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Glucocorticoid Therapy Increases Cox-2 Gene Expression in Nasal Polyps *in vivo*

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ABSTRACT

We aimed to evaluate the *in vivo* regulation of cyclooxygenase-2 in nasal polyps.

Sixty-five patients with nasal polyps, were randomly (3:1) treated (n=51, 33 with asthma) or not (n=14) with oral prednisone and intranasal budesonide for 2 weeks, plus intranasal budesonide for 10 additional weeks. Biopsies were obtained at baseline and after 2 and 12 weeks of treatment. All samples were analysed for cyclooxygenase-1 and cyclooxygenase-2 mRNA. Attempts were made to detect cyclooxygenase-2 protein.

At baseline, cyclooxygenase-1 and cyclooxygenase-2 expression did not differ between polyps from non-asthmatic and asthmatic patients. Cyclooxygenase-1 mRNA was unchanged by glucocorticoid treatment, while cyclooxygenase-2 mRNA increased in glucocorticoid-treated patients at week 2 ($p<0.01$) compared to baseline, and then decreased at week 12 ($p<0.05$ compared to week 2). Within subgroups, increased cyclooxygenase-2 mRNA was found at week 2 in polyps from non-asthmatic ($p<0.05$) and asthmatic patients ($p<0.05$) compared to baseline. At week 12, cyclooxygenase-2 expression remained high in non-asthmatics while it decreased in asthmatics ($p<0.01$ compared to week 2). Cyclooxygenase-2 protein was not detected under any circumstances.

Glucocorticoid therapy enhances cyclooxygenase-2 expression *in vivo* in nasal polyps, a finding that does not follow the generally accepted assumption that cyclooxygenase-2 expression is suppressed by glucocorticoids.

Word count: 198

Key words: Cyclooxygenase-2. Cyclooxygenase-1. Glucocorticoids , Nasal polyps.

INTRODUCTION

Prostaglandin (PG) endoperoxidase synthase, known as cyclooxygenase (Cox) is the rate-limiting enzyme in the biosynthesis of PG. There are two distinct enzymes, Cox-1 and Cox-2. While Cox-1 is constitutively expressed in most cells, the expression of Cox-2 is generally low under basal conditions, but is usually induced by inflammatory mediators, mitogens and growth factors. Many observations indicate that the expression of Cox-2 plays key roles in inflammation and tumorigenesis[1].

Over-expressed Cox-2 in response to stressful signals has been shown to be a major source of PG. PG can exert opposing effects on inflammatory response in the airways. Mast cells release PGD_2, which is partly responsible for the bronchoconstriction that usually occurs in immediate allergic responses. In contrast, PGE_2 can exert both beneficial and adverse effects. Inhalation of a high PGE_2 dose causes bronchoconstriction while a low dose prevents exercise-induced bronchoconstriction, early- and late-phase allergic airway reactions and aspirin-induced broncoconstriction[2,3,4]. All in all, the experimental data and clinical findings indicate that Cox-2 induction may be a friend or a foe in the lung[5,6].

Despite the fact that the Cox-2 enzyme is induced in states of inflammation, this is not the case in the nasal polyps of asthmatic patients, which show no increase in the expression of the enzyme[7,8]. This lack of up-regulation of Cox-2 in the inflamed tissue of nasal polyps is more marked in asthma patients with aspirin intolerance, where the level of Cox-2 expression is even lower than in aspirin-tolerant asthmatics[8-10]. The mechanisms responsible for this abnormal response are as yet unclear. Whether down-regulation of Cox-2 is involved in the formation of nasal polyps or is a collateral consequence of the nasal inflammatory process remains to be elucidated.

Glucocorticoids (GC) represent the most effective treatment for several clinical conditions, and they are used for their anti-inflammatory effects in the treatment of nasal polyps[11]. GC have been shown to modulate the expression of several genes involved in inflammatory responses. One of the target genes for GC action appears to be the Cox-2 gene, suppressed through both transcriptional and post-transcriptional mechanisms[12-14].

However, the ability of GC to decrease Cox-2 expression in the airways is not entirely clear. At least one group has found that GC can decrease Cox-2 expression in the bronchial mucosa of asthma patients[15], but others have reported no change in its expression in either bronchial mucosa of patients with asthma[16] or nasal polyps after GC therapy[10]. Similarly, *in vitro* studies also show disparate results, with some reports showing a decrease[12-14] and others an increase[17] in the expression of Cox-2 after the exposure of various cells and tissues to GC.

It is difficult to determine the true effect of GC on Cox-2 in airways, as the *in vivo* studies are comparison studies of different groups of patients in cross-sectional studies[10,15,16], and the *in vitro* studies vary widely with regard to the type of cells, the methodology used and the duration of exposure to treatment[10,12-14].

The only way to clarify whether GC increase or decrease Cox-2 expression *in vivo* is through a prospective study comparing the level of expression of the enzyme in tissues obtained from the same subjects before and after GC therapy. With this objective in mind, we performed serial nasal polyp biopsies on patients treated with GC and evaluated the local Cox-2 mRNA and protein expression.

SUBJECTS AND METHODS

Study subjects. A total of 65 patients (74% male, 51 ± 2 yr) with diagnosis of severe nasal polyposis were selected, comprising patients without asthma and patients with either aspirin-tolerant (ATA) or aspirin-intolerant asthma (AIA). Severe nasal polyposis, atopy, asthma and aspirin sensitivity were diagnosed as described elsewhere[18,19] (also see the online depository). All subjects agreed to participate in the study, which was approved by the Ethics Committee of the Hospital Clinic from Barcelona. Table 1 summarizes the subjects' demographic data and clinical characteristics.

Study design. The study design used herein has been previously reported[18]. After a 4-week washout period with no intranasal and oral steroids, patients were randomized (3:1) into two groups: 1) the GC-treated group included 51 patients who received oral prednisone (30 mg daily for 4 days followed by a tapering of 5 mg every two days) and intranasal budesonide (400 μg BID) for two weeks (w2), followed by intranasal budesonide (400 μg BID) alone for 10 additional weeks (w12); and 2) the non-treated group included 14 patients who did not receive any steroid treatment over a two-week period (w2). For ethical reasons, patients from the non-treated group were not kept for more than 6 weeks without any effective treatment. Nasal polyp biopsies were obtained from all patients at baseline, w2 (treated and non-treated groups), and w12 (treated group).

Histological analysis. The inflammatory content of biopsies, i.e. mononuclear cells, neutrophils, and eosinophils, was characterized by haematoxylin & eosin staining in 4 μm-thick paraffin sections. Sections were counted blindly using an Olympus microscope (x400 magnification). Between 1.6 and 2 sq. mm were counted for each

section and cell counts were expressed as the number of positive cells per square millimeter. Due to limitations in the amount of tissue obtained at three points (baseline, w2 and w12), and the preferential use of tissues for RT-PCR and protein analysis, the histological analysis could be completed only in 29 patients.

Reverse transcription and real-time PCR. The extraction of total RNA from the specimens and the reverse transcription step were performed as previously reported[9,10]. Quantification of Cox-1 and Cox-2 transcripts was achieved by extrapolation to a plasmid double-stranded DNA external standard curve added in each PCR run. The detailed protocol and validation of the real-time PCR assays have been reported elsewhere[9] (also see the online depository).

Western blot and ELISA of Cox-2. Total proteins from the nasal polyp biopsies were extracted as described elsewhere[20] and analyzed for the expression of Cox-2 protein through both Western blot[20] and ELISA using the ZYMED Cox-2 ELISA kit (ZYMED laboratories INC, San Francisco, CA). The detection range of the ELISA kit was from 2.15 to 275 ng/ml (see the online depository).

Statistical data analysis. The statistics and graphs were treated with the SPSS v12.0 and SigmaPlot 8.0 software packages, respectively. Data are expressed as median and 25th to 75th percentile. Non-parametric statistical analysis was performed by using the Friedman test and Wilcoxon rank test for within-group comparisons, and the Kruskal-Wallis test and Mann-Whitney U test for between-group comparisons. Spearman rank correlation was used when analyzing relationships between data. Statistical significance was set at $p < 0.05$.

The sample size of the study was established from practical considerations. The estimated statistical power for our sample size (51 GC-treated patients) to detect a 2-

fold change in the median of the main variable (Cox-2 mRNA) was 82% when analysed with a non-parametric approach (Wilcoxon test), with an alpha error of 5% two-sided.

RESULTS

Subjects. As expected, GC therapy significantly improved clinical symptoms, reduced nasal polyp size and relieved nasal obstruction. A detailed description of the clinical results has been previously reported elsewhere[18].

Histological findings. Except for neutrophils, at baseline, there were no significant differences in the number of inflammatory cells between GC-treated and non-treated control groups (Table 2).

There were no significant changes in the inflammatory cell counts between baseline and w2 in the non-treated group (Table 2). In contrast, at w2, GC-treated patients showed a marked down-regulation in the number of eosinophils, which remained low at w12, and an increase in the number of neutrophils compared to baseline (Table 2). The reduction in eosinophils at w2 was found in both non-asthmatic (20; 15-38; n = 8; $p < 0.05$) and asthmatic patients (18; 3-55; n = 12; $p < 0.01$), compared to baseline (no asthma: 199; 96-339; n = 9; asthma: 285; 126-808; n = 13). However, the reduction in eosinophils at w12 was more pronounced in the non-asthmatics (13; 3-71; n = 8; $p < 0.05$ compared to baseline) than in the asthmatic patients (41; 3-283; n = 10; ns compared to baseline). No differences in the response to GC treatment were apparently found between ATA and AIA patients. However, given the low number of patients available for histological analysis, caution has to be taken when comparing subgroup differences.

The increase in neutrophil numbers at w2 in all patients (Table 2) did not reach statistical significance when they were subdivided in non-asthmatics (w2: 17; 1-47 *vs* w0: 2; 1-13; $p = 0.07$) and asthmatics (w2: 20; 1-40 *vs* w0: 4; 2-30; $p = 0.07$), nor in ATA and AIA patients.**Basal expression of Cox-1 and Cox-2 mRNA.** When all patients were analysed at baseline, no significant differences in Cox-1 or Cox-2 mRNA

expression were found in nasal polyps between non-asthmatic and either ATA or AIA patients (Table 3).

Cox-1 and Cox-2 mRNA expression after treatment with glucocorticoids. At baseline, there were no significant differences in Cox-1 mRNA expression between treated and non-treated groups (Figure 1A). There were no significant changes in Cox-1 mRNA levels between baseline and w2 in the non-treated group, or between baseline and either w2 or w12 in the treated group (Figure 1A). Within the treated patients, no significant changes in Cox-1 expression were found at w2 and w12 compared to baseline in nasal polyps from both non-asthmatic and asthmatic patients (Figure 1B).

There were no significant changes in Cox-2 mRNA expression between baseline and w2 in the non-treated group, while Cox-2 mRNA was seen to be increased in treated patients at w2 compared to baseline. Cox-2 mRNA expression decreased significantly at w12, compared to w2 (Figure 2A and table 4). Interestingly, a different response to GC treatment was found between non-asthmatic and asthmatic patients (Figure 2B and table 4). Thus, in non-asthmatics, increased Cox-2 expression was found at w2 and w12 compared to baseline. However, in asthmatics, Cox-2 expression increased at w2 compared to baseline, and decreased to basal levels at w12. The same apparent pattern of regulation, i.e., increase in Cox-2 mRNA levels at w2 and return to basal levels at w12, was observed in both ATA and AIA patients, although changes did not achieve statistical significance (Table 4).

A weak but statistically significant negative correlation was found between Cox-2 mRNA levels and eosinophil counts ($r = -0.307$; $n = 73$; $p < 0.01$). However, changes in Cox-2 mRNA levels did not correlate with changes in eosinophil numbers after GC treatment. Changes in Cox-2 mRNA did not either correlate with the increase in neutrophil numbers at w2.

Cox-2 protein expression. Cox-2 protein was not detected through ELISA in any tissue or at any time point. Western blot analysis revealed absence of Cox-2 protein at baseline and occasional faint bands corresponding to Cox-2 protein after GC treatment in a few tissues, which were difficult to quantify (Figure 3).

DISCUSSION

It is generally accepted that Cox-2 is up-regulated under conditions of inflammation, and that the Cox-2 derived eicosanoids are mostly pro-inflammatory. Because eicosanoids are generally considered to be potent pro-inflammatory mediators, their inhibition by GC and nonsteroidal anti-inflammatory drugs (NSAID) has been considered a desirable therapeutic goal. However, many recent observations question these assumptions, particularly when they are applied to asthma and idiopathic interstitial lung fibrosis[5,6,21].

In asthma and nasal polyps, and most obviously in those patients with aspirin-sensitivity, there are intriguing data to support the existence of a substantial disregulation of the Cox-2 pathway. Cox-2 and/or PGE_2 levels have been reported to be down-regulated in nasal polyps[7-10,22], as well as in bronchial fibroblasts[23] and bronchial smooth muscle[24] from asthmatic patients. We did not find any significant differences in the expression of Cox-2 between ATA and AIA subjects, which concurs with previous reports showing that kinetic studies are sometimes necessary to demonstrate differences in the expression of Cox-2 between ATA and AIA asthma patients[9], and between healthy nasal mucosa and nasal polyps[7]. PGE_2 appears to be a modulator of the airway inflammatory response rather than to act as a pro-inflammatory substance[2-6]. Inhibition of the Cox pathway by NSAID in asthmatic patients generally has no beneficial effect. Indeed, NSAID can precipitate severe asthma reactions in some patients, which can be prevented by pre-treatment with PGE_2[3].

In contrast to what was expected, our study shows that GC increased Cox-2 mRNA in nasal polyps, a new intriguing finding to add to the altered regulation of Cox-2 in this inflamed nasal tissue. The initial intense therapy with systemic and intranasal GC caused a significant increase of Cox-2 mRNA in both non-asthmatic and asthmatic

patients, causing in the latter a return to baseline expression levels after prednisone withdrawal and treatment with intranasal GC alone for 10 additional weeks. In parallel, the 2-week oral and intranasal GC treatment provoked a marked reduction in eosinophil numbers and an increase in neutrophils. These differential effects of GC on inflammatory cells have been previously reported and ascribed to the opposing effects of GC on the regulation of apoptosis in eosinophils (increased apoptosis) and neutrophils (decreased apoptosis)[25]. When patients were treated with the less potent intranasal GC therapy, eosinophil numbers increased in nasal polyps of patients with asthma compared to oral GCs, while in non-asthmatic patients the eosinophilic infiltrate remained low. These findings suggest that the presence of a greater inflammatory activity in the nasal polyps of patients with asthma resulted in an earlier relapse of the eosinophilic infiltrate after prednisone withdrawal. Interestingly enough, Cox-2 mRNA expression appears to run in parallel with changes in the inflammatory cell infiltrate. Accordingly, a weak but significant negative correlation was found between Cox-2 mRNA levels and eosinophil counts, though changes in Cox-2 mRNA did not correlate with changes in eosinophils after GC treatment. Thus, changes in Cox-2 mRNA induced by GC treatment may involve a direct effect of GC on the regulation of the Cox-2 gene, but may also, at least in part, be a consequence of changes in the tissue cell composition.

Cox-2 protein was not detected either at baseline or post-glucocorticoid treatment. This is not an unexpected result because Cox-2 protein is not usually found by Western blot at baseline either in nasal mucosa or in bilateral nasal polyps[7,14]. Potent inflammatory stimuli are usually needed to induce the production of detectable levels of Cox-2 protein in *in vitro* studies[7]. Cox-2 protein can also be found *in vivo* in diseases with demonstrated high production of prostaglandins such as cystic fibrosis[20]. Since we did

not detect Cox-2 protein under any circumstances, we do not know whether the mild GC-induced increase in Cox-2 mRNA found in our study results in an increased translation to Cox-2 protein. We do not know either if GC treatment in our study has any effect on prostaglandin production.

It is unclear whether the GC effect on Cox-2 mRNA expression occurs only in inflamed nasal tissue or whether it can also take place in healthy nasal mucosa. For ethical reasons, we could not obtain nasal biopsies from healthy subjects before and after GC therapy.

Interestingly enough, our observation concurs with another previously reported by Dworski and co-workers[26], who unexpectedly also found that Cox-2 mRNA expression significantly increased in alveolar macrophages and blood monocytes from atopic asthma patients treated with 30 mg of prednisone for 7 days, while the same treatment resulted in a decreased expression of Cox-2 mRNA in the same cells from healthy subjects. In contrast with the results obtained *in vivo*, the expression of Cox-2 mRNA was significantly inhibited when stimulated monocytes from atopic asthmatics and healthy subjects were exposed to dexamethasone *ex vivo*.[26]

Thus, in spite of the fact that in our study the GC-induced increase of Cox-2 mRNA might be partly ascribed to the effects of GC on the inflammatory infiltrate, our results, together with those reported by Dworski and co-workers[26], support the hypothesis that GC therapy up-regulates Cox-2 expression *in vivo* in cells and airway tissues of patients with asthma and nasal polyps.

We cannot find an explanation for the increased expression of Cox-2 with GC therapy in asthma/nasal polyps. Based on the dichotomy in the effect of GC *in vivo* with respect to the *ex vivo* situation found by Dworski et al[26], we could hypothesise that components

of the inflammatory process that underlie asthma and nasal polyps might be responsible for the restricted expression of the Cox-2 gene. GC therapy might reduce the activity of one or more of these components, which would allow a partial release of the restrained Cox-2 gene expression.

In summary, we provide evidence that GC therapy can enhance the expression of Cox-2 mRNA in nasal polyps. These results, together with previous observations, support the notion that the regulation of Cox-2 in nasal polyps does not follow either the generally accepted notion that this enzyme is up-regulated under conditions of inflammation, or the assumption that Cox-2 expression is suppressed by GC. Identification of the mechanisms involved in these distinct responses may help to better understand the role of prostanoids in the regulation of inflammation in airway diseases.

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FIGURE LEGENDS

Figure 1. Regulation of Cox-1 mRNA in non-treated (n = 14) and GC-treated patients (n = 51) (**A**), and in GC-treated patients who either had no asthma (n = 18) or asthma (n = 33) (**B**). *Empty boxes*, *light grey boxes*, and *dark grey boxes* correspond to baseline, week 2, and week 12 of treatment, respectively. *See* methods for treatment regimens. Box plots show the 25th, 50th (median), and 75th percentile values. Whiskers show the 10th and 90th percentiles. No significant differences in Cox-1 mRNA expression were observed over time for any group (Friedman, Wilcoxon).

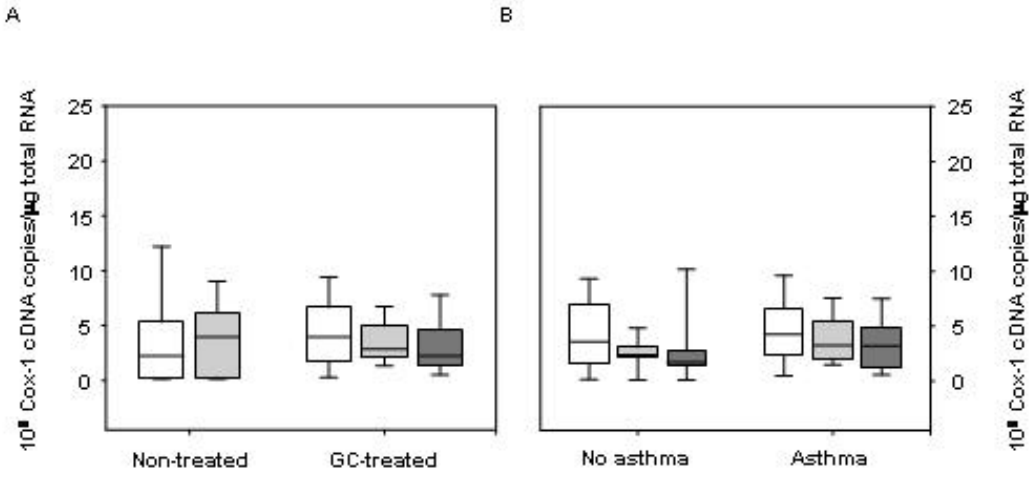

Figure 1

Figure 2. Regulation of Cox-2 mRNA in non-treated (n = 14) and GC-treated patients (n = 51) (**A**), and in GC-treated patients who either had no asthma (n = 18) or asthma (n = 33) (**B**). *Empty boxes*, *light grey boxes*, and *dark grey boxes* correspond to baseline,

week 2, and week 12 of treatment, respectively. *See* methods for treatment regimens. Box plots show the 25th, 50th (median), and 75th percentile values. Whiskers show the 10th and 90th percentiles. *p < 0.05 and **p < 0.01 (Wilcoxon test).

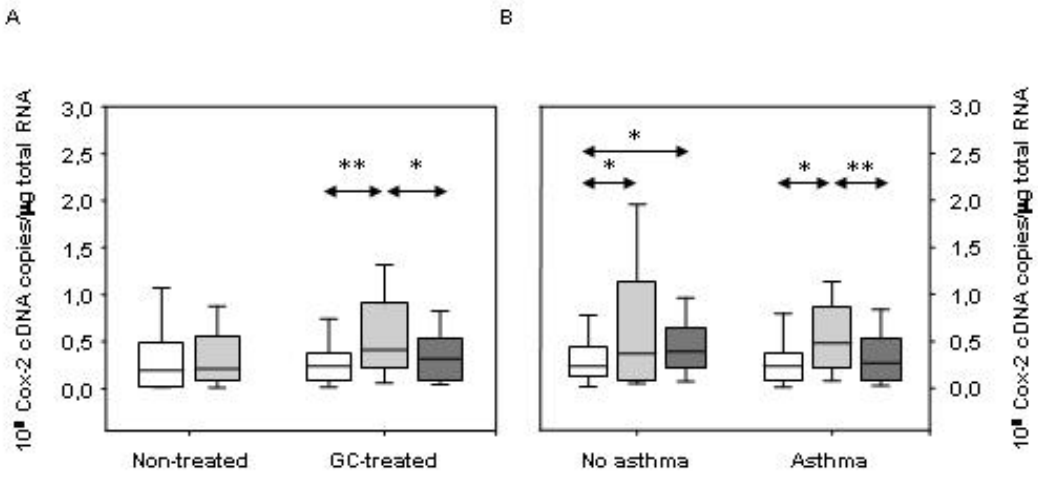

Figure 2

Figure 3. Representative image of Cox-2 protein in a nasal polyp detected through Western blot at baseline (basal) and after 2 (W2) and 12 weeks (W12) of GC treatment. 1, 2 and 3 denote serial dilutions of the Cox-2 standard (11, 5.5 and 2.75 ng, respectively). Note the absence of Cox-2 at baseline and W2, and at W12. Western blot for β-actin is shown below, demonstrating a similar protein load in all lanes.

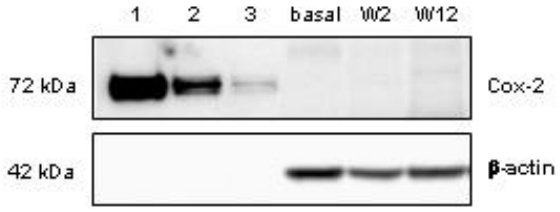

Figure 3

TABLES

Table 1. Demographic data and clinical characteristics of the study population.

		GC-treated group		
			With asthma	
	Non-treated group	Without asthma	ATA	AIA
Patients (n)	14	18	16	17
Gender (% female)	14.3	16.7	25.0	47.0
Age (yr)	52.5 ± 4.8	53.8 ± 3.8	48.6 ± 4.7	49.1 ± 2.9
Skin prick test (% positivity)	28.6	27.8	25.0	29.4

ATA, aspirin-tolerant asthma; AIA, aspirin-intolerant asthma; GC, glucocorticoid. Values of age are expressed as mean ± standard error of the mean.

Table 2. Inflammatory cell counts of nasal polyp biopsies from untreated or GC-treated patients.

Group	Time	N	Mononuclear cells	Eosinophils	Neutrophils	Total inflammatory cells
Non-treated	Baseline	7	928 (484-1893)	169 (86-215)	34 (13-69)	1127 (721-2531)
	Week 2	7	1277 (711-1482)	227 (117-1039)	42 (12-195)	1641 (1373-2181)
GC-treated	Baseline	22	892 (581-1351)	243 (100-480)	3 (1-14)††	1207 (832-2101)
	Week 2	20	1040 (582-1785)	20 (5-39)***	20 (1-43)*	1168 (634-1807)
	Week 12	18	1061 (540-1503)	22 (3-85)*	6 (1-26)	1294 (579-2191)

Results expressed as median and 25-75th percentile of cells/mm^2. N, number of cases. Total inflammatory cells correspond to the sum of mononuclear cells, eosinophils, and neutrophils. *$p < 0.05$ and ***$p < 0.001$, compared to baseline from GC-treated group (Wilcoxon test); ††$p < 0.01$ compared to baseline from non-treated group (Mann-Whitney U test).

Table 3. Basal expression of Cox-1 and Cox-2 mRNAs in the different subtypes of nasal polyps (all patients).

	N	Cox-1	Cox-2
Without asthma	24	3.29 (0.96-5.93)	0.21 (0.05-0.47)
With asthma	41	3.96 (2.02-6.66)	0.23 (0.06-0.37)
ATA	21	4.81 (2.32-7.18)	0.24 (0.08-0.35)
AIA	20	2.98 (1.65-6.01)	0.20 (0.05-0.61)

Cox data expressed as median and 25-75th percentile of the number ($x10^6$) of cDNA copies/μg total RNA. N, number of cases; ATA, aspirin-tolerant asthma; AIA, aspirin-intolerant asthma. No significant differences in either Cox-1 or Cox-2 mRNA expression among the different nasal polyp subtypes (Mann-Whitney U test, Kruskal-Wallis).

Table 4. Effect of glucocorticoid treatment on Cox-2 mRNA in the different subtypes of nasal polyps.

Group	N	Baseline	Week 2	Week 12
Non-treated	14	0.19 (0.02-0.48)	0.21 (0.08-0.55)	—
GC-treated	51	0.23 (0.08-0.38)	0.41 (0.20-0.90)**	0.31 (0.09-0.52)†
Without asthma	18	0.23 (0.13-0.44)	0.37 (0.08-1.12)*	0.39 (0.21-0.65)*
With asthma	33	0.23 (0.07-0.37)	0.47 (0.21-0.86)*	0.26 (0.07-0.51)††
ATA	16	0.24 (0.11-0.34)	0.55 (0.21-0.92)	0.19 (0.07-0.51)
AIA	17	0.18 (0.05-0.60)	0.40 (0.21-0.69)	0.27 (0.04-0.52)

Cox data expressed as median and 25-75th percentile of the number ($x10^6$) of cDNA copies/μg total RNA. N, number of cases; ATA, aspirin-tolerant asthma; AIA, aspirin-intolerant asthma. *$p < 0.05$ and **$p < 0.01$, compared to baseline (Wilcoxon test); †$p < 0.05$ and ††$p < 0.01$, compared to week 2 (Wilcoxon test).