

# Analysis of differential expression of protease-activated receptors in patients with allergic fungal rhinosinusitis

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## ABSTRACT

**Background:** Ever since its characterization in the 1970s, allergic fungal rhinosinusitis (AFRS) has been the subject of much controversy, especially regarding its pathogenesis. In this study, we analyzed the differential expression of genes that encode protease-activated receptors (PAR) in patients with AFRS and patients with chronic rhinosinusitis, and tried to understand the pathogenic basis of this disease.

**Objective:** To analyze the differential expression of PAR genes in patients with AFRS and in patients with chronic rhinosinusitis.

**Methods:** Mucosa from ethmoid sinuses of 51 patients (tests and controls) was biopsied and evaluated for messenger RNA expression of PAR genes by using reverse transcriptase–polymerase chain reaction. Each of the four PAR genes, i.e., par1, par2, par3 and par4 was amplified, the final gene products were run on 1.8% agarose gel and analyzed by densitometry to calculate differential expression. The significance level was determined as  $p \leq 0.05$ .

**Results:** It was observed that the expressions of all four par genes were higher in the test samples compared with the controls, but statistical significance was achieved only for par1 ( $p = 0.004$ ) and par2 ( $p = 0.05$ ). Comparative expression of the four PAR genes was also performed within the test and control groups, and a statistically significant difference was seen between par1 and par2 ( $p = 0.007$ ), par1 and par3 ( $p = 0.029$ ), par1 and par4 ( $p = 0.0001$ ), par2 and par4 ( $p = 0.002$ ), and par3 and par4 ( $p = 0.009$ ) in the test group. In the control group as well, par1, par2, and par3 exhibited a higher expression compared with par4 but the difference was significant between par3 and par4 genes only.

**Conclusion:** Patients with AFRS expressed increased levels of PAR genes in their nasal mucosa, and, of the four PAR genes, a higher expression of par1, par2, and par3 was observed in both the groups compared with par4. This information contributes toward our understanding of pathogenesis and possibly treatment of AFRS.

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Allergic fungal rhinosinusitis (AFRS) is a term introduced by Robson *et al.*<sup>1</sup> in 1989 to describe a constellation of unusual findings in a group of patients with chronic sinusitis. AFRS is a truly unique pathologic entity, defined largely by the presence of allergic fungal mucin, which is a thick green-to-gray lamellate of dense inflammatory cells, mostly eosinophils, in various stages of degranulation, Charcot-Leyden crystals, and fungal hyphae.<sup>2</sup> Since its initial characterization in the 1970s, AFRS has been the subject of much debate, controversy, and research

regarding its etiopathogenesis, diagnosis, classification, and appropriate management.

AFRS, in its strictest sense, is defined in a patient who is immunocompetent and with an allergy to fungus. The most common fungi reported are the dematiaceous species, e.g. *Biploaris*, *Curvularia*, *Alternaria*, *Aspergillus*.<sup>3</sup> The overall incidence of AFRS is 5–10% of patients undergoing surgery for chronic rhinosinusitis.<sup>4,5</sup> Patients who are afflicted are typically young (mean age at diagnosis, 21.9 years), atopic, and immunocompetent, who report long-standing chronic sinusitis despite prolonged medical therapy and multiple surgeries.<sup>6</sup> AFRS can be considered a refractory, more-severe form of chronic sinusitis. Masterson *et al.*,<sup>7</sup> in their retrospective review of 250 patients, found higher rates of revision surgeries in AFRS and chronic sinusitis with patients with polyposis compared with patients with chronic sinusitis without nasal polyposis, the difference being almost threefold, which thus highlights the spectrum of severity.

The pathogenesis of AFRS is not yet fully understood and is a subject of controversy. The immunologic theory presented by Manning and Holman<sup>8</sup> proposed a cycle of initial antigenic stimulus, followed by hypersensitivity reactions, *viz.*, Gell and Coombs types I and III, and a

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resultant self-perpetuating cycle of inflammation, obstruction of sinus ostia, and continuous antigenic exposure. This theory supports the allergic response of individuals susceptible to fungi. Stewart and Hunsaker<sup>9</sup> analyzed fungal-specific immunoglobulin E (IgE) and IgG levels in the controls who were nonatopic, patients with allergic rhinitis, non-allergic fungal rhinosinusitis polyp, patients with AFRS-like disease, and patients with AFRS. They also found increased levels of serum IgE and IgG to multiple fungi in patients with AFRS and patients with AFRS-like disease.

However, it was subsequently noted that some patients with the clinical picture of AFRS do not have allergies. This, along with the finding that fungi are ubiquitously present in the nose and paranasal sinuses, as suggested by Ponikau *et al.*,<sup>10</sup> raised the question as to why only some individuals develop AFRS and others do not. One plausible explanation to this involves an essential component of AFRS: hypersensitivity to environmental fungal allergens. Collins *et al.*<sup>11</sup> proposed a theory that AFRS is the result of a local, not systemic, hypersensitivity reaction. This study, which was based on finding fungus-specific IgE in the mucin of patients with AFRS as well as patients without AFRS, proposed evidence for a local type I response.

There are recent data that fungal proteases, which are an essential part of fungal physiology and development, are present in most airborne particles and can activate and enhance the innate immune response, which results in IgE-induced inflammation.<sup>12,13</sup> These fungal proteases mediate their actions by acting on protease-activated receptors (PAR), which are a family of proteolytically activated 7-transmembrane G protein coupled receptors that are widely expressed in airway epithelium, mast cells, eosinophils, neutrophils, macrophages, lymphocytes, smooth muscle, endothelium, fibroblasts, and neurons.<sup>12,14–16</sup> Cleavage within the extracellular amino terminus of these receptors activates them, which leads to G-signaling cascades that increase intracellular phospholipase C, which results in increased intracellular Ca<sup>++</sup> levels and eventually secretion and degranulation, which, in turn, promotes edema and angiogenesis.<sup>16</sup> PARs are of four types, *viz.*, PAR1, PAR2, PAR3, and PAR4, based on their structure as revealed by molecular cloning.<sup>17</sup> Genes encoding for *PAR1*, *PAR2*, and *PAR3* are present on the long arm of chromosome 5, whereas the *PAR4* gene is located on the short arm of chromosome 19.<sup>18,19</sup> Although the location of the four genes differs, there is high degree of structural similarity among them.

Even though the role of PARs in platelet activation and inflammation *per se* is well established, the significance in local hypersensitivity in airway inflammation is still a matter of active research. Park *et al.*<sup>20</sup> demonstrated increased airway allergic activity in the form of increased expression of chemokine genes in mouse

lung epithelial cells after inoculation of mice with *Acanthamoeba* trophozoites, which possess strong protease activity. Zhang *et al.*<sup>21</sup> extensively reviewed the role of PARs in allergic inflammation, and, in their review article, described the increased expression of all four PARs in mast cells, whereas PAR2 seemed to be the major influence in directing eosinophil functions. Despite extensive research, there is still an incomplete understanding of the pathophysiology of AFRS. Fungal proteases could play a role in susceptible individuals by activating PARs expressed in the nasal mucosa. There is no evidence yet that directly links the involvement of PARs in the pathogenesis of AFRS. We hypothesize that susceptible individuals overexpress the PARs in their airway epithelium. Protease inhibitors and PARs antagonists could prove to be an effective treatment for AFRS in the future.

## METHODS

The study was conducted in the Department of Otolaryngology and Head and Neck Surgery, in collaboration with the Department of Biochemistry, Post Graduate Institute of Medical Education and Research, Chandigarh. Institutional ethics committee approval was obtained before commencement of the study (Ref. NK/1753/MS/10589–90). Fifty-one patients, 25 cases and 26 controls, were included in the study. Patients diagnosed as having AFRS in a postoperative biopsy sample on histopathologic examination and who fulfilled all major parameters of the Bent and Kuhn criteria, *viz.*, type 1 hypersensitivity, nasal polyposis, characteristic CT findings, eosinophilic mucin without invasion, and positive fungal stain, were included. All biopsy samples in this study were evaluated by the same pathologist. The presence of allergic mucin, noninvading fungal hyphae, and eosinophilic inflammation were the necessary parameters for a histopathologic diagnosis of AFRS. For the control group, patients who were undergoing endoscopic sinus surgery for chronic rhinosinusitis with or without nasal polyposis were enrolled. Patients with Kartagener syndrome, cystic fibrosis, primary ciliary dyskinesia, immunodeficiency, HIV, concomitant autoimmune diseases, sinonasal malignancies, and invasive fungal sinusitis were excluded from the study.

All the patients were initially given a trial of maximal medical management in the form of steroid nasal sprays, antihistaminics, and oral steroids in tapering doses in refractory cases, and those who had persistence of disease were taken for surgery after providing well-informed consent. All the patients in both the groups were given an additional course of 0.5 mg/kg body weight oral prednisolone for a week before the surgery. Patients suspected of having AFRS underwent *Aspergillus*-specific skin-prick testing and *Aspergillus*-specific IgE testing to augment the diagnosis. During

Table 1 Demographic data of patients

	Test Group, AFRS (n = 25)	Control Group, CRS (n = 26)	p Value
Age, mean $\pm$ SD, y	27.16 $\pm$ 12.87	39.7 $\pm$ 17.35	<b>0.0052</b>
No. males to females	15:10	13:13	0.4731
Asthma, no./total no. (%)	11/25 (44)	6/26 (23.07)	0.1131
Skin-prick testing result, no.			
Positive	15	Not determined	—
Negative	4		
Not determined	5		
Peripheral eosinophilia, mean $\pm$ SD	6.32 $\pm$ 2.93	4.34 $\pm$ 1.89	<b>0.0059</b>
Lund-Mackay score, mean $\pm$ SD	18.56 $\pm$ 6.93	8.46 $\pm$ 6.33	<b>0.0001</b>

AFRS = Allergic fungal rhinosinusitis; CRS = chronic rhinosinusitis; SD = standard deviation.

Bold values indicated  $p < 0.05$ .

surgery, mucosal biopsy samples were taken from the anterior ethmoid sinuses in all the patients and were stored in RNAlater (Invitrogen, ThermoFisher Scientific, Waltham, MA) at  $-80^{\circ}\text{C}$  until additional experiments were conducted. Tissue, 15–30 mg of each sample, was transferred to a 1.5-mL microcentrifuge tube that contained 500  $\mu\text{L}$  of TRIzol reagent (Life Technologies Pvt Ltd, Carlsbad, CA). The samples were homogenized by using disposable homogenizer probes and were incubated at room temperature for complete dissociation of the nucleoprotein complex.

By using the TRIzol method according to the manufacturer's protocol, messenger RNA (mRNA) was isolated. The RNA was quantified by measuring absorbance at 260 nm with a Picodrop instrument (Picodrop Ltd., Cambridge, U.K.). RNA integrity was checked by running the samples on 2% agarose gel; 1–2  $\mu\text{g}$  of RNA was treated with DNase 1 kit (Thermo Fisher Scientific, Waltham, MA) to remove any DNA contamination. RNA was then reverse transcribed by following the protocol of Verso complimentary DNA synthesis kit (Thermo Fisher Scientific). Integrity of the complimentary DNA was confirmed by checking for the expression of the housekeeping gene  $\beta$ -actin. Each of the four genes were amplified by using specific primers, designed by using the NCBI/primer-BLAST (National Centre for Biotechnology Information/primer-Basic Local Alignment Search Tool) software and synthesized commercially. Polymerase chain reaction (PCR) was performed by following optimized assay in a thermal cycler: a denaturation program ( $95^{\circ}\text{C}$  for 10 minutes), an amplification program was repeated for 36 cycles ( $94^{\circ}\text{C}$  for 30 seconds,  $60^{\circ}\text{C}$  for 30 seconds,  $72^{\circ}\text{C}$  for 30 seconds), and a final extension ( $72^{\circ}\text{C}$  for 10 minutes). The amplified products were run on 1.8% agarose gel and, finally, quantified by densitometry and calculated by using imageJ software. All values were normalized to the expression of  $\beta$ -actin.

The raw data obtained were subjected to statistical analysis.

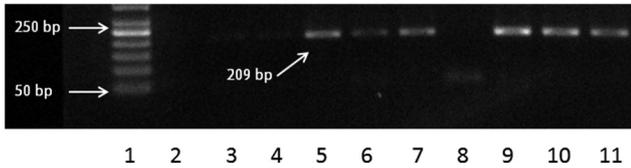
### Statistical Analysis

The statistical analysis was carried out by using Statistical Package for Social Sciences (SPSS for Windows, SPSS Inc., Chicago). Normality of the quantitative data was assessed by using the Kolmogorov-Smirnov test. The various parameters for the two groups were compared by using the Mann-Whitney test. The comparison of genes between each within the two groups was performed by using the Wilcoxon signed rank test. A  $p$  value of  $<0.05$  was considered to be statistically significant in all the tests.

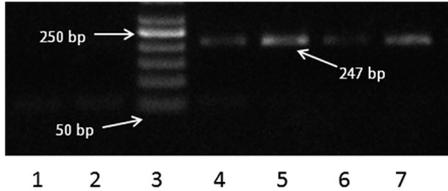
### RESULTS

In the test population, of a total number of 25 subjects, 15 were male and 10 were female subjects, and, in the control population, the total number of subjects was 26, 13 of whom were male and 13 were female subjects (Table 1). The mean age of the 25 subjects in the test population was  $27.16 \pm 12.87$  years, whereas that of the 26 patients of the control population was  $39.7 \pm 17.35$  years (Table 1). Eleven of 25 patients (44%) in the test group and 6 of 26 subjects (23.07%) in the control group had a history of asthma (Table 1). *Aspergillus*-specific skin-prick testing was performed only in patients who were clinically and radiologically suspected to have AFRS, and 15 patients in the AFRS group exhibited a positive response, 4 did not surmount a type-1 reaction, and 5 patients did not undergo the test due to logistic issues.

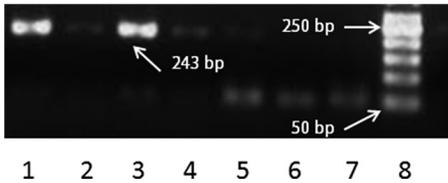
Representative gel images of the bands obtained for the four *PAR* genes are depicted in Figs. 1–4. Sample numbers 26 and 27 (controls) were excluded from the study because no RNA could be isolated from them. Ten samples (all controls) were excluded from *PAR4* analysis only



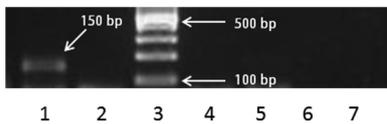
**Figure 1.** Representative agarose gel image for polymerase chain reaction–amplified products of the protease-activated receptor 1 (PAR1) gene. Product size = 209 base pairs (bp). Lane 1: 50-bp ladder; lanes 2–6: control samples; lanes 7–11: test samples.



**Figure 2.** Representative agarose gel image for polymerase chain reaction–amplified products of the protease-activated receptor 2 (PAR2) gene. Product size = 247 base pairs (bp). Lane 3: 50-bp ladder; lanes 1–2: control samples; lanes 4–7: test samples.



**Figure 3.** Representative agarose gel image for polymerase chain reaction–amplified products of the protease-activated receptor 3 (PAR3) gene. Product size = 243 base pairs (bp). Lane 8: 50-bp ladder; lanes 1–3: test samples; lanes 4–7: control samples.



**Figure 4.** A representative agarose gel image for polymerase chain reaction–amplified products of the protease-activated receptor 1 (PAR1) gene. Product size = 150 base pairs (bp). Lane 3: 100-bp ladder; lanes 1–2: test samples; lanes 4–7: control samples.

because no amplification could be seen in them after PCR. Due to the skewness of the data, as shown with the Kolmogorov-Smirnov test, the Mann-Whitney test was used for analysis, and it was observed that the expression of all four PAR genes was higher in the test samples but was statistically significant only for PAR1 ( $p = 0.004$ ) and PAR2 ( $p = 0.05$ ) (Table 2).

Box plots of the comparisons between cases and controls for all four PAR genes are depicted in Fig. 5. The expression of the four PAR genes against each other was also compared within the test and control groups by using the Wilcoxon signed rank test, and a statistically significant difference was seen between *par1* and *par2* ( $p = 0.007$ ), *par1* and *par3* ( $p = 0.029$ ), *par1* and *par4* ( $p =$

$0.0001$ ), *par2* and *par4* ( $p = 0.002$ ), and *par3* and *par4* ( $p = 0.009$ ) in the test group (Table 3). *par1* was observed to be significantly more expressed than the other PAR genes, whereas *par4* was found to be the least expressed. In the control group, as well, *par1*, *par2*, and *par3* exhibited higher expression compared with *par4* but achieved a statistically significant difference between *par3* and *par4* genes only (Table 4). Box plots of the expression of the four PAR genes in both the groups are depicted in Fig. 6.

## DISCUSSION

AFRS can be considered a refractory form of chronic rhinosinusitis characterized by intense eosinophilic response, which results in, e.g., allergic mucin, nasal polyps, hypersensitivity. In the most simple terms, it can be defined as an allergic reaction to environmental fungal allergens. However, various studies and evidence over the past 2 decades indicate a more-complex etiology. The role of PARs in airway inflammation has been extensively studied lately and the positive correlate triggered the possibility of a local hypersensitive reaction to fungal proteases, which act through PARs located on the cell membrane of sinonasal epithelium and trigger the inflammatory cascade. They increase the permeability of the surface epithelium by disrupting the tight junctions between epithelial cells, thereby providing access to the allergens. This was supported by the work of Tai *et al.*<sup>22</sup> who showed the breakdown of occludin, a tight junction protein, by the serine protease of *Penicillium chrysogenum*. Despite all the evidence that favors the role of PARs in the inflammatory pathway, there is a paucity of evidence that directly links AFRS and PAR expression. In our study, we compared the differential expression of the four PAR genes in 25 patients who had AFRS with 26 patients who had chronic rhinosinusitis with or without nasal polyposis.

After analyzing the results statistically, we found an increased expression of all four PAR genes in the test population compared with the controls as observed by the bands obtained on the agarose gels and their respective densitometric values. There was a statistically significant difference in the expression of PAR1 and PAR2 gene expression between the two groups and a borderline significance, with a  $p$  value of 0.059 for PAR3. In a study by Ebert *et al.*,<sup>23</sup> PAR expression was compared between patients with AFRS and controls without disease, which included patients who were undergoing endoscopic pituitary surgery, and between patients with AFRS and controls without disease who had chronic rhinosinusitis. It was concluded that a significant difference was seen in PAR3 expression in the AFRS versus the pituitary group. None of the genes in any of the other groups had a statistically significant difference. Although previous studies demonstrated the role of PAR2, primarily in the allergic pathway, there is evidence that PAR3 is similarly

Table 2 Mann-Whitney test for the four genes, comparing each of their expressions in the two groups

Serial No.	Gene Name	Case Group	Control Group	<i>p</i> Value
1	<i>par1</i>			<b>0.004</b>
	No.*	25	24	
	Median (IQR) Min-max	0.21 (0.04–0.57) 0.0–1.65	0.0 (0.0–0.21) 0.0–0.86	
2	<i>par2</i>			<b>0.050</b>
	No.*	25	24	
	Median (IQR) Min-max	0.03 (0.0–0.45) 0.0–0.97	0.0 (0.0–0.12) 0.0–0.65	
3	<i>par3</i>			0.059
	No.*	25	24	
	Median (IQR) Min-max	0.05 (0.0–0.44) 0.0–2.209	0.0 (0.0–0.04) 0.0–1.48	
4	<i>par4</i>			0.087
	No.*	25	14	
	Median (IQR) Min-max	0.0 (0.0–0.07) 0.0–0.28	0.0 (0.0–0.0) 0.0–0.97	

*par* = Protease-activated receptor; IQR = interquartile range; min = minimum; max = maximum.

\*The number of subjects analyzed in each group.

Bold values indicated  $p < 0.05$ .

involved. Shin *et al.*<sup>24</sup> demonstrated that nasal epithelial cells did not express PAR mRNA before stimulation; however, after stimulation with fungi, *viz.*, *Alternaria*, *Cladosporium*, and *Aspergillus*, they expressed PAR2 and PAR3 mRNA, as was evident in the reverse transcriptase-PCR analysis.

One possible explanation of conflict of the results with respect to the difference seen in the PAR1 gene could be the difference in the etiologic agent of fungal sinusitis in the Indian subcontinent. *Aspergillus flavus* is the most commonly isolated species in India compared with the dematiaceous fungi and *Aspergillus fumigatus* in the West.<sup>25–27</sup> In our study, 16 of 25 patients in the AFRS group were culture positive, with *A. flavus* isolated in 13 patients, *A. fumigatus* in 1 patient, and *Alternaria alternata* in 2 patients. To our knowledge, there are no data of the interaction of fungal proteases in different fungal species. Another factor that accounted for the difference could be the method of interpretation of gene expression. Although Ebert *et al.*<sup>23</sup> used the microarray technique, we performed PCR in our study for this purpose. The increased overall expression of all four *pars* in the test group, as shown in this study, is consistent with the literature that indicates the presence of PARs on epithelial cells, mast cells, lymphocytes, fibroblasts *etc.* The increased amount of inflammation, together with the activation of cells by fungal proteases in the test group, could be the reason for an increased expression seen.

However, when the expression of *par4* was compared with the other three *par* genes, it was found to be

clearly lower in both the test and the control groups, and the difference was significant in the test group. This was evident in the Wilcoxon signed rank test, in which a statistically significant difference was seen between *par1* and *par4*, *par2* and *par4*, *par3* and *par4* in the test group. These data also seemed to be consistent with the literature because of all the PARs, PAR4 was the least associated with airway inflammation. In the control group, however, an intergene comparison showed a statistically significant difference only between *par3* and *par4*. This suggested possible upregulation of the other *par* genes compared with *par4* in the test samples. Our study had some limitations. Although it clearly demonstrated a role of PAR expression in AFRS, a larger sample size and further validation of data with more confirmatory methods, *e.g.*, real-time PCR, would be required to conclusively link PAR expression with AFRS.

## CONCLUSION

This study was undertaken to improve our understanding of AFRS and its pathogenesis. The two major conclusions from our study were the following: first, a statistically increased expression of *par1* and *par2* in the test group, and, second, a decreased overall expression of *par4* compared with the other *par* genes in both the groups. Presently, there are limited options available to control the intense inflammatory response that occurs in this disease. PAR antagonists, by causing a disruption in this inflammatory pathway, could help in better

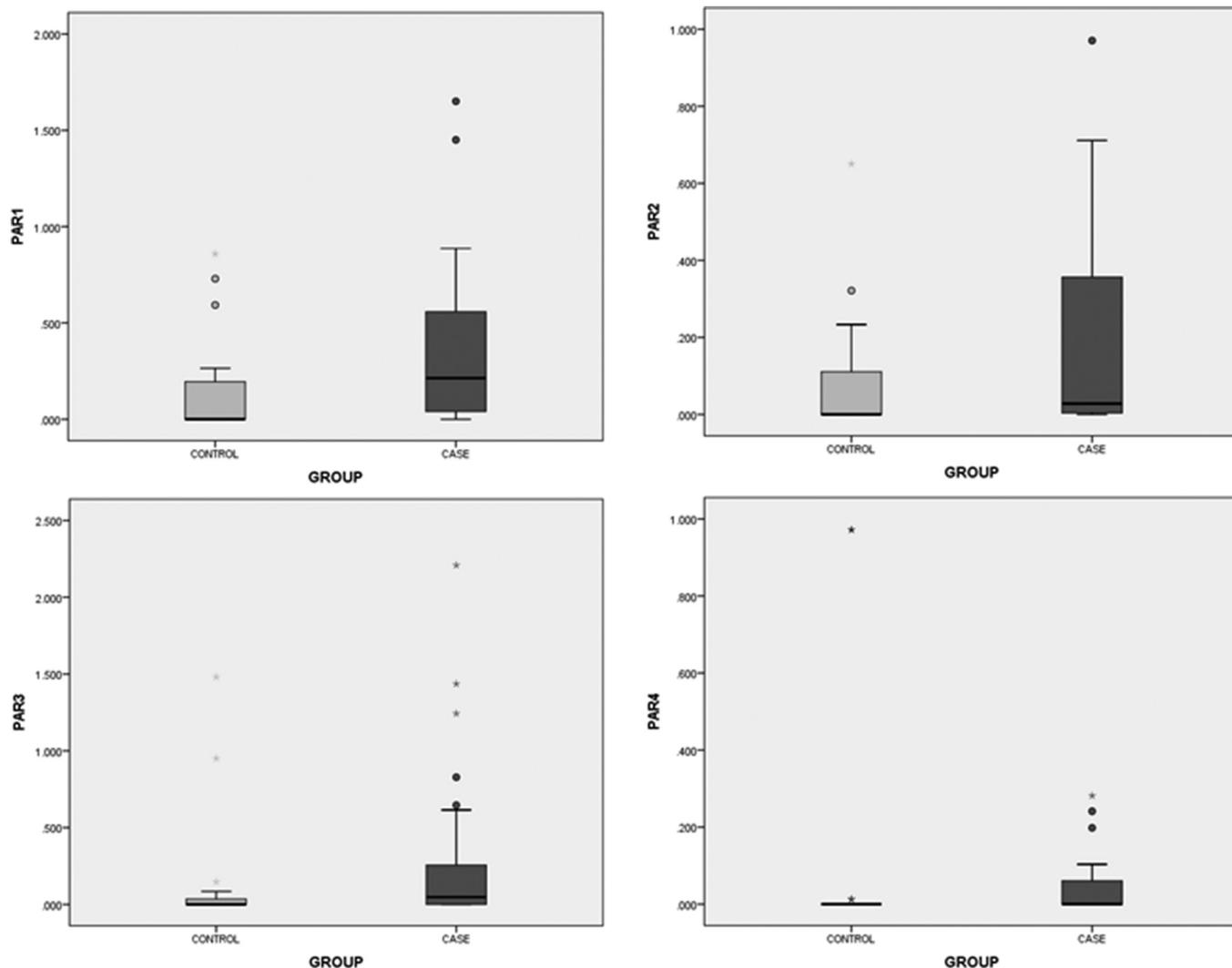


Figure 5. Box plots, depicting the comparison of the four genes in the two groups; it can be seen that the expression of all four genes is higher in the case allergic fungal rhinosinusitis (AFRS) group.

Table 3 Wilcoxon signed rank test, depicting intergene comparison within the case group

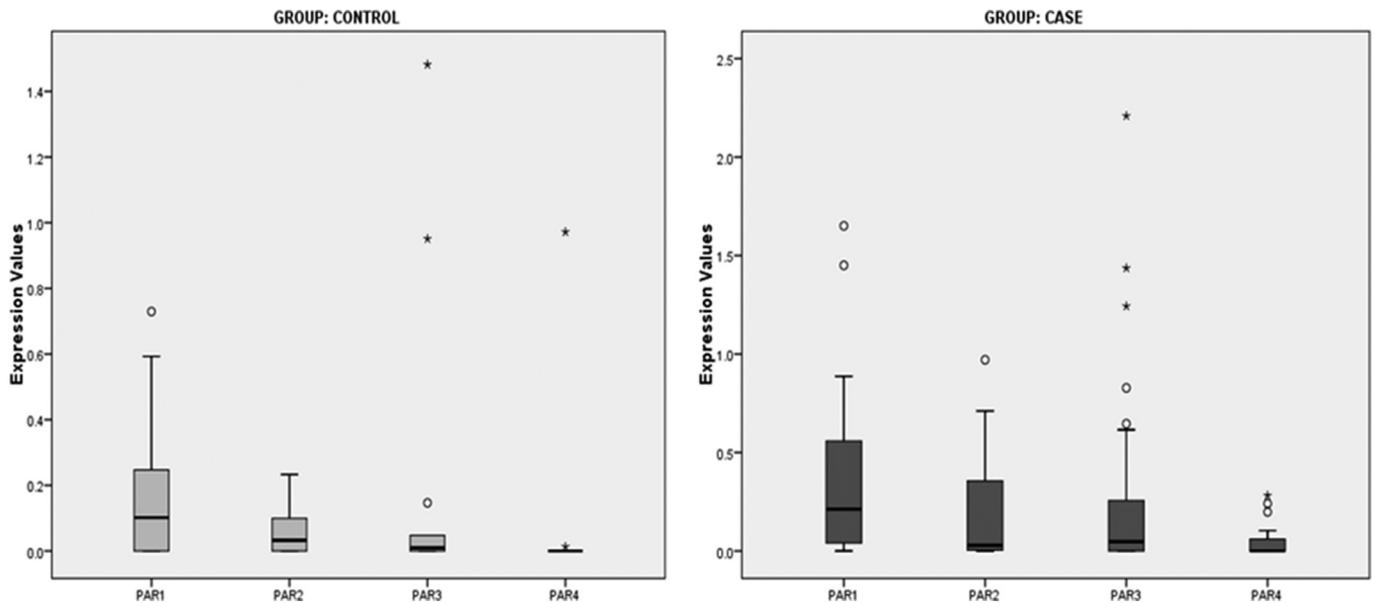
Serial No.	Gene Name	<i>p</i> Value
1	<i>par1</i>	<b>0.007</b>
2	<i>par1</i> <i>par3</i>	<b>0.029</b>
3	<i>par1</i> <i>par4</i>	<b>0.0</b>
4	<i>par2</i> <i>par3</i>	0.314
5	<i>par2</i> <i>par4</i>	<b>0.002</b>
6	<i>par3</i> <i>par4</i>	<b>0.009</b>

*par* = Protease-activated receptor.  
 Bold values indicated  $p < 0.05$ .

Table 4 Wilcoxon signed rank test, depicting intergene comparisons within the control group

Serial No.	Gene Name	<i>p</i> Value
1	<i>par1</i> <i>par2</i>	0.345
2	<i>par1</i> <i>par3</i>	0.209
3	<i>par1</i> <i>par4</i>	0.069
4	<i>par2</i> <i>par3</i>	0.363
5	<i>par2</i> <i>par4</i>	0.139
6	<i>par3</i> <i>par4</i>	<b>0.018</b>

*par* = Protease-activated receptor.  
 Bold values indicated  $p < 0.05$ .



**Figure 6.** Box plots, depicting intergene comparison within the two groups, showing protease-activated receptor 4 (PAR4) to be the least expressed in both the groups.

medical management of AFRS and result in fewer relapses.

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## ETHICAL APPROVAL

This study was approved by our institute's Ethics Committee (Ref:NK/1753/MS/10589-90).

## STATEMENT OF HUMAN AND ANIMAL RIGHTS

This study does not contain any animal subjects and the patients were duly enrolled in the study after a well informed consent.

## STATEMENT OF INFORMED CONSENT

All patients signed a consent form and were explained in detail about their participation before being enrolled in this study.

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