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Expression of transcription factors NF- κ B and AP-1 in nasal polyposis

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Clinical and
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Allergy

Summary

Background The treatment and prognosis of nasal polyposis (NP) may be influenced by transcription factors, but their expression is poorly understood.**Objective** To determine the expression of transcription factors [(nuclear factor- κ B) NF- κ B and (activator protein) AP-1], cytokines [IL-1 β , TNF- α and (granulocytes and macrophage colony-stimulating factor) GM-CSF], growth factor (b-FGF), chemokine (eotaxin-2) and adhesion molecule (ICAM-1) in NP in comparison with nasal mucosa controls.**Methods** Cross-sectional study. Twenty biopsies of nasal polyps were compared with eight middle turbinate biopsies. p65, c-Fos, IL-1 β , TNF- α , ICAM-1, b-FGF, eotaxin-2 and GM-CSF were analysed through RQ-PCR, and p65 and c-Fos were also analysed through Western blotting.**Results** NF- κ B expression was increased in patients with NP when compared with control mucosa ($P < 0.05$), whereas AP-1 expression did not differ significantly between groups. Expressions of IL-1 β , eotaxin-2 and b-FGF were also increased in patients with NP compared with controls ($P < 0.05$).**Conclusions** The transcription factor NF- κ B is more expressed in NP than in control mucosa. This is important in NP because NF- κ B can induce the transcription of cytokines, chemokines and adhesion molecules, which play an important role in the inflammatory process. Moreover, transcription factors influence the response to corticosteroids, which are the basis of NP treatment. Transcription factor AP-1 does not seem to have a significant role in the pathological process.**Keywords** AP-1, c-Fos, cytokines, nasal polyposis, NF- κ B, p65, transcription factors**Submitted** 20 February 2007; **revised** 19 February 2007; **accepted** 6 December 2007

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Introduction

Nasal polyposis (NP) is a chronic inflammatory disease with a prevalence of 0.5%–4% in the general population [1]. Its main histological alterations are thickening of the epithelium, increase of goblet cells, thickening of the basement membrane, oedema, increased fibroblast numbers and recruitment of inflammatory cells, especially eosinophils, into the stroma [1].

NP is induced and perpetuated by cytokines, basically through two mechanisms. One is the stimulation of local tissue growth, mainly mediated by basic fibroblast (b-FGF), TGF- α and TGF- β . The other one is the maintenance of a permanent inflammatory status, mediated by IL-1 β , TNF- α , IL-4, IL-5, IL-6, IL-10 and IFN- γ , among others [2].

Epithelial cells and fibroblasts are induced by the initial inflammatory process to produce inflammatory cytokines,

TNF- α and IL-1 β . Once stimulated by the cytokines, structural cells in the nasal mucosa produce a variety of other cytokines, chemokines and adhesion molecules such as granulocytes and macrophage colony-stimulating factor (GM-CSF), IL-3, IL-5, IL-8, TGF- β , eotaxin, eotaxin-2, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), in addition to TNF- α and IL-1 β themselves [3, 4]. These molecules in turn are able to recruit circulating cells such as T lymphocytes, eosinophils, mast cells and neutrophils. The complex interaction of cytokines produced by structural and inflammatory cells leads to the NP chronic inflammatory process, which, once started, apparently does not depend on a causal stimulus to perpetuate itself [5].

It is now known that cytokine production during the inflammatory process is induced by transcription factors. Once an inflammatory process (epithelial lesion,

production of cytokines IL-1 β and TNF- α , presence of bacteria, fungi or viruses) is initiated, transcription factors will be activated and translocated to the cell nucleus, inducing the transcription of some genes, particularly the ones related to inflammatory cytokines, chemokines and adhesion molecules [6]. Several genes involved in the inflammatory response have sites that activate both the transcription factors activator protein (AP-1) and nuclear factor- κ B (NF- κ B).

AP-1, a cytoplasmic dimer consisting predominantly of high transcription capacity c-Fos and c-Jun heterodimers [3, 6], when activated, induces the expression of cytokines such as IL-4, IL-5 and GM-CSF, as well as other pro-inflammatory genes [6]. AP-1 also regulates cell proliferation, differentiation, transformation and apoptosis.

NF- κ B is a heterodimer consisting of members of the p50 and p65 families. It coordinates and amplifies the immune and inflammatory response, in addition to regulating cell apoptosis, growth and differentiation. NF- κ B plays a fundamental role in the regulation of immune and inflammatory genes and its absence is incompatible to life [6]. When activated, NF- κ B translocates to the cell nucleus and its active fraction (p65) induces the transcription of cytokines, chemokines and adhesion molecules such as IL-1 β , TNF- α , IFN- γ , eotaxin, GM-CSF, ICAM-1, VCAM-1 and anti-apoptotic proteins [3, 6]. Because both IL-1 β and TNF- α may in turn activate NF- κ B, the perpetuation of the inflammatory process in NP could be explained easily [3].

NF- κ B and AP-1 may inhibit the action of glucocorticoids (GC) through a mechanism known as inhibitory cross-reaction [7]. Thus, the increased production of these transcription factors could not only induce the NP inflammatory process but also inhibit the action of GC used in its treatment.

Given the importance of transcription factors in the inflammatory processes and the poor description of transcription factors in NP, the objectives of the present study were (1) to evaluate mRNA expression by quantitative RQ-PCR and the concentration of proteins by Western blotting for the transcription factors AP-1 and NF κ B in NP patients compared with normal mucosa controls and (2) to correlate these findings to the expression of pro-inflammatory cytokines (IL-1 β , TNF- α and GM-CSF), chemokine (eotaxin-2), adhesion molecule (ICAM-1) and growth factor (b-FGF) determined by RQ-PCR in both groups of patients, NP and controls.

Patients and methods

This study was conducted with 20 patients showing bilateral NP attending at the Rhinosinusology Outpatient Clinic of the University Hospital of Ribeirão Preto, University of São Paulo. Patients who had used antihistamines, oral or topical GC for at least 2 months before admission were excluded from the study. Patients with

associated diseases such as asthma, Samter's triad, ciliary dyskinesia and cystic fibrosis were also excluded.

All patients gave written informed consent, and were then submitted to an outpatient nasofibroscope and to a polyp biopsy, which was immediately stored in liquid nitrogen.

The control group consisted of patients submitted to plastic nasal surgery, without nasal symptoms or changes in nasofibroscope. A biopsy from the mucosa of the middle nasal turbinate was obtained during surgery and stored as described previously. All patients gave written informed consent to participate in the study before surgery.

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

Western blotting

The proteins of each frozen sample were isolated and quantified by the method of Bradford, as described in the Amersham Biosciences guide for protein quantification (Little Chalfont, Buckinghamshire, UK). Briefly, 10 μ g from each sample was denatured and submitted to electrophoresis to separate the proteins according to molecular weight. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane (Amersham Biosciences) and immunodetected with anti-c-Fos (D-1) and anti-p65 (F-6) antibodies (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA). The secondary anti-mouse monoclonal antibody was then added (Amersham Biosciences) and the membranes were submitted to radiographic labelling in a dark chamber with the Enhanced Chemiluminescent Detection kit (ECL) (Amersham Biosciences) and left in contact with the solution and X-rays for 5 min. Semi-quantitation of the labelled proteins was performed with a computerized densitometer (GS 800 Calibrated Densitometer, BioRad, Hercules, CA, USA). The digitized images were analysed with a reading software (Quantity One Quantitation Software, BioRad), and the number of pixels/mm² was determined. This number is directly related to the concentration of separated proteins. Examples of digitalized images are illustrated in Fig. 1.

Real-time quantitative polymerase chain reaction

RNAs isolated from the frozen samples were treated with DNase (Invitrogen, Carlsbad, CA, USA). cDNAs were then synthesized from the isolated RNAs (2 μ g/reaction) using the Superscript III RNase H-reverse transcriptase kit (Invitrogen) for the TNF- α , p65, ICAM-1 and eotaxin-2 genes, and the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) for the GMCSF, IL-1 β , c-Fos and b-FGF genes.

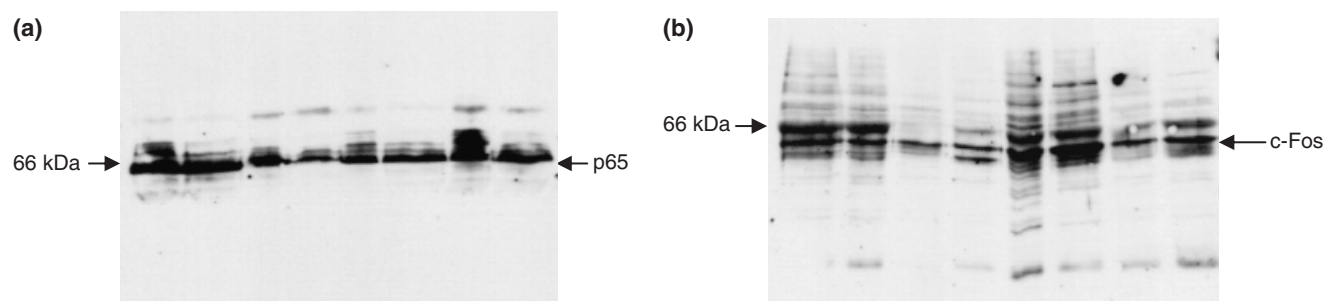


Fig. 1. Digitized images of the results obtained by Western blotting analysis of p65 (a) and c-Fos (b). Greater band intensity corresponds to higher protein concentration.

Table 1. Primers sequences (senses and anti-sense), NCBI numbers, size of amplicons (Amp) and expected melting temperatures (T_m), selected for RQ-PCR analysis of genes in the column at left

Primer	Sense (S) and anti-senses (AS)	NCBI numbers	Amp	T_m (°C)
IL-1 β	S: TCAGCCAATCTTCATTGCA AS: TGGCGAGCTCAGGTACTTCT	NM 000576.2 NM 000576.2	61	58
TNF- α	S: CTCCTTCTGCCTGCTGCACTT AS: GCCAGAGGGCTGATTAGAGA	NM 000594.2 NM 000594.2	82	62
GM-CSF	S: CAGCCTCACCAAGCTCAAG AS: TGACAAGCAGAAAGTCCTTCA	NM 000758.2 NM 000758.2	145	60
Eotaxin-2	S: CTGTCCAGCAGGAGCACAT AS: CTCTTGGTGGTGAAGAT	NM 002991.2 NM 002991.2	53	56
b-FGF	S: AGAGCGACCCTCACATCAAG AS: TTCCTTCATAGCCAGGTAACG	NM 002006.3 NM 002006.3	97	62
ICAM-1	S: GCCAACCAATGTGCTATTCA AS: GCCAGTTCCACCCGTTCT	NM 000201.1 NM 000201.1	93	58
c-Fos	S: ACTACCACTCACCCGAGAC AS: GTGGGAATGAAGTTGGCACT	NM 005252.2 NM 005252.2	102	62
p65	S: CCACGAGCTGTAGGAAAGG AS: CTGGATGCGCTGACTGATAG	NM 021975.2 NM 021975.2	144	62
GUS	S: GAAAATATGTGGTTGGAGAGCTCATT AS: CCGAGTGAAGATCCCTTTT	NM 000181.1 NM 000181.1	100	69

RQ-PCR, real-time quantitative PCR; GM-CSF, granulocytes and macrophage colony-stimulating factor; b-FGF, basic fibroblast growth factor; ICAM-1, intercellular adhesion molecule-1; GUS, Glucuronidase reporter gene.

The primers were selected from reference mRNA (acquired from the Ensembl program: <http://www.ensembl.org/index.html>) and constructed using the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) according to the specifications for RQ-PCR. Primers had a maximum size of 150 bp, a C/G concentration of 20%–80%, a dissociation temperature of around 60 °C and a maximum of 2 C/G nucleotides at the 3' end of the primer. To exclude DNA products, polymorphisms and alternative splicing primers were also synthesized in two different exons. The primers selected are described in Table 1.

The RQ-PCR procedures were performed in duplicate with the SYBR Green PCR Master MIX kit (Applied Biosystems), with the addition of 10 μ L SYBR Green PCR Master MIX, 4 μ L water, 0.5 μ L of each primer to be studied (sense and anti-sense, at a final concentration of 15 pm/20 μ L) and 5 μ L cDNA diluted 1 : 10. The RQ-PCR study was carried out using the Gene Amp[®] 7300

sequence detection system (PE, Applied Biosystems). PCR was standardized as follows: pre-heating at 50 °C for 2 min, denaturation at 95 °C for 10 min and 50 amplification and quantifying cycles (15 s at 95 °C and 60 s at 60 °C), followed by analysis of the dissociation curve of 60 s at 95 °C. PCR efficiency values were IL-1 β , 2.1; TNF- α , 1.9; GM-CSF, 1.9; eotaxin-2, 2.0; b-FGF, 2.0; ICAM-1, 2.1; c-Fos, 2.0; p65, 2.0; and GUS, 2.1.

An amplification curve was obtained for each sample, as well as a dissociation curve, which is specific for each gene. Figure 2 illustrates the amplification and dissociation curves for p65 and c-Fos.

The results were quantified and normalized, in relation to a standard curve constructed by using cell lines (K562-LUCENA cell line and bone marrow cells) that highly express the genes studied. cDNAs of these cell lines in four different dilutions (1 : 1, 1 : 10, 1 : 100, 1 : 1000) were used to obtain the standard curve, and

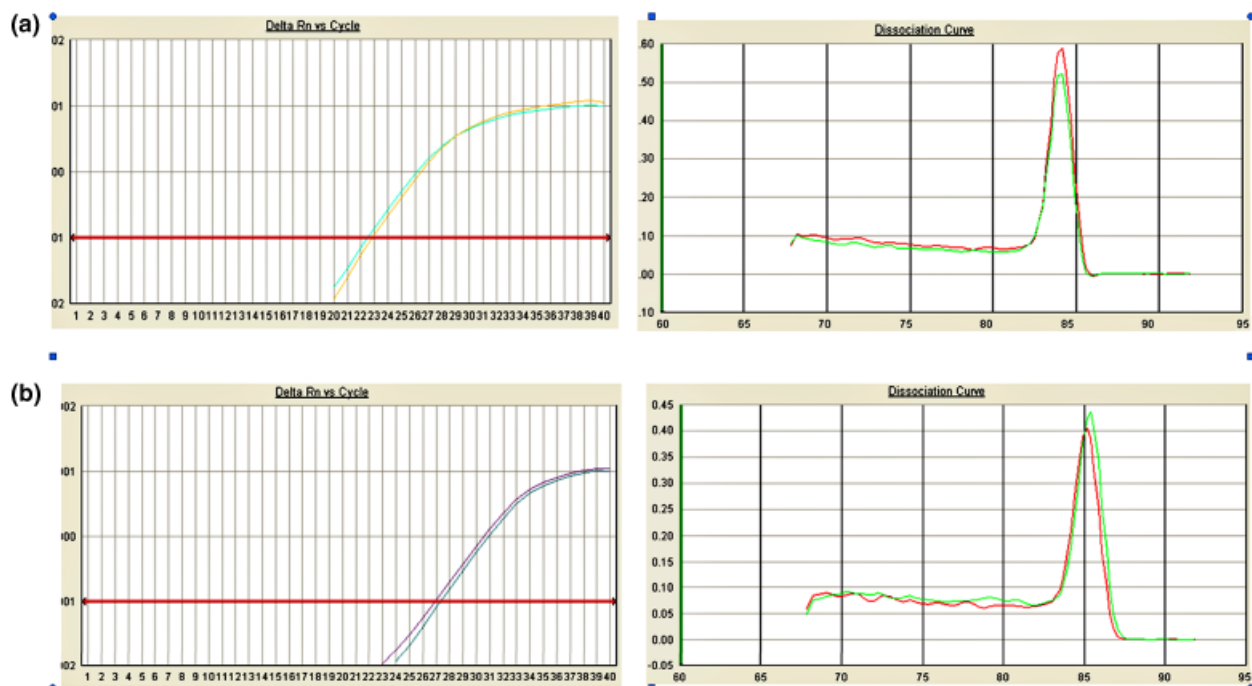


Fig. 2. Amplification (number of cycles obtained to reach the threshold) and dissociation (temperature at which the amplification curve is obtained) curve examples for genes p65 (a) and c-Fos (b).

Table 2. c-Fos and p65 protein expression in NP patients and control group, as measured by Western blotting

	NP patients			Control group			P
	Mean	Median	P25–P75	Mean	Median	P25–P75	
c-Fos	2.50	0.97	0.25–2.86	1.66	1.10	0.74–1.61	0.9999
p65	2.26	1.19	0.59–2.7	0.44	0.21	0.13–0.36	0.0039*

*Significant *P*.

P25–P75, percentile 25 to 75; NP, nasal polyposis.

sample expression was determined using the following equation:

Sample expression = (curve slope $\times$ *Ct*) + curve intercept, where *Ct* is the number of cycles necessary to achieve the threshold.

The β -glucuronidase reporter gene (*GUS*) was used as the housekeeping gene or the endogenous reference. Because it is similarly expressed in all cells, the *GUS* gene is used as a control for material validation and for normalization of cDNA concentration in the different samples [8]. To complete standardization, *GUS* was studied with both reverse transcriptases used in this study, as well as with both cell lines.

The following equation was used to obtain the final calibration and normalization of gene expression in different cell lines:

$$\text{Final calibration cell line} = \frac{\text{sample gene value}/\text{sample GUS value}}{\text{gene value}/\text{cell line GUS value}}$$

Statistical analysis

The non-parametric Mann–Whitney test was used for all analyses, with the level of significance set at $P < 0.05$. Mean and standard deviation were not appropriate because variables did not follow a normal distribution. Thus, the results were presented as median and P25–P75, representing values for the 25th and 75th percentiles, respectively.

Results

Twenty patients with NP were evaluated, 16 males (80%) and four females ranging in age from 15 to 59 years (mean $\pm$ SEM: 44.5 ± 15.09 ; median: 42.50). The control group consisted of eight patients submitted to nasal plastic surgery without nasal symptoms or any changes shown in nasal endoscopy. Of these, three were males and five were females ranging in age from 18 to 53 years (mean: 40.3 ± 10.23 ; median: 39.76).

Protein analysis by Western blotting exhibited higher p65 median values (1.19 vs. 0.21, $P < 0.005$) in patients with NP than in controls, whereas there was no significant difference for c-Fos between groups (Table 2 and Figs 3a and b).

By RQ-PCR analysis, higher median expressions of p65 (0.34 vs. 0.02, $P < 0.05$), IL-1 β (0.78 vs. 0.03, $P < 0.0005$), eotaxin-2 (17.05 vs. 1.27, $P < 0.05$) and b-FGF (0.15 vs. 0.005, $P < 0.05$) were observed in the NP group compared with the control (Figs 4a–d). The expression of the

remaining genes studied (ICAM-1, TNF- α , GM-CSF and c-Fos) did not differ significantly between groups (Table 3).

Discussion

Quantitative PCR indicated that expression of the IL-1 β , eotaxin-2 and b-FGF genes was higher in patients with NP when compared with controls. These results stress the importance of cytokines, chemokines and growth factors in the genesis of NP, as proposed by Min and Lee [9] and Fokkens et al. [10]. IL-1 β has a fundamental role in the induction and perpetuation of the chronic inflammatory process; eotaxin-2 is a potent eosinophil recruiter and b-FGF stimulates the growth of fibroblasts, cells that are potent producers of various other cytokines, in addition to inducing stroma proliferation in NP.

Lennard et al. [11], using *in situ* hybridization, reported a significant increase in IL-1 β and TNF- α expression in nasal polyps compared with the mucosa of the middle turbinate. Similarly, Voegels and Pádua [12], using ELISA, reported a greater quantity of IL-1 β in patients with NP compared with the control middle turbinate mucosa, although the difference was not statistically significant.

In vitro studies have emphasized the importance of the pro-inflammatory cytokines IL-1 β and TNF- α in airway inflammatory processes. Nonaka et al. [13] observed that IL-1 β induced the production of GM-CSF and the Regulated upon Activation, Normal T-cell Expressed and Secreted chemokine (RANTES) in cultures of nasal fibroblasts from both nasal polyps and the unciform process. Wilson et al. [14] reported that TNF- α -induced the expression of GM-CSF, ICAM-1 and IL-8 in respiratory epithelial cells via NF- κ B. Ohori et al. [15] observed the TNF- α induced expression of VCAM-1 in an NF- κ B-dependent pathway in cultures of nasal polyp fibroblasts.

Although our results confirm a higher IL-1 β expression, the nonsignificant TNF- α results hinder observations on

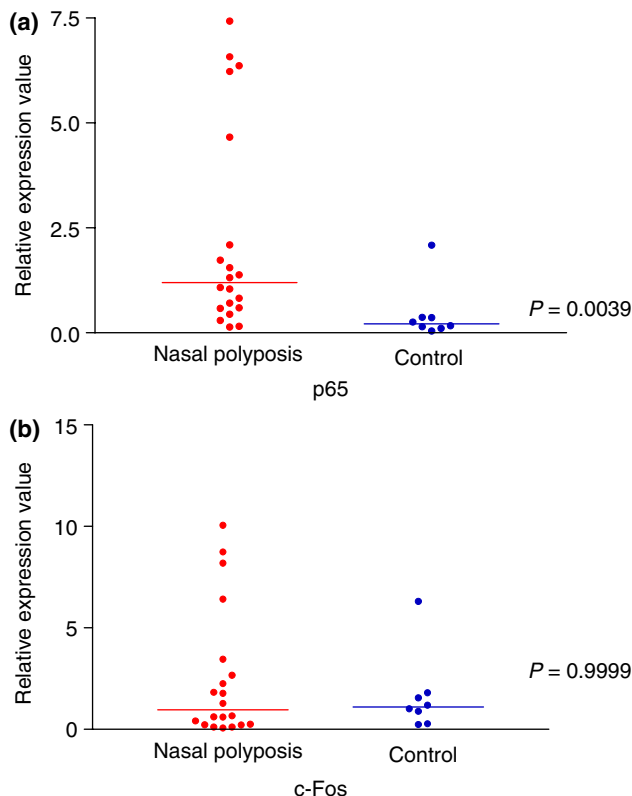


Fig. 3. Expression of p65 (a) and c-Fos (b) proteins. Dispersal curve and median values for patients with nasal polyposis and control.

Table 3. mRNA expression of genes listed in the left column in NP patients and control group as measured by RQ-PCR

	NP patients			Control group			<i>P</i>
	Mean	Median	P25–P75	Mean	Median	P25–P75	
c-Fos	55.62	1.00	0.35–4.80	24.26	1.87	0.22–50.32	0.9801
p65	40.33	0.34	0.12–1.09	0.33	0.02	0.02–0.70	0.0487*
TNF- α	145.34	0.44	0.13–9.32	17.64	0.58	0.06–6.77	0.5665
IL-1 β	145.49	0.78	0.15–3.37	0.03	0.03	0.01–0.04	0.0004*
GM-CSF	45.60	0.23	0.10–0.82	0.33	0.14	0.05–0.38	0.2584
ICAM-1	117.61	0.62	0.09–2.25	4.38	0.63	0.03–2.12	0.6005
Eotaxin-2	328.19	17.05	9.70–91.50	31.26	1.27	0.31–24.13	0.0375*
b-FGF	68.52	0.15	0.02–0.68	0.03	0.005	0.002–0.005	0.037*

*Significant *P*.

P25–P75, percentile 25 to 75; NP, nasal polyposis; RQ-PCR, real-time quantitative PCR; GM-CSF, granulocytes and macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; b-FGF, basic fibroblast growth factor.

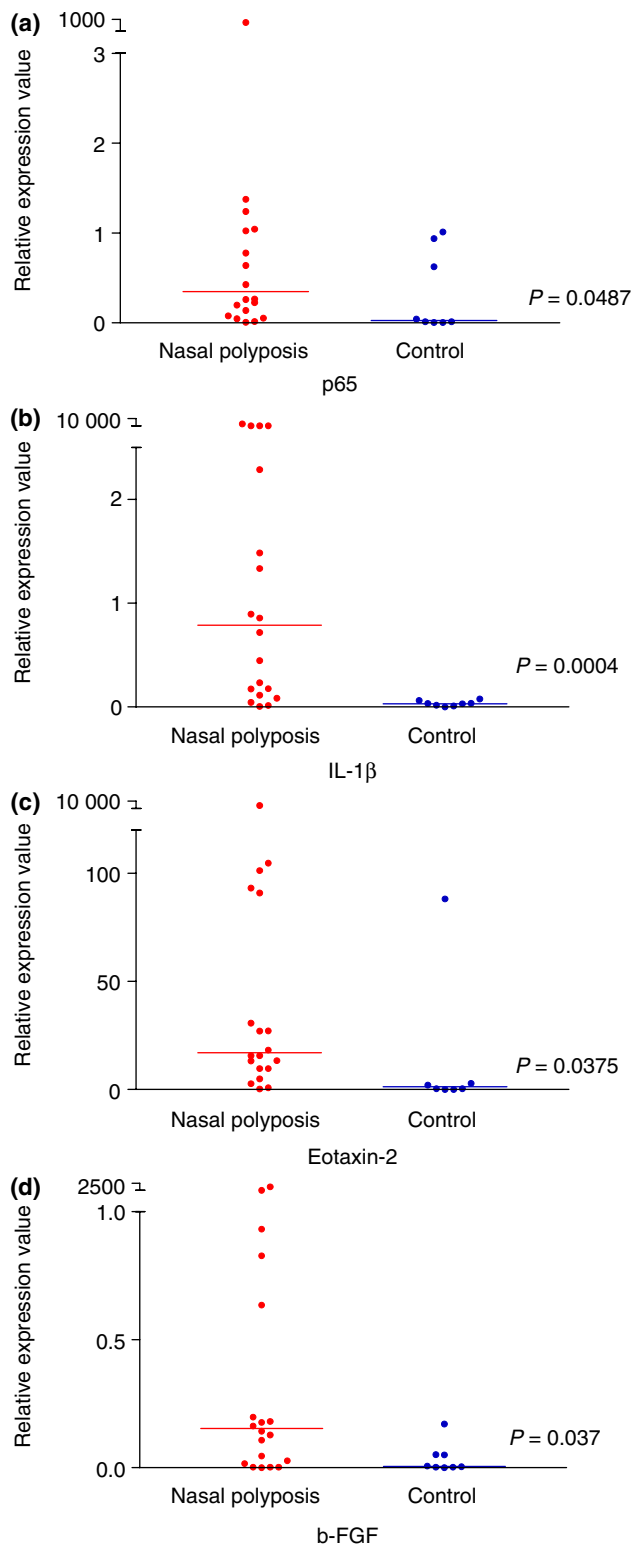


Fig. 4. Expression of mRNA p65 (a), IL-1 β (b), eotaxin-2 (c) and (b-FGF) basic-fibroblast growth factor (d). Dispersal curve and median values for patients with nasal polypsis and control.

this pivotal cytokine and are probably due to the small number of patients in each group in this study. Regarding chemokines, we observed a significant increase in eotax-

in-2 mRNA, a result similar to that reported by Jahnsen et al. [16] and Olze et al. [17], using semiquantitative PCR and an ELISA assay. Jahnsen et al. [16] observed a significantly higher expression of eotaxin than RANTES in nasal polyps and concluded that eotaxin chemokines are the most important recruiters of eosinophils in NP.

In the present study, RQ-PCR showed that the expression of growth factor b-FGF was increased in nasal polyps compared with control mucosa. This result is in agreement to those reported by Cayé-Thomansen et al. [18] using immunohistochemistry. Silvestri et al. [19] also observed a high b-FGF expression in cultures of nasal polyp fibroblasts and proposed that this increase may induce cell proliferation in polyps through NF- κ B.

As confirmed by Jahnsen et al. [16], this study did not show differences in the expression of the adhesion molecule ICAM-1 in nasal polyps when compared with control mucosa. In contrast, Papon et al. [20] observed a significantly higher concentration of the ICAM-1 protein in nasal polyps than in control mucosa, in measurements by immunochemistry methods. These authors and Silvestri et al. [19] reported that this increase in ICAM-1 is mediated by NF- κ B and stimulated by TNF- α .

The expression of transcription factors has been poorly evaluated in NP. In the present study, higher levels of mRNA and p65 protein, the transcriptional fraction of NF- κ B, were observed in patients with NP compared with controls. There was no detectable difference in c-Fos, the active fraction of AP-1, between groups.

Twenty-eight NP patients not treated by GC were analysed through immunochemistry by Takeno et al. [4], who observed an increase of the NF- κ B p50 fraction in these patients. The authors also reported that the increase in p50 was correlated to an increased expression of IL-8, IL-16 and eotaxin, suggesting that NF- κ B may have a central role in the perpetuation of the inflammatory process in NP. However, these authors focused on the constitutional fraction of NF- κ B, p50, and not on the fraction that actually induces the inflammatory process, i.e. p65. In the present study, a quantitative assay confirmed the importance of p65 in NP genesis, because this NF- κ B constituent was significantly more expressed in nasal polyps not treated with GC than in the control nasal mucosa.

No significant increase in c-Fos expression was observed in the present study, in contrast to results reported by Baraniuk et al. [21]. However, these authors evaluated the presence of c-Fos by immunohistochemistry and PCR in a qualitative manner, observing a significantly higher frequency, but not a greater amount, of c-Fos in polyps than in control mucosa.

Because transcription factors are of great importance in cell cycles apoptosis and induction of inflammation, it may be speculated that increased expression is an important co-factor in the genesis and perpetuation of NP. Significantly increased transcription of cytokines, such

as IL-1 β , eotaxin-2 and b-FGF shown in the present study, supports the importance of p65 in NP, because they can be NF- κ B -induced. As AP-1 levels did not differ in gene or protein expression between groups, this transcription factor is probably not as essential as NF- κ B in this process.

Transcription factors are important in glucocorticoid resistance, and NP clinical treatment could be impaired by their increased expression. Thus, the study of these molecules is fundamental for a better understanding of their influence on NP physiology and on responses to medical treatment, although isolated expression data are not sufficient to inform about their activated status.

Conclusion

There was a higher gene and protein expression of the transcription factor NF- κ B in NP, but not of AP-1. There was also an increased gene expression of the cytokines IL-1 β , eotaxin-2 and b-FGF in NP.

These results stress the importance of transcription factors in NP because they are able to generate and exacerbate the inflammatory process by inducing the transcription of pro-inflammatory cytokines.

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