using the LightCycler 480 Software. *PTGDR* expression levels were normalized to *GAPDH* and *TBP* mRNA levels using the Livak Method [22]. All procedures were performed following MIQE guidelines [23]. The analysis of expression was not performed on 15 samples that did not have the RIN value> 7 (8 controls, 7 patients).

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Statistical analysis

Chi-square, Fisher's exact, and Monte Carlo (10^4 simulations) tests were used for qualitative variables; ANOVA, Kruskall Wallis and U Mann Whitney were employed for continuous variables across each genotype. Homoscedasticity was analyzed accordingly. Logistic regression was employed to model the effects of multiple covariates. Age and sex were included as potential covariates in multivariate analysis. Analyses of allelic and genotypic distributions, as well as a study of haplotype distribution and the Hardy-Weinberg equilibrium were performed using the Shesis *on line* platform [24]. Correction for multiple comparisons, false-positive report probability (FPRP), and statistic power were also calculated as statistic control. Statistical analyses were performed by using SPSS 17.0 software (IBM, Chicago, USA). ROC analysis was applied for sensitivity and specificity study.

RESULTS

Characteristics of the study population

Phenotypic characteristics of the study population are shown in Table 1. Briefly, age was significantly lower in patients than in controls (p-value<0.001) and total IgE levels were significantly lower in controls (p-value<0.001). In addition, patients diagnosed with both allergic asthma and allergic rhinitis had significantly higher IgE levels than patients suffering only from rhinitis (p-value= 0.013).

Asthma severity was evaluated according to GINA guidelines [25]. Intermittent asthma was the most common (48%), followed by moderate persistent asthma (32%). Rhinitis

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classification was made following ARIA guidelines modified by Valero and cols [26], being moderate persistent rhinitis (41%) and moderate intermittent rhinitis (22%) the more frequent types. The most common aeroallergen sensitization was to pollen (146 patients), followed by mites (139 patients).

Selection of Reference Genes

A general recommendation in gene expression studies is to choose reference genes with a similar expression level to the target gene [27]. According to this and due to its lowest variability among all analyzed genes, *TBP* was the selected reference gene (see Table 1 of Supplementary material). Following MIQE guidelines two housekeeping genes should be included. In this sense, *GAPDH* was also included because it has been broadly used in gene expression studies of asthma [28, 29]. Before evaluating expression data, correlation of the two selected reference genes was confirmed (Spearman´s rho of 0.783, $p<0.001$).

Expression Analysis

PTGDR expression levels were significantly higher in the population of allergic patients than in controls ($p < 0.001$). This highly statistical difference was also observed in all subgroups of allergic patients. No significant differences in *PTGDR* gene expression levels were observed between the different subgroups of allergic patients, although *PTGDR* expression levels were slightly higher in patients sensitized to pollens (see Table 2).

We have considered possible influences of anti-inflammatory medications in the expression of *PTGDR*. No patient was taking NSAID or oral corticosteroids at blood sampling. In addition, 32 patients were receiving allergen immunotherapy (AIT) and 19 more patients had previously received AIT. No statistically significant differences (p=0.436) in the expression levels of *PTGDR* of patients receiving AIT respect to patients not receiving AIT were found. Concerning pollen allergy, the sample was obtained during the pollen season in a total of 58 patients out of 152. Again, no statistically significant differences were observed when the sample was obtained during the pollen season or out of it (p=0.624). This also implies that medication used to treat pollen allergy, in particular nasal and/or inhaled corticosteroids have no effect in the expression of *PTGDR*. In addition, the bioavailability of inhaled corticosteroid, which are administered at higher doses that nasal corticosteroids, is very low of even negligible for some of them [30]. The exposition to the rest of aeroallergens was not seasonal.

***PTGDR* expression levels as a potential biomarker of allergy sensitization**

We compared *PTGDR* mRNA expression levels with serum IgE levels as allergy biomarkers. A ROC curve was performed, obtaining an area under the curve of 0.654, 95% CI (0.588-0.721) providing a cut off for the *PTGDR* expression of 0.509, results are shown in Table 3.

Total IgE levels significantly increased according to the number of sensitization groups; however, these differences were not observed for *PTGDR* expression levels (see Table 4).

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Genetic Association Analysis

In the association study of the 9 *PTGDR* promoter polymorphisms, statistically significant differences between allergic patients and controls were observed for the allelic and the genotypic frequencies of -1289G>A and -1122T>C SNPs. As it happened with the *PTGDR* gene expression, the differences in the genotype distribution of these SNPs between allergic patients and controls were also observed for the subphenotypes of Atopic Asthma and Atopic Rhinitis. When the type of allergic sensitization was taken into account, significant differences were observed in patients with allergy to pollens (p=0.028). Also, in the group of patients sensitized to mites and pollens, statistically significant differences were found for the -1289G>A and -1122T>C polymorphisms after adjusting by sex and age, [p = 0.009 (OR: 0.24. 95% CI: 0.08- 0.70; FPRP = 14.4%)]. The linkage study carried out for -1289G>A and -1122T>C, provided an ID´I = 1, r^2 = 0.985. The Hardy-Weinberg equilibrium was fulfilled in all cases, except in the case of the 441C>T SNP in the control group (see Table 5). No statistically significant differences were observed for the rest of the SNPs between patient and controls populations (see Table 5), nor for the general haplotype distribution (see Table 6).

Association between *PTGDR* promoter polymorphisms and expression levels

In the total population of patients and controls, the mutant homozygous genotypes AA and CC of polymorphisms -1289G>A and -1122T>C respectively, were both associated to statistically significantly lower *PTGDR* expression levels, p=0.034 (see Table 7). These differences were especially confirmed in the subgroup of patients simultaneously

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sensitized to pollen and mites, where genotypes carrying the mutant alleles are associated with significant lower *PTGDR* expression, p=0.009. Interestingly these mutant alleles associated to lower *PTGDR* levels were significantly more frequent in controls than in any of the allergic groups (see Table 7).

DISCUSSION

In the present study, highly significant differences in *PTGDR* expression levels between controls and allergic patients were observed. Thus, allergic patients showed increased *PTGDR* expression levels respect to non-allergic subjects. *PTGDR* expression levels were independent of the aeroallergen group sensitization and/or clinical manifestations, although expression levels were slightly higher in patients sensitized to pollens. This suggests that expression levels of *PTGDR* could be a general trait of atopy. In this sense, arachidonic acid derivatives such prostaglandins and leukotrienes are important mediators in allergic reactions and are responsible of great part of symptoms of rhinitis and asthma. Thus, PGD2 is the most abundantly produced cyclooxygenase metabolite of arachidonic acid in response to environmental allergens and has been proposed as a mast cell activation marker [31]. PGD2 produces bronchoconstriction, vasodilation, increasing of capillary permeability and mucus production in allergic patients [32].

Expression studies require rigorous quality controls with respect to the samples collection and characterization, qPCR primers, nucleic acid extraction, RT-PCR, target gene information, qPCR protocol, qPCR validation and analysis of data [23]. So, all

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these considerations should be followed in order to provide reliable results. In this sense, we carefully followed all MIQE recommendations and performed a previous deep analysis in order to select the most appropriate constitutive genes from 19 candidates. In this study, the expression of *PTGDR* is able to clearly differentiate between allergic patients and controls with robustness. In addition, it can be detected by a simple and rapid technique from an easy to obtain biological sample and has a better sensitivity that IgE levels. However, the scarce specificity and NPV make limit its potential for differential diagnosis. We found that total IgE levels increased according to the number of groups of sensitization [33]. This is not the case for *PTGDR* levels, which did not exhibit significant differences regarding the number of sensitization groups. These results may indicate that *PTGDR* could be a good indicator of the inflammation activation mechanism of allergy rather than of severity. In addition, our results show that *PTGDR* expression determination could be consider as a complementary diagnostic test when it is suspected that IgE determination was not sensitive enough. With regard to the genotyping analysis, we found that the mutant alleles of -1289G>A and -1122T>C SNPs were significantly more frequent in controls than in any of the groups of allergic patients. In addition, these mutant alleles were associated to lower levels of *PTGDR* expression suggesting that the presence of these SNPs could be associated with a decreased risk to develop the disease. Both SNP are in linkage disequilibrium suggesting that the analysis of only one of them could be informative enough.

Some polymorphisms analyzed in this study have been previously associated with asthma. Thus, the -549T>C variant has been associated with asthma in Caucasians and

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African Americans, and the -441T>C variant in a Caucasian population [4]. In a Spanish population, the SNP -197T>C was associated with asthma, particularly allergic asthma [11]. In another study, the SNP -613C>T was associated with allergy, mainly with co-sensitization to mites and pollens, whereas the SNP -549T>C was close to significance ($p = 0.09$) in patients sensitized to both pollen and mites [11]. However, studies in Mexican, Puerto Rican and African American populations showed no involvement of SNP -549T>C, -441T>C and -197T> C [13] in the development of asthma, as well as studies on an Australian population [12]. These discrepancies may be due to small ethnic differences between populations or to environmental factors, since only in Caucasian population these polymorphisms were associated with asthma. In our work we have not found any significant association for these SNPs, but it should be taken into account that that our work focuses on allergic patients and not on asthma. Most of these studies lack of functional analysis. In our study, the expression analysis suggests that the association of both -1289G>A and -1122T>C SNPs could be related to their transcriptional role. We emphasized that expression analysis can contribute to identify the mechanisms underlying the inflammatory pathways related to allergic disease and to check the functional role of SNPs suggested in association studies. We have shown that *PTGDR* gene expression seems to be determined in part by the presence of the -1289G>A and -1122T>C SNPs. However, it was also observed, that for the same genotype the expression levels still having some differences between controls and allergic patients, which indicate that additional factors such as other SNPs and/or epigenetic factors also seem to be acting over *PTGDR* expression.

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Potential limitations of this study could be initially related to the sample size that was solved by analyzing the statistical power. Distribution of sex between patient and control groups was solved by logistic regression. The probability of a type 1 error was solved by applying the Bonferroni correction and the False Positive Report Probability. Finally, the putative selection of an inappropriate constitutive gene was solved by analyzing 19 genes in both control and patients.

This study opens interesting insights on *PTGDR* as a potential predictor marker of allergy, which could deeper explain how it is involved in the disease and its consequences on the activation of the inflammation pathway. In addition, this could contribute to the analysis of PTGD receptors as therapeutic targets highlighting the potential role of genetic variants that impaired the expression of *PTGD* receptors, as possible pharmacogenetic predictors of therapeutic response to their antagonists [34].

Further studies including validation of these results in different populations would be of great interest to determine the specific mechanisms by which the increased expression of *PTGDR* is related to the occurrence of allergic phenotype and can impact on therapeutic strategies.

CONCLUSIONS

PTGDR expression levels were associated with allergy. The -1289G>A and -1122T>C promoter SNPs are candidates to explain some of the variation in the expression levels observed in patients. *PTGDR* could have a potential role as biomarker and pharmacogenetic factor in allergy.

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Conflict of Interest Statement

The authors have declared that they have no conflict of interest.

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Table 1. Characteristics of the study population.

	Population	Controls	Allergic Patients	Patients with allergic asthma	Patients with allergic rhinitis	Patients with allergic rhinitis without asthma
Subjects	307	112	195	137	189	58
Sex (%)						
Female	53.4	57.1	51.3	51.8	50.3	50
Male	46.6	42.9	48.7	48.2	49.7	50
Age (AM±SD)	41.5±19.1	56.7±17.4	32.9±14.1	32.7±13.9	32.5±13.6	33.5±14.5
IgE(kU/l) (AM±SD)	269.6±382.1	99.6±178.5	361.5±428.9	407.7±460.5	365.9±432.1	258.1±327.9
p-value*			p<0.001	p<0.001	p<0.001	p<0.001
p-value†				p=0.013		p=0.010

AM: arithmetic Mean. SD: standard deviation. *p*-value*: p-value of each group of patients vs controls. *p*-value†: p-value of allergic patients without asthma vs patients with allergic asthma; patients with allergic rhinitis and asthma vs patients with allergic rhinitis only.

Table 2. *PTGDR* expression levels according to diagnosis and sensitization.

	N	Ct Mean ± SD	*p*-value	SP
Controls	104	0.68 ±0.52		
Allergic patients	188	0.92±0.52	p<0.001	95.4 %
Allergy to mites (at least)	133	0.87±0.49	p<0.001	80.6 %
Allergy to mites (only)	37	0.94±0.63	p=0.016	72.2%
Allergy to pollens (at least)	140	0.92±0.51	p<0.001	94.7%
Allergy to pollens (only)	34	0.98±0.42	p=0.03	96.2%
Allergic asthma	131	0.92±0.55	p<0.001	92.2 %
Allergic asthma (polysensitized)	89	0.90±0.54	p<0.001	81.3%
Allergic asthma (monosensitized to mites)	21	0.95±0.70	p=0.044	38.6%
Allergic asthma (monosensitized to pollens)	21	0.96 ±0.43	p= 0.003	75.1%
Allergic rhinitis	182	0.91 ±0.50	p< 0.001	94.4 %
Allergic rhinitis (polysensitized)	114	0.89 ±0.52	p< 0.001	84.9 %
Allergic rhinitis (monosensitized to mites)	34	0.87 ±0.50	p= 0.016	46.5 %
Allergic rhinitis (monosensitized to pollens)	34	0.98 ±0.42	p< 0.001	92.8 %

SP: Statistical Power. SD: standard deviation. For each group of patients, the p-value results from comparing the expression values in that group of patients with the values of the control group.

Table 3. Comparison between *PTGDR* mRNA expression levels and peripheral blood total IgE levels.

	PTGDR (Ct Mean)	IgE (kU/l)
Cut Off	0.509	100 kU/l
Sensitivity	81.4%	67%
Specificity	42.3%	75%
Positive Predictive Value	72%	83%
Negative Predictive Value	55%	55%

Table 4. Total IgE and *PTGDR* expression levels according to the number of sensitization groups in patients.

* Numbers of sensitizations	Total IgE (kU/l)	*PTGDR*
	Mean ± SD	Ct Mean ± SD
1 (n=71)	246.3 ± 313.6	0.960 ± 0.533
2 (n=58)	363.2 ± 446.1	0.889 ± 0.572
3 or more (n= 59)	490.8 ± 490.6	0.885 ± 0.461
p-value	†p = 0.005	¥p = 0.652

* Number of sensitizations refers to the number of groups of aeroallergens (pollens, mites, epithelia and molds) to which patients were sensitized. SD: standard deviation. †p: p-value obtained for the comparison of IgE levels among patients sensitized to 1, 2, 3 or 4 groups of allergens. ¥p: p-value obtained for the comparison of *PTGDR* expression levels among patients sensitized to 1, 2, 3 or 4 groups of allergens.

Table 5. Genotypic and allelic frequencies of *PTGDR* promoter SNPs.

Phenotype		Genotype				Allele			
-1289G>A	*n*	GG	GA	AA	**p*-value	G	A	*p*-value	OR(95% CI)
Controls	112	0.38	0.52	0.10		0.64	0.36		
All allergic patients	195	0.53	0.39	0.08	0.044/0.045	0.73	0.27	0.027	0.67(0.47-0.96)
Allergic asthma	136	0.54	0.40	0.06	0.045/0.062	0.74	0.26	0.019	0.63(0.43-0.92)
Allergic rhinitis	188	0.53	0.39	0.08	0.054/0.047	0.72	0.28	0.035	0.68(0.48-0.97)
Pollen allergy (at least)	145	0.56	0.37	0.08	0.019/0.028	0.74	0.26	0.014	0.62(0.42-0.91)
Mites + pollen allergy (at least)	90	0.57	0.33	0.10	0.023/0.031	0.73	0.27	0.048	0.65(0.42-0.99)
Mites + pollen allergy (only)	32	0.63	0.31	0.06	0.050/0.009	0.78	0.22	0.035	0.50(0.26-0.96)
-1122T>C	*n*	TT	TC	CC	**p*-value	T	C	*p*-value	OR(95% CI)
Controls	112	0.38	0.52	0.10		0.64	0.36		
All allergic patients	195	0.53	0.39	0.08	0.053/0.047	0.72	0.28	0.038	0.69(0.48-0.98)
Allergic asthma	136	0.53	0.40	0.07	0.066/0.064	0.73	0.27	0.030	0.65(0.45-0.96)
Allergic rhinitis	188	0.52	0.39	0.09	0.064/0.048	0.72	0.28	0.049	0.70(0.49-0.99)
Pollen allergy (at least)	145	0.55	0.37	0.08	0.025/0.029	0.73	0.27	0.023	0.64(0.44-0.94)
Mites + pollen allergy (at least)	90	0.57	0.33	0.10	0.023/0.031	0.73	0.27	0.048	0.65(0.42-0.99)
Mites + pollen allergy (only)	32	0.63	0.31	0.06	0.050/0.009	0.78	0.22	0.035	0.50(0.26-0.96)
-881C>T	*n*	CC	CT	TT	*p*-value	C	T	*p*-value	OR(95% CI)
Controls	112	0.80	0.20	0.00		0.90	0.10		
All allergic patients	195	0.78	0.21	0.01	0.544	0.88	0.12	0.545	0.85(0.49-1.45)
Allergic asthma	136	0.75	0.23	0.02	0.372	0.87	0.13	0.323	0.75(0.43-1.32)
Allergic rhinitis	188	0.78	0.21	0.01	0.529	0.88	0.12	0.523	0.84(0.49-1.44)
Pollen allergy (at least)	145	0.76	0.23	0.01	0.392	0.87	0.13	0.335	0.76(0.43/1.33)
Mites + pollen allergy (at least)	90	0.80	0.18	0.02	0.276	0.89	0.11	0.718	0.89(0.47-1.69)
Mites + pollen allergy (only)	32	0.69	0.28	0.03	0.101	0.83	0.17	0.114	0.53(0.24-1.17)
-834C>T	*n*	CC	CT	TT	*p*-value	C	T	*p*-value	OR(95% CI)
Controls	112	0.59	0.35	0.06		0.76	0.24		
All allergic patients	195	0.50	0.42	0.08	0.354	0.71	0.29	0.182	0.77(0.53-1.13)
Allergic asthma	136	0.51	0.43	0.06	0.416	0.72	0.28	0.351	0.82(0.55-1.24)
Allergic rhinitis	188	0.51	0.42	0.07	0.405	0.72	0.28	0.221	0.79(0.54-1.15)
Pollen allergy (at least)	145	0.49	0.44	0.07	0.303	0.71	0.29	0.197	0.77(0.52-1.15)
Mites + pollen allergy (at least)	90	0.49	0.43	0.08	0.392	0.71	0.29	0.208	0.75(0.48-1.17)
Mites + pollen allergy (only)	32	0.50	0.41	0.09	0.650	0.70	0.30	0.345	0.74(0.40-1.38)
-613C>T	*n*	CC	CT	TT	*p*-value	C	T	*p*-value	OR(95% CI)
Controls	112	0.79	0.21	0.00		0.90	0.10		
All allergic patients	195	0.78	0.21	0.01	0.557	0.88	0.12	0.640	0.88(0.51-1.50)
Allergic asthma	136	0.75	0.23	0.02	0.396	0.87	0.13	0.389	0.78(0.45-1.37)
Allergic rhinitis	188	0.78	0.21	0.01	0.545	0.88	0.12	0.615	0.87(0.51-1.49)
Pollen allergy (at least)	145	0.76	0.23	0.01	0.418	0.87	0.13	0.403	0.79(0.45-1.37)
Mites + pollen allergy (at least)	90	0.80	0.18	0.02	0.260	0.89	0.11	0.809	0.92(0.49-1.74)
Mites + pollen allergy (only)	32	0.69	0.28	0.03	0.109	0.83	0.17	0.137	0.56(0.25-1.21)

-549T>C	*n*	CC	CT	TT	*p*-value	C	T	*p*-value	OR(95% CI)
Controls	112	0.36	0.46	0.18		0.59	0.41		
All allergic patients	195	0.27	0.51	0.22	0.235	0.53	0.47	0.125	0.77(0.55-1.07)
Allergic asthma	136	0.27	0.52	0.21	0.329	0.53	0.47	0.204	0.79(0.55-1.13)
Allergic rhinitis	188	0.27	0.52	0.21	0.270	0.53	0.47	0.167	0.79(0.56-1.10)
Pollen allergy (at least)	145	0.24	0.55	0.21	0.117	0.51	0.49	0.086	0.73(0.52-1.04)
Mites + pollen allergy (at least)	90	0.28	0.51	0.21	0.457	0.53	0.47	0.254	0.79(0.53-1.18)
Mites + pollen allergy (only)	32	0.31	0.47	0.22	0.834	0.55	0.45	0.537	0.84(0.48-1.47)
-441C>T	*n*	CC	CT	TT	*p*-value	C	T	*p*-value	OR(95% CI)
Controls	112	0.70	0.23	0.07		0.82	0.18		
All allergic patients	195	0.70	0.25	0.05	0.874	0.82	0.18	0.942	1.01(0.66-1.56)
Allergic asthma	136	0.67	0.29	0.04	0.456	0.82	0.18	0.999	1.00(0.63-1.58)
Allergic rhinitis	188	0.70	0.25	0.05	0.807	0.82	0.18	0.886	1.03(0.67-1.59)
Pollen allergy (at least)	145	0.68	0.27	0.05	0.691	0.81	0.19	0.861	0.96(0.61-1.51)
Mites + pollen allergy (at least)	90	0.70	0.27	0.03	0.579	0.83	0.17	0.722	1.09(0.65-1.85)
Mites + pollen allergy (only)	32	0.75	0.19	0.06	0.852	0.84	0.16	0.657	1.19(0.56-2.53)
-197T>C	*n*	TT	TC	CC	*p*-value	T	C	*p*-value	OR(95% CI)
Controls	112	0.81	0.18	0.01		0.90	0.10		
All allergic patients	195	0.77	0.21	0.02	0.707	0.88	0.12	0.408	1.25(0.73-2.14)
Allergic asthma	136	0.77	0.21	0.02	0.617	0.87	0.13	0.366	1.30(0.73-2.29)
Allergic rhinitis	188	0.77	0.21	0.02	0.684	0.88	0.12	0.387	1.27(0.74-2.17)
Pollen allergy (at least)	145	0.79	0.20	0.01	0.861	0.89	0.11	0.594	1.17(0.66-2.06)
Mites + pollen allergy (at least)	90	0.72	0.27	0.01	0.329	0.86	0.14	0.163	1.53(0.84-2.81)
Mites + pollen allergy (only)	32	0.72	0.28	0.00	0.405	0.86	0.14	0.346	1.49(0.65-3.41)
-95G>T	*n*	GG	GT	TT	*p*-value	G	T	*p*-value	OR(95% CI)
Controls	112	0.99	0.01	0.00		0.99	0.01		
All allergic patients	195	0.97	0.03	0.00	0.310	0.97	0.03	0.310	0.34(0.03-2.97)
Allergic asthma	136	0.98	0.02	0.00	0.419	0.99	0.01	0.421	0.41(0.04-3.93)
Allergic rhinitis	188	0.97	0.03	0.00	0.295	0.99	0.01	0.297	0.34(0.04-2.89)
Pollen allergy (at least)	145	0.99	0.01	0.00	0.724	0.99	0.01	0.775	0.65(0.06-7.23)
Mites + pollen allergy (at least)	90	0.98	0.02	0.00	0.442	0.99	0.01	0.444	0.40(0.03-4.48)
Mites + pollen allergy (only)	32	0.97	0.03	0.00	0.345	0.98	0.02	0.347	0.28(0.02-4.62)

**p*-value: Fisher´s p-value/logistic regression p-value adjusted by age and sex covariates. OR(95%CI): Odds Ratio (95% Confidence Interval). For each group of patients, the p-values and OR were obtained by comparing the genotypic and allelic distributions in that group of patients with the ones of the control group. Allergic asthma: most of these patients also had allergic rhinitis. Allergic rhinitis: Two thirds had concomitant asthma. Pollen allergy (at least): Patients with allergy to at least pollens (most of them may have other sensitizations). Mites + Pollen (at least): Patients with allergy to at least pollens and mites (some of them may have other sensitizations). Mites + pollen allergy (only): Patients with allergy to pollens and mites (but not to any other group of aeroallergens).

Table 6. Haplotypic frequencies.

Haplotype	Control	Allergic patients	Allergic asthma	Allergic rhinitis	Pollens allergy (at least)	Mites + Pollen allergy (at least)	Mites + Pollen allergy (only)
ACCCCCCTG	0.36	0.27 p=0.017	0.25 p=0.013	0.27 p=0.023	0.25 p=0.009	0.26 p=0.027	0.22 p=0.032
GTCCCCCCG	0.10	0.11 p=0.673	0.11 p=0.485	0.11 p=0.635	0.10 p=0.728	0.13 p=0.302	0.13 p=0.507
GTCCCCCCT	0.00	0.01 p=0.404	0.01 p=0.495	0.01 p=0.384	0.01 -	0.01 p=0.475	0.02 p=0.259
GTCCCCCTG	0.03	0.02 p=0.400	0.02 p=0.513	0.02 p=0.438	0.02 p=0.447	0.02 p=0.563	0.02 p=0.485
GTCCCTTTG	0.17	0.18 p=0.735	0.18 p=0.691	0.18 p=0.789	0.19 p=0.546	0.17 P=0.976	0.16 P=0.799
GTCTCTCTG	0.23	0.29 p=0.117	0.28 p=0.241	0.29 p=0.144	0.29 p=0.117	0.29 p=0.151	0.30 p=0.306
GTTCTCCTG	0.10	0.11 p=0.591	0.13 p=0.365	0.11 p=0.568	0.12 p=0.364	0.11 p=0.713	0.17 p=0.121

Data represent the percentage of each haplotype in each phenotypic group and the corresponding Fisher's p-value. Haplotypes with a frequency >1% among either controls or patients are included. The order of the SNPs in each haplotype is -1289G>A, -1122T>C, -881C>T, -834C<T, -613C>T, -549T>C, -441C>T, -197T>C, and -95G>T. Please see the legend of Table 5.

Table 7. *PTGDR* expression levels according to -1289G>A and -1122T>C genotypes.

		-1289G>A				-1122T>C			
		GG	GA	AA	p-value	TT	TC	CC	p-value
Controls	Frequency	0.38	0.52	0.10		0.38	0.52	0.10	
	Ct Mean ± SD	0.76±0.62	0.67±0.47	0.43±0.27	0.160	0,75±0,62	0,67±0,46	0,42±0,26	0.161
Allergic Patients	Frequency	0.53	0.39	0.08	0.045	0.53	0.39	0.08	0.045
	Ct Mean ± SD	0.93±0.47	0.91±0.59	0.79±0.51	0.580	0.93±0.47	0.91±0.59	0.79±0.48	0.600
Pollen allergy (at least)	Frequency	0.56	0.37	0.07	0.028	0.55	0.37	0.08	0.029
	Ct Mean ± SD	0.93±0.41	0.94±0.62	0.79±0.52	0.540	0.93±0.42	0.94±0.62	0.79±0.49	0.541
Pollens + mite allergy (at least)	Frequency	0.57	0.33	0.10	0.031	0.57	0.33	0.10	0.031
	Ct Mean ± SD	0.92±0.44	0.75±0.42	0.87±0.55	0.340	0.92±0.44	0.75±0.42	0.87±0.55	0.340
Pollens + mite allergy (only)	Frequency	0.63	0.31	0.06	0.009	0.63	0.31	0.06	0.009
	Ct Mean ± SD	1.06±0.54	0.48±0.41	0.58±0.32	0.008	1.06±0.54	0.48±0.41	0.58±0.32	0.010

Pollens + mite allergy (at least) group includes patients with allergy to both pollens and mites regardless that they could have others sensitizations. The group pollens + mite allergy (only) includes patients who only have allergy to pollens and mites but not to any other group of aeroallergens.