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COX-1 AND COX-2 ARE UP-REGULATED IN NASAL POLYPS IN CYSTIC FIBROSIS

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ABSTRACT

Background. Since abnormalities in prostanoid metabolism occur in the lower airway of CF patients, they should also be detected in the nose.

Methods. We examined the degree of mRNA and protein expression of cyclooxygenase (Cox) enzymes 1 (Cox-1) and 2 (Cox-2) using a quantitative reverse competitive polymerase chain reaction (RT-PCR) and Western Blot in nasal polyps from 10 CF patients, 10 nasal polyps from non-CF patients and 11 nasal mucosa. Results are presented as 10^6 cDNA molecules/μg total RNA and densitometric ratio between protein and β-actin.

Results. We found that Cox-1 mRNA levels were significantly higher in CF nasal polyps (median 2.34, 25^{th} to 75^{th} percentiles 1.6-3.2) than in nasal mucosa (0.78, 0.11-1.21) while there was no difference with non-CF nasal polyps (1.11, 0.80-3.15). Similarly, CF nasal polyps Cox-1 protein levels (3.63,2.71-4.27) were also significantly higher than in nasal mucosa (1.55,0.66-2.33) and non-CF nasal polyps (2.19,1.72-3.68). Cox-2 mRNA in CF nasal polyps (3.34, 2.42-7.05) was significantly higher than in nasal mucosa (1.69,0.19-3.50). No differences were found in Cox-2 mRNA expression between CF and non-CF polyps (1.38, 0.12-6.07). Cox-2 protein levels were also significantly higher in CF nasal polyps (0.23, 0.04-0.34) than in both non-CF nasal polyps (0.011,0.009-0.016) and nasal mucosa (0.014, 0.014-0.016).

Conclusions. Up-regulation in the expression of Cox-1 and Cox-2 could explain the high production of prostanoids reported in CF. These findings raise questions regarding the potential use of selective or non-selective Cox-2 nonsteroidal anti-inflammatory therapy in CF.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal-recessive disorder of the exocrine-glandular function. The basic metabolic derangement is related to a mutation in the gene regulating the CF transmembrane conductance regulator (CFTR), which acts as a chloride channel, regulating salt and water exchange in the apical membrane of epithelial cells. The genetic alteration results in excessive bronchial mucus secretion, reduced pancreatic enzyme secretion and malabsorption in the gastrointestinal tract (1).

Progressive lung disease characterized by chronic airway infection is the prime cause of morbidity and determines the prognosis and vital evolution (1). Chronic rhinosinusitis with or without nasal polyps also commonly occurs in CF (2,3). Because the defective mucous secretion affects exocrine glands along the entire respiratory tract, including the nose, it is generally accepted that this finding contributes to the high incidence of chronic rhinosinusitis in CF patients (1).

The traditional hypothesis postulates that pro-inflammatory substances produced by various cells stimulated by infectious agents are responsible for CF lung disease (1). However, recent studies have suggested that inflammation may even occur in the absence of infection. An increase in markers of inflammation has been found in the lungs of patients with mild disease, as well as in those in stable clinical conditions (4-7).

In response to infectious agents, high levels of interleukin 8 (IL-8), 1 (IL-1), 6 (IL-6) and tumor necrosis factor-α (TNF-α) have been detected in CF sputum and bronchioalveolar lavage (BAL) (8,9). Since the intensity of the inflammatory reaction appears to be, at least in part, independent of the infectious stimulus, it has been suggested that early and excessive inflammation may be related to constitutive abnormalities, associated with a defective CFTR or otherwise (4, 7,10,11,12).

In addition to an increased release of pro-inflammatory cytokines, several studies have also demonstrated over-production of leukotrienes (13,14) and prostaglandins E_2 and $F_{2\alpha}$ (15,16,17). Prostaglandin endoperoxide synthase, commonly called cyclooxygenase (Cox), is the key enzyme required for the conversion of arachidonic acid into prostaglandins (PGs). Two Cox isoforms have been identified, Cox-1 and Cox-2. Cox-1 is constitutively expressed in most tissues, where it maintains the physiological processes (18). Cox-2 is highly inducible at inflammatory sites and is considered the main target for anti-inflammatory therapies (18,19). Ibuprofen, a non-steroidal anti-inflammatory drug (NSAID) that works by inhibiting both Cox enzymes, has been shown to be an effective drug in CF patients. High-dose ibuprofen slowed lung function decline in patients with CF, the benefit being most evident in patients between 5 and 13 years old (20).

Because there is an overproduction of PGs in CF, and because PG production under conditions of inflammation is dependent on the induction of Cox-2, we hypothesized that an up-regulated Cox-2 enzyme should account for the increased production of prostanoids. The purpose of this study was to examine the degree of expression of Cox-1 and Cox-2 in the nose of patients with CF. This study was designed as the first step in the assessment of the potential therapeutic effects of selective Cox-2 inhibitors in CF patients.

SUBJECTS AND METHODS

Subjects.

Endoscopy sinus surgery and polypectomy was offered to 10 CF patients with nasal polyps filling up the nasal cavity and without any substantial clinical and endoscopy response after at least one year of intranasal glucocorticoid therapy and short courses of oral glucocorticoid. Glucocorticoid therapy was discontinued at least two weeks before surgery.

A definitive diagnosis of CF was made on the basis of raised sweat electrolytes (CL^- level >60 mmol L^{-1}) and identification of associated CFTR mutations.

Nasal polyps were obtained from 10 aspirin-tolerant patients undergoing polypectomy. Nasal mucosa was also obtained from 11 patients undergoing nasal corrective surgery (healthy control group). The 10 patients with nasal polyps had not received glucocorticoids for at least 2 weeks before surgery. None of the healthy subjects and non-CF patients had suffered from upper respiratory infection during the 4 weeks before surgery, nor had any of the CF patients suffered an acute upper airway infective exacerbation of their chronic process within the 4 weeks prior to polypectomy.

The subjects or their parents agreed to participate in the study, which was approved by the Ethics Committee of our institutions.

Methods

Study of atopy

Atopic status was assessed by skin prick testing to a panel of common aeroallergens present in our geographic area (Bial Aristegui Laboratories, Bilbao, Spain) according to standard methods. Patients were considered atopic if at least one of the allergens tested was positive.

Microscopic analysis

Surgical specimens were examined using standard hematoxylin-eosin staining methods by a pathologist who was blinded to the clinical data. Inflammatory cells, including eosinophils, lymphocytes, plasma cells and polymorphonuclear cells, were quantified and results were related to 100 inflammatory cells.

Reverse transcriptase (RT) - competitive polymerase chain reaction (PCR).

RNA extraction and reverse transcription. A specimen obtained at the time of surgery was immediately snap-frozen in liquid nitrogen and kept at –80º C until analyzed. Total RNA from nasal tissue specimens was isolated using a rapid extraction method (TRI-Reagent), as previously described (21). Total RNA (4 μg) from nasal mucosa and nasal polyps was reverse transcribed to cDNA using random hexanucleotide primers and SuperScript II Rnase H^- reverse transcriptase (Invitrogem, Paisley, United Kingdom).

Primer design. Cox-1 and Cox-2 primers were designed to span introns. Their sequences were as follows: 5´ TGCCCAGCTCCTGGCCCGCCGCTT 3´ (Cox-1 sense, nucleotides:516-539) and 5´ GTGCATCAACACAGGCGCCTCTTC 3´ (Cox-1 antisense, nucleotides:769-819), 5´ TTCAAATGAGATTGTGGGAAAATTGCT 3´ (Cox-2 sense, 574-600), 5´ AGATCATCTCTGCCTGAGTATCTT 3´ (Cox-2 antisense, nucleotides:855-878).

*Competitive polymerase chain reaction***.** To measure Cox-1 and Cox-2 mRNA expression we developed a previously reported competitive PCR technique, in which, after the reverse transcription step, known amounts of an exogenous DNA (external standard) were added to the amplification mixture (RT-competitive PCR). The exogenous molecule, called competitor, was co-amplified in competition with the target in the same test tube. Since the initial amount of internal standard was known, this technique permitted the determination of the initial amount of target cDNA. The reverse transcription and PCR reaction conditions have been previously reported elsewhere (21).

Western Blot

Frozen nasal samples were placed in cold, sterile tubes with 200 µl of lysate buffer (a Complete™ protease inhibitor cocktail tablet in 50 mL of 0.05 M Hepes buffer solution, 0.05% v/v Triton X-100, and 625 µM PMSF). Tissue samples were sonicated twice, 15 s., in a sonifier from Branson (Danbury, CT, ISA). Samples were centrifuged at 12.000 g for 10 min at 4ºC. Supernatants were removed and kept at -80ºC until used. Total proteins were measured using a modified Lowry method in which the absorbencies were read at 630 nm in a microplate reader from Biotek Instruments (Winooski, VT, USA). Total protein concentrations were interpolated from a standard curve prepared with bovine serum albumin, using the Delta soft II 4.0 software package. The assay range was 50 to 400 µg/ml. Ten µL (50 µg) of total protein was added to 5 µl of loading buffer (NuPAGE LDS sample buffer) and spun down. Samples were then heated in a thermocycler to 70ºC for 10 min. Rainbow molecular weight marker (15 µl, Amersham) or samples were loaded in 7% TRIS-acetate gels and ran (125 V, 90 min.) in a Novex XCell II Mini-Cell (San Diego, CA, USA). Following electrophoresis, proteins were transferred (30 V, 1 h.) to a 0.45-µm pore size nitrocellulose membrane using a Novex XCell II Blot Module. To check for equal loading and transfer efficiency, membranes were stained with Ponceau S (0.5% in 1% acetic acid). Membrane non-specific binding sites were blocked using blocking buffer (5% nonfat dry milk, 0.1 % Tween 20, in 10 nM PBS), for 1 h. at room temperature in an orbital shaker. Membranes were then incubated with goat polyclonal IgG anti-Cox-1 or anti-Cox-2 antibodies in blocking buffer (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then washed four times in 0.5 % Tween 20 in 10 nM PBS. Membranes were incubated with peroxidase-conjugated antigoat IgG in blocking buffer (1:3000). After a new series of washes, immunoreactive bands were visualized using a light-emitting chemoluminiscent method (Supersignal West Pico Chemiluminescent Substrate) and the light emissions were detected by the CCD Camera System LAS 3000 (Fujufilm). The bands' intensities were quantified with Fujifilm Image Gauge 4.0 Software. The membranes were re-probed with a mouse anti-human β-actin monoclonal antibody (Sigma) at a dilution of 1:7.500. The bands' intensities were quantified and used as loading control.

Statistical analysis. Results are presented as medians and 25th to 75th percentiles and as mean±standard deviation (SD). Nonparametric statistical analysis was performed by using Kruskal-Wallis and Mann-Whitney U tests. A p value of less than 0.05 was considered statistically significant.

RESULTS

Subjects. The demographic characteristics of the three groups are shown in table 1. CF patients were significantly younger than non-CF subjects.
Histology. The microscopic study showed a higher percentage of eosinophils in non-CF nasal polyps (29%, $p<0.05$) than in NM (3%) and CF nasal polyps (5%). There were no significant differences in the number of lymphocytes, polymorphonuclear and plasma cells in the different tissues (Table 2).
Cox-1 mRNA and protein. The mean levels of Cox-1 mRNA expression in nasal polyps from CF patients were significantly higher ($p<0.01$) than in nasal mucosa. There was no difference in Cox-1 mRNA levels between CF and non-CF nasal polyps and between non-CF nasal polyps and nasal mucosa (Figure 1). Up-regulation of mRNA in CF nasal polyps was also found in Cox-1 protein levels since the Western Blot analysis showed statistically significant higher levels in CF nasal polyps with respect to non-CF nasal polyps ($p=0.05$) and nasal mucosa ($p<0.01$)(Figure 1).
Cox-2 mRNA and protein. The mean levels of Cox-2 mRNA expression in nasal polyps from CF patients were significantly higher ($p<0.05$) than in nasal mucosa from healthy subjects. No differences were found between nasal mucosa and non-CF nasal polyps (Figure 2). Cox-2 protein was detected in CF nasal polyps but it was almost undetectable in nasal mucosa and non-CF nasal polyps (Figure 2).

DISCUSSION

Previous studies have reported high levels of prostanoids in the BAL, saliva and urine of CF patients (15,16,17). On the basis of these findings, we hypothesised that high levels of prostanoids should result from an up-regulated Cox-2, which is the enzyme responsible for the increased production of prostanoids under conditions of inflammation (18).

Because there is compelling evidence to support the notion that nasal respiratory mucosa shares the histological characteristics and ion transport abnormalities of lower airways mucosa in CF patients (22), we used nasal respiratory samples to test our hypothesis.

Our study shows that CF nasal polyps are characterized by a marked activation of Cox-1 and Cox-2, which was reflected in the high expression of both mRNA and protein. The increased expression of Cox-2 detected in our study concurs with the generally accepted theory that predicts an up-regulation of this enzyme in inflamed tissues (18,19). However, it is interesting to note that previous studies have reported an abnormal down-regulation of Cox-2 mRNA in nasal polyps originating in patients with inflammatory pathologies other than CF (21-23-29). Accordingly, Cox-2 protein is not usually found, or is detected in low amounts by Western Blot, in both healthy tissues and in non-CF nasal polyps (21,24). These abnormalities in Cox-2 regulation are associated with a reduced production of prostaglandin E_2 in polyps from aspirin-intolerant patients (23, 29).

In a previous study we reported a significantly lower expression of Cox-2 mRNA in nasal polyps with respect to nasal mucosa (26). We also found that these differences were detectable only when nasal samples were frozen immediately after surgery. We could not replicate these findings in the present study, probably because the nasal samples were collected from various

hospitals dome distance away and the protocol for handling specimens could not be followed strictly.

The mechanism involved in the different regulation of Cox-2 in nasal polyps obtained form non-CF and CF patients is as yet unclear. Although CF patients were significantly younger than non-CF subjects, differences in age cannot explain the marked differences in the level of expression of Cox-2 protein found in our study. In fact, Cox-2 was undetectable in all subjects from the two non-CF groups including several patients with ages similar to CF patients.

The detection of high levels of Cox-2 protein in CF polyps supports the notion that the increased levels of prostanoids in urine, BAL and sputum reported in CF patients are the consequence of the marked activation of the Cox-2 enzyme.

The increased expression of Cox-1 mRNA and protein in CF nasal polyps suggests that this enzyme may also contribute to the increased production of prostanoids in patients with CF. This was an unexpected finding because Cox-1 is usually considered an enzyme with merely physiological roles that is not involved in inflammatory responses. However, this is not always the case, since up-regulation of Cox-1 has been reported in some inflammatory situations, such as in the gastric mucosa (30).

Our observations raises important questions regarding the origin of the reported benefits of drugs capable of inhibiting the two Cox, such as ibuprofen, in the treatment of CF. Is the efficacy of ibuprofen related to its capacity to inhibit both Cox-1 and Cox-2? Would a selective Cox-2 inhibitor be more or less effective?.

It is generally accepted that the main physiological regulator of CFTR is cAMP. Prostanoids such as PGE_2 stimulate the increase of this intracellular signal and thereby activate the CFTR and increase chloride efflux. Recent studies have shown that pro-inflammatory cytokines such as interleukin-1β can reduce cAMP accumulation and chloride efflux in response to PGE_2. This effect appears to be mediated by an autocrine loop involving induction of Cox-2 and endogenous PGE_2 production. It is likely that by breaking this loop using Cox-2 inhibitors the deleterious effects of inflammation on PGE_2 and on chloride efflux can be reduced (31).

Advances in NSAID research have permitted the development of a new class of drugs with selective Cox-2 inhibitory effects (19). Whether selective Cox-2 inhibitors could be more effective and better tolerated than unselective Cox inhibitors by CF patients is something that needs to be tested in a comparative study of the two types of drugs. However, the use of selective Cox-2 inhibitors as anti-inflammatory drugs in CF and other chronic inflammatory diseases has recently been questioned, due to the observation of a significant increase in the cardiovascular risk in patients on long-term therapy with these new drugs (32).

However, the role of prostanoids in CF is far from clear. The traditional view generally gives prostanoids a negative and pro-inflammatory role in some inflammatory diseases, which justifies the need to limit their production through the use of NSAIDs. However, recently reported findings suggest that the role of prostanoids is much more complex, to the point that they may also act by controlling inflammation in some chronic lung inflammatory processes (33). Interestingly enough, these potential salutary effects of prostanoids have been

demonstrated in CF. The administration of misoprostol, a synthetic prostaglandin E_1 analogue, improves fat malabsorption in CF, -an observation which suggests that the inhibition of prostanoids synthesis may not always be beneficial to CF patients (34).

In summary, our study shows up-regulation in the expression of Cox-1 and Cox-2 in nasal polyps of CF patients. This finding could explain the high production of prostanoids reported in this disease and detected in BAL, saliva and urine. Our results also raise questions regarding the potential use of selective or non-selective Cox-2 non-steroidal anti-inflammatory therapy in CF.

Table 1. Demographic characteristics

Group	N	Age (yr)	Gender (M/F)	Atopy (yes/no)
NM	11	32± 1.9 (18-54)	8/3	2/8
Non-CF NP	10	51± 4 (40 - 63)	7/3	3/7
CF NP	10	14.5± 7(7 -31) *	7/3	3/7

Age is expressed as mean±SD and range; NM, nasal mucosa; Non-CF NP, nasal polyps from non cystic fibrosis patients; CF NP, polyps from cystic fibrosis patients;*P < 0.05 compared with CF NP and NM.

Table 2. Microscopic analysis

Tissue	Lymphocyte (%)	Plasma cells (%)	Polymorphonuclear (%)	Eosinophils (%)
NM	42 (32.5 - 40)	50 (47.5 - 55)	5 (4 - 5)	3 (2– 9)
Non-CF NP	35 (15 - 45)	32 (25 - 40)	4 (4 - 10)	29 (10 - 50)*
CF NP	34 (20 - 36)	52 (49- 61)	9 (5 - 16)	5 (3 - 10)

Results are expressed as median and (25-75th) percentile; NM, nasal mucosa; Non-CF NP, nasal polyps from non cystic fibrosis patients; CF NP, polyps from cystic fibrosis patients;*P < 0.05 compared with NM and CF NP.

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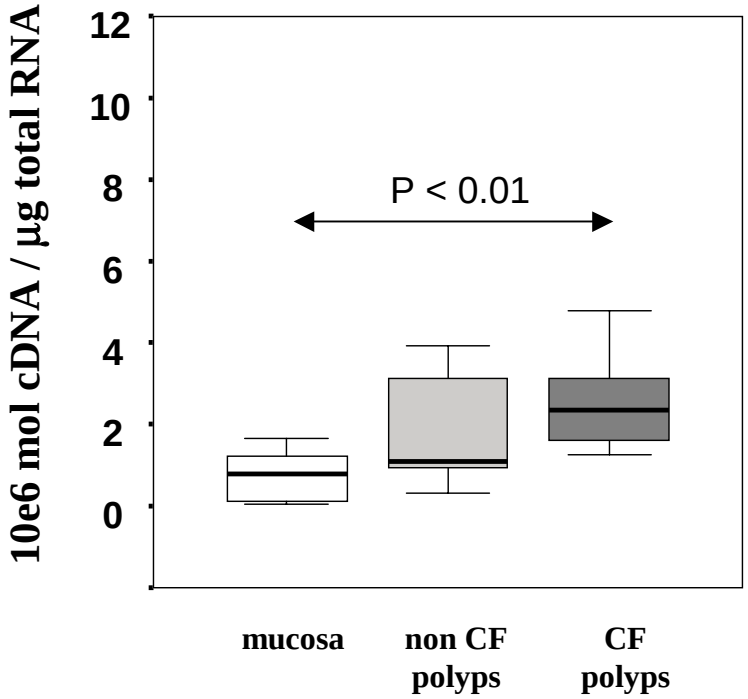
Figure legends

Figure 1. RT-PCR (10^6 cDNA molecules/μg total RNA) and Western Blot (densitometric ratio between protein and β-actin) analysis of Cox-1 in CF nasal polyps, non-CF nasal polyps and nasal mucosa. *Box plots* show the 25th, 50th (median), and 75th percentile values. *Whiskers* show the minimum and maximum values. CF= cystic fibrosis.

Figure 2. RT-PCR (10^6 cDNA molecules/μg total RNA) and Western Blot (densitometric ratio between protein and β-actin) analysis of Cox-2 in CF nasal polyps, non-CF nasal polyps and nasal mucosa. *Box plots* show the 25th, 50th (median), and 75th percentile values. *Whiskers* show the minimum and maximum values. CF= cystic fibrosis.

Cox-1 mRNA



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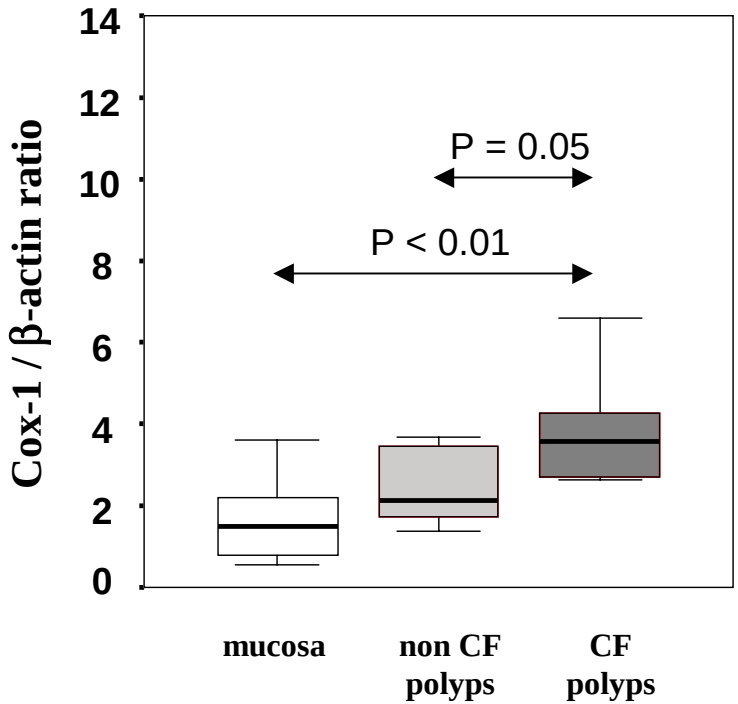

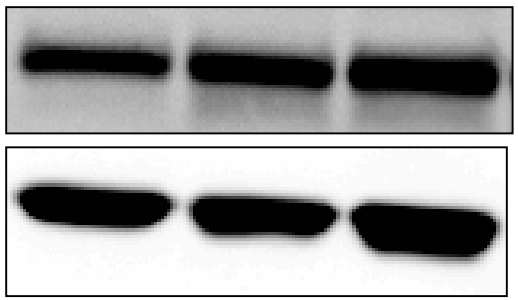

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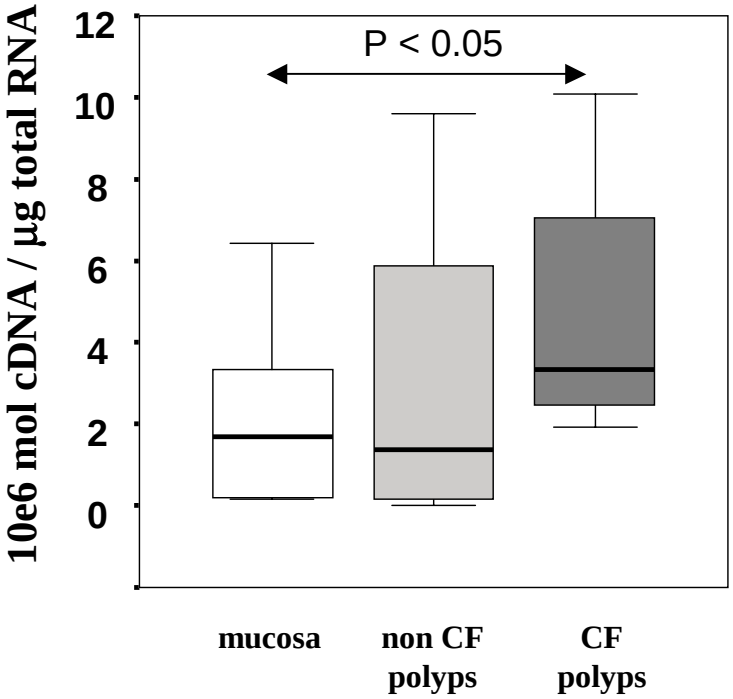

Cox-2 protein