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NF-κb expression predicts clinical outcome for nasal polyposis*

F. Cardoso Pereira Valera[1], R. Queiroz[2], C. Scrideli[2], L. Gonzaga Tone[2], W.T. Anselmo-Lima[1]

[1] Department of Ophthalmology, Otorhinolaryngology and Head and Neck Surgery, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil
[2] Department of Pediatrics, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil

SUMMARY

Objective: *To correlate clinical prognosis of patients with nasal polyps to the expression of p65, c-Fos, GRα and GRβ.*

Methods: *A biopsy was obtained at the first evaluation of patients with nasal polyps, and at rhinoplasty for control mucosa. Patients with nasal polyps were treated with glucocorticoids and followed for at least 60 months. The expression of p65, c-Fos, GRα and GRβ was determined by Real Time-PCR and correlated to clinical outcome. The end-point of resistance to glucocorticoid therapy was considered when surgery was indicated.*

Results: *Patients with nasal polyps presented a higher expression of p65, a lower expression of GRα, and a lower GRα/GRβ ratio than control mucosa. The patients with nasal polyps who had a higher expression of p65 correlated with a poorer response to glucocorticoids, with a 3.5-fold higher risk for surgery.*

Conclusion: *Patients with a higher p65 (NF-κB) expression at diagnosis were associated to a worse response to clinical treatment, suggesting one of the mechanisms of cell resistance to glucocorticoid treatment in patients with nasal polyps.*

Key-words: glucocorticoid, nasal polyps, glucocorticoid receptor, transcription factors, NF-κB

INTRODUCTION

Topical glucocorticoid (GC) is considered the drug of choice for the initial treatment of Nasal Polyposis (NP) [1,2]. Nevertheless, the rate for remission of NP with GC alone has been reported to be between 60.9 and 80% [2]. There are at least two main causes for GC treatment failure: a limited action of topical GC, either due to polyps extension [3], or due to poor compliance to treatment [4], and cell/molecular resistance to the response to GC [5,6].

When GC binds to its cytoplasmic GC Receptor (GR), the GC-GR complex translocates into the nucleus. Once inside the nucleus, the GC-GR complex may exert two main forms of action: binding to glucocorticoid response elements (GRE), which are specific DNA domains that induce or inhibit transcription [7,8]; or by inactivation of transcription factors (TF) such as NF-κB and AP-1 by direct interaction [8]. Since these TFs can also inhibit GR, this phenomenon is known as mutual antagonistic cross-action [9]. It is recognized that the main mechanism of anti-inflammatory action of GC is through TF inhibition [8], and the transactivation of anti-inflammatory genes is considered to be of minimal importance in the anti-inflammatory process.

There are at least two well-described isoforms for GR [5,8], which differ themselves only in their hormone-binding domain: GRα, the predominant isoform, which has a high affinity to GC and an elevated transcriptional activity, and GRβ, which is uncapable of interacting with GC, but interacts with GRα in the nucleus [8]. GRβ is considered to be an endogenous inhibitor of GRα [5], and its increased expression has been reported in immune-mediated diseases such as ulcerative colitis, GC-resistant asthma and rheumatoid arthritis.

There is still some controversy about the real impact of GR isoform expression on GC resistance [10-12], but higher expression of GRβ, lower expression of GRα, or lower GRα/GRβ relation have been implicated as responsible for the increased cell resistance to GC [13]. Among these different mechanisms, an increased expression of GRβ has been consistently and extensively reported [5,6,14] in NP. Conversely, a lower GRα expression in nasal polyps compared to control nasal mucosa has only recently been described [15].

Cell resistance to GC may also be mediated by other molecular pathways [12], such as: activation of p38 mitogen activated protein (MAP) kinase; histone deacetylase dysfunction; and a reciprocal inhibitory effect between GR and TF. As GR and TF have this specific but reciprocal inhibitory interaction, higher activity of TF would increase the inflammatory response and could also promote cell resistance to GC.

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AP-1 is a cytoplasmic dimer predominantly consisting of c-Fos/c-Jun heterodimers, with c-Fos having high transcriptional activity [8,14]. When activated, AP-1 induces the expression of several cytokines and of other pro-inflammatory genes [8], regulating cell proliferation, differentiation, transformation, and apoptosis.

NF-κB is a heterodimer consisting of members of the p50 and p65 family, which coordinates and amplifies immune and inflammatory responses, as well as cellular apoptosis, growth and differentiation. NF-κB is pivotal to the regulation of immune and inflammatory genes. When activated, NF-κB translocates to the cell nucleus and p65 can directly bind to DNA and induce gene transcription of cytokines, chemokines and adhesion molecules such as IL-1β, TNF-α, IFN-γ, eotaxin, GM-CSF, ICAM-1, VCAM-1, as well as anti-apoptotic proteins (8,13). It is remarkable that some cytokines activated by NF-κB, such as IL-1β and TNF-α, can also activate this TF, and this circuit may explain the perpetuation of the local inflammatory process observed in some inflammatory diseases [13], as in the case of NP.

The objective of the present study was to correlate the expression of p65, c-Fos, GRα and GRβ, as well as the GRα/GRβ relation, to the prolonged clinical response to topical GC treatment in patients with NP.

MATERIALS AND METHODS

Patient population

Twenty patients with bilateral NP had their first evaluation at the Rhinosinusology Outpatient Clinic - University Hospital of School of Medicine of Ribeirão Preto - University of São Paulo. To confirm the presence of moderate NP, a CT scan was performed prior to inclusion in the study, and the extensiveness of disease was assessed through the Lund and Mackay score [16]. Exclusion criteria were associated diseases (such as Samter triad, ciliary dyskinesia and cystic fibrosis) and the prior use of antihistamines, antileukotrienes or oral or topical GC for the last two months before evaluation.

After giving written informed consent, patients answered a clinical questionnaire, grading each symptom from 0 to 4, according to intensity. The questionnaire assessed the symptoms of nasal obstruction, hyposmia/anosmia, cacosmia, anterior and posterior rhinorrhea, headache or facial pain, itching, and sneezing. All patients were then submitted to outpatient endoscopy to obtain an endoscopic score according to Lund [17], during which a polyp biopsy was obtained with the aid of a forceps, without previous anesthetic or vasoconstrictor. The biopsy was immediately identified and stored in liquid nitrogen.

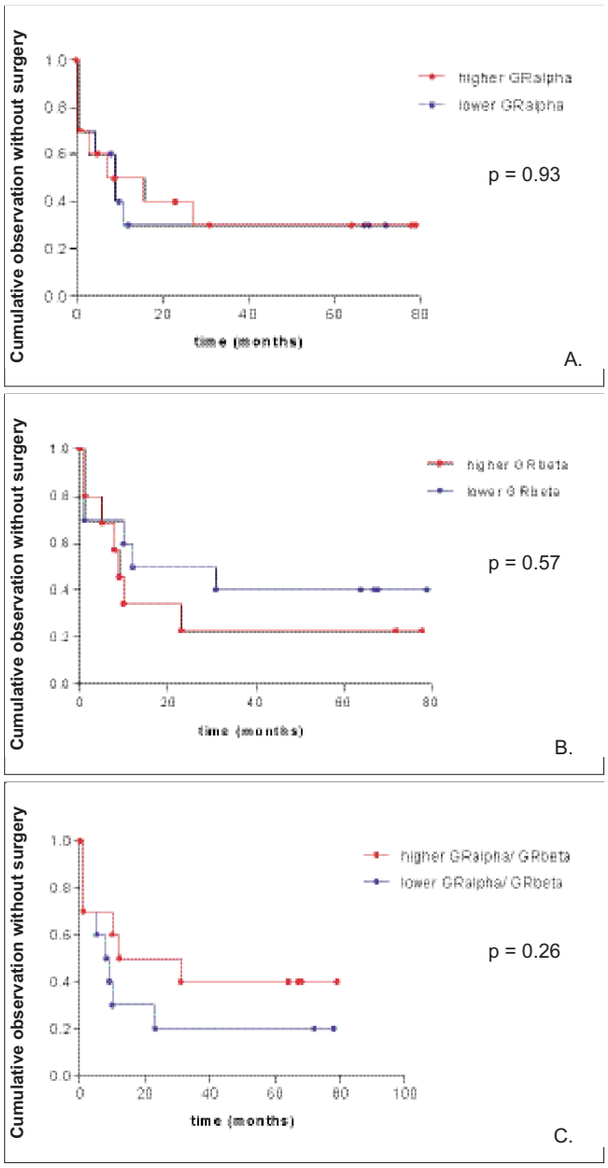

Figure 1. Comparison of clinical outcome (without surgical need) between patients with higher (G1) vs. lower (G2) expression of GRα(1a), GRβ (1b) and GRα/GRβ ratio (1c). Statistics by the Gehan Breslow-Wilcoxon test.

Topical glucocorticoids (Budesonide. Astra Zeneca, Cotia, Brazil) were then provided, and patients were instructed to use them in a dose of 256 mcg (or 2 sprays/nostril/BD) for two months. The patients were reassessed at the end of this period, and those with a good response to topical GC continued this treatment, while those considered with a poor response (assessed by maintenance or worsening of symptoms and/or endoscopic signs) were submitted to a more aggressive treatment, which included oral GC (prednisolone, 1 mg/kg/day, maximum dose at 60 mg, for 15 days) and antibiotics (either amoxicillin/clavulanate or levofloxacin for 15 days), if necessary. If the patient persisted symptomatic after the observational 4 month-period of medical treatment (which includ-

ed in all cases the topical GC during the whole period, with additional oral GC and antibiotics, if needed, for 15 days), an "immediate" endoscopic sinus surgery (ESS) was performed.

If the patients had an immediate response to topical GC but, during the 5-year period of evaluation, worsened their symptoms, they were submitted to the same aggressive medical treatment mentioned above. If patient persisted symptomatic, or frequently needed aggressive medical treatment to control the recurrent relapses, they were submitted to what was called "late" ESS.

The control group consisted of patients who were submitted to rhinoplasty with no nasal symptoms or changes observed by nasal endoscopy. All patients gave written informed consent before surgery, during which a biopsy was obtained and stored as described above. As nasal polyps are generally located in the middle meatus, we chose to obtain the biopsy of the middle nasal turbinate, even though they have a discrepant morphology.

This study was approved by the Ethics Committee of the Clinics Hospital of School of Medicine of Ribeirão Preto - University of São Paulo (process 10909/2003) and by the National Research Ethics Committee (CONEP, process 25000.000240/2004-81).

Real Time-PCR

RNA was extracted from the samples by the Trizol® technique and cDNA was synthesized from the isolated RNA using the Superscript III RNAse H-reverse transcriptase kit (Invitrogen, Carlsbad, USA) for GRα and p65, and the high capacity cDNA archive kit (Applied Biosystems, Carlsbad, USA) for GRβ and c-Fos. Since different RTases were used, an endogenous reference was performed for adequate normalization.

The primers were selected from the genomic DNA sequence (acquired from the Ensembl program: http://www.ensembl.org/index.html), according to Real Time-PCR specifications. The following primers, with their respective NCBI access numbers, were selected: c-Fos (S: ACTACCACTCACCCGCAGAC, AS: GTGGGAATGAAGTTGGCACT; NCBI: NM 005252.2), p65 (S:CCACGAGCTTGTAGGAAAGG, AS:CTGGATGCGCTGACTGATAG; NCBI: NM 021975.2), GRα (S:GAAGGAAACTCCAGCCAGAA, AS:TGTTTGGAAGCAATAGTTAAGGA; NCBI: NM001018074.1), GRβ (S:GAAGGAAACTCCAGCCAGAA, AS:GCCAAGATTGTTGGGATGAA; NCBI: NM001020825.1) and GUS (S:GAAAATATGTGGTTGGAGAGCTCATT, AS: CCGAGTGAAGATCCCCTTTTTA; NCBI: NM 000181.1).

Real Time-PCR studies were performed in duplicate with the SYBR Green PCR Master MIX kit (Applied Biosystems), using the sequential detection system Gene Amp® 7300 (PE Applied Biosystems). An amplification curve was obtained for each sample, as well as a dissociation curve specific for each gene. Efficiency PCR values were: c-Fos: 2.0; p65: 2.0; GRα: 1.91; GRβ: 1.92; GUS: 2.1.

The results were analyzed by the absolute quantification, through standard curve equation [18,19]. For this, a standard curve was constructed with four different dilutions (1:1, 1:10, 1:100, 1:1000) of cDNAs from cell line K562-LUCENA and bone marrow, both are known to have high expression of the genes under study.

The *GUS* gene was used as a housekeeping gene, as it is not influenced by the inflammatory process [20]. Since different RTases were used, *GUS* was analyzed for both of them, as well as for both cell lines, to complete normalization.

Statistical analysis

The expression for each gene was compared between patients with NP and controls. For this evaluation, we used the non-parametric Mann-Whitney test, with $p < 0.05$ considered significant.

To verify if the expression of each gene influenced the clinical outcome, we have separated the patients with NP based on the median value of each gene expression. As a result, the 20 patients were allocated in two groups of 10: G1, which was composed of those patients with higher expression of the specific gene to be analyzed, and G2, composed of those with lower expression for each studied gene. The same patients were differently distributed over these groups based on each gene expression.

All patients with NP were followed-up for at least 60 months. The event was considered unfavorable when the patient had undergone ESS, because this demonstrated resistance to clinical treatment. For each of the studied genes, the normalized groups of patients (G1 vs. G2) were compared, based on the surgical need. The event-free cumulative observation curve (Kaplan-Meyer method) was used to compare the outcome of each group. Log-Rank (Mantel-Cox) test was used for statistical analysis, and the level of significance was set at $p < 0.05$. If significant, the Hazard Ratio was also calculated.

RESULTS

Twenty patients with NP were studied, consisting of 16 males (80%) and 4 females with an age range from 15 to 59 years (mean ± SD: 44.5 ± 15.09; median: 42.50). The control group consisted of 8 patients with 3 males and 5 females, ranging in age from 18 to 53 years (mean: 40.3 ± 10.23; median: 39.76).

The clinical score showed that the most frequent symptoms experienced by patients with NP were: hyposmia/anosmia (median score: 4.0 out of 4), nasal obstruction (median score: 3.0 out of 4) and posterior rhinorrhea (median score: 3.0 out of 4). At endoscopy, the patients presented a median score of 4.0 (out of 6), and the median CT score was 16.0 (out of 24), showing that the majority of patients presented an extensive NP. The clinical, endoscopic and CT scan scores obtained at first evaluation are listed in Table 1, and were described in details elsewhere [3].

The molecular studies with Real Time-PCR revealed that patients with NP showed a significantly lower expression of GRα (median: NP: 0.33 vs. control: 2.68; $p < 0.0001$) and a lower GRα/GRβ ratio (median: NP: 0.86 vs. control: 2.74; $p < 0.0001$), as well as a significantly higher expression of p65 (median: NP: 0.34 vs. control: 002; $p < 0.05$) when compared to the control group (Table 2). No significant difference in expression was observed between these two groups for GRβ or c-Fos.

All 20 patients with NP were followed up for a mean period of 72.8 ± 5.4 months. During this long-term observation, 6 patients showed a considerable improvement of symptoms throughout the

Table 1. Score for each symptom. Endoscopic findings and CT scans (median and P25-P75) for patients with NP both during the initial evaluation and after treatment. Statistical analysis (initial versus after treatment) by Mann-Whitney's test.

	Initial evaluation		After treatment		p
Variable	median	P25-P75	median	P25-P75	
Nasal obstruction	3.00	2.00-4.00	1.00	0.00-2.00	0.0011*
Post nasal drip	3.00	2.00-3.00	0.00	0.00-1.00	0.0019*
Cacosmia	0.00	0.00-0.25	0.00	0.00-0.00	0.6949
Frontal headache	2.00	0.75-2.00	0.00	0.00-1.00	0.017*
Hyposmia	4.00	3.00-4.00	2.50	0.00-4.00	0.0214*
Nasal itching	1.00	0.00-3.00	0.00	0.00-1.00	0.1039
Anterior rhinorrhea	1.00	0.00-3.00	0.00	0.00-1.00	0.0431*
Sneezing	2.50	1.75-3.00	1.00	0.00-1.25	0.0026*
Endoscopy	4.00	3.75-5.00	3.00	2.00-4.00	0.0064*
Computed Tomography	16.00	12.0-23.5	----------	----------	----------

*: statistically significant

Table 2. Comparison of the expression of genes GRα, GRβ, c-Fos and p65, as well as the GRα/GRβ ratio in patients with nasal polyposis in comparison to controls. Statistical analysis by Mann-Whitney's test.

	Nasal polyposis		Control		
Gene	median	P25-P75	median	P25-P75	p
GRα	0.33	0.12-0.86	2.68	1.41-6.02	<0.0001*
GRβ	0.49	0.20-2.21	0.54	0.15-1.02	0.7086
GRα/GRβ relation	0.86	0.12-1.69	2.74	2.13-44.46	<0.0001*
c-Fos	1.00	0.35-4.80	1.87	0.22-50.32	0.9801
p65	0.34	0.12-1.09	0.02	0.01-0.70	0.0487*

*: statistically significant

Table 3. Groups 1 and 2. Expression and clinical outcome of each patient based on the median value for each studied gene. Time for recurrence expressed in months.

	GRα		GRβ		GRα/GRβ		c-Fos		p65	
	Expression	Recurrence	Expression	Recurrence	Expression	Recurrence	Expression	Recurrence	Expression	Recurrence
G1 (higher	0.911	23	142.95	23	17.665	1	225.99	23	39.737	8
expression)	0.848	9	999.22	1	1.434	1	824.27	1	0.427	1
	1.838	5	16.229	5	1.872	10	1.586	5	0.777	5
	0.427	1	0.775	1	0.894	1	1.973	1	8.404	1
	0.83	No	0.718	1	20.74	No	2.829	No	1.044	No
	0.811	No	6.634	No	1.637	No	10.727	No	750.64	12
	0.393	No	0.520	No	2.186	No	1.697	10	1.024	9
	7.288	1	1.544	10	1.412	12	1.153	No	1.238	1
	1.112	1	0.793	8	1.072	No	23.968	12	0.639	1
	499.78	31	4.21	9	1697.88	31	14.266	9	1.376	31
G2 (lower	0.271	1	0.477	1	0.006	23	0.195	1	0.053	23
expression)	0.189	1	0.040	No	0.0002	1	0.82	1	0.078	No
	0.117	1	0.228	1	0.113	5	0.855	1	0.197	No
	0.102	10	0.413	1	0.122	No	0.461	1	0.264	1
	0.064	No	0.054	10	0.827	1	0.024	10	0.046	1
	0.116	10	0.146	No	0.163	1	0.014	No	0.261	10
	0.128	8	0.120	No	0.123	No	0.296	8	0.016	No
	0.239	No	0.018	12	0.075	10	0.329	No	0.006	10
	0.262	No	0.366	No	0.161	8	0.564	No	0.224	No
	0.026	12	0.294	31	0.201	9	0.361	31	0.136	No

follow-up period with clinical treatment alone, so they continue with topical GC up to the present time. Six patients have worsened or maintained their symptoms after the first 2 months of evaluation, and were thereafter submitted to ESS. Eight other patients had an initial improvement of symptoms and were maintained in the medical treatment group, but their remaining symptoms or their frequent recurrences led us to opt for surgical treatment, and ESS was performed on average 12 months after the initial evaluation.

Based on the median of the expression of each studied gene, patients were allocated into G1 or G2. The details are demonstrated on Table 3. The comparison between patients with higher (G1) or lower (G2) expression of each gene, by the Kaplan-Meyer

method and Log-rank (Mantel-Cox) analysis, revealed that patients with higher expression of p65 had a significant poorer clinical outcome (p = 0.039, Figure 2b). The Hazard Ratio for p65 overexpression was also calculated, and the value obtained was HR = 3.446 (95% CI: 1.063 to 11.16). This result demonstrates that patients with higher expression of p65 on nasal polyps at diagnosis presented a 3.5-fold higher chance to need surgery to control their symptoms due to clinical treatment failure when compared to those with lower expression of p65. No differences in Kaplan-Meyer curves were observed between subgroups in relation to the other genes.

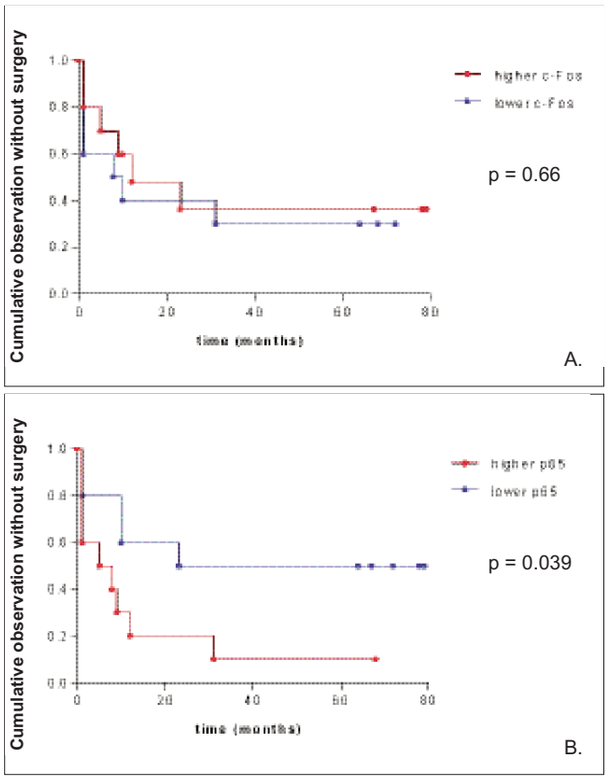

Figure 2. Comparison of clinical outcome (without surgical need) between patients with higher (G1) vs. lower (G2) expression of c-Fos (2a) and p65 (2b). Statistics by the Gehan Breslow-Wilcoxon test.

DISCUSSION

Glucocorticoids have been used for a long time for the treatment of several inflammatory diseases, including NP. Nevertheless, only recently the mechanism of GC action is being clarified. As GC is the most effective treatment for NP, it is essential to understand its mechanism of action, as well as how the cells act to inhibit the drug. This knowledge will confer the development of new pharmacotherapies that, separately or in combination with GC, might be more effective for the treatment of NP.

In the present study, we observed that untreated nasal polyps presented a lower GRα/GRβ ratio than control nasal mucosa due to lower GRα expression, with no difference in GRβ expression between the two groups. Since GRα is the GR isoform responsible for GC translocation into the nucleus and for its interaction with DNA, we assume that lower GRα expression could have negatively interfered with GC action in this group of patients. This could, at least in part, explain the incomplete response to treatment occasionally observed in NP patients.

Lower GRα/GRβ ratios has been previously reported in nasal polyps and has been considered to be related to inflammation [5], although in that study, as well as in many others [6,14,21], this lower ratio occurred due to a higher expression of GRβ. However, in two recent reports employing quantitative PCR (the same method employed in the present study) conducted by Li et al. [15] and Pujols et al. [22], lower expression of GRα was observed in nasal polyps when compared to control mucosa, with no difference in GRβ expression. With these results, we hypothesized that, if GC resistance is strongly related to GR expression, it seems to occur due to a lower GRα/GRβ ratio, irrespective of the expression status of each isoform in particular.

Higher expression of TF has been largely described as a pivotal mechanism of GC resistance in many diseases, since these factors bind to GR and inhibit the nuclear translocation of the GR-GC complex. In the present study, patients with NP similarly expressed c-Fos when compared to control mucosa, whereas p65 was significantly more expressed in nasal polyps than in control.

There are few studies demonstrating the importance of these TF (c-Fos and NF-κB) on nasal polyps. Baraniuk et al. [23] observed by qualitative PCR a consistent higher expression of c-Fos in nasal polyps than in control mucosa. Takeno et al. [24] observed by immunohistochemistry a higher expression of the NF-κB subunit p50, its constitutional fraction, in patients with NP compared to control. We opted to use quantitative methods, and the analysis of our results confirmed the importance of p65 (the active fraction of NF-κB), but not of c-Fos (the fraction of AP-1) in the genesis and perpetuation of inflammatory responses in NP.

Prospective studies correlating gene expression in response to GC or to therapeutic prognosis are even more scarce. Two recent articles [22,25] found no correlation between the expression of GR isoforms and the clinical resistance to GC in nasal polyposis. To our knowledge, this is the first reference about TF expression and its association to GC resistance in nasal polyps.

Our group has previously described the clinical response to topical budesonide to gene expression at diagnosis and after a short-term (over a 2 month period) follow-up of clinical treatment [26]. We observed that the patients with a poor response to topical GC presented higher expression of p65, IL-1β and ICAM-1 at first evaluation. After treatment, poor responders persisted with higher expression of IL-1β and developed a higher expression of GRβ when compared to good responders.

In the present study, we evaluated the influence of the initial expression of GR and TF genes on long-term clinical outcome, and we observed that patients with higher expression of p65 at diagnosis turned to be poor responders to clinical treatment, with a consequent 3.5-fold higher risk of surgical need to control their

symptoms.

NF-κB is a central transcription factor on gene regulation, and its expression is reported to be involved in various auto-immune and inflammatory diseases, as well as in neoplasias. This is the first study to evaluate NF-κB expression and to correlate it to prognosis in NP. Our results reinforce the importance of NF-κB among the mechanisms of resistance to clinical treatment in NP, especially when glucocorticoids are involved. This finding may be of particular importance because NF-κB inhibitors are currently being developed. These new compounds, either isolated or in combination with topical GC, may turn to be a rationale therapeutical option towards the improvement of the clinical treatment on NP.

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Fabiana Cardoso Pereira Valera, MD
Department of Ophthalmology
Otorhinolaryngology and Head and Neck Surgery
School of Medicine of Ribeirão Preto
University of São Paulo
Av. Bandeirantes, 3900
14049-900 Ribeirão Preto
São Paulo
Brazil

Tel: +55-16-3602 2862
Fax: +55-16-3602 2860
E-mail: facpvalera@uol.com.br